University of Glasgow

McKie, Amanda Elaine (1997) *Molecular and biochemical characterisation of Methionine γ-lyase from Trichomonas vaginalis.* PhD thesis.

http://theses.gla.ac.uk/1808/

Copyright and moral rights for this thesis are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Glasgow Theses Service
http://theses.gla.ac.uk/
theses@gla.ac.uk

# Molecular and Biochemical Characterisation of Methionine γ-lyase from *Trichomonas vaginalis*

Amanda Elaine McKie

Divison of Infection and Immunity
Institute of Biomedical and Life Sciences
University of Glasgow
Glasgow
G12 8QQ

This thesis is presented in submission for the degree of
Doctor of Philosophy in the Faculty of Science

May 1997

GLASGOW

## SUMMARY

Two methionine γ-lyase gene homologues, *mgl1* and *mgl2* have been isolated from *Trichomonas vaginalis* using a degenerate oligonucleotide/PCR approach. Degenerate oligonucleotides designed against cystathionine γ-lyase from yeast, rat and human were used in the PCR experiments. *mgl1* and *mgl2* are present at single copy in the *T. vaginalis* genome and are expressed to give 1.3kb mRNAs. The two genes have extremely short 5' untranslated regions. The predicted molecular mass of MGL1 and MGL2 are 42.9 and 43.1 kDa, respectively. High homology exists at the amino acid level between the two *T. vaginalis* methionine γ-lyase gene homologues and methionine γ-lyase from *Pseudomonas putida* and cystathionine γ -lyase from a range of organisms and other related sulphur amino acid-metabolising enzymes.

The two methionine γ-lyase homologues were cloned into expression vectors and recombinant proteins purified and subsequently characterised. Biochemical characterisation of rMGL1 and rMGL2 revealed that both recombinant proteins were able to break down methionine, catabolise homocysteine at high rate and also able to metabolise cysteine and O-acetyl L-serine. Interestingly, the recombinant proteins were not able to break down cystathionine. The two proteins were expressed in *T. vaginalis*, as judged by ~43 kDa proteins being detected in *T. vaginalis* lysates and soluble extracts by Western blot analysis.

*Tritrichomonas foetus* and *Tritrichomonas augusta* do not possess the ability to breakdown homocysteine and are thought not to contain methionine γ-lyase. Interestingly, however, antibodies raised against rMGL1 and rMGL2 recognised

proteins of various molecular weights and upon immunostaining with intact parasites, it is probable that the Golgi apparatus of these parasites were recognised by the anti rMGL1 and rMGL2 sera. For *T. vaginalis*, immunostaining using the anti rMGL1 and rMGL2 sera revealed a staining of the nucleus and a more general staining of the cytoplasm of these parasites.

# LIST OF CONTENTS

## CHAPTER 2.
## MATERIALS AND METHODS.

## LIST OF FIGURES

## LIST OF TABLES

# ABBREVIATIONS

## Chemicals

| | |
|---|---|
| BSA | -bovine serum albumen |
| cDNA | -complementary DNA |
| CIP | -calf intestinal phosphatase |
| DEPC | -diethyl pyrocarbonate |
| DNA | -deoxyribonucleic acid |
| DNase | -deoxyribonuclease |
| ECL | -enzyme-linked chemoluminescence |
| EDTA | -ethylene diamine tetraacetic acid (disodium salt) |
| EtBr | -ethidium bromide |
| FITC | -fluorescein isothiocyanate conjugate |
| HCl | -hydrochloric acid |
| HRP | -horse radish peroxidase |
| IPTG | -isopropyl thiogalactoside |
| mRNA | -messenger RNA |
| PLP | -pyridoxal 5-phosphate |
| RNA | -ribonucleic acid |
| RNase | -ribonuclease |
| RT | -reverse transcriptase |
| SDS | -sodium docecyl sulphate |
| SSC | -standard saline citrate |
| TEMED | -N,N,N',N' tetramethyl ethylenediamine |
| Tris | -tris (hydroxymethyl) amino methane |
| X-gal | -5-bromo-4-chloro-3-indolyl-beta-D-galactosidase |

## Measurements

| | |
|---|---|
| Å | -angstrom |
| bp | -base pair(s) |
| *g* | -centrifugal force equal to gravitational acceleration |
| M | -molar |
| ml | -millilitre |
| mM | -millimolar |
| mmol | -millimoles |
| µg | -microgramme |
| µl | -microlitre |
| µM | -micromolar |
| w/v | -weight/volume |
| % | -percent (w/v unless otherwise indicated) |

## Miscellaneous

| | |
|---|---|
| Km | -Michaelis constant |
| PAGE | -polyacrylamide gel electrophoresis |
| PCR | -polymerase chain reaction |

## DECLARATION

The results presented in this thesis are my own work except where there is an explicit statement to the contrary. Dr Paul Appleton carried out the immunostaining and fluorescence microscopy described in Chapter 6 of this thesis.

## ACKNOWLEDGEMENTS

I wish to thank Professor Graham Coombs for supervising this project. A big thank you goes to Dr. Jeremy Mottram for becoming an unofficial second supervisor of this project and for all his help, encouragement and support throughout the duration of my studies. I also wish to thank Professor Andy Tait for so generously providing me with a second home at the Wellcome Unit of Molecular Parasitology. I posthumously thank Dr. Barbara Lockwood for giving me the opportunity to work on this project.

A huge thank-you goes to Mrs Dorothy Armstrong for catering to my every whim in the Laboratory of Biochemical Parasitology, for keeping my bench tidy and for her friendship. Also I wish to thank Helcia Lepitak for so patiently washing what must have been hundreds, maybe even thousands of cuvettes and test tubes from my beloved enzyme assay work! I also thank David Laughland for so generously giving me some of his trichomonad pellets and litres of MDM.

I also wish to thank Dr. Joe Irvine for his advice on protein purification and for keeping me entertained at coffee time, along with Mrs Caroline Morrison who also a stalwart of Biochemical Parasitology Coffee Times.

I wish to thank Dr. Karen Grant for teaching me something about the art of protein purification and for endless hours of gossiping across the bench, which did include some useful discussions about my work (honest) and for her friendship. I also wish to thank Phil McCready for passing on the secrets of her cloning successes to me and for teaching me how to quantify DNA in gels. I thank the other members of Jezza's group

who managed to cope with my presence at W. U. M. P., especially Ragnar. I am grateful to Dr. John Gilleard for rescuing me from GCG hiccups! I thank Dr. John Walker for some stimulating discussions on sulphur amino acid metabolism and for being the only other person who at times, I felt knew, what I was wittering on about!

I wish to thank Kathryn Brown, Helen Denton and Mhairi Frame for their much valued friendship over the past four years, and for delicious teas which did much to comfort the stresses and strains of doing and then writing up a PhD. In particular I wish to thank Kathryn for providing me with accommodation when I needed to stop over in Glasgow, especially after Zoology barbecues and to Helen for help and advice with enzyme assays and for yummy onion soup, chilli, cakes and ice cream.

Finally, I wish to thank Paul Appleton for everything, without whose patience, support, help with figures and his computer and printer, this thesis would never have been written.

As a sign of my true appreciation of Paul, I dedicate this thesis to him.

This work was funded by the Wellcome Trust.

# CHAPTER 1. INTRODUCTION.

## 1.1 General biology of Trichomonads.

Species of the genus *Trichomonas* identified by Donne in 1896 are protists that occur in the intestines of mammals including man, birds, reptiles, amphibia, molluscs (slugs) and termites; in the mouth of man and monkeys; and the urinogenital tract of man and cattle. No cyst is produced, and no sexual process is known. Reproduction is solely by binary fission. There are three species of trichomonads that infect human beings, and they are extremely site specific (Honigberg, 1989). *Trichomonas tenax* lives only in the oral cavity, *Pentatrichomonas hominis* only in the intestinal tract and *Trichomonas vaginalis* only in the lower genitourinary tract. Only *T. vaginalis* is considered pathogenic (Thomason *et al.*,1984, Honigberg, 1989, Graves and Gardner, 1993).

*Tritrichomonas foetus* is the bovine equivalent to *T. vaginalis*. *T. foetus* lives in the vagina and uterus of the cow and in the prepucial sheath of the bull. As with the human equivalent the disease is sexually transmitted. Trichomoniasis in cows could be considered to be more serious than the human condition because if *T. foetus* is present in a pregnant uterus the organism invades the foetus and an early abortion often results. This aspect of the biology of *T. foetus* is obviously important from an economic perspective. In cows the infection is self limiting with all trophozoites being expelled with the foetus and placenta, this is fortunate since no reliable treatment is known.

*Trichomonas gallinae* is a species of trichomonad that infects birds, primarily the domestic pigeon *Columba livia*. The trophozoites inhabit the mouth, pharynx,

oesophagus and crop. *T. gallinae* produces a severe disease in some young pigeons, which may be fatal. The disease is variously known as canker, frounce, roup or more scientifically, avian trichomoniasis. Finally, *Trichomitus augusta* is a species of trichomonad that infects the intestine of the leopard frog *Rana pipiens*!

*T. vaginalis* is the organism of choice for the area of research covered by this thesis, but there will also be some mention of *T. foetus* and *T. augusta*.

## 1.2 Biology of *Trichomonas vaginalis.*

*Trichomonas vaginalis* is a motile, ovoid, 10 to 20μm in length, flagellated, anaerobic protozoon. The organism has four anterior flagella and a fifth recurrent flagellum which is attached to thin fin-like extension of the body to form an undulating membrane. The undulating membrane covers the upper two thirds of its body, beneath which is located a cytoplasmic costa. All five flagella are involved in locomotion. Although capable of rapid motility *T. vaginalis* also shows amoeboid movement. An axostyle, a skeletal organelle of longitudinally arranged parallel microtubules, runs the entire length of the organism and protrudes posteriorly.

Internally, *T. vaginalis* has a large nucleus characteristic of eukaryotic cells as well as a highly developed golgi complex (Diaz *et al.*, 1996) The cytoplasm is rich in glycogen granules and ribosomes but contains no mitochondria, peroxisomes or glycosomes. Electron dense chromatic granules, called hydrogenosomes (Muller, 1980, 1993), which occur in other anaerobic protozoa and fungi (Brul *et al.*,1994) are found within the cytoplasm and play an important role in metabolism.

### 1.2.1 Nutrition.

*T.vaginalis* is an aerotolerant anaerobe, optimal growth and reproduction occurs in anaerobic conditions. An extremely fastidious organism, *in vitro* cultivation requires a large number of essential nutrients, including carbohydrates (Read, 1957), amino acids (Rowe and Lowe, 1986), purines (Heyworth *et al.*, 1982) and pyrimidines (Wang and Cleng, 1984), fatty acids (Roitman *et al.*,1978), vitamins (Hollander and Leggett, 1985) and iron (Gorrell, 1985). *In vivo*, the vagina and its secretions contain all of the nutrients required for growth (Huggins and Petri, 1981). *T. vaginalis,* also engulfs and digests bacteria, possibly helping to fulfil nutritional requirements (Francioli *et al.*, 1983).

*T. vaginalis* is known to attach to mucous membranes via specific adhesins. This action is mediated by surface proteinases (Arroyo and Alderete, 1989) and is iron regulated, as lactoferrin (the major iron source at mucosal surfaces) upregulates adhesin protein expression (Lehker *et al,* 1991). For a review of the mechanisms and molecules involved in cytoadherence and pathogenesis of *T. vaginalis* see Alderete *et al.*, (1995).

### 1.2.2 Immunity to *T. vaginalis.*

Protective immunity does not occur with trichomoniasis. Specific anti-*T. vaginalis* serum antibody (Coyne *et al*, 1975), usually at low titres, has been identified in both men and women, but local mucosal antibody has been isolated in women only (Ackers *et al.*, 1975). No convincing evidence supports a major role for antibodies preventing or clearing trichomonal infection (Ackers, 1985). Absence of an effective antibody response highlights the roles of non specific host immune responses. These include activation of the alternative pathway of complement (Demes *et al.*, 1988), neutrophil

chemotaxis and activation induced by the organism itself (Mason and Forman, 1982), and, possibly, locally increased zinc levels (Krieger and Rein, 1982). These non-specific defences may not be completely effective in the treatment, both asymptomatic and symptomatic infections can persist for prolonged periods.

### 1.2.3 Trichomoniasis occurrence.

Trichomoniasis is the most prevalent non-viral sexually transmitted disease (McLellan *et al.*, 1982). World-wide, there are approximately 180 million cases with 2.5 to 3 million infections occurring in the United States annually (Thomason and Gelbart, 1989). The prevalence of disease varies widely in different populations. In asymptomatic patients attending family planning clinics, 5% of women had disease, whereas 50% to 75% of prostitutes were infected (Rein and Muller, 1989). In sexually transmitted disease clinics, the prevalence of trichomoniasis ranges from 0.9% to 39.6% (Lossick, 1989). Multiple sexual partners, black race, previous history of sexually transmitted diseases, coexistent infection with *Neiserria gonorrohoeae,* and non use of either barrier or hormonal contraceptives are known risk factors for acquisition of *Trichomonas* (Lossick, 1989). Previously thought risk factors such as age, coexistent infection with chlamydia and seasonality are thought to play much less important roles in *T. vaginalis* acquisition (Lossick, 1989).

### 1.2.4 Transmission.

Trichomoniasis is a sexually transmitted disease. This was demonstrated by Hasseltine in 1942 when he inoculated the vagina of pregnant women with viable trichomonads and created a symptomatic disease (Hasseltine, 1942). Transmission rates appear higher from men to women, as 70% of men have the disease within 48 hours of exposure

(Weston, 1963), compared to 85% of exposed women (Honigberg, 1989). Interestingly, the percentage of infected men decreases to 33% after exposure, suggesting that the disease is more apt to be spontaneously cleared in men (Weston, 1963). The organism lives only the genitourinary tract on the squamous epithelial cells lining the anterior fornix of the vagina, Skene glands and urethra in women, and in the urethra and prostate gland in men. It can also live under the foreskin in uncircumcised males (Weston, 1963). It is transferred during penile-vaginal sexual intercourse. Although *T. vaginalis* will survive for short periods on moist objects, for example toilet seats, damp towels, (Whittington, 1957, Burch *et al.*, 1959) or in exposed bodily fluids (Whittington, 1951 Gallai and Sylvester, 1966), no cases of transmission by indirect or formite exposure have been documented.

**1.2.5 Symptoms.**

Persons infected with *T. vaginalis* may not show any signs of infection. Up to 50% of women and over 90% of men who harbour the organism will have no noticeable discomfort. However, 30% of infected women have the acute onset of profuse vaginal discharge, vulvar pruritis, cystitis, dysuria, or mild dyspareunia. Symptoms may worsen after menses. The most common symptom, increased vaginal discharge, is found in more than half of symptomatic cases. The nature of the discharge depends on the presence of associated bacteria. Trichomoniasis is strongly associated with bacterial vaginosis because *T. vaginalis* creates an anaerobic environment, thereby changing the vaginal flora (Thomason *et al.*, 1988). The classically described green, frothy, foul smelling discharge is found in fewer than 10% of symptomatic infected women, and punctate heamorrhagic lesions (strawberry spots) are found in only about 2% of cases (Wolner-Hanssen *et al.*, 1989). Some patients may have lower abdominal discomfort,

but *T. vaginalis* has not been shown to cause pelvic inflammatory disease. Phagocytosis or attachment of cervicovaginal bacteria, including, *N. gonorrhoeae,* may be a cause of upper reproductive tract infection associated with *T. vaginalis.* Increased vaginal concentrations of iron salts (abundant in menstrual blood ) may facilitate overgrowth and clinical worsening of symptoms associated with menses (Rein and Chepel, 1975). In these circumstances other sexually transmitted disease should be suspected.

In symptomatic men, common complaints include scanty, clear to mucopurulent urethral discharge, dysuria, mild pruritis or burning immediately after intercourse. The prostate gland is infected in 40% of symptomatic males (Kreiger, 1981). Zinc, which has anti-trichomonal activity *in vitro*, is found in high concentrations in normal prostatic fluid and may inhibit multiplication of *T. vaginalis* (Krieger and Rein, 1982). This may account for the lower number of organisms found in males. Men with non-specific urethritis unresponsive to usual antimicrobial therapy should be treated for *T. vaginalis* because 15-20% will harbour this organism (Kreiger, 1981).

### 1.2.6 Diagnosis.

Clinic-based laboratory evaluation of *T. vaginalis* remains unsatisfactory. The vaginal pH is elevated above 4.5 in as many as 90% of cases (Rein, 1990). This finding, however, is non specific as 90%of women with bacterial vaginosis also have an elevated pH (Pheifer *et al*, 1978). The presence of a fishy odour after application of 10% potassium hydroxide (whiff test) is present in 50% patients (Chen *et al.*, 1982), but this test is non-specific for *Trichomonas*. A helpful test in differentiating trichomoniasis from bacterial vaginosis is evaluation of the wet smear for polymorphonuclear cells (PMNs) and "clue cells". The ratio of PMNs to vaginal epithelial cells is greater than 1

in 75% of trichomonal infections, whereas in bacterial vaginosis this ratio is less than 1 in 90% of patients (Rein, 1990).

The time honoured approach for the diagnosis of trichomonal infections has been microscopic ("wet prep") evaluation. A sample of vaginal fluid is placed in normal saline and viewed under phase-contrast microscopy. The diagnosis of trichomoniasis is made by directly observing the motile parasite. This procedure detects 60% to 80% of cases and is more sensitive if symptoms are present.

Newer methods of identification based on the nucleic acid of *T. vaginalis* are being rapidly developed. For example the detection of variable DNA repeats in *T. vaginalis* by a single set of polymerase chain reaction primers holds promise in for the development of a typing scheme for *T. vaginalis* (Riley *et al.*, 1991). A method for the identification of *T. vaginalis* by fluorescent in situ hybridisation (FISH) has also been developed (Muresu *et al.*, 1994). Also rapid nucleic acid hybridisation test have been developed for the diagnosis of *T. vaginalis* and other causative agents of vaginitis such as *Gardnerella vaginalis* and *Candida sp* (Ferris *et al.*, 1995). A detection method for *T. vaginalis* in pregnant women based on PCR has also been developed recently (Witkin *et al.*, 1996).

### 1.2.7 Treatment.

Trichomoniasis should not be ignored even if the patient has no symptoms. Treatment is necessary to prevent transmission to sexual partners and to avoid later problems. The only drugs proved to be effective are the 5-nitroimidazoles. These include

metronidazole, ornidazole and tinidazole. If both partners are treated, cure rates approach 100% (Lossick, 1982).

Alternative treatments are considered palliative because the 5-nitroimidazoles are the only drugs with documented efficacy. Clotrimazole (an anti-fungal cream) given intravaginally for 6 days cures 48% to 66% of patients (Schell, 1974). Some authors, however, report cure rates of 25% or less (Rein and Muller, 1989, Lossick,1990). Nonoxynol-9 (a spermicidal agent) was anecdotally effective in a refractory case (Livengood and Lossick,1991). Other potential treatments requiring further investigation include mebendozole and taxol (Juliano *et al.*, 1987).

It has been shown that MDL 63 604, a semi synthetic derivative of purpuromycin, had an in vitro activity similar to that of metronidazole against *T. vaginalis* (Goldstein *et al.*, 1995). It has also been shown that furazolidone, a nitrofuran presently used to treat giardiasis and infections with some anaerobic resistant enteric bacteria, may be a good candidate for treating metronidazole resistant trichomoniasis (Narcisi and Secor, 1996). Neo-Penotran® pessaries, 500mg metronidazole and 100mg miconazole nitrate have been shown to be a novel and effective formulation for the treatment of common types of vaginitis and the pessary may be of particular value in the treatment of resistant or recurrent vaginitis (Kukner *et al.*, 1996). Finally, it has been shown that geneticin (G418), an aminoglycoside was effective at killing *T. vaginalis* cells at doses intermediate between those previously effective against prokaryotes and eukaryotes (Riley and Kreiger, 1996).

Concerns continue regarding use of relatively high oral doses of metronidazole during pregnancy. The toxic metronidazole intermediate, which is cidal to *T. vaginalis*, interacts with bacterial and mammalian DNA (Lossick, 1982). Despite this, no human data demonstrate increased risks or teratogenesis or carcinogenesis associated with metronidazole use (Beard *et al.*, 1979, Rosa *et al.*, 1987). Most practitioners, however, prefer to delay treatment until after embryogenesis is complete. Use of topical clotrimazole may be effective in treating patients in the first trimester.

### 1.2.8 Phylogenetic position of *T. vaginalis.*

Phylogenetic trees based on small subunit ribosomal RNA (SSU rDNA) (Sogin, 1991), reveals three protist lineages which consistently branch most deeply; the Parabasala, Metomonada and Microspora (see Corliss, 1994 for nomenclature), all lack mitochondria. One possible evolutionary inference is that all three of these protist groups are primitively amitochondriate, having diverged from the main eukaryotic trunk before the endosymbiotic event that led to mitochondria took place (Patterson and Sogin, 1993). In addition, trichomonads and microspora possess 70S rather than 80S ribosomes, which has been interpreted as the retention of ancestral state present in prokaryote outgroups, providing additional support for their divergence early in eukaryotes history (Champney *et al.*, 1992 and Cavalier-Smith, 1993). The relative order of these taxa has not been clearly resolved, as it varies with the taxon composition or method of analysis, and all three groups are plausible candidates for the deepest branch in the SSU rDNA tree (Liepe *et al.*, 1993 and Gunderson *et al.*, 1995).

Trichomonads (Parabasala) are unique among the three basal taxa in that they do possess another double membraned organelle, the hydrogenosome.

While many authors suggest that the deep phylogenetic position of trichomonads indicates that they primitively lack mitochondria (Patterson and Sogin, 1992 and Muller, 1993), others have argues that they may have secondarily lost mitochondrial functions (Cavalier-Smith, 1987). The precise question of hydrogenosome evolution in trichomonads, in particular *T. vaginalis,* is addressed in section 1.3.1.1 of this introduction.

## 1.3 Biochemistry of *T. vaginalis.*

### 1.3.1 Energy metabolism of *T. vaginalis.*

*T. vaginalis* and other anaerobic protozoa eg. *Giardia lamblia* and *Entamoeba histolytica* have been shown to be fermentative and produce a mixture of acids (eg. lactate, acetate, succinate, butyrate and propionate), alcohols (eg. ethanol and hydrogen) as major end products (Muller, 1988). For all of the anaerobic protozoa investigated, including *T. vaginalis*, energy production is based upon glycolysis with the mixture of end products providing a means of maintaining redox balance at the same time as generating energy, which is additional to energy resulting from glycolysis (for reviews see Muller, 1988, 1991 and Coombs and Muller, 1995). A common feature is the excretion of both acetate and carbon dioxide. These result from the catabolism of pyruvate, catalysed by pyruvate:ferredoxin oxido-reductase, to give acetyl CoA, reduced ferredoxin and carbon dioxide. In *T. vaginalis* the reduced ferredoxin is reoxidised by hydrogenase to yield hydrogen. The production of hydrogen by *T. vaginalis* and other anaerobic protozoa, such as rumen protozoa, is strictly correlated with the presence, in the organism, of a peculiar microbody-like organelle; and it has subsequently been established that hydrogen production does occur in these organelles which have consequently been given the name hydrogenosomes (Muller, 1980, 1993).

### 1.3.1.1 Metabolism and Evolutionary Origins of Hydrogenosomes of *T. vaginalis.*

The hydrogenosomes of trichomonads have been studied in most detail. This organelle is surrounded by two membranes and appears to contain no genetic material (Muller, 1993). Metabolic studies on cell fractions enriched for hydrogenosomes have shown that the organelle is involved in the oxidation of pyruvate to acetate, carbon dioxide and hydrogen. This process is accompanied by ATP formation via substrate level phosphorylation (Muller, 1980, 1993).

Metabolically, hydrogenosomes could be said to be the anaerobic equivalent of mitochondria. This, as well as the fact that cells which contain hydrogenosomes lack mitochondria (Muller, 1988), has lead to the proposals that hydrogenosomes are degenerate mitochondria (Cavalier Smith, 1987) or that these two organelles are derived from a common progenitor organelle (Johnson *et al.*, 1990). Analysis of *T. vaginalis* ribosomal RNA (rRNA) (Sogin, 1989) indicates that the evolutionary line leading to trichomonads diverged from the main line of eukaryotic evolution prior to the appearance of mitochondria. This is consistent with the theory of a common progenitor organelle that ultimately gave rise to mitochondria in aerobic organisms and hydrogenosomes in certain anaerobic organisms. Alternatively, hydrogenosomes may have arisen completely independently of mitochondria, via an endosymbiotic relationship with an anaerobic bacterium (Whately *et al*, 1981). The presence of hydrogenase in hydrogenosome, as well as other proteins which are typically found in anaerobic bacteria (Muller, 1980, 1993), supports this latter hypothesis.

Very recently, there have been a number of publications that have addressed the question of hydrogenosome evolution. Genes have been isolated from *T. vaginalis* that encode chaperone proteins, HSP70, HSP60, HSP10 (Germot *et al.*, 1996 and Bui *et al.*, 1996) and CPN60 (Roger *et al.*, 1996 and Horner *et al.*, 1996), proteins that in other lineages are present in the mitochondrial compartment. It is suggested by all of the above authors that the presence of the genes for the above proteins in *T. vaginalis* provide evidence that the ancestors of trichomonads perhaps harboured the endosymbiotic progenitors of mitochondria, but these evolved into hydrogenosomes early in trichomonad evolution.

### 1.3.2 Amino acid metabolism of *T. vaginalis.*

*T. vaginalis* requires amino acids for protein synthesis but they may also be involved in other aspects of cell metabolism. Amino acid metabolism in *T .vaginalis* has been relatively well studied. This introduction concentrates on the aspects of amino acid metabolism of *T. vaginalis* that are most relevant to this study.

#### 1.3.2.1 Proteinases of *T. vaginalis.*

It has been shown that *T. vaginalis* contains high levels of proteinase activity. For an extensive review of proteinases of *T. vaginalis* see North, 1991. The especially high levels of proteinase activity in *T. vaginalis* and the continuous release from cells suggests that proteolysis is likely to be important for the organism. Possible roles include the utilisation of host proteins for nutrition, the destruction of components of the host immune system, the release of amino acids for amine production to counter the low pH of the vagina, and direct effects on host cells to assist cytoadherence.

### 1.3.2.2 Glutamate Dehydrogenase and Aminotransferases.

Glutamate dehydrogenase (GDH) and aminotransferases have a central role in the catabolism and biosynthesis of amino acids. Aminotransferases redistribute nitrogen from amino acids to keto acids. When amino acids are catabolized, the first step is frequently catalysed by an aminotransferase which transfers the amino group to ketoglutarate to form glutamate which can be deaminated by GDH to give α-ketoglutarate and ammonia. For the biosynthesis of amino acids, these steps are reversed. Glutamate dehydrogenase was isolated from *T. vaginalis* and characterised by Turner and Lushbaugh (1988). The enzyme is NADP-linked and is able to catalyse the amination of α-ketoglutarate and the deamination of glutamate.

A number of different amino transferases have been identified in *T. vaginalis*. The aspartate aminotransferase of *T. vaginalis*, although the name implies a high level of specificity, is able to use a broader range of amino acids as amino acid donors. Indeed the enzyme will also catalyse the transamination of the aromatic amino acids, phenylalanine, tryptophan and tyrosine (Lowe and Rowe 1985, 1986 and 1987). In contrast the mammalian enzyme will only catalyse the transamination of aspartate or glutamate with oxaloacetate or α-oxoglutarate.

Lowe and Rowe (1986b) published a survey of aminotransferase activities in *T. vaginalis*. They found α-ketoglutarate, oxaloacetate or phenylpyruvate acted as effective amino acceptors with ten amino acids. Activities of aspartate, aromatic amino acid, branched chain amino acid and ω-amino acid aminotransferases were

demonstrated. The high levels of activity with branched chain and aromatic amino acids and aspartate confirmed the earlier findings of Jaroszewicz and May Iszko (1965).

### 1.3.2.3 Alanine Metabolism.

Alanine is an end product of glucose catabolism in *T. vaginalis* (Zuo *et al.*, 1995). Its formation is linked to redox balance, alanine aminotransferase and glucose catabolism. When alanine is a major end product of glucose catabolism, substantial amounts of nitrogenous compounds must be available to contribute the amino group. In *T. vaginalis* arginine catabolism is a likely provider of nitrogen for alanine synthesis (Linstead and Cranshaw, 1983). It has also been shown that alanine is a major end product of metabolism by *Giardia lamblia*. Edwards *et al.*, 1988 have shown by proton nuclear magnetic resonance that alanine is the major end product formed during log phase growth of these anaerobic parasites. Ethanol is also produced by *G. lamblia* but its formation lags behind that of alanine until the parasites enter stationary phase. Acetate is also produced by these parasites but at a constant rate throughout growth.

### 1.3.2.4 Amino acids as energy sources in *T. vaginalis*.

*T. vaginalis* relies heavily on glycolysis for energy production (For reviews see, Muller 1988, 1991; Coombs and Muller 1995). However, it has also been suggested that amino acids can be used as a source of energy in *T. vaginalis*. The initial step of amino acid catabolism frequently involves the loss of the amino nitrogen to yield a carbon skeleton in the form of $\alpha$ keto acid, either as a result of transamination or by deamination. In general, energy is derived from amino acids by the oxidation of the carbon skeletons after entry into the intermediary metabolic pathways.

### 1.3.2.5 Arginine and Ornithine Metabolism.

Arginine is broken down by *T. vaginalis* to ornithine, ammonia and $CO_2$. The dihydrolase pathway is involved in the breakdown of arginine which consists of the three enzymes: arginine deiminase, catabolic ornithine transcarbarmoylase and carbamate kinase (Linstead and Cranshaw, 1983) Arginine is present at relatively high levels in the extracellular fluid of mammals and it may represent a major energy source for *T. vaginalis in vivo*. The dihydrolase pathway is unusual in eukaryotes. The pathway includes a substrate-level phosphorylation catalysed by carbamate kinase, and production of ATP means that arginine can act as an energy source under anaerobic conditions. It is thought that the major role of the dihydrolase pathway in *T. vaginalis* would be to produce ornithine for biosynthesis of polyamines.

The arginine dihydrolase pathway also exists in *G. lamblia* (Edwards *et al.*, 1992). Due to the high activity of the enzymes of this pathway in *G. lamblia* it is thought that arginine catabolism could contribute significantly to giardial energy production, by substrate level phosphorylation at the terminal step catalysed by carbamate kinase.

### 1.3.2.6 Leucine and Threonine Catabolism.

Threonine dehydratase, which converts threonine into α-ketobutyrate and ammonia has been detected in *T. vaginalis*. The enzyme is involved in threonine catabolism, rather than isoleucine biosynthesis (Lockwood and Coombs, 1989). Addition of leucine and threonine to culture of *T. vaginalis* growing in vitro results in the production and release of α-hydroxyisocaproate and α-hydroxybutyrate; respectively. They are synthesised via the corresponding α-keto acid.

The functional significance of the catabolism of these amino acids is not clear, but it is likely that hydroxy acid secretion is simply a reflection of the high concentrations of keto acids produced and the broad specificity of lactate dehydrogenase. Isoenzymes of lactate dehydrogenase in *T. vaginalis* have been shown to be active towards both α-ketobutyrate and α-ketoisocaproate (Lockwood and Coombs, 1989).

### 1.3.2.7 Sulphur amino acid metabolism in *T. vaginalis*.

Sulphur amino acid metabolism in *T. vaginalis* forms the major area of interest and research in this thesis. The pathways of sulphur amino acid metabolism in *T. vaginalis* differ significantly from those of the host. It is the differences in some of the pathways that have initiated a long term study of the sulphur amino acid metabolism of this parasite, with a view to evaluating some of the peculiar pathways as potential targets for chemotherapeutic attack.

Sulphur amino acid metabolism in *T. vaginalis* may be considered to be made up of two separate parts, the methionine cycle and the reverse transulphuration pathway, the two pathways are linked by the sulphur containing amino acid homocysteine (see Figure 1.1).

#### 1.3.2.7.1 The methionine cycle in *T. vaginalis*.

L-methionine is required by *T. vaginalis* for the biosynthesis of S-adenosylmethionine (SAM) (Lawrence and Robert-Gero,1991). This compound is involved in a number of synthetic processes such as transmethylations and polyamine biosynthesis. Living trichomonads have been shown to incorporate exogenous labelled methionine into

intracellular S-adenosylmethionine (SAM) (Thong *et al.*, 1986 and 1987). The labelled methyl carbon was also detected in nucleic acids and lipids presumably owing to the subsequent role of SAM as a one carbon donor in transmethylation reactions (Lawrence and Robert-Gero, 1991). These results suggest that trichomonads contain SAM synthetase and SAM dependent methyltransferases, and both of these enzymes have been detected in *T. vaginalis* (Yarlett, 1988 and Thong *et al.*, 1987), respectively. S-adenosyl homocysteine hydrolase activity has been detected in *T. vaginalis* (Thong *et al.*, 1985) and recently the gene for this enzyme from *T. vaginalis* has been characterised (Bagnara *et al.*, 1996).

**1.3.2.7.2 Methionine γ-lyase of *T. vaginalis*.**

In *T. vaginalis* methionine is rapidly broken down to α-ketobutyrate, methanethiol and ammonia by the activity of methionine γ-lyase (Thong and Coombs 1987a, Lockwood and Coombs, 1991). Methionine γ-lyase of *T. vaginalis* catalyses a simultaneous deamination and dethiomethylation of methionine, whereas mammals and aerobic organisms catabolize methionine by a two step reaction (i.e. transamination and then dethiomethylation ). The enzyme is also responsible for the breakdown of homocysteine to α-ketobutyrate, hydrogen sulphide and ammonia, (Thong and Coombs, 1985, 1987). A reaction catalysed by homocysteine desulphurase, (a synonym for methionine γ-lyase). The formation of α-ketobutyrate from methionine may generate an additional source of energy. It is probable that pyruvate: ferredoxin oxidoreductase catalyses the conversion of α-ketobutyrate to propionyl CoA which could be used for substrate level phosphorylation. Methionine γ-lyase is absent from mammalian cells and, although homocysteine desulphurase activity has been detected, it was shown to be

due to cystathionine γ-lyase and thought to be of little or no significance in mammals (Cooper, 1983). Thus it appears that methionine γ-lyase of *T. vaginalis* performs a function peculiar to this parasite, and clearly the presence of the enzyme offers opportunities for chemotherapeutic exploitation.

**1.3.2.7.3 Reverse transulphuration pathways in *T. vaginalis*.**

In *T. vaginalis* methionine sulphur metabolism occurs by the methionine cycle coupled to the transulphuration pathway, as well as by the direct breakdown by methionine γ-lyase. The transulphuration pathway results in the transfer of the sulphur of methionine via the thioether cystathionine to produce cysteine. The pathways of methionine and cysteine metabolism and transulphuration pathways are summarised in Figure 1.1. Points of interest in the sulphur amino acid metabolism of *T. vaginalis* that arise from Figure 1.1 are as follows.

Cystathionine β-synthase from several organisms (mammals, nematodes, yeast and fungi) catalyses the synthesis of cystathionine from homocysteine and serine, the penultimate step in the cysteine biosynthetic pathway from methionine. The enzyme also catalyses the reversible replacement of the β-SH group of cysteine with water to form serine and hydrogen sulphide. The enzyme of *T. vaginalis* and a similar enzyme found in nematodes displays 'activated' serine sulphydrase activity in which the production of hydrogen sulphide from cysteine is stimulated by a second thiol substrate, a property which distinguishes them from their mammalian counterpart (Thong and Coombs 1985b, Walker and Barrett, 1992). Cystathionine β-synthase activity in *T.*

*vaginalis* has not been looked at, but it probably exists due to the presence of activated serine sulphydrase activity in parasite lysates.

Cystathionine γ-lyase (γ-cystathionase) catalyses the final step in the transulphuration pathway, (which serves to transfer sulphur from dietary methionine to cysteine). The enzyme has been identified in mammals, yeast, fungi, nematodes and *T. vaginalis*. For *T. vaginalis* there has only been one report of the presence of cystathionine γ-lyase in parasite lysates, which was reported to have a very low specific activity (Thong, 1985), and since then very little attention has been given to this enzyme.

In order to compare the metabolism of sulphur amino acids in *T. vaginalis* with other organisms, I find that Figures 1.2, 1.3 and 1.4 illustrate the fundamental differences between this parasite and other general classes of organism.

### 1.3.2.8 Sulphur amino acid metabolism in bacteria and plants.

Soda, 1987 and Giovanelli, 1987 have presented reviews of microbial and plant sulphur amino acid metabolism, also Flavin, 1975 has presented a detailed review of methionine biosynthesis in micro-organisms. Figure 1.2 summarises the metabolism of sulphur containing amino acids in plants and bacteria based on the information provided in these reviews and more recent publications. Various interesting points arise from this pathway which have relevance to this thesis.

In bacteria and plants, homoserine O-phosphate is the last intermediate of the aspartate pathway that is common to both methionine and threonine biosynthesis. It can either be methylated to threonine by threonine synthetase, or undergo displacement of the

phosphate by an acyl group as the first step of methionine biosynthesis. Homoserine O-phosphate is esterified by acyl transfer from acyl CoA, displacing the phosphate. In *Escherichia coli* and *Salmonella typhimurium* a succinyl ester is formed, while in non-enteric bacteria it is an acetyl group that is transferred from acetyl CoA. In higher plants a variety of acyl groups, including acetyl and oxalyl, may be used depending on species.

As can be seen from Figure 1.2 the acyl of O-acylhomoserine is displaced by cysteine forming cystathionine, a reaction catalysed by cystathionine γ-synthase, the first step in the transulphuration pathway of bacteria and plants. Cystathionine γ-synthase has been purified and characterised for a number of plants. Cystathionine γ-synthase from wheat (*Triticum aestivum*) was found to have a native molecular mass of 155 kDa and be composed of four identical subunits of ~34.5 kDa (Kreft *et al.*, 1994) The same enzyme from spinach (*Spinacea oleracea*) has also been characterised and was shown to catalyse the formation of cystathionine or homocysteine depending on the sulphur containing substrate, cysteine or sulphide (Ravanel *et al.*, 1995). The gene for cystathionine γ-synthase from *A. thaliana* has been cloned (Ravanel *et al.*, 1995) and has subsequently been purified and characterised by overexpression in *E. coli* (Ravanel *et al.*, 1996).

The second step of the transulphuration pathway in bacteria and plants is the β-cleavage of cystathionine which results in the formation of homocysteine, pyruvate and ammonia. In recent years, there has been considerable research activity into cystathionine β-lyase from a variety of plant and bacterial sources, that has lead to the detailed characterisation of this enzyme. Interest in cystathionine β-lyase of spinach was initiated in the early 70's by Giovanelli and Mudd, 1971 and the enzyme was reported to

have been purified from this plant some sixteen years later (Giovanelli, 1987). Further, more detailed characterisation of the enzyme from spinach was reported a couple of years ago (Droux *et al.*, 1995). Around the same time of reports on the characterisation of the spinach enzyme, there were several reports of the enzyme being purified and characterised from garlic (Nock and Mazelis, 1986), onion (Nock and Mazelis,1987) and leek (Won and Mazelis, 1989). Analogous enzymes have also been purified from cabbage (Hall and Smith, 1983) and broccoli (Hamamoto and Mazelis (1986). More recently the gene for cystathionine β-lyase has been isolated from *Arabidopsis thaliana* (Kim and Leustek, 1996) and this will probably lead onto the biochemical characterisation of this protein from this particular organism.

As for cystathionine β-lyase in bacteria. The enzyme from *E. coli* was purified and characterised in the early 80's (Dwivedi *et al.*, 1982). The pyridoxal 5'-phosphate binding region of this enzyme was characterised by Martel *et al.*, (1987). In 1996 the enzyme was overexpressed in *E. coli*, purified and crystallised (Laber *et al.*, 1996). The crystal structure of cystathionine β-lyase from *E .coli* has now been resolved to 1.83Å (Clausen *et al.*, 1996). The enzyme has also been characterised from *Bordetella avium* (Gentry-Weeks *et al.*, 1995), the aetiological agent of an upper respiratory disease in birds which, symptomatically and pathologically, resembles bordetellosis in humans.

Homocysteine synthesis in bacteria occurs by a γ-replacement reaction in which the substrate O-succinylhomoserine reacts with cysteine to form homocysteine and succinate, the same reaction in plants uses O acetyl homoserine in combination with sulphide to produce homocysteine. Bacteria are also able to use O-acetyl homoserine as

a substrate in the synthesis of cystathionine. Several species of bacteria use the O-acetyl form of homoserine for the synthesis of cystathionine and homocysteine, for example *Bacillus subtilus* (Brush and Paulus, 1971) *B. polymyxa* (Wyman and Paulus, 1975), *Corynebacterium acetophilum* (Murooka *et al.*, 1970), *Brevibacterium flavum* (Brush and Paulus, 1973) and *Arthrobacter paraffineus* (Nakayama *et al.*, 1969).

Homocysteine is then methylated to methionine in a methyl tetrahydrofolate dependent reaction.

In bacteria and plants, cysteine is synthesised by a two step reaction. Serine is O-acetylated by a reaction with acetyl CoA, catalysed by the pyridoxal phosphate dependent serine acetyltransferase. The acetyl group is then displaced by a reaction with inorganic sulphide, either free in solution or bound to a protein such as thioredoxin to produce cysteine. This reaction is catalysed by O-acetyl serine sulphydrylase. The pyridoxal 5'-phosphate binding region for this enzymes has been resolved as has the reaction mechanism for the enzyme (Schnackerz and Cook, 1995, Cook *et al.*, 1996). The pyridoxal 5'-phosphate binding region of the spinach enzyme has also been characterised (Roland *et al.*, 1996).

The synthesis of cystathionine in bacteria may represent a means of storing organic sulphur in a readily usable and relatively non toxic form. Sulphide ions are rare in the environment and the reduction of sulphate to sulphide is closely linked to cysteine synthesis.

#### 1.3.2.8.1 Methionine γ-lyase of anaerobic bacteria.

Methionine γ-lyase has only been found in anaerobes, namely *Clostridium sp*, (Wiesendanger and Nisman 1953) *Pseudomonas* (Miwatani *et al.*, 1954; Kallio and Larson, 1955; Ito *et al.*, 1975; Tanaka *et al.*, 1977), *Aeromonas* (Nakayama, 1984), some rumen bacteria (Merricks and Salsbury, 1974) and *Entamoeba histolytica* (Lockwood and Coombs, 1989). As in *T. vaginalis*, methionine breakdown in these organisms occurs by a simultaneous deamination and dethiomethylation. Recently the gene for methionine γ-lyase from *P. putida* has been isolated and characterised (Inoue *et al.*, 1995 and Hori *et al.*, 1996)

### 1.3.2.9 Sulphur amino acid metabolism in yeast and fungi.

Figure 1. 3 gives a summary of the metabolism of sulphur containing amino acids in yeast and fungi. There are a number of points of interest that arise from the metabolism of sulphur amino acids in yeast and fungi that are relevant to the area of study of this thesis.

A characteristic feature of sulphur metabolism of yeast and fungi, as can be seen from Figure 1.3, is the existence of alternative pathways for cysteine and homocysteine synthesis. Cysteine can be synthesised from O-acetylserine (OAS) by O-acetylserine sulphydrylase and then be used for the synthesis of homocysteine in two reactions catalysed by cystathionine γ-synthase and cystathionine β-lyase, respectively. Alternatively, homocysteine is synthesised directly from O-acetyl homoserine (OAS) and sulphide by O-acetylhomoserine sulphydrylase and serves as a precursor of both cysteine and methionine. The physiological role of these pathways may differ considerably in various yeast and fungi (for a review see Yamagata, 1989). The OAS-OAH sulphydrylase of *S. cerevisiae* was shown to be a bifunctional sulphydrylase, it

was able to synthesise both cysteine and homocysteine (Yamagata *et al.*, 1975). The enzyme is also able to catalyse the synthesis of methionine and ethionine with OAH when the sulphide was replaced with methylmercaptan and ethylmercaptan, respectively (Yamagata, 1971). It has been shown that the fission yeast *Schizosaccharomyces pombe* catalyses OAH sulphydrylation but not cystathionine synthesis (Yamagata, 1984). *Sacharomycopsis lipolytica* also has a OAS-OAH sulphydrylase which is very similar to that described for *S. cerevisiae* (Paszewski and Grabski, 1976).

OAH sulphydrylase, which functions in homocysteine synthesis in fungi has been characterised in *Neurospora crassa* and *Aspergillus nidulans*. (Piotrowska *et al.*, 1980 and Paszewski *et al.*, 1984). Figure 1.3 shows that the reverse transulphuration pathway, which consists of cystathionine β-synthase and cystathionine γ-lyase, also exists in yeast and fungi. Cystathionine β-synthase from *S. cerevisiae* has been purified and characterised (Ono *et al.*, 1994). The gene for cystathionine γ-lyase from *S. cerevisiae* has been cloned, expressed in *E. coli* purified and subsequently characterised (Yamagata et al.,1993). The enzyme has also been purified and characterised from *Neurospora* (Flavin and Slaughter, 1964 and Flavin and Segal, 1964).

### 1.3.2.10 Sulphur amino acid metabolism in mammals.

Figure 1.3 summarises the pathways of sulphur amino acid metabolism that are known to exist in mammals. The metabolism of sulphur amino acids in mammals can be considered in two parts. Firstly, the methionine cycle in which S-adenosyl methionine, as in *T. vaginalis*, transfers its methyl group to a variety of acceptors and the resultant S-adenosylhomocysteine is deadenylated by S-adenosylhomocysteinase. The resultant homocysteine may be either remethylated to methionine or metabolised by way of

cysteine, which forms the second part of sulphur amino acid metabolism in mammals, the reverse transulphuration pathway. The first step of reverse transulphuration in mammals is catalysed by cystathionine β-synthase. Cystathionine β-synthase has been purified and characterised from rat and human (Nakagawa and Kimura, 1968 and Kraus and Rosenberg, 1983). The final step of mammalian reverse transulphuration is catalysed by cystathionine γ-lyase. Cystathionine γ-lyase from rat has also been purified and extensively characterised. (Matsuo and Greenberg1959a/b). The gene sequences for cystathionine γ-lyase from rat and human have also been isolated and characterised (Erickson *et al.*, 1990 and Lu *et al.*, 1992).

## 1.4 Molecular biology of *T. vaginalis.*

The molecular biology of *T. vaginalis* is an area of study which has received very little attention. Since 1990 there have been several publications on different aspects of the molecular biology of *T. vaginalis*. However, this is an area which has the potential to yield more information on the molecular aspects of this ancient eukaryote. In this introduction my aim, simply, is to give an overview of what is currently known about the molecular biology of *T. vaginalis*.

### 1.4.1 *T. vaginalis* DNA.

The DNA from *T. vaginalis* and has been thoroughly characterised by Wang and Wang, 1985. The unique sequences of the *T. vaginalis* genome constitute 2.5 x$10^7$ bp, the value comparable with the complexity of *Trypanosoma brucei* (Borst *et al.*, 1980) and *Plasmodium berghei* (Dore *et al.*, 1980). Although the genome of trichomonads is relatively small, it contains a high proportion of repetitive sequences, representing approximately 53.3% of *T. vaginalis* DNA. (Wang and Wang, 1985). The *T. vaginalis*

genome contains an additional highly repeated DNA fraction comprising 13.3% (Wang and Wang, 1985). Due to the presence of repetitive sequences, distinct banding patterns can be seen after digestion of trichomonad DNA with restriction endonucleases and separation of restriction fragments by agarose gel electrophoresis.

Further characterisation of a repetitive sequence specific for *T. vaginalis* was carried out by Paces *et al.*, 1992. A family of 650bp long repeats from the *T. vaginalis* genome, designated the *T. vaginalis*-E650 family, were cloned and sequenced. The repetitive DNA sequence is A+T- rich (73.3% A+T). The *T. vaginalis* E650 repeats were conserved in all *T. vaginalis* strains examined, regardless of their diverse geographical origin. Hybridisation of the E650 repeats showed they are highly conserved between different isolates. A dot blot hybridisation protocol was developed which does not required the isolation of DNA. Use of the protocol allowed the detection of DNA released from approximately $10^3$ *T. vaginalis* cells per dot. These observations suggest that the *T. vaginalis* E650 probe is potentially applicable to the identification and detection of *T. vaginalis*.

### 1.4.2 *T. vaginalis* genes.

To date, April 1997, a number of genes have been characterised from *T. vaginalis*. The genes that have been isolated and characterised are as follows:-

Firstly, ferredoxin (Johnson *et al.*, 1990), secondly, β Succinyl Coenzyme A (Lahti, *et al.*, 1992), thirdly β tubulin encoding genes (Katiyar and Edlind, 1994), fourthly, α subunit of succinyl CoA synthetase (Lahti *et al,* 1994), fifthly, cysteine proteinase genes

(Mallinson *et al.*, 1994), and finally P-glycoprotein, a 70kDa cytosolic heat shock protein (HSP 70) and α tubulin encoding genes (Quon *et al.*, 1994).

A number of different strategies have led to the isolation of the above *T. vaginalis* genes, in the case of ferredoxin and the β subunit of succinyl CoA synthetase genes, antibodies raised against purified protein and an enriched bydrogenosomal fraction, respectively were used to isolate clones from *T. vaginalis* cDNA expression libraries (Johnson *et al.*, 1990, Lahti *et al.*, 1992).

Use of a *Giardia lamblia* β tubulin cDNA as a heterologous probe, along with degenerate primers designed to conserved regions of β tubulin amino acid sequence, was the approach used to isolate the β tubulin genes from *T. vaginalis* (Katiyar and Edlind, 1994). An α tubulin cDNA probe from *T. brucei* was used as heterologous probe to isolate α tubulin genes from *T. vaginalis* (Quon *et al*,. 1994). Glycoprotein, 70kDa cytosolic heat shock protein and the α subunit of succinyl CoA synthetase (Johnson *et al.*, 1994), plus the cysteine proteinase genes of *T. vaginalis* were isolated using PCR, in which the degenerate primers for amplification were based on the relevant genes which had already been characterised from a variety of sources (Quon *et al.*, 1994) (Mallinson et al, 1994). Seventeen ubiquitin genes from *T. vaginalis*, including an eleven repeat fragment were isolated using a degenerate primer/ PCR approach (Keeling and Doolittle, 1995).

The degenerate oligonucleotide/PCR technique has been the approach most widely used for the isolation and subsequent molecular characterisation of several *T. vaginalis* genes,

in the past year. Bui and Johnson, 1996 used the technique to isolate two genes encoding [Fe] hydrogenases from *T. vaginalis*. Five superoxide dismutase genes were also isolated from *T. vaginalis* using the same approach (Viscogliosi *et al.*, 1996). Interestingly, the first gene encoding a sulphur amino acid metabolising enzyme *from T. vaginalis* has been isolated and characterised at the molecular level. Bagnara *et al.*, 1996 have cloned the gene encoding S-adenosyl homocysteine hydrolase (sahh), an enzyme involved in the methionine cycle of sulphur amino acid metabolism in *T. vaginalis*, the authors also used a degenerate oligo/PCR approach.

Genes have been isolated from *T. vaginalis* that have helped address the question of hydrogenosome evolution. The genes were all isolated by a degenerate oligo/PCR approach, they were HSP70, HSP60 and HSP10 (Germot *et al.*, 1996 and Bui *et al*,. 1996) and chaperonin 60 (cpn60) (Horner *et al.*, 1996 and Roger *et al.*, 1996). The gene for the largest subunit of RNA polymerase II in *T. vaginalis* has been isolated (Quon *et al.*, 1996) as have genes for histone H3 and H4 (Marinets *et al.*, 1996), the approach used for the isolation of these genes was the same as outlined above. Finally a gene for N- acetylneuraminate lyase was isolated from *T. vaginalis* using a polyclonal antisera that had been raised against *T. vaginalis* extracellular proteases (Meysick *et al.*, 1996)

A survey of the *T. vaginalis* genes isolated and characterised to date indicates that they appear not to contain introns, and they have unusually short 5` and 3` untranslated regions (Quon *et al.*, 1994). The hydrogenosomal genes characterised from *T. vaginalis* have been found to encode, a 8-9 amino acid sequence, which is absent from the purified proteins. The short leader sequence is thought to play a role in targeting the protein of the hydrogenosome, as these amino terminal sequences are characterised by properties typical of mitochondrial presequences.

### 1.4.3 Transcriptional control of *T. vaginalis* genes.

An attempt has been made to identify regulatory elements that play a role in transcription initiation of *T. vaginalis*. Characterisation of seven protein coding genes from this protist invariably revealed the presence of a highly conserved DNA sequence motif immediately upstream of the coding region (Quon *et al.*, 1994). This 13 nucleotide motif was shown to surround and contain precise sites for transcription initiation. No typical TATA boxes, positioned at 25-30nt upsteam of the transcription start sites of these genes, were found (Quon *et al.*, 1994).

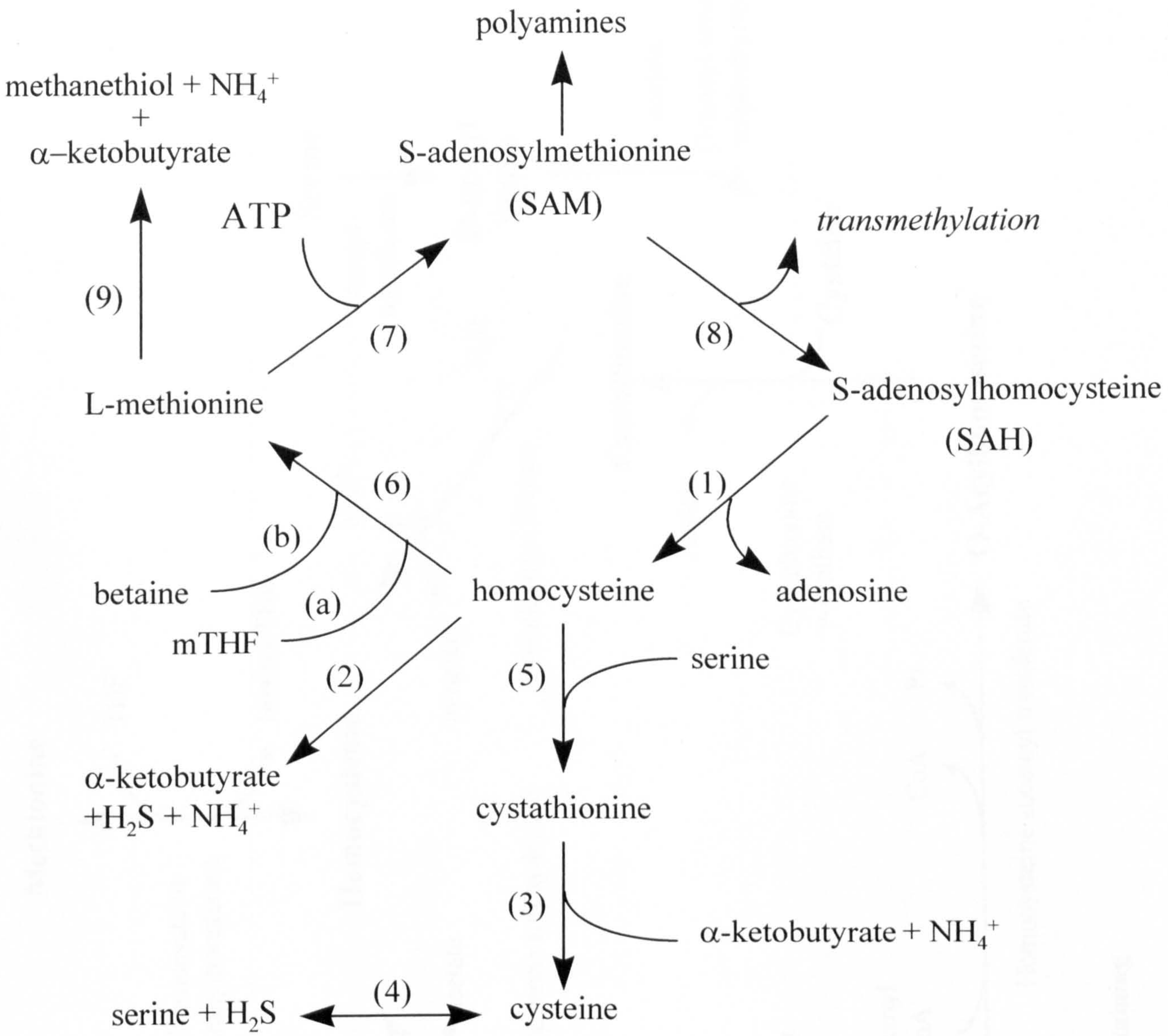
This start site regions from a seven *T. vaginalis* genes impart strong specific initiation of transcription in a mammal *in vitro* transcription assay. The consensus promoter element found in *T. vaginalis* is structurally and functionally similar to elements found in promoters of higher eukaryotes (Smale and Baltimore, 1989).

Recently, transcription in *T. vaginalis* was examined by analysing the effect of the fungus derived toxin α-amanitin on the transcription of protein coding genes in this ancient eukaryote. It was found that the RNA polymerase that transcribes the protein-coding genes was relatively resistant to α-amanitin and molecular characterisation of the gene encoding the large subunit of the RNA polymerase II shows that it lacks many of the conserved residues present in the putative α-amanitin binding site of other RNA polymerase II genes, including the typical heptapeptide repeat carboxy-terminal domain (CTD) that is a hallmark of higher eukaryotic RNA polymerase IIs (Quon et al., 1996).

## 1.5 Aims of the project

There were three main project aims at the onset of this study:

1. To isolate and characterise the gene encoding methionine γ-lyase from *T. vaginalis.*
2. To clone the methionine γ-lyase gene of *T. vaginalis* into a protein expression vector, so that recombinant protein could be produced and be biochemically characterised.
3. To gain more insight into the function of methionine γ-lyase and other sulphur amino acid metabolising enzymes of *T. vaginalis*.




Enzymes: (1) S-adenosylhomocysteine hydrolase; (2) homocysteine desulphurase; (3) cystathionine γ-lyase; (4) serine sulphydrase; (5) cystathionine β-synthetase; (6a) methyltetrahydrofolate: homocysteine methyltransferase; (6b) betaine: homocysteine methyltransferase; (7) S-adenosylmethionine synthetase; (8) S-adenosylmethionine-dependent methyltransferases; (9) methionine γ–lyase

**Figure 1.1 Metabolism of sulphur-containing amino acids in *T. vaginalis***

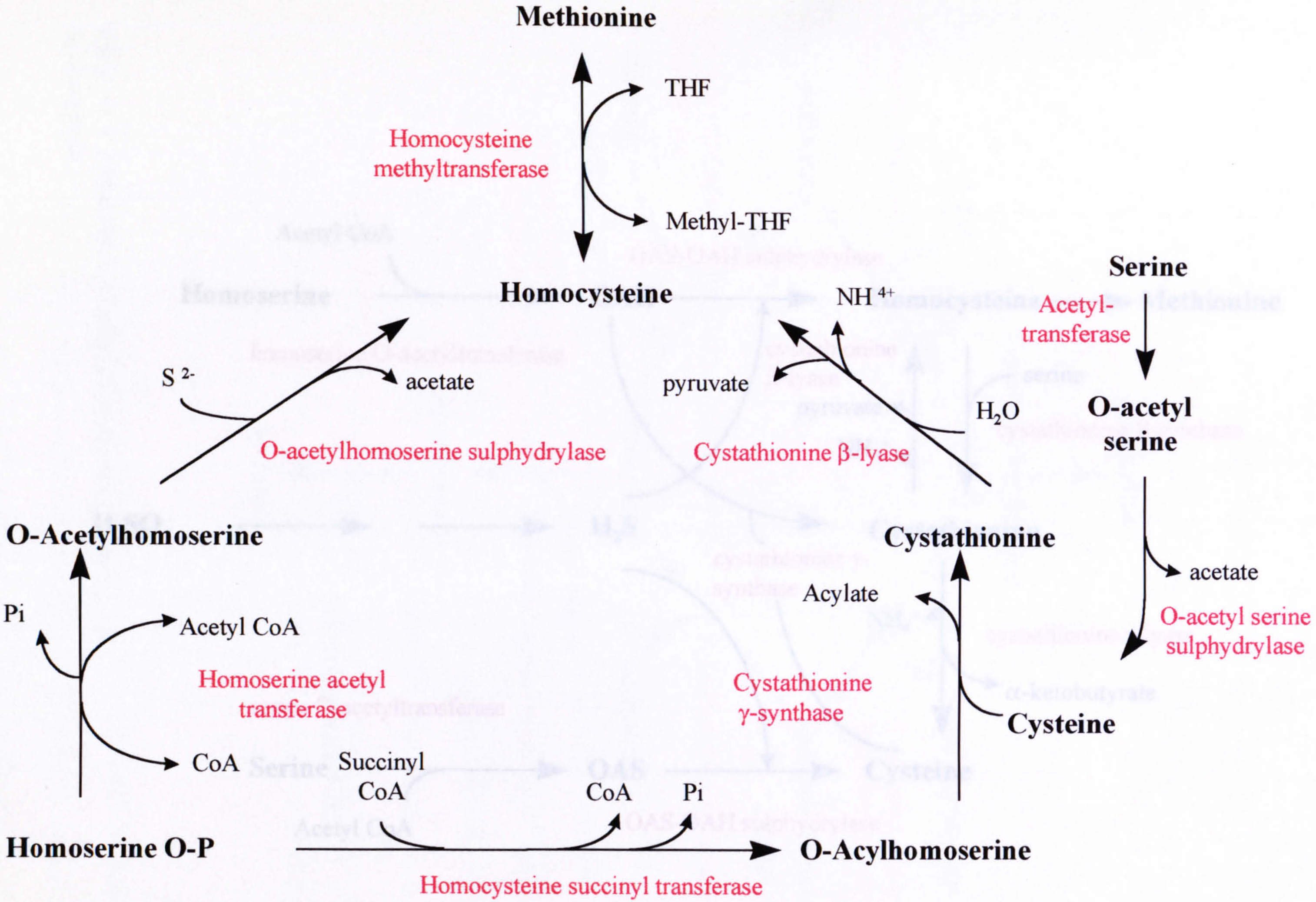



**Figure 1.2 Metabolism of sulphur-containing amino acids in plants and bacteria**

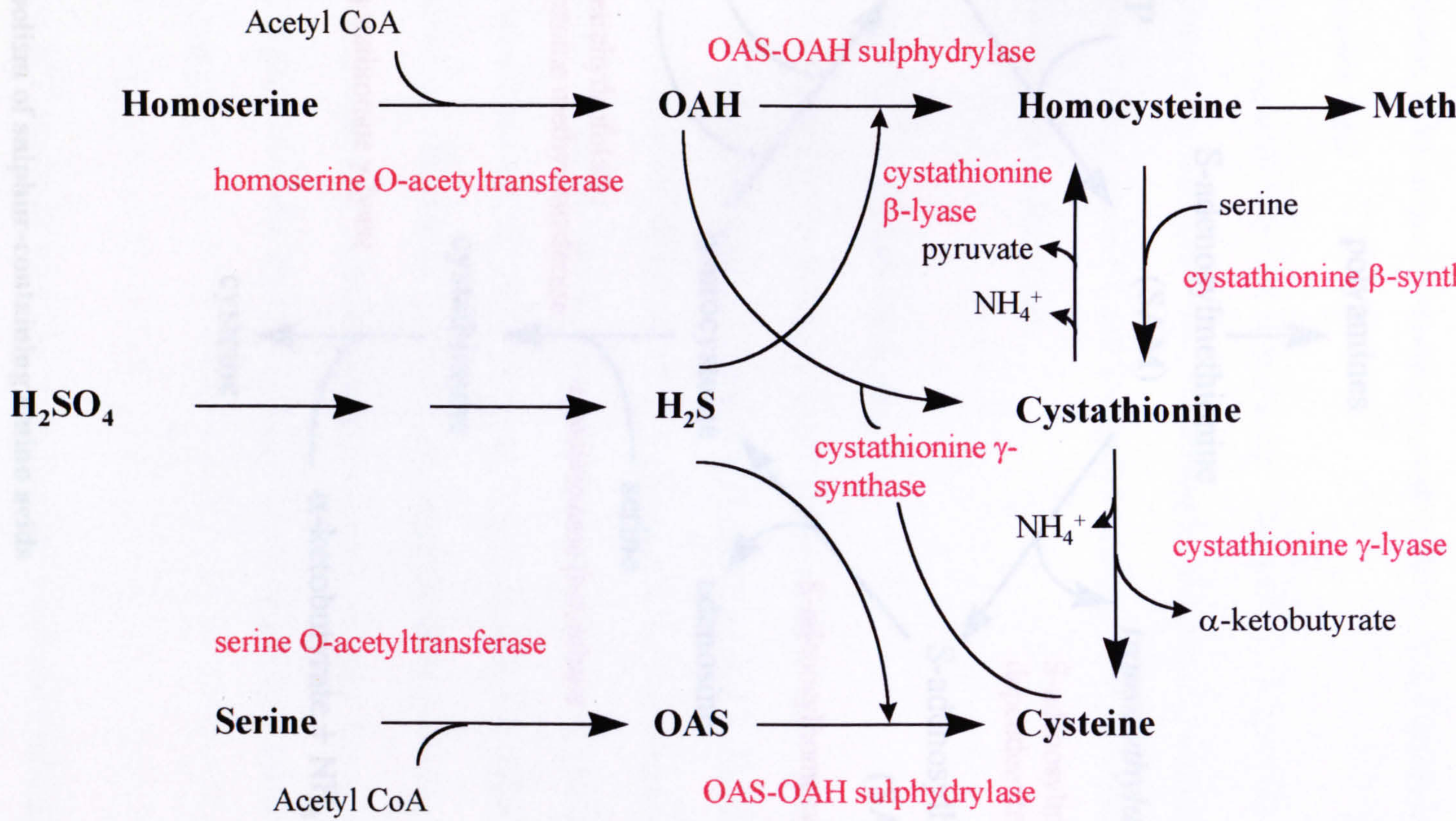



**Figure 1.3 Metabolism of sulphur amino acids in yeast and fungi**

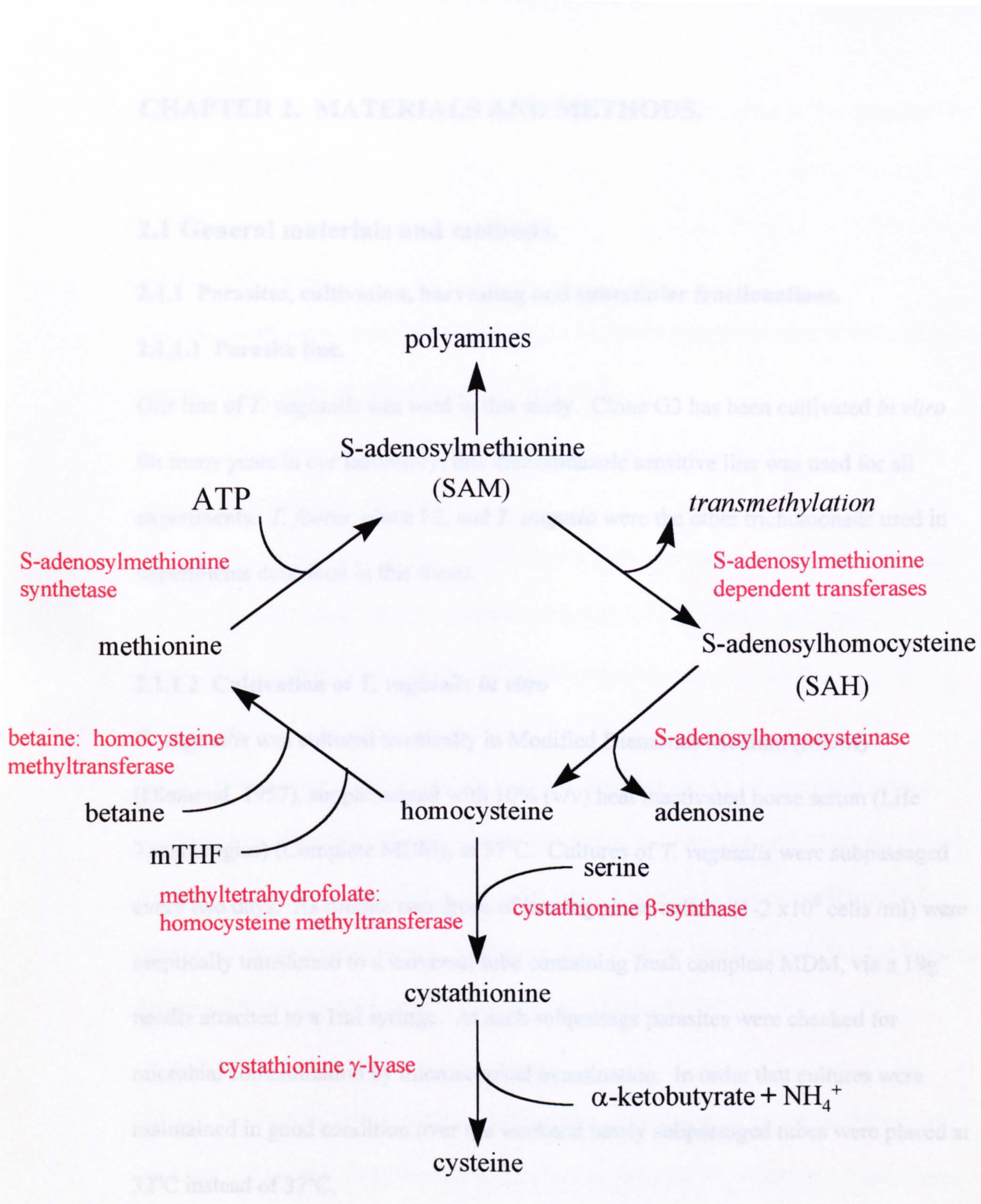



**Figure 1.4 Metabolism of sulphur-containing amino acids in mammals**

# CHAPTER 2. MATERIALS AND METHODS.

## 2.1 General materials and methods.

### 2.1.1 Parasites, cultivation, harvesting and subcellular fractionations.

#### 2.1.1.1 Parasite line.

One line of *T. vaginalis* was used in this study. Clone G3 has been cultivated *in vitro* for many years in our laboratory, this metronidazole sensitive line was used for all experiments. *T. foetus*, clone F2, and *T. augusta* were the other trichomonads used in experiments described in this thesis.

#### 2.1.1.2 Cultivation of *T. vaginalis in vitro*

*T. vaginalis* was cultured axenically in Modified Diamonds Medium (MDM) (Diamond, 1957), supplemented with 10% (v/v) heat inactivated horse serum (Life Technologies) (Complete MDM), at 37°C. Cultures of *T. vaginalis* were subpassaged every two days. As routine two drops of late log phase culture (1-2 x$10^6$ cells /ml) were aseptically transferred to a universal tube containing fresh complete MDM, via a 19g needle attached to a 1ml syringe. At each subpassage parasites were checked for microbial contamination by microscopical examination. In order that cultures were maintained in good condition over the weekend newly subpassaged tubes were placed at 32°C instead of 37°C.

Bulk cultures of *T. vaginalis* would be set up from the most recent tube of subpassaged cells. Parasites would be set up at an initial density of 1 x $10^4$cells/ml and grown for ~40-42 hours at 37°C and harvested at late log phase. Alternatively the parasites would

be initiated at 1 x $10^5$cells/ml and grown for 24 hours at 37°C, prior to harvesting at late log phase of growth.

### 2.1.1.3 Cell counts.

All cell counts were made using an improved Neubauer hemocytometer at x40 objective magnification under phase contrast ( x400 overall ).

### 2.1.1.4 Harvesting *T. vaginalis.*

Cells at late log phase of growth (1-2 x$10^6$ cells /ml) were harvested by centifugation at 2300 x g for 15 minutes at 4°C, washed twice with 0.25 M sucrose and used immediately or stored as pellets at -70°C until required.

### 2.1.1.5 Production of crude lysates, cell free homogenates and soluble fractions.

Crude lysates of *T. vaginalis* in 0.25M sucrose were produced using 0.25% (v/v) Triton X-100. Lysates were centrifuged at 13,000 x g in a bench top microcentrifuge for 5 minutes to pellet cellular debris. The supernatant fraction was the crude soluble fraction that was frequently used in enzyme assays and Western blots.

Soluble fractions for native enzyme purifications were produced by resuspending ~$10^9$ parasites in 5 ml of 0.25 M sucrose and disrupting them by 30 strokes of a Potter tissue homogeniser fitted with a serrated Teflon plunger type A (A. H. Thomas Co., Philadelphia, USA) operating at 2500 rpm. This caused disruption of more than 95% of the parasites but functioned to keep organelles such as hydrogenosomes and lysosomes intact. The parasite homogenate was centrifuged at 100 000 x g for 1 hour at 4°C in order to obtain soluble and particulate fractions, the former was used as the starting

material for native methionine γ-lyase purifications. The proteinase inhibitors E64 (10 μM), 1,10 phenanthroline (2 mM), pepstatin A (4 μM) and PMSF (1 mM) were added to all types of *T. vaginalis* lysates and homogenates produced, at the final concentrations indicated.

#### 2.1.1.6 Cryopreservation of *T. vaginalis.*

*T. vaginalis* was cryopreserved using a procedure which involved adding 10% (v/v) sterile dimethylsulphoxide (DMSO) in complete MDM to late log cultures (ie. 500 μl 10% (v/v) DMSO in MDM + 500 μl late log. *T. vaginalis*) 1 ml samples were transferred to screw capped cryopreservation tubes (Nunc) and the lids tightened. The tubes were wrapped in cottonwool and transferred to a polystyrene box and placed at -70°C for 18-24 hours. The tubes were immersed in liquid nitrogen for long term storage. *T. vaginalis* was removed from long term storage by thawing the contents of a single tube as quickly as possible using warm water and transferring the contents (1 ml) aseptically to 25 ml of MDM in a sterile universal tube.

## 2.2 Molecular Materials and Methods.

### 2.2.1 Bacterial maintenance, cultivation and harvesting.

The strains of *Escherichia coli* used in this study were: XL1-Blue (Stratagene), M15pREP4 (Qiagen) and TOP 10 (Ingenius). The plasmids used in this study were pBluescript (SK-) (Ampicillin$^R$) (Stratagene). pQE30 and pQE60 (Ampicillin$^R$) (Qiagen) and pTAg (Ampicillin$^R$, Kanamycin$^R$) (Ingenius).

### 2.2.1.1 Long-term maintenance of bacterial strains.

Bacterial strains containing plasmids of interest were maintained as a glycerol stabilate at -70°C. An overnight culture of bacteria was diluted 1:1 with 40% glycerol and peptone and stored in screw cap vials at -70°C.

### 2.2.1.2 Short-term maintenance of bacterial strains.

Bacteria were made available for inoculation into liquid cultures by streaking out from a glycerol stabilate onto solid L-agar media containing the appropriate antibiotic for plasmid selection. Discrete colonies were produced by the streaking out procedure. Bacteria maintained on solid L-agar plates remained viable for a period of 4-6 weeks when kept sealed at 4°C.

### 2.2.1.3 Bacterial cultivation.

Bacterial cultures to be used for small scale preparations of plasmid DNA were cultivated as follows. Between 3 and 10ml of LB liquid media plus appropriate antibiotic (ampicillin was used at a final concentration of 100 μg/ml, kanamycin was used at a final concentration of 25 μg/ml and tetracycline was used at a final concentration of 12.5 μg/ml), were transferred aseptically to sterile glass tubes with caps. The liquid media were inoculated with a single bacterial colony taken from an agar plate via a long wooden sterile stick. Inoculated cultures were incubated in a rotary shaker operating at 200rpm at 37°C overnight.

Larger cultures for plasmid preparation or recombinant protein expression work were prepared in the same manner as outlined above except 50mls of LB broth plus

appropriate antibiotic were placed in a 250 ml sterile conical flask plugged with cottonwool and inoculated with a single bacterial colony taken from an L-agar plate.

### 2.2.1.4 Bacterial harvesting.

Bacterial cultures for small scale preparations of plasmid DNA were transferred to 1.5ml Eppendorfs and centrifuged at 13 000 x g in a microfuge for 3 minutes to pellet cells. Large volumes of bacterial cultures were decanted into sterile centrifuge pots and centrifuged at 6000 x g for 10 minutes in a Beckman J2 centrifuge using a JA14 rotor.

## 2.2.2 Isolation of plasmid DNA from *E. coli.*

### 2.2.2.1 Small scale plasmid preparations-'mini-preps'.

Plasmid DNA was routinely isolated from 3 ml of the appropriate overnight bacterial culture using Wizard mini-preps (Promega), according to manufacturer's instructions. Briefly, the bacterial cells were resuspended, lysed and neutralised. The bacterial chromosomal DNA was centrifuged out and the cleared supernatant containing the plasmid DNA was loaded on to a column containing a DNA binding resin, the column was washed prior to elution of the DNA in sterile water or TE buffer pH 8.0.

### 2.2.2.2 Medium scale plasmid preparations.

'Midi-preps' of plasmid DNA were prepared using Qiagen tip 100 according to manufacturer's instructions. The procedure was essentially the same as for Wizard 'mini-preps' as outlined above. Yield was approximately 100 μg DNA. The principle of Qiagen Tip 100 is basically the same as outlined above for the Wizard mini-preps.

### 2.2.3 Polymerase Chain Reaction (PCRs) Amplification of cystathionine γ-lyase homologues.

Degenerate primers were used in PCR reactions with *T. vaginalis* first strand cDNA. The sequences of the primers used in the degenerate PCRs and for gene sequencing are shown in Table 2.1. The 50 µl reaction contained 5 ng *T. vaginalis* cDNA, each of the primers at a final concentration of 5 ng, nucleotides at a final concentration of 250 µM and 5U Taq polymerase (Promega). The following amplification protocol was employed: An initial denaturation step of 4 minutes at 95°C was followed by 30 cycles at 94°C for 1 minute, 42°C for1 minute and 72°C for1 minute. A final step of 5 minutes at 72°C was used to complete the extension.

PCRs for experiments other than degenerate PCR, for example the engineering of restriction sites onto the ends of DNA fragments for cloning experiments, contained the same PCR constituents as outlined above, with template and primers at approximately the same concentration. If amplified DNA was required in which base changes introduced by *Taq* polymerase were minimised, then *Pfu* polymerase (Stratagene), which has proof reading activity was used (for example, when *mgl1* was cloned into pQE60). The amplification protocol was basically the same but with higher annealing temperatures for oligonucleotides of known sequence. Less amplification cycles were used if the DNA template had been previously amplified.

### 2.2.4 5' RT PCR-RACE.

The 5' ends of the mRNA transcribed from *mgl1* and *mgl2* were determined using a 5' RACE system kit (Life Technologies). For RACE-PCR (Loh *et al.*, 1989) total cellular

RNA from *T. vaginalis* (1 μg) was reverse transcribed into single stranded cDNA (ssDNA) using the components of the 5' RACE system kit, with 20ng of oligo dT as primer for the cDNA synthesis. Excess dNTPs and primer were removed from the ssDNA using a Glassmax cartridge and associated reagents (Life Technologies) and a homopolymeric tail of dCs were added to the 3' end of the sscDNA. 5μl of tailed cDNA was added to 50 μl PCR reactions containing the components of the 5' RACE System Kit, 2U Taq (Promega), anchor primer (which included a sequence complementary to the poly dC tail) and a gene specific primer. See Table 2.1 for details of the gene specific primers used in the 5'RACE-PCR experiments.

### 2.2.5 *T. vaginalis* λZAPII cDNA library screening.

Screening of the *T. vaginalis* λZAPII cDNA library (Mallinson *et al.*, 1994) with oligonucleotide probes or polyclonal antisera was carried out in accordance to the Stratagene protocol, the suppliers of the cDNA library components. Information on screening procedures and the subsequent lambda purification procedures were from Sambrook *et al.*, (1989). Briefly, 100, 000 λZAP phage particles and XL1-Blue in BBL top agarose were plated on to BBL agar in 18 cm square petri dishes. The plates were incubated at 37 °C until the lambda plaques just touched one another. The DNA from the lambda clones was transferred to Hybond N (Amersham) and the lambda DNA denatured, neutralised and washed using the same procedure as for Southern hybridisations. Hybridisation of radioactive gene specific probes was carried out as for Southern hybridisations.

Once pure lamba clones containing the DNA insert of interest were obtained, the inserts were rescued directly into the pBluescript phagemid using helper phage R408 (Stratagene) (Short *et al.*, 1987 and Stratagene protocol).

### 2.2.6 Isolation of genomic DNA from *T. vaginalis.*

Genomic DNA from *T. vaginalis* was isolated using the Nucleon II protocol (Scotlab) according to manufacturer's instructions. Briefly, the parasites were resuspended and then lysed using an SDS based solution, the RNA was degraded using RNase and the lysed cells deproteinised using sodium perchlorate. The genomic DNA was extracted using chloroform and a silica based resin and finally precipitated using ethanol. Approximately 0.5mg of genomic DNA was obtained from $3 \times 10^8$ *T. vaginalis*.

### 2.2.7 General manipulation of nucleic acids.

#### 2.2.7.1 Concentration of nucleic acids.

DNA and RNA was routinely precipitated by the addition of x 2.5 volumes of 100% ethanol and $1/10^{th}$ volume 3M NaAc pH 5.2, when the volume of the nucleic acid solution to be precipitated was taken into consideration. The precipitation process was aided by a minimum incubation of 15 minutes at -70°C. The nucleic acid precipitant was then centrifuged at 13 000 x g for 15 min at 4°C in a microfuge. The supernatant was discarded, the precipitated nucleic acid washed with 70% ethanol and resuspended in a suitable volume of TE buffer pH 8 or sterile distilled water.

#### 2.2.7.2 Restriction endonuclease digestion of plasmid DNA.

Plasmid DNA was routinely digested using an excess of restriction endonuclease. For digestion of 1 μg of plasmid DNA in a final volume of 10μl, 10U of restriction enzyme

was used. The restriction cocktail consisted of: ~1 µg plasmid DNA, 1µl 10 x React buffer (Life Technologies), 1 µl restriction enzyme, 10 U (Life Technologies) and sterile distilled water made up to a final volume of 10 µl. The restriction was incubated for 1-2 hours at 37°C and the reaction stopped by adding 1/5th volume agarose gel loading buffer (40% sucrose (w/v), 0.5% (w/v) SDS, 0.1M EDTA pH8, 0.05% bromophenol blue (w/v)). If higher concentrations of plasmid DNA were required to be restricted, the restriction cocktail was simply scaled up.

### 2.2.8 *T. vaginalis* genomic DNA restriction digests.

*T. vaginalis* genomic DNA restriction cocktails routinely consisted of the following: 2 µg *T. vaginalis* genomic DNA, 2 µl 10x React buffer (Life Technologies), 2 µl restriction enzyme, 20 U (Life Technologies), and made up to a final volume of 20 µl with sterile distilled water. Genomic DNA restrictions were incubated at 37°C for 5 hours. Restrictions were terminated using 1/5th restriction volume agarose gel loading buffer.

### 2.2.9 Calf Intestinal Alkaline Phosphatase treatment (CIP treatment).

For plasmids digested with only one restriction enzyme, religation was prevented by the removal of the 5'terminal phosphates through CIP treatment. 5U of CIP(Boehringer) was added directly to restriction enzyme cocktail mixes and incubated for 15 minutes at 37°C. Phosphatase treatment was completed by the addition of a further 5U of CIP and incubation for 15 minutes at 37°C, prior to the addition of gel loading buffer.

### 2.2.10 Ligations.

Ligation of plasmid DNA and DNA inserts (50ng of each) was performed using the Amersham ligation kit according to the manufacturer's instructions, The kit contained a ligase buffer and ligase. Ligations were for 30 minutes at 16°C.

### 2.2.11 Bacterial transformations with plasmid DNA.

Supercompetent and competent XL1-Blue were purchased from Stratagene for important cloning experiments. Competent strains of bacteria used for routine cloning experiments were also made in the laboratory following the calcium chloride method as outlined in Sambrook *et al.*, (1989). The transformation protocol used for all experiments, regardless of whether the cells had been bought from Stratagene or made in the laboratory, was as exactly as outlined in the Stratagene Supercompetent and Competent cell protocol. Briefly, ligated plasmid and insert were added to the competent cells in a Falcon 15 ml polypropylene tube and left on ice for 30 minutes. The cooled cells were heat shocked for 42°C, left on ice for a further 2 minutes and 500 µl SOC medium added to the cells which were the incubated at 37°C for 1 hour with shaking at 200 rpm. 200 µl of the transformed cells were aseptically spread on to plate containing the appropriate antibiotic for plasmid selection and in the case of XL1-Blue, also IPTG (6.5µl 0.5M stock per plate) and X-gal (40µl 20mg/ml stock per plate).

### 2.2.12 Agarose Gel electrophoresis.

Two types of buffer system were used for agarose gel electrophoresis:

1. TBE agarose gel electrophoresis was used for the routine visualisation of DNA after restriction endonuclease digestion.
2. TAE agarose gel electrophoresis was used when DNA was subsequently excised from the agarose gel and used for subsequent DNA manipulations such as ligations.

### 2.2.12.1 Mini gels

Two sizes of agarose gel were used for DNA electrophoresis: For mini gels, 50ml of the desired percentage agarose gel would be prepared by dispensing the appropriate amount of agarose powder (BRL-Ultrapure (Life Technologies) for routine work, Seakem GTG (FMC Bioproducts) when gel purification was required) and buffer into a 250ml conical flask. The agarose would be melted by microwaving at medium power for approximately 3 minutes. The boiled agarose solution was cooled to hand hot temperature and ethidium bromide (EtBr) solution added to a final concentration of 0.5 μg/ml. The cooled, molten agarose was poured into the gel tray with the gel formers and comb in position of a mini gel kit (Life Technologies). Approximately 35mls of molten agarose solution was sufficient to fill the gel tray. Gels were run at constant 80V until the bromophenol blue dye front had migrated two thirds of the way down the gel.

### 2.2.12.2 Midi gels.

For midi gels, 150mls of agarose gel solution were used with a midi gel kit (Life Technologies), from which ethidium bromide would be omitted. Gels were run at a constant 26 V overnight or at constant 120 V for 4 to 5 hours.

### 2.2.12.3 DNA size markers.

DNA size standards would be run alongside DNA samples under investigation during agarose gel electrophoresis. 1 kb ladder and λ*Hin*dIII markers (λ DNA digested with *Hin*dIII (Life Technologies) were routinely used.

## 2.2.13 Visualisation of DNA and photography.

Agarose mini gels in which EtBr had been incorporated could be examined immediately over a source of UV light. However, midi gels and mini gels containing DNA fragments less than 300 base pairs in size were stained for 30 minutes in a 0.5µg/ml EtBr solution in electrophoresis buffer or water and destained under running tap water prior to exposure to UV light. Short wave UV light (254nm) was used to visualise DNA in agarose gels and the results recorded using a Polaroid camera with a Wratten 2A and gelatin filter, plus 667 film or by using an Appligene Imager (Appligene Oncor) in which the captured image could be stored to disk or reproduced on paper. Long wave UV light (365nm) was used to visualise gels from which DNA was to be extracted and used for subsequent DNA manipulations. This was to minimise UV light induced damage of the DNA.

### 2.2.14 Extraction of DNA from agarose gels.

DNA to be used in further DNA manipulations (eg. ligations, random priming) was extracted from the gel using a clean scapel blade and the DNA removed from the gel plug using one of two procedures.

1. If the DNA to be extracted from the gel plug was of a fairly high concentration ie. greater than1 µg, Spin X columns (Costar) were used according to the manufacturer's instructions. Spin X columns contain a membrane which allows the passage of DNA whilst the components of the agarose gel are retained

2. If the DNA to be extracted from the gel plug was less than 1µg, the Qiaex gel extraction kit (Qiagen) was used according to the manufacturer's instructions. Briefly, the agarose gel containing the DNA to be extracted is solubilised, the solubilised material is applied to a column that has DNA binding capacity and after column washing the DNA is eluted in sterile water or TE buffer pH 8.0.

### 2.2.15 Manual DNA sequencing.

Manual DNA sequencing was carried out using the Sequenase Quick Denature Plasmid Sequencing Kit (Amersham). This DNA chain terminating kit utilises Sequenase™ Version 2.0 DNA polymerase and $^{35}S$ methionine (NEN Dupont0. DNA fragments were resolved on 6% acrylamide/urea gels using the buffer system appropriate to the conditions in which the DNA was originally denatured. A sequencing gel kit (Life Technologies) or Base Ace gel kit (Stratagene) was used to run wedged spaced sequencing gels. Short run gels were run at a constant 60W for ~2 hours or until the bromophenol blue dye front was at the bottom of the gel. This allowed the first 200 base pairs of sequence from the primer to be read. Long run gels were run at a constant 60W for ~5 hours or until the xylene cyanol dye was at the bottom of the gel. This allowed 200-500bp of sequence from the primer to be read. After electrophoresis the gels were fixed in 10% (v/v) methanol, 10% (v/v) acetic acid for 30 minutes prior to transfer to Whatman 3MM chromatography paper which was overlaid with Saran wrap and dried for 1.5 hours. Dried gels with the Saran wrap removed, were exposed to Fuji RX film at room temperature, overnight to 36 hours.

### 2.2.16 DNA sequence analysis.

The University of Wisconsin Genetic Computer Group (GCG) Version 7.0 and associated programs were used for all nucleic and amino acid analyses.

### 2.2.17 Primers for DNA sequencing and PCRs

For subclones made in pBluescript, T3, T7 and -20 reverse primers (Stratagene) were used, for pTAg, the M13 reverse primer was used (Applied Biosystems). Gene specific primers for sequencing and PCRs were designed by taking into consideration the

nucleotide sequence already obtained. Gene specific primers/oligonucleotides were commercially synthesised by the Molecular Biology Laboratory, University of Strathclyde, Glasgow. The sequence of gene specific primers and degenerate primers used in this study are shown in Table 2.1.

### 2.2.18 Radioactive probes.

Radioactive DNA probes (ie. 20 ng of restriction fragments from $p4_{100}$ and $p5_{100}$) used in Southern and Northern blot hybridisations were synthesised using the Prime It kit (Stratagene) according to the manufacturer's protocol. The kit uses random hexamers and Exo (-) Klenow DNA polymerase to incorporate a radioactive nucleotide (dCTP, 3000mCi/mmol, NEN Dupont) along with other non radioactive nucleotides into a newly synthesised strand of DNA. The radioactive probes were purified using NucTrap® probe purification columns and the push column beta shield device (Stratagene) prior to use in hybridisation experiments. Before addition to hybridisation experiments, the radioactive probes were boiled for 10 minutes to make them single stranded.

### 2.2.19 Southern blotting and hybridisations.

Prior to transfer to Hybond N membrane (Amersham) agarose gels containing *T. vaginalis* genomic DNA were depurinated by immersing the gel in 0.25 M HCl for 30 minutes with shaking at room temperature, this was followed by denaturation of the genomic DNA in denaturation solution for 30 minutes with shaking , followed by a final neutralisation step for 30 minutes in neutralisation solution, prior to the Southern blot being set up. Restricted *T. vaginalis* genomic DNA was transferred to Hybond N membrane (Amersham) as described by Sambrook *et al.*, (1989). High stringency

hybridisations of immobilised *T. vaginalis* genomic DNA with radioactive fragments of the DNA of interest were carried out at 65°C. Hybond N membranes were prehybridised in glass tubes with screw capped lids (Hybaid) with 15mls of hybridisation solution (6x SSC, 10x Denhardt's, 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate and 100µg/ml herring sperm denatured and sheared DNA) for 1.5 to 2 hours at 65°C. The prehybridisation solution was then discarded and 10mls of fresh hybridisation solution was added to the tubes and the single stranded radioactive fragment of DNA added directly. The filters were hybridised for approximately 18 hours (overnight) at 65°C with rotation of the glass tubes. After hybridisation the radioactive probe was decanted off and the filter removed for washing. The filters were washed in a total of two litres of 0.1x SSC and 0.1% SDS (wash solution) for 1 hour at 65°C with shaking, one litre of wash solution was replaced after 30 minutes with another litre of pre-warmed wash solution.

After washing the filters were sealed in a polythene bag and exposed to autoradiographic film (Fuji RX) with intensifying screen at -70°C for 18 to 36 hours. Exposed autoradiographic films were automatically developed using a Kodak Xomat film processor.

### 2.2.20 RNA isolation from *T. vaginalis.*

Total RNA was isolated from *T. vaginalis* using the commercially available reagent TRIzol™ (Gibco), which is a mono-phasic solution of phenol and guanidine isothiocyanate, according to manufacturers instructions. Poly $[A]^+$ RNA was isolated from the total RNA of *T. vaginalis* using Poly $[A]^+$Quik columns (Stratagene),

according to manufacturers protocol. The concentration of the Poly $[A]^+$ RNA was spectrophotometrically determined at A 280/260 nm.

### 2.2.21 First strand cDNA synthesis from *T. vaginalis* poly $[A]^+$ RNA.

1µg of poly $[A]^+$ RNA isolated from *T. vaginalis* was primed with oligo dT (22ng) and made up to a final volume of 22.5 µl with diethylpyrocarbonate (DEPC) treated water. The tube was heated at 65°C for 10 minutes to make the RNA single stranded. The tube was chilled on ice and spun for 5 seconds in a microfuge to collect the contents at the bottom of the tube. Reverse transcriptase buffer was added to a final 1x concentration, also DTT (6 mM), dNTPs (0.3 mM) and RNase-in were added The tube was incubated at 42°C for 2 minutes. 1 µl (10 U) Superscript reverse transcriptase (Moloney, Murine Leukaemia Virus (MMLV), Life Technologies) was added and the tube incubated for a further 30 minutes at 42°C to allow cDNA synthesis to occur. After first strand cDNA synthesis had taken place 480 µl of TE pH 8.0 was added and the cDNA heated to 65°C for 10 minutes to denature the reverse transcriptase.

### 2.2.22 RNA gels.

1.4% formaldehyde agarose gels were used to analyse *T. vaginalis* total and poly $[A]^+$ RNA. A MOPS/ formaldehyde buffer system was used to run the RNA samples through the gel after they had been combined with formaldehyde and formamide according to Sambrook *et al.*, (1989). RNA markers (9.5 kb-0.24 kb, Life Technologies) were run on the gel so as to provide an estimation of the size of the *T. vaginalis* RNA.

### 2.2.23 Northern blots and hybridisations.

*T. vaginalis* total RNA was transferred directly to Hybond N membrane (Amersham), using 20x SSC as the transferrant as outlined in Sambrook *et al.*, (1989). RNA transfer was allowed to proceed overnight. Transfer of RNA was checked by staining the filter in 5% methylene blue for 20 minutes at room temperature with shaking and then destaining with 20% ethanol until the background of the filter became almost white once more, destaining was completed by washing with 2% SDS. If ribosomal RNA subunits were visible upon destaining, as well as the RNA size markers, then this was an indication that successful transfer of RNA had taken place.

### 2.2.24 Commonly used buffers, reagents and media in molecular biology.

Buffers, autoclavable solutions and media were sterilised using the following conditions: 120°C, 15lbs $in^{-2}$ for 15 minutes.

#### 2.2.24.1 Commonly used buffers.

**SM Buffer:** 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 8 mM $MgSO_4$, 0.01% (v/v) gelatin in distilled water. Autoclaved and stored at room temperature.

**STE buffer:** 100 mM NaCl, 20 mM Tris-HCl pH 7.5, 10 mM EDTA. Stored at room temperature.

**TAE (50x):** 40 mM Tris-acetate, 1 mM EDTA in distilled water. Stored at room temperature.

**TBE (10x):** 0.9 M Tris-HCl, 0.9 M Boric acid, 25 mM EDTA in distilled water. Stored at room temperature.

**TE buffer:** 10 mM Tris pH 7.6 or pH 8.0,1 mM EDTA in distilled water. Autoclaved and stored at room temperature.

### 2.2.24.2 Commonly used reagents.

**Agarose gel loading buffer:** 40% (w/v) sucrose, 0.1 M EDTA, 0.5% (w/v) SDS, 0.5% bromophenol blue. Stored at room temperature.

**Ampicillin:** 100mg/ml in distilled water, filter sterilised through 0.22 μM membrane (Millipore). Stored at -20°C.

**Denaturation buffer:** 1.5 M NaCl, 0.5 M NaOH in distilled water. Stored at room temperature.

**Denhardt's solution (50x):** 1% (w/v) BSA, 1% (w/v) ficoll, 1% (w/v) polyvinyl pyrolidine in sterile distilled water.

**Ethidium Bromide:** 10 mg/ml stock in distilled water. Final working concentration 0.5 μg/ml. Stored at room temperature.

**IPTG:** 0.5 M stock in sterile distilled water. Stored at -20°C.

**Herring sperm DNA:** 10 mg/ml stock in sterile distilled water, sheared with a hypodermic needle and syringe (19 gauge), boiled and snap frozen at -20°C.

**Kanamycin:** 25 mg/ml stock in distilled water, filter sterilised through 0.22 μM membrane (Millipore). Stored at -20°C.

**Magnesium sulphate:** 1 M and 10 mM Magnesium sulphate in distilled water. Autoclaved and stored at room temperature.

**Neutralisation buffer:** 1.5 M NaCl, 0.5 M Tris-HCl pH 8.0 in distilled water. Stored at room temperature.

**SDS:** 10% (w/v) stock solution in distilled water. Stored at room temperature.

**SSC (20x):** 3 M NaCl, 0.3M tri-sodium citrate pH 7.0 (using NaOH) in distilled water. Stored at room temperature.

**Tetracycline:** 12.5 mg/ml in 100% ethanol. Stored at -20°C.

**2.2.24.3 Commonly used media.**

**BBL agar:** 1% (w/v) trypticase peptone, 0.5% (w/v) NaCl, pH 7.2, 1% (w/v) agar. Autoclaved and stored at room temperature.

**BBL agarose:** 1% (w/v) trypticase peptone, 0.5% (w/v) NaCl, pH 7.2, 0.25% $MgSO_4$ $7H_20$, 0.65% (w/v) agarose (Sigma Type I).

**Luria-Bertani agar (LB agar):** As LB broth with 0.8% (w/v) agar (Difco).

Autoclaved and stored at room temperature.

**Luria-Bertani broth (LB broth):** 1% (w/v) bactotryptone (Difco), 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl in distilled water. The broth was sterilised by autoclaving and stored at room temperature.

**SOB medium:** 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl.

Autoclaved and stored at room temperature. When required add $MgSO_4$ and $MgCl_2$ to a final concentration of 10 mM and filter sterilised prior to use.

**SOC medium:** As SOB medium, plus 0.4% (w/v) final concentration glucose. Filter sterilised prior to use.

**YT broth:** 0.8% (w/v) bactotryptone, 0.5% NaCl, 0.5% yeast extract. Autoclaved and stored at room temperature.

Suppliers of the components of the reagents, buffers and media used in this study were Sigma, BDH, ICN Flow and Difco.

## 2.3 Biochemical Materials and Methods.

### 2.3.1 Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE).

*T. vaginalis* native and recombinant proteins were evaluated using discontinuous SDS-PAGE as described by Laemmli, 1971. Mini (0.75 mm thickness) 12% (w/v) acrylamide resolving gels, unless indicated otherwise, were cast, loaded and run using Bio-Rad Mini-Protean II dual slab cell (see manufacturer's protocol for instruction on how to assemble, cast and run polyacrylamide gels). SDS 7 (Sigma), Rainbow markers (Amersham) and Biorad PAGE molecular weight markers were routinely run on polyacrylamide gels so that estimates of protein molecular weights could be made.

After electrophoresis was complete, gels were stained for 20 minutes to 2 hours in Coomassie Blue stain (0.1% (w/v) Coomassie Blue R250, 7% (v/v) acetic acid, 40% (v/v) methanol) and then destained with 7% (v/v) acetic acid and 40%(v/v) methanol until the background of the gel was clear.

### 2.3.2 Native gel electrophoresis.

Native gels were cast, loaded and run as for SDS-PAGE gels except that SDS was omitted from all gel buffers. Also reducing agents, such as β-mercaptoethanol, were omitted from the sample buffer. After electrophoresis the gels were submerged in the relevant enzyme activity stain. Simply, native gels containing *T. vaginalis* proteins were placed in an enzyme assay mixture and the enzymatic reaction allowed to take place *in situ*.

The homocysteine catabolising capabilities of *T. vaginalis* was investigated using native PAGE, the native gel was placed in 30 ml of enzyme assay mixture containing: 3.3 mM homocysteine, 0.33 mM lead acetate, 28.4 mM β-mercaptoethanol in 0.1 M sodium phosphate buffer pH 7.5 at 37°C. The breakdown of homocysteine by *T. vaginalis* methionine γ-lyase in this system allows the detection of enzymatic activities by a deposit of lead sulphide at the position of the protein responsible for the homocysteine catabolism. Hydrogen sulphide, one of the products of the breakdown of homocysteine, combines with the lead acetate in the assay to form lead sulphide, which is a brown precipitate, that is deposited at the site of homocysteine breakdown. The reaction was stopped by replacing the assay components with water.

### 2.3.3 Western blotting.

#### 2.3.3.1 Transfer of proteins from SDS-PAGE gels to solid supports.

Proteins from SDS-PAGE gels were transferred to a solid support such as Hybond-ECL membrane (Amersham) essentially as described by Towbin *et al.*, 1979. The Bio-Rad mini transblot cell was used for the transfer of proteins to solid supports in accordance with the manufacturer's instruction booklet.

After transfer of proteins to the solid support, the membranes to which the protein had been blotted would sometimes be stained in Ponceau S stain (2% (w/v) Ponceau S, 30% (w/v) trichloroacetic acid and 30% (w/v) sulphosalicyclic acid, 10% stock diluted to ) which enabled one to establish if the blotting process had been successful and localise tracks of protein should the membrane have to be cut prior to incubation with primary antibody. Membranes were simply placed in ~20 ml of Ponceau S stain for 15 minutes with shaking and then destained with 5% acetic acid until the background of the

membrane was clear once more. On some occasions it was not necessary to Ponceau stain the membrane as the Rainbow molecular weight markers (Amersham) were clearly visible if the blotting process has been successful.

### 2.3.3.2 Blocking binding sites for immunoglobulins in the solid support.

Once the blotting process had taken place, the sites not occupied by *T. vaginalis* proteins or other proteins in question are blocked so as to prevent non-specific binding of the immunological reagents used in the subsequent steps of the Western blotting process. The blocking reagent used in all Western blots described in this study was 5% non-fat dried milk (Boots own brand), 10% (v/v) heat inactivated horse serum (Life Technologies) in 1x Tris Buffered Saline (TBS) (20 mM Tris, 137 mM NaCl pH 7.6). Membranes were placed in ~20ml of blocker and incubated at 4°C overnight, with shaking.

### 2.3.3.3 Binding of the primary antibody to the target protein.

After the membranes were incubated overnight in blocker, they were incubated with the primary antibody in question. Most of the Western blots described in this thesis involved the use of the polyclonal antisera raised against recombinant MGL1 and MGL2, there were also some results of Western blots in which antiserum raised to native methionine γ-lyase from *T. vaginalis* was used. The incubation of the membranes and the primary antibodies were routinely carried out small heat sealed bags that contained 5 ml of blocker and primary antibody. For anti rMGL1 and rMGL2 serum a dilution of 1 in 500 was used for the primary antibody incubation. For antiserum raised against native methionine γ-lyase from *T. vaginalis* a 1 in 50 dilution was used. The

membranes and primary antibody were incubated for 2 hours at 4°C with shaking. After incubation with primary antibody, the membranes were washed four times with ~200 ml of TBST (TBS + 0.1% Tween 20) for 15 minutes with shaking.

### 2.3.3.4 Binding of the secondary antibody to the primary antibody.

Primary antibody incubations involving anti rMGL1and rMGL2 sera, used horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Scottish Antibody Production Unit, SAPU) at a dilution of 1 in 2500 in blocker. Incubations with antisera to native methionine γ-lyase from *T. vaginalis* used alkaline phosphatase conjugated goat anti rabbit antibody (Stratagene) at a dilution of 1 in 3000. Secondary antibody incubations were carried out in 20 ml of blocker for 1 hour at room temperature with shaking. After incubation with the relevant secondary antibody, the filters were washed four times with ~200 ml of TBST at room temperature with shaking.

### 2.3.3.5 Development of Western blots.

For blots in which the horseradish-peroxidase conjugated secondary antibody was used (i.e., those blots that were incubated with anti rMGL1 and rMGL2 sera), detection of the target protein and primary antibody interactions was by a procedure known as enhanced chemiluminescense (ECL). The washed membranes were incubated in the components of the ECL kit (Amersham). The ECL kit consists of luminol solutions that are cleaved by the horseradish peroxidase which emits light, at the location of the antigen, primary and secondary antibody interactions, which is detected by a fogging of autoradiographic film. Various exposures to autoradiographic film in a cassette holder were carried out, and the exposed film was automatically developed using a Kodak Xomat film processor.

For blots that were incubated with the antisera raised against native methionine γ-lyase and the alkaline phosphatase-conjugated secondary antibody, the components of the picoBlue immunoscreening kit (Stratagene) were used to detect target protein and primary antibody interactions, according to the manufacturer's instructions.

### 2.3.4 Metabolic labelling of *T. vaginalis* proteins.

Two cultures of *T. vaginalis* were set up as in 2.1.1.2, however one culture was grown in the presence of 10 μM DL-propargylglycine. Two cell pellets of $1x10^7$ cells were harvested and resuspended in 1ml minimal Eagles medium (MEM) with Earles salts without methionine (Life Technologies), supplemented with 10%(v/v) heat inactivated dialysed foetal bovine serum (glutamine was added back to the minimal Eagles medium to a final concentration of 1x glutamine, as the original medium does not contain glutamine). The resuspended cells were then placed in 20 ml of the above medium which contained 125 μCi Expre$^{35}$S$^{35}$S protein labelling mix (the composition of which was 72% L-[$^{35}$S] methionine and 18% L-[$^{35}$S] cysteine) (NEN, Dupont). The viability of the cells was checked by microscopic observation as soon as they were placed in the radioactive culture medium. Incubation of the cells was for six hours at 37°C. Checks on viability of the cells were made every two hours.

After incubation the cells were pelleted by centrifugation at 2300 x g and washed twice with 0.25 M sucrose. Resuspended aliquots of cells were centrifuged at 2300 x g, the supernatants removed and the radioactive cell pellets stored at 70°C until required.

### 2.3.5 Preparation of radioactive *T. vaginalis* cell extracts.

Radioactive *T. vaginalis* cell pellets were resuspended in 50mM MOPS (3-[N-Morpholino]propane sulphonic acid ) pH 7.2, 100 mM NaCl, 0.25% (v/v) Triton X100, 100 μg/ml leupeptin, 50 μg/ml PMSF and 1 mM 1, 10, phenanthroline (Lysis solution with inhibitors, LSI) and incubated on ice for 10 minutes. The lysed cells were centrifuged at 13 000 x g for 15 minutes at 4°C. The resulting supernatants were transferred to fresh tubes and the pellets resuspended in 0.5ml LSI.

Incorporation of $^{35}S$ methionine into the newly synthesised proteins of *T. vaginalis* was evaluated using SDS-PAGE as described in section 2. 2. 1. After electrophoresis one gel was stained with Coomassie R250 as described in section 2.2.1. and the other gel was fixed with 10% acetic acid for 30 minutes, submerged in Entensify Part A, precipitating reagent (NEN-Dupont) for 30 minutes and finally submerged in Entensify Part B, aqueous fluor solution (NEN-Dupont) for 30 minutes. Both gels were placed onto Whatman 3M chromatography paper, covered in clingfilm and dried for approximately 30 minutes (Bio-Rad gel drier). Gels were then placed in an autoradiography cassette, overlaid with film and exposed for approximately 17 hours at room temperature. The fluorograph was developed automatically by a Kodak Xomat film processor.

### 2.3.6 Immunoprecipitation of *T. vaginalis* proteins with various antisera.

Protein-A sepharose beads were washed extensively with 50m M MOPS pH 7.2, 100 mM NaCl, 0.25% (v/v)Triton X100 (lysis solution, LS). Immunoprecipitations were carried out using 2 ml select columns (CP Labs) as the protein-A beads could be retained in the column as they were being washed.

Polyclonal antisera (1. pre-immune anti-methionine γ-lyase, 2. immune anti-methionine γ-lyase and 3. anti VSG of *Trypanosoma brucei*) were added to protein-A sepharose beads and incubated at 4°C with rocking for 1 hour. The beads were washed with two column volumes of LS. Bovine serum albumin and LSI was added to the beads and incubated at 4°C for 2 hours with rocking.

$^{35}$S labelled cell extract supernatants of *T. vaginalis* were added directly to the beads and incubated overnight at 4°C on a roller. After overnight incubation the beads were washed extensively with five column volumes of LS.

The beads from each immunoprecipitation were boiled with 2x reducing sample buffer (0.5 M Tris-HCl, pH 6.8, 10% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.05% bromophenol blue) to release immunoprecipitated proteins. Analysis of immunoprecipitated proteins was made by SDS-PAGE and fluorography as outlined in section 2.3.1 and 2.3.5.

### 2.3.7 Enzyme assays.

All assays were carried out at 37°C in a final volume of 1 ml. To confirm the specificity of the reaction control assays were carried out lacking one or more of the assay components. Experiments were also conducted to ensure a linear relationship between reaction rate and sample size over the normal working range.

### 2.3.7.1 Methionine γ-lyase assays and other assays which determined α-keto acid production.

α-keto acid production from a variety of substrates, including methionine was determined for rMGL1 and rMGL2. The production of α-ketobutyrate was determined as described by Soda, (1968), but with the following exceptions. Methionine breakdown by the two recombinant proteins was investigated by using saturating amounts of substrate at a concentration of 25 mM. Homocysteine breakdown was also measured by using saturating levels of homocysteine (30 mM for rMGL1 and 40 mM for rMGL2). Cystathionine breakdown by the two recombinant proteins used a substrate concentration of 5 mM. Also, the colour development part of the assay with methylbenzoylthiolhydrazone (MBTH) used half the volume of reagents, compared to Soda, (1968). The principle of the assay is that the α-keto acids combine with the MBTH reagent to form azines that absorb maximally at 320nm

Specific activities for the breakdown of methionine and other substrates by rMGL1 and rMGL2 were determined from a standard curve of α-ketobutyrate. The standard curve for α-ketobutyrate that was determined using the MBTH reagent is shown in Figure 2.3.1.

The breakdown of cysteine and O-acetyl L-serine by the two recombinant proteins was determined by assaying for the production of pyruvate, using LDH as a coupling enzyme with NADH, as described (Bergmeyer, 1965) with substrate concentrations at 10 mM for cysteine and O-acetyl L-serine. All assays were carried out using 0.1 M imidazole buffer pH 6.5 and were conducted at 37°C.

### 2.3.7.2 Homocysteine desulphurase assays.

The breakdown of homocysteine by *T. vaginalis* crude homogenates, soluble fraction and rMGL1 and rMGL2 was determined as described by Thong and Coombs, (1985). The assay works on the principle that the hydrogen sulphide produced from the breakdown of homocysteine combines with the lead acetate in the assay to produce lead sulphide, which is a brown precipitate, the production of which can be monitored at 360 nm. For rMGL1 and rMGL2 homocysteine concentrations were 30 mM and 40 mM.

#### 2.3.7.2.1 Microtitre plate homocysteine desulphurase assays.

As a matter of convenience a microtitre plate assay for the catabolism of homocysteine was used for assaying column fractions that were produced during native methionine γ-lyase purifications from *T. vaginalis* (Lockwood and Coombs, 1991). The reaction mixture contained a final concentration of 2 mM homocysteine, 1 mM lead acetate, 0.1 M potassium phosphate buffer pH 7.5 and 50 µl of fraction in a final volume of 150 µl. Incubation was at 37°C, and the $A_{340}$ nm after 10 minutes was measured with a Titertek Multistage MCC plate reader

### 2.3.7.3 Activated serine sulphydrase assays.

The activity of activated serine sulphydrase in *T. vaginalis* crude homogenates and soluble fractions were determined as described by Thong and Coombs,1985b.

## 2.3.8 Kinetic calculations.

Kinetic calculations, including the determination of Michaelis constants (Km) were performed using the computer program 'Grafit' (Leatherbarrow, 1992).

### 2.3.9 Protein estimations.

Protein concentrations were determined using the Pierce BCA protein assay kit (microtitre protocol) with bovine serum albumin as standard.

### 2.3.10 Native methionine γ-lyase purification from *T. vaginalis.*

The purification of methionine γ-lyase from *T. vaginalis* was carried out as described by Lockwood and Coombs,1991. The purification protocol is briefly summarised below.

#### 2.3.10.1 Anion exchange chromatography

The non-sedimentable fraction of a *T. vaginalis* homogenate as produced in 2.1.5 was clarified through a 0.22 μm pore GS membrane (Millipore). Samples (0.5ml) were injected onto a Mono Q HR5/5 (Pharmacia) anion exchange column, pre-equilibrated with 20 mM potassium phosphate buffer pH7.5, containing 15 μM β-mercaptoethanol, 1mM ethylenediaminetetra acetic acid (EDTA) and 20 μM pyridoxal 5'-phosphate (PLP). Protein was eluted with a 0-350 mM NaCl gradient in the same buffer. Fractions containing the major peak of methionine γ-lyase activity were pooled and concentrated using centricon microconcentrators (Amicon) with a molecular weight cut-off of 30 kDa.

#### 2.3.10.3 Hydrophobic interaction chromatography.

Solid ammonium sulphate was added to the concentrated fractions to give a final concentration of 2.4 M, the sample was filtered to remove any precipitated proteins. Filtered sample (1ml) was injected onto an Alkyl Superose HR5/5 (Pharmacia) hydrophobic interaction chromatography column, equilibrated with the same buffer as used for anion exchange chromatography. Bound protein was eluted by linearly decreasing the ammonium sulphate concentration.

### 2.3.10.4 Gel filtration chromatography.

Fractions containing methionine γ-lyase eluted from the Alkyl Superose column were pooled and injected onto a Superose 12 HR 10/30 gel filtration column (Pharmacia), equilibrated with 0.2 M potassium phosphate buffer pH 7.5 with the same additions as in previous steps. Protein was eluted with the same buffer.

### 2.3.11 Production of rMGL1 and rMGL2.

Briefly, 50ml of an overnight culture of *E. coli* containing pQMGL1 or pQMGL2 were inoculated into 400ml of LB and 100 μg/ml ampicillin and 25 μg/ml kanamycin and grown until they reached an $OD_{600nm}$ of 0.6-0.8. The culture was then induced with 2 mM and 0.2 mM IPTG respectively and grown for a further 2.25 hours before harvesting, as described in 2.2.1.4. The *E. coli* were resuspended in 5mls of sonication buffer (50 mM Na-phosphate pH 8.0, 300 mM NaCl) and pyridoxal 5' phosphate added to a final concentration of 20 μM.

The cells were lysed by 10 rounds of sonication (1 round = 10 second duration, pulse on, 1 second, pulse off, 1 second) (Jencons sonicator) and the soluble fraction was recovered by centrifugation at 10,000 x g for 30 min at 4°C. Soluble fraction was

loaded on to a 4ml $Ni^{2+}$-NTA resin column (Qiagen) which had been pre-equilibrated with sonication buffer overnight. After sample application the column was washed for 60 minutes with 30 ml of sonication buffer and 60 minutes with 30 ml of wash buffer (50 mM Na-phosphate pH 6.0, 300 mM NaCl, 10% glycerol). Recombinant protein was eluted from the column by an FPLC generated linear gradient of 0-500mM imidazole in wash buffer over 100 minutes (Waters LC Chromatography System).

Fractions containing recombinant rMGL1 or rMGL2 were identified by $OD_{280}$ and by Coomassie Blue-R250 staining of the fractions following SDS-PAGE on a 12% gel as described in 2.3.1. Recombinant protein eluted from the $Ni^{2+}$-NTA resin column were combined with Laemmli sample buffer but only heated to 37°C, due to the presence of imidazole. Peak fractions were pooled and dialysed against 1 litre of dialysis buffer (100 mM Na-phosphate buffer pH6.5, 300 mM NaCl, 20 μM pyridoxal 5' phosphate and 15 μM DTT) for 18h at 4°C. Dialysed rMGL1 or rMGL2 was combined 1:1 with enzyme stabilisation solution (80% glycerol, 300 mM NaCl, 20 μM pyridoxal 5' phosphate, 15 μM DTT in 100 mM Na-phosphate buffer pH6.5) and stored at -20°C.

### 2.3.12 Gel filtration chromatography of rMGL1 and rMGL2.

A Superose 12 HR 10/30 column (Pharmacia) was equilibrated with 50mM Na-phosphate buffer pH6.5, 100mM NaCl and 0.05% $NaN_3$ overnight. The column was calibrated with standards of known molecular mass (ferritin, 450,000 Da; catalase, 240,000 Da; aldolase, 158,000 Da; bovine serum albumin, 68,000 Da; ovalbumin, 45,000 Da; cytochrome c, 12,400 Da, Boeringher Mannheim) and used for the estimation of the native molecular mass of rMGL1 and rMGL2.

### 2.3.13 Immunisation regime for polyclonal antisera production.

The production of polyclonal antisera to rMGL1 and rMGL2 was carried out as described in Harlow and Lane, 1988. Briefly, 200 μg/ml rMGL1 and rMGL2 in Freund's complete adjuvant was injected into female New Zealand White rabbits. A secondary boost of 100 μg/ml rMGL1 and rMGL2 in Freund's Incomplete adjuvant was injected into the rabbits one month later. A tertiary boost with the same components as the secondary boost was given another month later. Sera was taken from the rabbits between the 2° and 3° boosts and tested against the antigen and *T. vaginalis* lysates. Ten days after the 3° boost, the rabbits were exsanguinated, the serum separated from the blood cells and aliquoted and stored at -70°C.

### 2.3.14 Commonly used buffers and reagents in biochemistry.

Details of many of the common buffers and reagents used in biochemistry are given in the reference source, Data for Biochemical research, 3rd edition, Dawson *et al.*, 1989, Oxford University Press. Unless otherwise stated constituents of biochemical buffers were Analar grade. The main suppliers of chemicals and buffer components used in this study were, Sigma, BDH and ICN Flow.

## 2.4 Immunofluorescence of intact trichomonads using anti rMGL1 and rMGL2 sera.

*T. vaginalis*, *T. foetus* and *T. augusta* were harvested and washed as described 2.1.1.4. Parasite pellets (~2.5 x$10^7$) were fixed in 2% paraformaldehyde in 0.1M phosphate

buffer pH 7.2 and were smeared onto clean glass slides and air dried.. The smears incubated in phosphate buffered saline (PBS)(pH 7.2) with 0.2% Tween 20 and either 3% bovine serum albumen (BSA) or 10% foetal calf serum for 15 minutes. Slides were incubated with primary anti-rMGL1 or anti-rMGL2 sera diluted to 1:100 with PBS/BSA, for 1 hour at room temperature. They were then washed with 3 changes of PBS/0.1% BSA for 10 min [each] and incubated with secondary donkey anti-rabbit fluorescein labelled antibody (Scottish Antibody Production Unit) at a dilution of 1:100 with PBS/BSA plus 10μg $ml^{-1}$ 4',-6-diamidino-2-phenylindole (DAPI) (Sigma) as counter stain, for one hour. Finally, smears were washed thoroughly with PBS/0.1% BSA. Control slides were exposed to PBS/BSA containing no rabbit immune serum during the first incubation, or were incubated in pre-immune rabbit serum. Slides were mounted in 10% PBS in glycerol with 25mg $ml^{-1}$ 1'4-diazabicyclo-[2.2.2] octane (DABCO) antifadant and examined using a Zeiss Axioskop compound microscope with phase contrast and ultraviolet (UV) epifluorescence through fluorescein and DAPI filter sets.

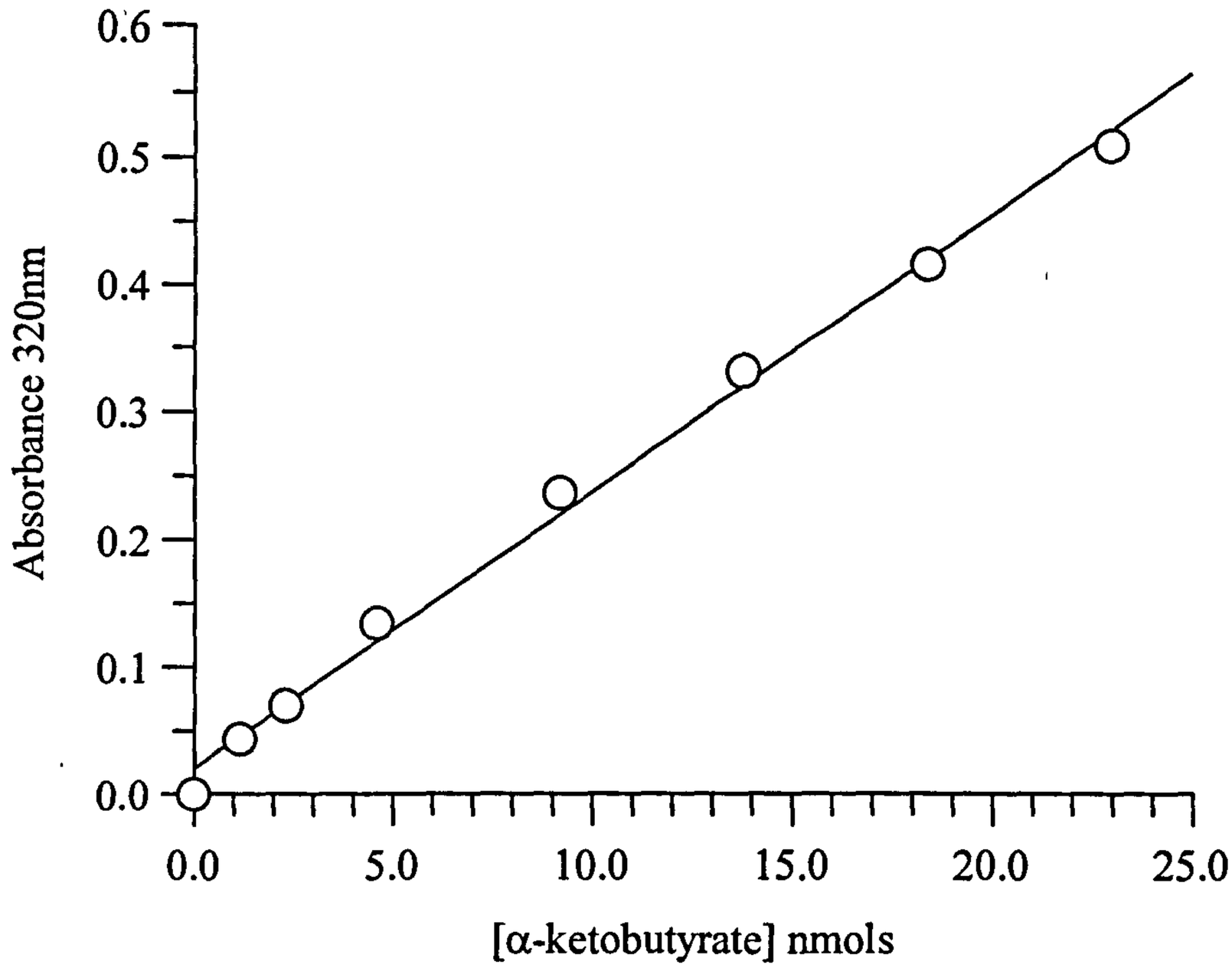



**Figure 2.3.1 Standard curve for α-ketobutyrate using MBTH reagent.**

| Oligonucleotide name | Oligonucleotide sequence |
|---|---|
| Cyst 5′ | 5′-GCAAGCTTGTITGGATTGAGACICCIACGAA-3′ |
| Cyst 3′ | 5′-GCCTCGAGCCGTTIATGTACTTIGTAGC-3′ |
| AM9501 | 5′-CGCCATGGCTCACGAGAGAATGAC-3′ |
| AM9502 | 5′-GCAGATCTTAAAAGAGCGTCAAGGCCC-3′ |
| AM9503 | 5′-CGAGACACCAGCCAACCCAACACTC-3′ |
| AM9504 | 5′-ATCGGAGATTAAGTGATCTCCGGCC-3′ |
| AM9505 | 5′-CTGGCATGGGTGCTATTGCTGCTTC-3′ |
| AM9506 | 5′-GCAGCAATAGCACCCATGCCAGAAG-3′ |
| AM9507 | 5′-GAAGCAGATGAAGATGTTCGGCTC-3′ |
| AM9508 | 5′-AACAACGTGGGAGGTGTCAAGGAC-3′ |
| AM9509 | 5′-GCAATGGCACCCATGCCAGAAGATG-3′ |

**Table 2.1 Oligonucleotide sequences used for gene specific sequencing and cloning.**

# CHAPTER 3. EVALUATION OF *T. VAGINALIS* METHIONINE γ-LYASE POLYCLONAL ANTIBODY AND PURIFICATION OF NATIVE METHIONINE γ-LYASE FROM *T. VAGINALIS*.

## 3.1 Introduction.

The work described in this chapter is considered in two separate, but related sections. As has already been outlined in the aims of this project, it was a priority that the gene for methionine γ-lyase from *T. vaginalis* be isolated and characterised. The results of this chapter outline the various problems that were encountered during the initial period of this project with regards to polyclonal antiserum that had been raised against purified methionine γ-lyase and the purification of native methionine γ-lyase from the parasite.

## 3.2 Results.

I started working on methionine γ-lyase from *T. vaginalis* with a view to initiating a molecular characterisation of this interesting enzyme. The tools available to me to start this work were: a *T. vaginalis* λZAPII cDNA expression library, some polyclonal antiserum which had been raised against purified methionine γ-lyase (Lockwood and Coombs, 1991) and a published protocol for the purification of the enzyme from *T. vaginalis* (Lockwood and Coombs, 1991). A starting point for the isolation of the gene for methionine γ-lyase from *T. vaginalis* was an evaluation of the polyclonal antiserum that had been raised against purified enzyme (Lockwood and Coombs, 1991). I felt that this was a sensible option as I had not been involved in the

purification of the enzyme that had been used to create the antiserum, also I wanted to familiarise myself with techniques that I would be using throughout the course of my studies.

### 3.2.1 Western blot analysis of *T. vaginalis* soluble proteins using anti serum raised against *T. vaginalis* methionine γ-lyase.

Firstly, I used the polyclonal antisera that had been raised to methionine γ-lyase from *T. vaginalis* for Western blot analysis with the soluble fraction of parasite lysates. Figure 3.1 shows the results of Western blot analysis of *T. vaginalis* soluble proteins with the polyclonal antiserum raised against purified methionine γ-lyase.

As can be seen from Figure 3.1, there were two *T. vaginalis* proteins that were predominately recognised by the antiserum against purified methionine γ-lyase. The major protein that was recognised was ~66 kDa, with a second protein of ~43 kDa also being recognised by the antiserum. It must be mentioned that there were several other, more faintly staining bands, being recognised by the antiserum that could not be reproduced in this figure because of their faintness. The pre-immune serum that was available from the rabbit into which the pure methionine γ-lyase was injected, was of very poor quality, (ie. the serum had been freeze dried and it proved difficult to reconstitute the lyophilised serum) but at 1 in 50 dilution for Western blots the pre-immune serum did not recognise any *T. vaginalis* proteins.

The result obtained for the *T. vaginalis* soluble proteins and the antiserum raised against methionine γ-lyase gave some cause for concern. The subunit size of

methionine γ-lyase from *T. vaginalis* had been calculated as being 43 kDa, so it was expected that a band of 43 kDa would be recognised by the methionine γ-lyase in *T. vaginalis* lysates. As for the band at 66kDa and the various other faintly staining bands recognised by the antibody, it seemed probable that they were contaminants of the supposedly pure methionine γ-lyase that was injected into the rabbits.

### 3.2.2 Primary screen of the *T. vaginalis* cDNA expression library with the *T. vaginalis* methionine γ-lyase.

Even though the antiserum against methionine γ-lyase was recognising several *T. vaginalis* proteins it was still used to conduct a primary screen of the cDNA expression library, as it was expected that one of the proteins that the antibody was recognising was methionine γ-lyase from *T. vaginalis*. However, the primary screen of the library with the antibody failed to detect any reactive bacteriophage plaques. This result of the library screen was disappointing but several options were still available to enable the cloning and characterisation of the methionine γ-lyase gene from *T. vaginalis*.

The first option was to evaluate further the usefulness of the polyclonal antiserum that had been raised against methionine γ-lyase from *T. vaginalis*. It was decided that immunoprecipitation studies would be carried out using the antiserum that had been raised against the trichomonal enzyme. Immunoprecipitation is a sensitive procedure which allows a qualitative analysis of proteins that are recognised by the antisera in question. The aim of this experiment was to characterise further the *T. vaginalis* proteins that were being recognised by the methionine γ-lyase antiserum. To perform

Immunoprecipitations newly synthesised polypeptides of the organism in question have to be labelled in order that they can be detected after the immunoprecipitation has taken place. Detection of the *T. vaginalis* proteins after immunoprecipitation would be by fluorography, as *T. vaginalis* proteins were labelled with $S^{35}$-methionine.

### 3.2.4 Metabolic labelling of *T. vaginalis* proteins.

Metabolic labelling studies were therefore undertaken for *T. vaginalis* based on a system used for *Leishmania mexicana* (see Chapter 2 for details of this experiment). Figure 3.2 shows the result of a metabolic labelling experiment with *T. vaginalis*. As can be seen from Figure 3.2 the labelling of newly synthesised proteins in *T. vaginalis* was successful. There has been a good level of incorporation of the radioactive methionine in to all proteins present in the supernatant (lane 1) and pellet fractions of *T. vaginalis* (lane 2).

When this experiment was being set up, the high rate with which methionine is metabolised by *T. vaginalis* (Thong and Coombs, 1985a and b and Thong *et al.*, 1987) was taken into consideration. It was thought that it may have been possible that the radioactive methionine and cysteine may have been lost through volatile thiol production as a result of their catabolism by methionine γ-lyase and serine sulphydrase, respectively. Therefore, metabolic labelling experiments were set up in the presence of propargylglycine, a known inhibitor of the enzymes mentioned above. Lanes 3 and 4 are the labelled *T. vaginalis* proteins that were labelled in the presence of this inhibitor. As can be seen from these lanes in comparison to lanes 1 and 2 in which the inhibitor was absent, there appears to be no qualitative difference in the *T. vaginalis* proteins that have been labelled. The metabolic labelling experiment did,

however, reveal that the system used for labelling the *T. vaginalis* proteins would be suitable for detection of immunoprecipitated proteins.

### 3.2.5 Immunoprecipitation of *T. vaginalis* labelled proteins using methionine γ-lyase antiserum.

Immunoprecipitation experiments were carried out using the metabolically labelled proteins of *T. vaginalis* and three different sera. The results of this experiment are shown in Figure 3.2b. Lane 1 shows the *T. vaginalis* proteins immunoprecipitated with pre-immune serum, lane 2, proteins immunoprecipitated with immune serum and lane 3, proteins immunoprecipitated with *Trypanosoma brucei* anti-VSG sera. As can be seen from lanes 1 and 2, there are many $S^{35}$-labelled *T. vaginalis* proteins immunoprecipitated by both the pre-immune and immune sera. There appears to be no difference in the proteins recognised by the pre immune and immune sera. This experiment, I feel, confirms quite clearly that the antibodies raised against 'purified' methionine γ-lyase were not able to recognise a specific protein of 43 kDa, which hopefully would have been the methionine γ-lyase of *T. vaginalis*. Instead numerous proteins are immunoprecipitate by the antibody, which confirms earlier fears that the methionine γ-lyase used as antigen in the immunisation experiments was not purified to homogeneity, as was thought. There was no recognition of *T. vaginalis* proteins with the *T. brucei* anti-VSG serum.

As the antibodies raised to purified *T. vaginalis* methionine γ-lyase were in no way specific for the protein in question, it was decided that more methionine γ-lyase would be purified by an established procedure that had been published (Lockwood and

Coombs 1991). Purified methionine γ-lyase would provide material to which new polyclonal antibodies could be raised and it would also allow N terminal and/or internal amino acid sequence of the enzyme to be determined, which would provide the basis for the design of oligonucleotide probes that could be used alternatively to screen the *T. vaginalis* cDNA library. The purification of methionine γ-lyase from *T. vaginalis* was therefore undertaken according to the procedures of Lockwood and Coombs, 1991).

### 3.2.6 Purification of native methionine γ-lyase from *T. vaginalis.*

#### 3.2.6.1 Anion exchange chromatography of *T. vaginalis* soluble proteins, the first stage of the native methionine γ-lyase purification from *T. vaginalis.*

Anion exchange chromatography was carried out exactly as described in Lockwood and Coombs (1991). Figure 3.3 shows a representative A280nm profile obtained from a Mono Q anion exchange chromatography column (Pharmacia), when soluble *T. vaginalis* proteins were applied. As can be seen from Figure 3.3, there are two peaks of homocysteine desulphurase activity eluted from the column due to the action of a linearly increasing gradient of NaCl to 350 mM. The purification of methionine γ-lyase was monitored by homocysteine breakdown as this was more convenient and it had been shown previously that methionine γ-lyase was responsible for the breakdown of homocysteine in *T. vaginalis* (Lockwood and Coombs, 1991). The major peak of homocysteine desulphurase activity was eluted at a lower salt concentration than the second. For a typical purification run of methionine γ-lyase, the Mono Q column run was repeated four times with 1 ml of soluble *T. vaginalis* proteins being applied to the

column. The fractions of the major peak of homocysteine desulphurase activity from each Mono Q run were pooled (~12mls) and subsequently concentrated to 1ml using Centricon 30 concentrators (Amicon). The anion exchange chromatography step of the methionine γ-lyase purification was very consistent in the A280 nm profiles and separation of homocysteine desulphurase activities that it produced and was in agreement with the results of this column step as outlined in Lockwood and Coombs (1991).

### 3.2.6.2 Hydrophobic interaction chromatography of Mono Q eluant.

Hydrophobic interaction chromatography of the concentrated post Mono Q fractions was carried out using an Alkyl Superose column (Pharmacia) according to the method of Lockwood and Coombs (1991). The post Mono Q fractions were cut with solid ammonium sulphate and unprecipitated proteins applied to the column. Bound proteins were eluted by a linearly decreasing gradient of ammonium sulphate. Figure 3.4 shows a typical A280 nm trace obtained from a hydrophobic interaction column chromatography run. As can be seen from Figure 3.4 there was hardly any protein eluted from the column and there were three main peaks of homocysteine desulphurase activity and not one as reported by Lockwood and Coombs (1991).

### 3.2.6.3 Gel filtration of alkyl superose eluant.

Peak fractions containing homocysteine desulphurase activity were concentrated using Centricon 30 concentrators (Amicon) prior to application to a Superose 12 gel filtration column, protein was eluted in the same buffer in which the column had been equilibrated. Figure 3.5 shows a typical A280 nm profile obtained from the gel filtration column stage of the methionine γ-lyase purification. It appears from Figure

3.5 that there has been very little separation of the post alkyl superose fractions on the basis of native molecular weight. There are still several peaks of homocysteine desulphurase activity detected in the fractions eluted from this column. As this was the last column in the protein purification protocol, one may expect at this stage only one peak of activity would be detected.

### 3.2.6.4 SDS-PAGE analysis of proteins eluted from the three different columns used in the purification of methionine γ-lyase from *T. vaginalis.*

Figure 3.6 shows the polypeptide constituents of the homocysteine desulphurase-containing fractions that were eluted from the three columns used in the purification of methionine γ-lyase from *T. vaginalis*. The most important feature to note from this Figure is that homocysteine desulphurase containing fractions eluted from the Superose 12 column, the last column of the purification scheme, contain numerous proteins of varying molecular weight. There appears to be no particular enrichment of a protein around ~43 kDa which would be the subunit size of the purified methionine γ-lyase. The Mono Q column procedure resulted in the removal of some of the major *T. vaginalis* proteins between 64 and 36 kDa. The hydrophobic interaction chromatography step resulted in many *T. vaginalis* proteins being removed from the post Mono Q fraction, but many faintly silver stained proteins remained after this step, which was difficult to reproduce in Figure 3.6. Qualitatively there was no difference between proteins eluted from the hydrophobic interaction column and the gel filtration column.

Table 3.1 shows the results of a typical purification of methionine γ-lyase from *T. vaginalis*. As can be seen the recovery of enzyme was very low, only 1.5% after the gel filtration step. The recovery of the enzyme from the attempted purifications was approximately 5-fold lower than the purification of the protein as reported by Lockwood and Coombs (1991).

In essence the purification protocol as published by Lockwood and Coombs, (1991), failed in my hands, to produce methionine γ-lyase that was homogeneous. The purification was carried out many times with changes and variations, but all efforts were in vain, I simply could not purify methionine γ-lyase from *T. vaginalis* based on the purification scheme of Lockwood and Coombs, 1991.

## 3.4 Discussion.

A considerable period of time was spent trying to purify methionine γ-lyase from *T. vaginalis* according to Lockwood and Coombs, 1991. Unfortunately, I was not able to purify the protein to homogeneity, which was required for the production of more polyclonal antibodies to the protein and for amino acid sequence. The reasons for not being able to purify the protein are still a mystery. Every possible stage of the purification protocol was checked.

The problems that were experienced with the purification of methionine γ-lyase meant that the approach of the project had to be re-thought. The results of the project rethink are outlined in the next chapter of this thesis.

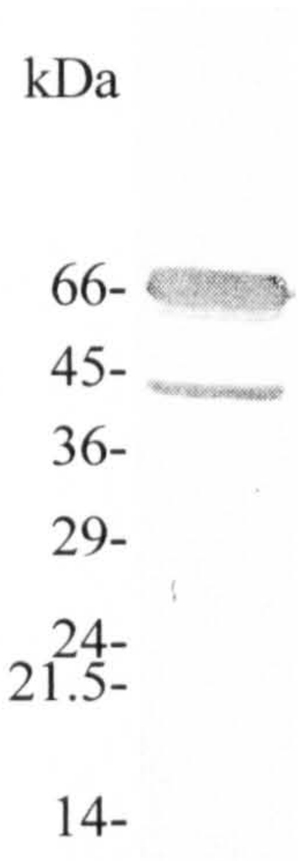



**Figure 3.1 Western blot analysis of *T.vaginalis* soluble fraction proteins with antiserum raised against purified methionine γ-lyase from *T. vaginalis*.**

Molecular weight markers (Sigma, SDS 7) are indicated on the left hand side of the figure

**Figure 3.2 A/B Fluorographs of *T. vaginalis* proteins.**

**Panel A**: Fluorograph of metabolically-labelled *T. vaginalis* proteins

Lane 1: Supernatant fraction of labelled proteins of *T. vaginalis*

Lane 2: Pellet fraction of labelled proteins of *T. vaginalis*

Lane 3: Supernatant fraction of labelled proteins of *T. vaginalis* when treated with 10 μM propargylglycine

Lane 4: Pellet fraction of labelled proteins of *T. vaginalis* when treated with 10 μM propargylglycine

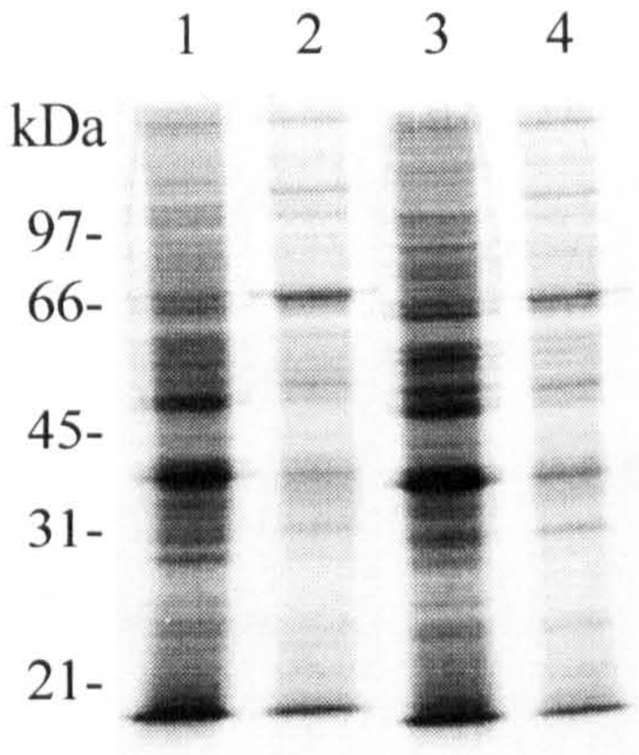
**Panel B**: Immunoprecipitation of *T. vaginalis* labelled proteins with various antisera

Lane 1: *T. vaginalis* proteins immunoprecipitated with pre-immune methionine γ–lyase serum

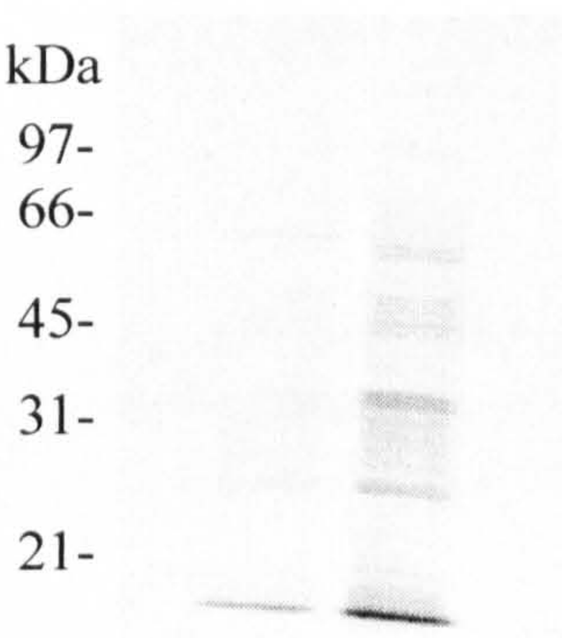
Lane 2: *T. vaginalis* proteins immunoprecipitated with immune methionine γ-lyase serum

Lane 3: *T. vaginalis* proteins immunoprecipitated with *T. brucei* anti-VSG serum

A




B



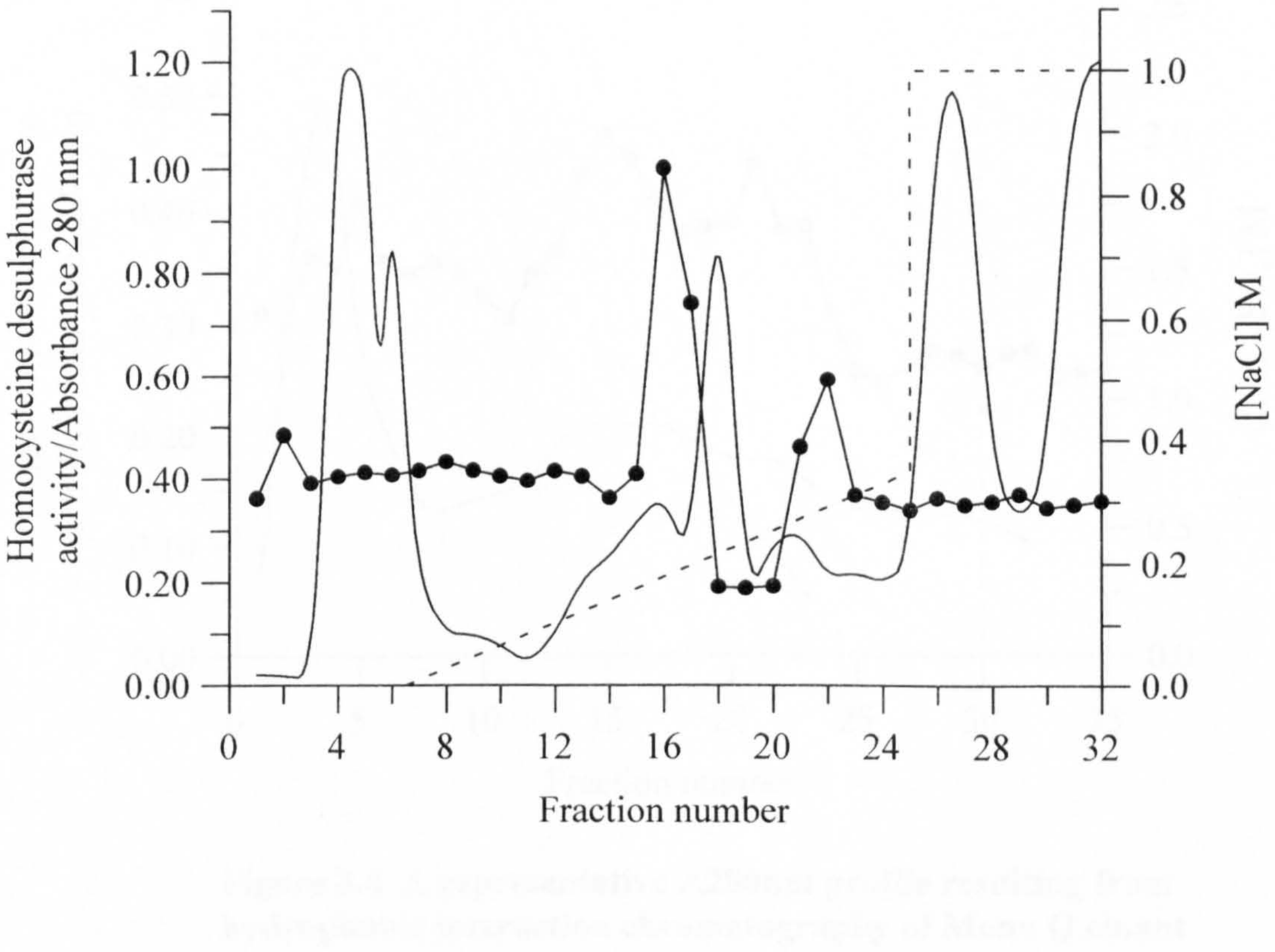



**Figure 3.3 A representative A280 nm profile resulting from anion exchange chromatography *T. vaginalis* soluble proteins using a Mono Q column**

—— A280 nm

—●— Homocysteine desulphurase activity (ΔA340 nm/5 min)

- - - [NaCl] M

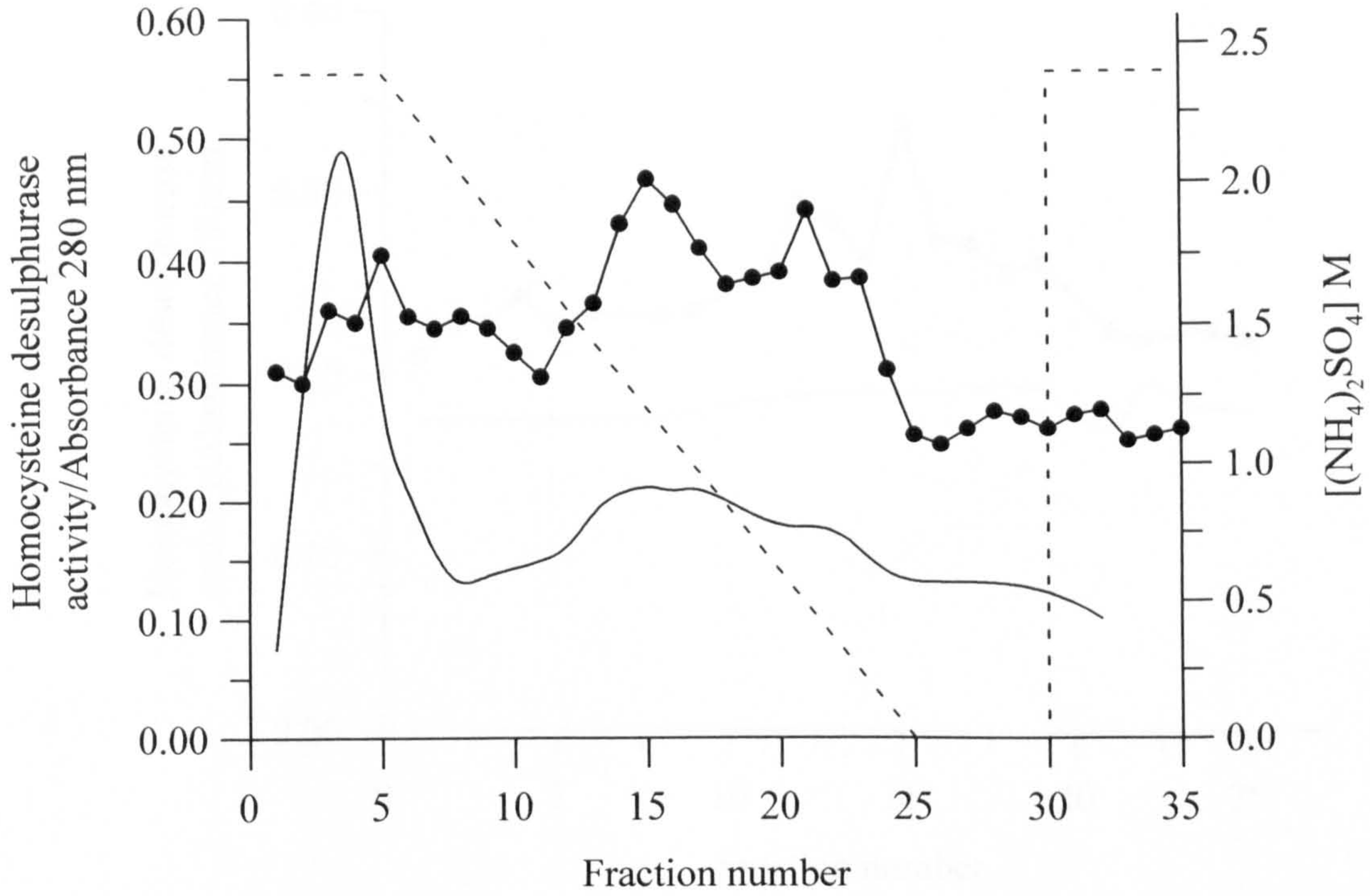



**Figure 3.4 A representative A280nm profile resulting from hydrophobic interaction chromatography of Mono Q eluant using an Alkyl Superose column**

—— A280 nm

—●— Homocysteine desulphurase activity (Δ A340 nm/5 min)

- - - $[(NH_4)_2SO_4]$ M

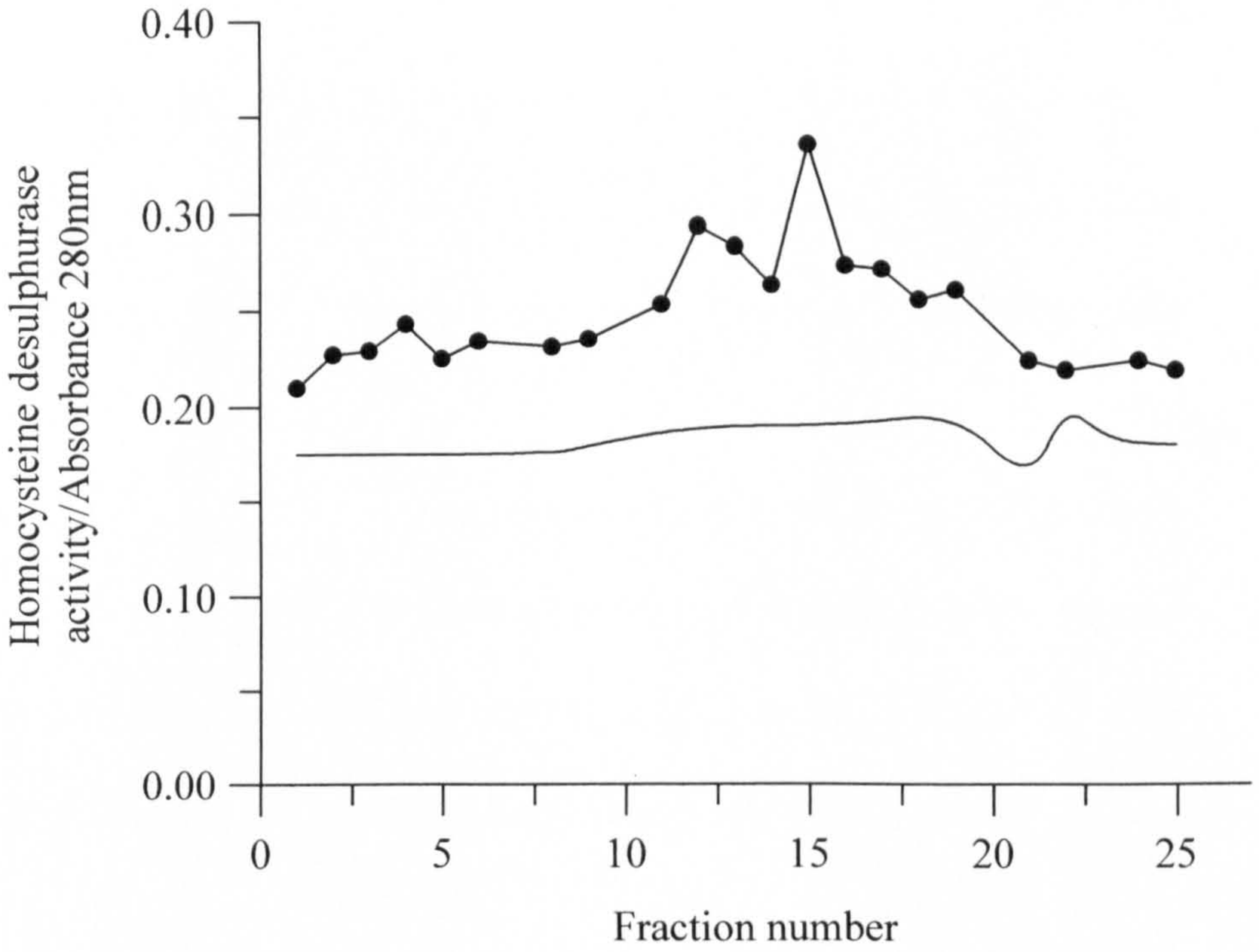



**Figure 3.5 A representative A280nm profile resulting from gel filtration chromatography of Alkyl Superose eluant using a Superose 12 column.**

—— A280 nm

—●— Homocysteine desulphurase activity (ΔA340 nm/5min)

**Figure 3.6 A/B SDS-PAGE analysis of proteins from a native methionine γ-lyase purification.**

Proteins eluted from the columns used in the different stages of the purification of methionine γ-lyase from were combined with Laemmli sample buffer, boiled, electrophoresed and stained with Coomassie R250 or silver stained.

**Panel A** Silver-stained-10% acrylamide gel

Lane 1: *T. vaginalis* non-sedimentable fraction

Lane 2: Peak homocysteine desulphurase fractions eluted from the Mono Q column

Lane 3: Peak homocysteine desulphurase fractions eluted from the Alkyl Superose column

Lane 4: Peak homocysteine desulphurase fractions eluted from the Superose 12 column

Lane 5: Molecular weight markers

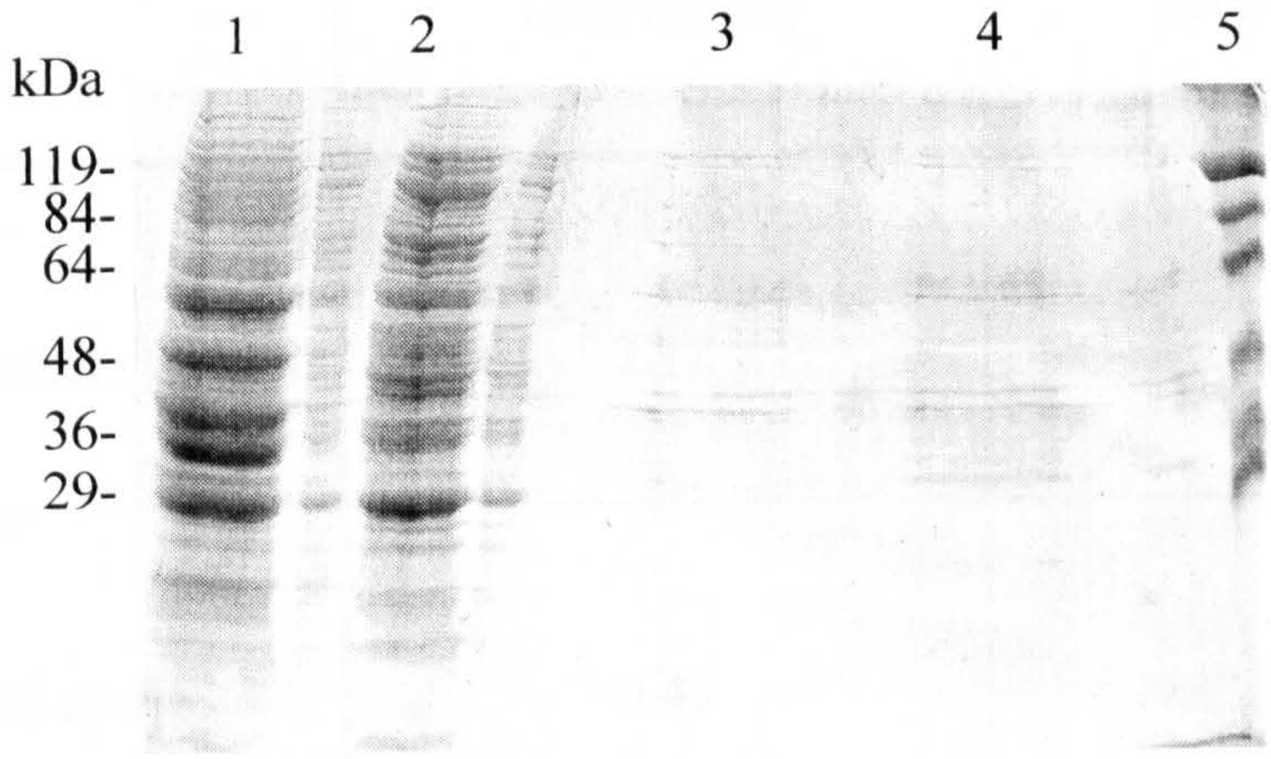
**Panel B** Coomassie Blue R250-10% stained acrylamide gel

Lane 1: *T. vaginalis* non-sedimentable fraction

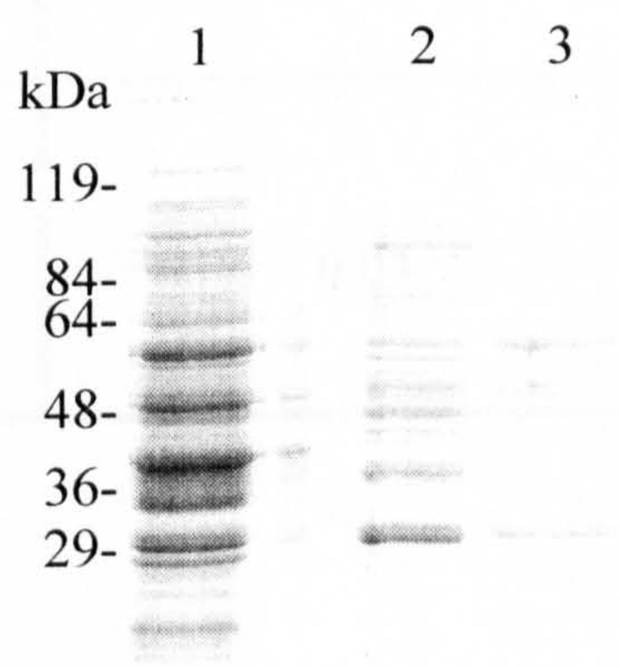
Lane 2: Peak homocysteine desulphurase fractions eluted from the Mono Q column

Lane 3: Peak homocysteine desulphurase fractions eluted from the Superose 12 column

A




B



| | Total activity | Recovery (%) |
|---|---|---|
| Non sedimentable fraction | 2.12 | 100 |
| Mono Q eluate | 0.45 | 21 |
| Alkyl Superose eluate | 0.05 | 2.5 |
| Superose 12 eluate | 0.03 | 1.5 |

**Table 3.1 Purification of methionine γ-lyase from *T. vaginalis.***

The above table shows the results of a typical purification of the several that were carried out. Activity was measured with homocysteine as the substrate. Total activity is in μmol PbS produced /min

# CHAPTER 4. ISOLATION AND MOLECULAR CHARACTERISATION OF METHIONINE γ-LYASE GENE HOMOLOGUES FROM *T. VAGINALIS.*

## 4.1 Introduction.

The aim of this project was to clone the gene encoding the sulphur amino acid metabolising enzyme, methionine γ-lyase, from *T. vaginalis*. Three different approaches were attempted to try and isolate the gene which encodes this interesting enzyme. The first approach was to screen a cDNA *T. vaginalis* expression library with polyclonal antibodies that had been raised previously by Dr. B. C. Lockwood against purified methionine γ-lyase from *T. vaginalis* (Lockwood and Coombs, 1991). However, these polyclonal antibodies raised against methionine γ-lyase from *T. vaginalis* were found to be relatively unspecific and therefore unsuitable for isolating the gene encoding the protein in question (see Chapter 3.).

A second approach involved the purification of more methionine γ-lyase from *T. vaginalis* according to the published methods (Lockwood and Coombs., 1991), in order that N-terminal amino acid sequence and/or proteolytic cleavage fragment sequence of the enzyme could be obtained. This sequence information would subsequently be used to design oligonucleotides, that could be used as probes to screen a *T. vaginalis* λZAPII cDNA library. In addition it was hoped that more specific polyclonal antibodies would be raised against the purified *T. vaginalis* methionine γ-lyase. This approach was also unsuccessful as purification of methionine γ-lyase from *T. vaginalis* using a

combination of methods, both published and novel, failed to yield the molecule in high enough purity or quantity (see Chapter 3). A new approach was therefore sought in order to advance the project.

Methionine γ-lyase as well as being present in *T. vaginalis* is also found in various anaerobic bacteria namely, *Pseudomonas* (Miwatani *et al.*, 1954; Kallion and Larson, 1955; Ito *et al.*, 1975 and Tanaka *et al.*, 1977), *Clostridium* (Wiesendanger and Nisman, 1953 and Kreis and Hession, 1977) and *Aeromonas* (Nakayama *et al.*, 1984), some rumen bacteria (Merricks and Salsbury, 1974) and the anaerobic parasitic protist *Entamoeba histolytica* (Lockwood and Coombs, 1989). There was at the time of embarking upon this project no nucleotide sequence available for methionine γ-lyase from these organisms. There was a small amount of protein sequence available for methionine γ-lyase from *P. putida* derived from cyanogen bromide and tryptic fragment treatment (Nakayama *et al.*, 1988a). Mammals do not possess the ability to breakdown methionine in a single catalytic step (Cooper, 1983). Methionine γ-lyase in *T. vaginalis* may represent a potential new target for chemotherapy.

Cloning the methionine γ-lyase gene from *P. putida* using oligonucleotides designed to the available protein sequence, was a considered option. The *P. putida* methionine γ-lyase gene could then have been used as a heterologous probe to try and isolate the gene encoding methionine γ-lyase from *T. vaginalis*. However, such considerations were soon abandoned as the available protein sequence data was very limited and little was known about the similarity between bacterial and trichomonal methionine γ-lyases.

The third option taken was to utilise the fact that the sulphur amino acid metabolising enzyme cystathionine γ-lyase, has many biochemical features in common with methionine γ-lyase. Cystathionine γ-lyase was the candidate enzyme to study for the following reasons. Firstly, *T. vaginalis* has been shown to possess both methionine γ-lyase and cystathionine γ-lyase activities (Thong *et al.*, 1987, Thong and Coombs, 1985a), but it was not known whether the methionine γ-lyase and cystathionine γ-lyase were activities due to distinct enzymes or whether one protein is able to catalyse the breakdown of both methionine and cystathionine.

Secondly, both methionine γ-lyase and cystathionine γ-lyase catalyse γ-elimination reactions, in which the two enzymes catabolise similar substrates by identical reaction mechanisms. Thus, it was not unreasonable to assume that these two enzymes would be structurally similar at the primary amino acid level. The isolation of the cystathionine γ-lyase gene(s) from *T. vaginalis* and then relating the gene(s) and the encoded enzyme activities would reveal whether or not the methionine γ-lyase and cystathionine γ-lyase activities in *T. vaginalis* could be attributed to the same or two different molecules, and thus shed more light on what may or may not be exploitable drug targets in *T. vaginalis*.

Thirdly, nucleotide sequence data (and hence encoded protein sequence) was available for cystathionine γ-lyase from a range of different organisms, thus providing the opportunity to clone cystathionine γ-lyase and cystathionine γ-lyase like genes from *T. vaginalis*. The following approach was adopted:

Firstly, multiple sequence alignment of the protein sequences for cystathionine γ-lyase from human, rat and yeast were used to identify regions of amino acid conservation within the sequence. Degenerate oligonucleotides were designed to the best regions of homology and were subsequently used in polymerase chain reactions (PCRs) to amplify the equivalent region of a cystathionine γ-lyase homologue from *T. vaginalis* cDNA.

Figure 4.1a shows the multiple protein sequence alignment for cystathionine γ-lyase from human, rat and yeast. Black boxes are the regions of homology chosen for the design of degenerate oligonucleotides to be used as primers in PCRs. The first region of homology chosen for the design of the 5' oligonucleotide primer was a region in which the sequence between the different cystathionine γ-lyase molecules was highly homologous,V154-T160,. The second region chosen for the design of the 3' primer was the pyridoxal 5` phosphate (PLP) binding domain, A210-H217. The sequences of the degenerate oligonucleotides are outlined in Figure 4.1b. In order to facilitate cloning, restriction sites for the enzymes *Hin*dIII and *Xho*I were tagged onto the end of the two degenerate oligonucleotides, respectively.

When the positions of the degenerate primers were taken into consideration and assuming the *T. vaginalis* cystathionine γ-lyase gene would have the same basic primary sequence characteristics as the cystathionine γ-lyase genes of yeast, rat and human and not have any insertions or deletions, a region of some 200bp of *T. vaginalis* DNA would be expected to be amplified.

The plan was if the PCRs were successful, the amplified fragments of *T. vaginalis* DNA would be cloned, with the aid of the restriction sites engineered onto the ends of the

fragment by the amplification process. The cloned PCR fragments would then be sequenced to ascertain their identity and if indeed they had sequence and predicted peptide homology to cystathionine γ-lyase from yeast, rat and human. If a *T. vaginalis* cystathionine γ-lyase homologue was isolated then the PCR fragment would be used as a probe to screen a *T. vaginalis* λZAPII cDNA library, with a view to isolating full length *T. vaginalis* cystathionine γ-lyase homologues, which would then be sequenced and further analysed.

## 4.2 Results.

### 4.2.1 RNA Isolation.

Total RNA was successfully isolated from *T. vaginalis* in a single step using the commercially available TRIZOL™ reagent (Gibco), which is a monophasic solution of phenol and guanidine isothiocyanate. The total RNA produced from *T. vaginalis* using this reagent was analysed both spectrophotometrically and by agarose gel electrophoresis, in order to determine quantity and purity of the RNA. Spectrophotometric analysis of the total RNA at 260 nm/280 nm gave ratios between 1.8 and 2.0. A ratio of >1.8 indicates relatively pure RNA. Poly $[A]^+$ RNA was isolated from total RNA, as this type of RNA was deemed to be more suitable for cDNA synthesis. Figure 4.2 shows the results of non-denaturing gel electrophoresis of total *T. vaginalis* RNA and poly $[A]^+$ RNA. Lanes 2 and 3 show 1μg and 4μg of *T. vaginalis* total RNA respectively. The electrophoretic analysis confirmed the purity of the RNA as there appears to be little or no DNA contamination of the RNA. Also the electrophoretic process has separated the various components of *T. vaginalis* total RNA.

It can be seen that there are two distinct bands present towards the top of the gel and these correspond to the large subunit rRNAs. Towards the bottom of the gel there are higher mobility RNAs and these correspond to smaller species RNAs such as tRNAs and 5S rRNA. The *T. vaginalis* poly $[A]^+$ upon spectrophotometric analysis had a A260/280 nm ratio of 1.5.

### 4.2.2 cDNA Synthesis and PCR using degenerate primers.

1μg of poly $[A]^+$ RNA from *T. vaginalis* was used to synthesise first strand *T. vaginalis* cDNA. The cDNA was used as a template in PCRs with the degenerate oligonucleotides (Figure 4.1b). A control cDNA synthesis was performed by omitting reverse transcriptase.

Figure 4.3 shows the result of a number of test PCRs using the degenerate oligonucleotides, Cyst5' and Cyst3', either singly or in combination, and *T. vaginalis* cDNA synthesised from *T. vaginalis* poly $[A]^+$ RNA, plus a control without reverse transcriptase. Conditions of the PCRs used in this experiment were as follows: Initial denaturation 94°C for 4 minutes followed by 30 amplification cycles consisting of 94°C for 1 minute, 42°C for 1 minute and 72°C for 1minute and a final extension cycle at 72°C for 5 minutes. The products of PCRs when only the Cyst 5' primer was used along with *T. vaginalis* cDNA resulting from synthesis with reverse transcriptase and a control synthesis without enzyme are shown in Lanes 2 and 3, respectively. Lanes 4 and 5 are the same as above except the Cyst 3' primer was used in the PCRs.

No amplified DNA was detected in lanes 2, 3, 4 and 5 indicating that the degenerate primers on their own and coupled with the control reaction does not give spurious DNA

amplification. The diffusely staining regions near the bottom of the gel are monomer oligonucleotide primers.

No DNA was amplified when PCR was performed with both degenerate oligonucleotides and the control reaction, in which the reverse transcriptase was omitted (lane 7). The PCR under these conditions confirmed that the *T. vaginalis* poly $[A]^+$ RNA was free from any contaminating *T. vaginalis* genomic DNA. PCR performed with both degenerate oligonucleotides and *T. vaginalis* first strand cDNA, amplified two DNA fragments (lane 6). The first fragment was approximately 200bp in size (lower band fragment), and the second fragment was approximately 250bp in size (upper band fragment). The 200bp amplified fragment of approximately was about the size of fragment expected to be amplified, taking into consideration the location of the regions used in the design of the degenerate primers. For human and rat cystathionine γ-lyase the two regions are 64 amino acid residues apart and a 208bp fragment would be amplified. For yeast cystathionine γ-lyase the two regions are 67 amino acid residues apart (there is a three amino acid residue insertion in the yeast cystathionine γ-lyase sequence when compared to the amino acid sequence of the cystathionine γ-lyase of human and rat, Figure 4.1b), and thus a 217bp fragment would be amplified. As PCR using the two degenerate oligonucleotides and *T. vaginalis* cDNA amplified the expected size fragment, several more identical PCRs were performed in order to obtain enough material for cloning.

A positive control PCR was also carried out after the original degenerate PCRs to confirm that the *T. vaginalis* first strand cDNA was of suitable quality and integrity to amplify fragments of *T. vaginalis* transcripts and that the conditions of the PCR being

used were suitable for the amplification of such *T. vaginalis* DNA fragments. Degenerate primers previously used in the successful isolation of cysteine proteinase genes from *T. vaginalis* (Mallinson *et al.*, 1994), were used in PCRs with *T. vaginalis* first strand cDNA under the same conditions as outlined previously, the results are shown in Figure 4.4, lane 3. A DNA fragment of TvCP1 of approximately 500 bp was amplified, which was in complete agreement with Mallinson *et al.* 1994. The result from the positive control PCR appeared to confirm that the template *T. vaginalis* first strand cDNA and the conditions of PCR being used, should successfully amplify genuine gene fragments of *T. vaginalis* cystathionine γ-lyase, if sufficient homology exists between the cystathionine γ-lyase genes of different species.

The results of PCRs used to generate enough material for cloning are shown in Figure 4.4. Five *de novo* PCR reactions were set up under the exact conditions described previously. The products of the five identical PCRs were combined and an aliquot run on an agarose gel (Figure 4.4, lane 3). Once again the *de novo* PCRs gave both an upper band and lower band amplified product of 250bp and 200bp respectively.
Figure 4.4 also shows that when previously amplified upper band (lane 4) and lower band DNA (lane 5) which were gel purified and subsequently used as a template, in PCRs with less amplification cycles, a large amount of product was observed for both sizes of template. The upper band DNA template (lane 4), gave a mixture of amplified products, consisting mainly of 4 bands, between 200bp and 250bp in size. The lower band template (lane 5) gave two amplified products of around 200bp in size.

### 4.2.3 Cloning of PCR fragments amplified using degenerate oligonucleotides for methionine γ-lyase.

The PCR reaction containing amplified DNA was precipitated to remove contaminating nucleotides and subsequently phenol chloroform/chloroform extracted to remove the Taq polymerase. The amplified DNA was then restricted to completion with *Hin*d III and *Xho*I restriction enzymes. These enzymes were chosen as their restriction sites had been engineered onto the ends of the amplified DNA by their inclusion at the termini of the Cyst 5'and Cyst 3'degenerate oligonucleotides respectively. The DNA was further purified by gel electrophoresis. DNA was run on a 2% TAE agarose gel, stained with EtBr to allow visualisation of the amplified DNA under a UV light source, the amplified bands of interest excised from the gel using a clean scalpel blade. The DNA was eluted from the agarose gel slice using commercially available Spin X columns (Costar).

The restricted, purified DNA (individual upper band and lower band products and a combination of the two) arising from the PCRs with the degenerate primers, was then combined with pBluescript (Stratagene) which also had been restricted with *Hin*d III and *Xho*I and gel purified. The pBluescript and amplified PCR fragments of approximately 200-250bp were ligated in quantities of 200 ng insert plus 200 ng vector using the Amersham ligation kit.

The ligations were then transformed into Ultracompetent XL1 Blue (Stratagene). Approximately 30-50ng of ligated DNA was used to transform the competent bacterial cells. Transformation mixes were plated out onto LB plates containing ampicillin, X-gal and IPTG and incubated overnight at 37°C. Colonies of transformed XL1 Blue bacteria, when plated onto LB-amp X-gal, IPTG plates take on either a white or deep blue appearance, after growth. The presence of white colonies allowed blue/white selection of plasmids with inserts to be carried out.

After transformation mainly white bacterial colonies were selected from LB amp, X-gal, IPTG plates for further analysis. Plasmid DNA was isolated using the mini prep procedure and subjected to selective restriction analysis to ascertain whether cloning of the amplified DNA into pBluescript had been successful.

### 4.2.4 Restriction based screening of Cysta clones to ascertain successful cloning of the 200bp amplified fragment into pBluescript.

*Pvu*II restriction analysis of pBluescript is a useful procedure to adopt when screening this vector for the successful insertion of small DNA fragments into the multiple cloning site (MCS) of pBluescript. pBluescript possesses two *Pvu*II restriction sites that flank the MCS and about 400bp apart. If pBluescript is restricted with *Pvu*II, a fragment of 400bp is cleaved from the plasmid. However, if a DNA fragment is cloned successfully into the MCS of pBluescript and then cut with *Pvu*II, the size of the *Pvu*II fragment represents a sum of 400bps plus the size of the fragment that was cloned into the vector. This *Pvu*II screening procedure therefore allows a more accurate sizing of smaller DNA fragments and it also avoids the visualisation of small DNA fragments on high percentage agarose gels. The principles of this *Pvu*II screening process are demonstrated in Figure 4.5. When pBluescript was restricted with *Pvu*II, two fragments of 2.5 kb and 400 bps were produced (lane 2). However, when plasmid DNA from different single white transformants (in which lower band PCR amplified material was cloned into pBluescript) was restricted with *Pvu*II, two fragments of 2.4kb and ~600bp were produced which indicated that the plasmid had an insert of ~200bp in the multiple cloning site and that the cloning of the PCR amplified material into pBluescript had been successful (lanes 3-7).

Figure 4.6 illustrates *Pvu*II restriction analysis of plasmid from individual white transformants in which PCR amplified material from all bands was 'shot-gun' cloned into pBluescript. Two clones produced a 650bp fragment (lanes 6 and 7), probably corresponding to the upper band, whereas three clones gave a 600bp fragment probably corresponding to the lower band (lanes3, 4 and 5).

The cloned PCR products then became known as 'Cysta clones'. A total of 17 Cysta clones were obtained. Sequencing and subsequent analysis of the Cysta clones would reveal whether a genuine fragment of a cystathionine γ-lyase homologue had been amplified from the *T. vaginalis* cDNA.

### 4.2.5 Sequencing and analysis of the Cysta clones.

All of the Cysta clones were sequenced on one or both strands, using T7 and - 21 mer primers (Stratagene), and Sequenase Quick Denature Plasmid sequencing Kit (Amersham) as outlined in Chapter 2.

Sequence analysis of the Cysta clones revealed that they fell into three groups. Firstly one group consisted of Cystas 2, 9, 10 and 15 which were similar to each other and encoded peptides with homology to yeast cystathionine γ-lyase. A second group consisted of Cystas 8, 14 and 16,which were similar to each other but different to Cystas 2, 9, 10 and 15 also had peptide homologies to yeast cystathionine γ-lyase. A third group of Cystas had no homology to yeast cystathionine γ-lyase and were thought to be non-specific PCR amplification products. A correlation was found between the Cysta clones that had genuine homology to cystathionine γ-lyase and the lower band PCR

products. For example Cysta 2, a representative clone from one of the groups was 68% identical and 84% similar, at the amino acid residue level to cystathionine γ-lyase from yeast. Cysta 16, a Cysta clone from the other group was 58% identical and 74% similar to yeast cystathionine γ-lyase in terms of amino acid residues.

### 4.2.6 Southern blot analysis of *T. vaginalis* genomic DNA using Cysta based radioactive probes.

It was important to show that the PCR products were genuine *T. vaginalis* sequences and not PCR artefacts derived other organisms, therefore Southern blot analysis was performed.

An electrophoretic analysis of *T. vaginalis* genomic DNA after digestion with various restriction enzymes is shown in Figure 4.7. *T. vaginalis* genomic DNA was restricted with *Eco*RI, *Hin*cII, *Hin*dIII and *Xba*I for 5 hours at 37°C prior to being electrophoresed on a 0.7% agarose gel (lanes 2-5 and 7-10) A distinct banding pattern was observed after staining with EtBr. The distinct fragments are thought to be highly repetitive nucleotide sequences that occur in the *T. vaginalis* genome (Wang and Wang, 1985).

The DNA shown in these gels was transferred to Hybond N filters (Amersham) using the method of Southern (1975) and subjected to hybridisation with the 200bp amplified fragments of Cysta 8, 9, 14 and 16. DNA for probes specific to Cysta 8, 9, 14 and 16 probes was generated by PCR, using the appropriate 600bp *Pvu*II fragment from each clone as template and the degenerate oligonucleotides as used in the original PCR. The probes were labelled using a random priming method.

The hybridisation pattern obtained when restricted *T. vaginalis* genomic DNA was hybridised with either Cysta 9 (Panel A) or Cysta 8 (Panel B). For Cysta 9, three hybridising bands of different sizes were detected. Firstly, a *Eco*RI fragment of 7.5kb (lane 1), secondly a 6.5kb *Hin*d III fragment (lane 3) and thirdly a >23kb *Xba*I fragment (lane 4). There was no hybridisation of the Cysta 9 amplified fragment with *Hinc*II restricted *T. vaginalis* genomic DNA. The probable explanation for the lack of hybridisation with the Cysta 9 amplified fragment and the *Hinc*II digested genomic DNA is that there was a *Hinc*II site in the middle of the probe region (See Figure 4.17) and therefore it was likely that the hybridising fragments were too small to be detected. (Cysta 2-gave same size bands as Cysta 9, data not shown). Cysta 8 hybridised to a 7.5kb *Eco*RI fragment (Panel B, lane 1), a 2.5kb fragment with *Hinc*II (Panel B, lane 2), a 15kb fragment with *Hind* III (Panel B, lane3) and finally a 18kb fragment with *Xba*I (Panel B, lane 4).

Figure 4.8 illustrates the hybridising patterns of restricted *T. vaginalis* genomic DNA with the Cysta 14 (Panel C) and Cysta 16 (Panel D) amplified DNA. (The filters used in these hybridisation experiments were duplicates of those used in the above experiments, ie., two gels with the same samples were run and subsequently blotted). Lanes 1 to 4 represent *Eco*RI, *Hinc*II, *Hind* III and *Xba*I restricted *T. vaginalis* genomic DNA. Cysta 14 and Cysta 16 gave an identical hybridisation pattern as those obtained with Cysta 8 and restricted *T. vaginalis* genomic DNA (Figure 4.8, Panel B). The hybridisation patterns obtained for the different Cysta clones, together with nucleotide/peptide sequence data confirmed that they fell into two distinct groups, both of which had sequence homology to yeast cystathionine γ-lyase. Southern blot analysis also confirmed that the amplified fragments were of sufficient size to detect the gene from

the genomic DNA of *T. vaginalis* and could be used for the isolation of the relevant full length genes from the *T. vaginalis* cDNA library.

### 4.2.7 Northern blot analysis of *T. vaginalis* total and poly $[A]^+$ RNA using a 600bp radioactive fragment from Cysta 2.

In order to investigate expression of the putative *T. vaginalis* cystathionine γ-lyase homologue Northern blot analysis of *T. vaginalis* RNA was also performed with the Cysta 2 clone (Figure 4.9). *T. vaginalis* total RNA (lane 1) and poly $[A]^+$ RNA (lane 2) was probed with the 600bp *Pvu*II restriction fragment from the Cysta 2 plasmid. A transcript size of approximately 1.3kb was detected in both total and poly $[A]^+$ RNA, which has the coding capacity of a protein of approximately 43kDa. This corresponds to the subunit size of cystathionine γ-lyase from a yeast (Ono *et al.*, 1992), rat (Erickson *et al.*, 1990) and (Lu *et al.*, 1992).

### 4.2.8 Isolation of full length *T. vaginalis* methionine γ-lyase homologues.

A representative clone from each group of Cysta clones, Cysta 2 and Cysta 16 were used to isolate the corresponding full length cDNA clones from a *T. vaginalis* λZAP II cDNA library (Mallinson *et al.*, 1994). A total of 100,000 cDNA clones were screened. Briefly, 100,000 bacteriophage were plated in L-top agarose, along with the host cells *E. coli* XL1 Blue and the bacteriophage plaques were allowed to propagate at 37°C until they were just touching one another in the bacterial lawn. The bacteriophage plaques were then transferred to a Hybond N filter, the DNA of the 'phage was denatured *in situ* and subsequently hybridised with either the Cysta 2 or Cysta 16 200bp cystathionine γ-lyase cDNA fragment. The Cysta 2 and Cysta 16 probe material was produced by PCR

using the appropriate 600bp *Pvu*II fragment as template, along with the degenerate oligonucleotides used in the original PCRs.

A second round of plaque purification was carried out and the pure clone chosen for further analysis. Positively hybridising λ clones from the secondary screens with Cysta 2 and Cysta 16 200bp fragments that were separate from other plaques were selected as these could be isolated as pure clones. The cDNA present in the λZAP II vector was 'rescued' directly into the plasmid pBluescript using a f1 helper bacteriophage mechanism (see Short *et al*, 1987).

### 4.2.9 Restriction enzyme analysis of Cysta 2 and Cysta 16 clones.

Two lambda clones named $1_{100}$ and $4_{100}$, which hybridised with the Cysta 2 probe were rescued into pBluescript using the f1 helper bacteriophage mechanism. The two pBluescript phagemids were named $p1_{100}$ and $p4_{100}$. Restriction analysis of the DNA contained in pBluescript was then undertaken for each clone. Figure 4.10 illustrates the results of restriction analysis of the $1_{100}$ and $4_{100}$ DNA, which hybridised with the Cysta 2, 200bp probe. $1_{100}$ and $4_{100}$ plasmids were restricted with *Eco*RI and *Xho*I alone and as a double digest. These enzymes were chosen as the original *T. vaginalis* cDNA was cloned into these sites in the vector. Also $1_{100}$ DNA was restricted with both *Spe*I and *Kpn*I. $1_{100}$ plasmid DNA restricted with *Eco*RI and *Xho*I released three fragments of ~2.5kb, ~1.4kb and ~1kb (lane 2). The first and largest fragment was the pBluescript DNA, the 1.4kb fragment was thought to be uncut supercoiled plasmid and the 1kb fragment was thought to be the true size of the insert contained within the $1_{100}$ plasmid. $1_{100}$ plasmid DNA cleaved with *Xho*I alone (lane 3) or *Eco*RI alone (lane 4) released no DNA fragment from the plasmid. When $1_{100}$ DNA was restricted with *Spe*I and *Kpn*I,

two restriction enzymes present in the multiple cloning site of pBluescript, out with the *Eco*RI and *Xho*I sites into which the *T. vaginalis* cDNA was originally cloned, four fragments were produced of ~3 kb, ~2 kb, ~1.5 kb and ~1 kb. The largest fragments were thought restricted pBluescript and uncut supercoiled plasmid and the 1 kb fragment was thought to be the true size of fragment contained in the multiple cloning site of pBluescript.

A series of restriction digests for $4_{100}$ plasmid are also shown in Figure 4.10. A double digest of $4_{100}$ plasmid with both *Eco*RI and *Xho*I produced two insert DNA fragments, one of 1kb and the second of 300bp. This result indicates that one of the restriction enzymes used was cutting within the insert. A 300bp fragment was cleaved from the $4_{100}$ plasmid when restricted with *Xho*I, which would indicate that there is an *Xho*I site 300bp from the end of the cDNA which was cloned into the *Xho*I site of pBluescript. No insert was released from the $4_{100}$ plasmid when it was restricted with *Eco*RI alone (lane 8). As a result of the restriction analyses of $1_{100}$ and $4_{100}$ plasmids, clone $4_{100}$ became the focus of further study as it had the larger insert totalling about 1.3kb. An insert of this size should be sufficient to encode a protein of 43 kDa, which is the subunit size of cystathionine γ-lyase from a variety of organisms.

Five lambda clones that hybridised with Cysta 16 were rescued into pBluescript using the f1 helper bacteriophage. The rescued plasmids were subsequently analysed by restriction with the enzymes *Eco*RI and *Xho*I. Plasmid clones $1_{100}$a (lane 2) and b (lane 3) and $3_{100}$ (lane 4) were digested with *Eco*RI and *Xho*I.

Two fragments of approximately 550bp and 450bp were observed together with 3kb of pBluescript plasmid showing these plasmids had an internal *Eco*RI or *Xho*I site, but making an insert size of 1000bp. Plasmids $5_{100}$ (lane 6) and $6_{100}$ (lane 7) when digested

with *Eco*RI and *Xho*I gave fragments of approximately 800bp and ~470bp in size. Thus the insert was nearly 1.3kb in total and contained either an internal *Eco*RI or *Xho*I site.

Figure 4.12 illustrates the results of restriction analysis undertaken to establish which restriction enzyme sites are present internally within the inserts of $1_{100A}$ and $5_{100}$.plasmids. When $1_{100A}$ plasmid was restricted with *Eco*RI a 550bp fragment was released (lane 3), indicating that the internal restriction site in the $1_{100A}$ clone DNA is that of *Eco*RI. No fragment was released after digestion with *Xho*I (lane 2) A 800bp fragment was observed when the $5_{100}$ plasmid was restricted with *Eco*RI (lane 5), whereas *Xho*I did not cut within the insert (lane 4). When $1_{100A}$ (lane 6) and $5_{100}$ (lane7) plasmids were digested with *Kpn*I and *SpeI*, restriction enzymes that cut out with the *Eco*RI and *Xho*I sites of pBluescript, the intact insert was released This restriction analysis showed that the $1_{100A}$ plasmid had an insert size of was 1000bp (lane 6) whereas the insert size of the $5_{100}$ plasmid was 1.3kb (lane 7).

As a result of restriction analyses of the plasmids isolated with Cysta 2 or Cysta 16 200bp probes, a Cysta 2 clone ($p4_{100}$) and a Cysta 16 clone (p5100), a Cysta 16 hybridiser were selected to be fully sequenced. These two clones were chosen as they had the largest inserts and were thought to be of sufficient size to encode a full length copy of the cystathionine γ-lyase gene homologue from *T. vaginalis*.

At this point the genes encoding the mRNA which gave the cDNA were named *T. vaginalis mgl1* for the $4_{100}$ cDNA clone and *T. vaginalis mgl2* for the $5_{100}$ cDNA clone (to be referred to as *mgl1* and *mgl2*).

### 4.2.10 Sequencing of p4100 and p5100, two *T. vaginalis* methionine γ-lyase homologues.

Restriction analysis of p4100 and p5100 coupled with sequence information from the 5' and 3' ends of each gene allowed the construction of a preliminary restriction map of each *mgl1* and *mgl2*, which allowed the basis of a subcloning strategy that assisted in obtaining the full nucleotide sequence of each gene.

The results of the digests with $p4_{100}$ and $p5_{100}$ are shown in Figures 4.13 and 4.14 respectively. It is important to note at this point that the cDNA contained in $p4_{100}$ was reversed ie., the *Xho*I site was present at the 5' end and the *Eco*RI site was present at the 3'end, which is the reverse of the norm. The reversal of the cDNA insert into pBluescript may have partly occurred due to incomplete digestion of vector/insert by *Xho*I during the synthesis of the cDNA library. $p4_{100}$ restricted with *Eco*RI and *Xho*I and *Xho*I alone (lanes 2 and 3, respectively) gave fragments of 1kb and 300bp as previously determined (Figure 4.10, lanes 6 and 7). $p4_{100}$ digested with *Eco*RV and *Xho*I gave three fragments 500bp, 300bp and 200bpin size (lane 4). $p4_{100}$ digested with *Eco*RV alone gave a fragment of 200bp (lane 5), whereas a double digest of the plasmid with both *Eco*RI and *Eco*RV gave two fragments of 200bp and 350bp (lane 6).

When $p5_{100}$ was digested with *Eco*RI and *Xho*I two fragments of 800bp and 450bp were observed (lane 2), whereas the same plasmid digested with *Eco*RI alone gave a fragment of 800bp (lane 3) which suggests an internal *Eco*RI site, as seen previously (Figure 4.12, lane 5). With respect to the digestion of $p5_{100}$ with *Eco*RV, it must be remembered that there is an *Eco*RV site in the polylinker of pBluescript that is close to the *Xho*I site used

in the cloning of the cDNA. This *Eco*RV site is not present in the cDNA clones therefore all of the other *Eco*RV sites are internal in the cDNA insert. $p5_{100}$ digested with *Eco*RI and *Eco*RV gave four fragments which were approximately 550, 350, 120 and 80 bp in size (lane 4), whereas the same plasmid digested with *Eco*RV alone gave three fragments of 450, 120 and 80 bps in size (lane 5). Finally when $p5_{100}$ was digested with *Eco*RV and *Xho*I (lane 6) the same size fragments were obtained as for the single digest with *Eco*RV.

### 4.2.11 Restriction mapping and subcloning strategy of *mgl1* and *mgl2*.

The information generated from the above experiments allowed the restriction maps to be drawn up for each gene ( Figures 4.15 and Figure 4.16) which allowed a subcloning strategy to be made to facilitate the full sequencing of the two genes.

The subclones of $p4_{100}$ and $p5_{100}$ used for the sequencing of *mgl1* and *mgl2* utilised restriction sites present in *mgl1* and *mgl2* that were present at intervals of reasonable base pair length. The restriction sites used for the production of the respective subclones are indicated in Figures 4.15 and 4.16. The restriction sites used to produce the subclones of $p4_{100}$ and $p5_{100}$ can be correlated to Figures 4.17 and 4.18, where the sites are indicated by pink text. Where it was not possible to produce a subclone for $p4_{100}$ or $p5_{100}$ in order to obtain overlapping sequence, then oligonucleotide primers were designed to fill in the gaps of nucleotide sequence for the two genes. The oligonucleotides used to complete the sequencing of *mgl1* and *mgl2* are indicated in blue in Figures 4.15 and 4.16 and their positions are able to be seen in Figures 4.17 and 4.18, as indicated by the blue text.

Sequencing of the $p4_{100}$ and $p5_{100}$ and the respective subclones (which were made in pBluescript) was achieved using Sequenase Quick Denature Plasmid sequencing kit (Amersham) with T7 and T3 primers (Stratagene). The complete nucleotide sequence and predicted amino acid sequence of the cDNA clone $4_{100}$ is shown in Figure 4.17, indicated in this figure are the relevant restriction sites used in subcloning and oligonucleotides used to complete the sequencing of the cDNA. The complete nucleotide sequence and predicted amino acid sequence of the cDNA $5_{100}$ is shown in Figure 4.18, with the relevant restriction sites and oligonucleotide sequences indicated as outlined above.

### 4.2.12 5' RACE of *T. vaginalis mgl1* and *mgl2.*

In order to obtain the sequence covering the 5' untranslated regions (UTRs) of *mgl1* and *mgl2* and to determine the position of the start codons, 5' Reverse Transcriptase Rapid Amplification of cDNA Ends (RT-RACE) was performed. The 5' ends of *mgl1* and *mgl2* were obtained using a commercially available 5' RACE system for the Rapid Amplification of cDNA Ends (Life Technologies) coupled with *T. vaginalis* total RNA that had been isolated from the parasites using the commercially available reagent TRIzol (Life Technologies). The methodology of 5' RACE is summarised in Figure 4.19. Figure 4.20 summarises the 5' RACE protocol performed for *mgl1* and *mgl2.* The location of the gene specific primers used in the two rounds of the RACE-PCR are shown in Figure 4.17 and 4.18. Figure 4.21 shows the amplified 5' RACE products for the *mgl1* and *mgl2* and genes after the second round of amplification using gene specific primers 9 and 4 respectively. Two independent amplifications of the 5' ends of *mgl1* and *mgl2* were performed (lanes 3 and 4, lanes 6 and 7 respectively). Control amplifications using the appropriate gene specific primers for each gene but without reverse

transcriptase were performed for *mgl1* and *mgl2* (lanes 2 and 5, respectively). Both of the *mgl1* and *mgl2* 5'-RACE products were approximately 350bp in size, the size of product expected when the location of each gene specific primer was taken into consideration (see Figures 4.17 and 4.18).

The 350bp 5'-RACE products were cloned directly into pTAg vector of the ligATor kit (Ingenius). This system exploits the feature that PCRs performed in the presence of Taq polymerase have an adenosine residue 5' overhang on the ends of the amplified fragment. This facilitates the cloning of PCR products into a vector which has a complementary thymidine residue overhang. Restriction analysis of transformants revealed that the 5' RACE products had been cloned into pTAg. Sequencing of the RACE clones was carried out on both strands using the -20 primer and the M13 reverse primer (Perkin Elmer). The sequence of the 5' RACE products were found to be identical to the cDNA clones $4_{100}$ and $5_{100}$, but with additional 5' sequence as outlined in Figure 4.17 and 4.18, as indicated by red text.

$4_{100}$ ATTTTTAGACAACATG

$5_{100(1)}$ ACTTTATATAAAAGATG

$5_{100(2)}$ AAAGATG

Sequence analysis of the *mgl1* and *mgl2* RACE clones showed that the 5' ends of each of the genes had been amplified. Only one RACE clone for *mgl1* was obtained. However, the sequence of this clone was identical to the cDNA sequence isolated from

the cDNA library and showed that the ATG codon present at the beginning of this cDNA probably does encode the start methionine of this gene. The 5' RACE of *mgl1* also revealed that the 5' UTR is very short, being only some 13 nucleotides long.

For *mgl2* two independent 5' RACE products were obtained. Both clones possess the methionine start codon, which was absent from the copy of the gene isolated from the cDNA library. The 5' RACE clones for *mgl2* show that the clone isolated from the cDNA library was only 12 base pairs short of the nucleotides encoding methionine start codon. As found with the *mgl1* RACE clone there was a very short 5' UTR region which was only 14 nucleotides in length.

### 4.2.13 Analysis of *mgl1* and *mgl2* sequence.

Once the full nucleotide sequence of each clone was obtained a number of analyses were conducted using various programmes available from the University of Wisconsin, Genetics Computer Group (GCG). The nucleotide sequences of the open reading frames of *mgl1* and *mgl2* were converted into peptide sequence using the TRANSLATE program, and these sequences were compared to two available sequences of methionine γ-lyase from *Pseudomonas putida* and to cystathionine γ-lyase from yeast and human. Both GAP and PILE-UP applications were used to establish the percentage identity and the percentage similarity between the different sequences of interest and also illustrate regions of homology and dissimilarity in peptide sequence. It must be remembered that the methionine γ-lyase sequences of *P. putida* was not available when this project was initiated and they were not used to determine the sequence of the degenerate oligonucleotides used in PCRs. Figure 4.22 shows the pile-up comparison of the peptide sequences of the two *T. vaginalis* methionine γ-lyase homologues, TvMGL1 and

TvMGL2 with methionine γ-lyase sequences from *P. putida* (PpMGL1 and PpMGL2, Inoue *et al.*,1995 and Hori *et al.*, 1996 respectively) and cystathionine γ-lyase from yeast (ScCYS3) (Ono *et al.*,1992) and human (HsCGL) (Lu *et al.*, 1990). Regions of amino acid identity found in all sequences are indicated by black boxes. Amino acid residue identity between the two trichomonad gene products and the two methionine γ-lyases of *P. putida* are shown by a #. Points of interest that arise from the peptide sequence comparisons are as follows:

Firstly, there is significant amino acid identity in the regions to which the degenerate oligonucleotides were designed for the PCRs, as indicated by a solid black line in Figure 4.22. This feature undoubtedly contributed to the success of cloning *T. vaginalis* homologues for cystathionine γ-lyase. Analysis of the amino acid sequence over which the 3'Cyst degenerate oligonucleotide was designed reveals the following features. The amino acids between residues A207 and G213 (numbering is based on that of the TvMGL1 sequence) are identical in all of the sequences compared except for the amino acid residues at positions at 211 and 212. Amino acid residue identity between all of the sequences also extends outside region to where the Cyst 3'degenerate was designed, with residues being identical at S206, H214 and D216. There is such a high degree of conservation in sequence between species within this region because it represents the pyridoxal 5' phosphate (PLP) binding domain of the enzyme (Nakayama *et al.*, 1988). The region between residues V151 and N158 also has a high degree of amino acid identity between the different species, but not as much as the PLP binding region of the enzyme. This second region was selected for the design of the Cyst 5' degenerate oligonucleotides for PCR, as a region which had a high degree of peptide sequence identity between the relevant sequences. It can be seen that there is identity in all

residues of the all of the sequences at positions E153, P155 and N158, however, there are also differences. At positions Y152, F153 and A156 there is identity in all of the residues between the four known methionine γ-lyase sequences, but not in the cystathionine γ-lyases, whereas at position V151 there is identity between the two *T. vaginalis* sequences and the cystathionine γ-lyase sequences of yeast and human.

Secondly, other regions of identity between all of the sequences include the DNT motif between residues 184 and 186, the YPG motif between residues 289 and 291, the SLG motif beginning at residue 338, the ESL motif starting at residue 343 and the SVG motif between 475 and 478. As such regions of peptide sequence are conserved between the methionine γ-lyases of *P. putida*, the cystathionine γ-lyase of yeast and human and the *T. vaginalis* homologues this would intimate that such residues are important for the function of the enzyme. They could for example be important in maintaining a certain tertiary structure that is important in the function of γ-elimination enzymes.

Thirdly, there are also amino acid residues that are exclusively conserved between the methionine γ-lyases of *T. vaginalis* and *P. putida* (as indicated in Figure 4.22). There are 81 residues that are identical between the methionine γ-lyases of *T. vaginalis* and *P. putida* in addition to those conserved in both the cystathionine and methionine γ-lyases, this represents a 20% identity of the residues between the methionine γ-lyases when expressed as a percentage of the total. For example, there is a three amino acid motif beginning at Y30 along with a region between G236 and T241 in which four of the six amino acid residues located in this region are identical between the methionine γ-lyase sequences. There are also identical single amino acid residues present throughout the

methionine γ-lyase sequences, one of these such residues is that of C113 (as indicated by a * in Figure 4.23), which has been implicated in being catalytically important, but not essential (Nakayama *et al.*, 1988) to pyridoxal 5' phosphate binding in the so called γ class of PLP-linked enzymes, as defined by Alexander *et al.*, 1994.

Fourthly, there is a seven amino acid insertion between residues G49 and I55 in all of the methionine γ-lyase sequences when compared to cystathionine γ-lyase from yeast and human.

Table 4.1 shows in tabulated form the % identity and % similarity in amino acid residues between TvMGL1and TvMGL2, the methionine γ-lyases from *P. putida* and cystathionine γ-lyase from yeast and human. Also included in this table are the % identities and similarities between the former amino acid sequences and sequences from other proteins involved in sulphur amino acid metabolism in yeast and *E. coli*, namely *S. cerevisiae O*-acetyl homoserine sulphydrylase (Kerjan *et al.*, 1986) and *E. coli* cystathionine γ-synthase (Duchange *et al.*, 1983) and cystathionine β-lyase (Belfaiza *et al.*, 1986). TvMGL1and TvMGL2 have more sequence identity to each other (69%) than to the methionine γ-lyase and cystathionine γ-lyase from other species (42-44%). It is interesting to note the two methionine γ-lyase gene homologues from *T. vaginalis* are really no more identical, in terms of amino acid residues to methionine γ-lyase from *P. putida* than to cystathionine γ-lyase from yeast or human The percentage identity between TvMGL1 and *P. putida* MGL1 and yeast cystathionine γ-lyase (CTL) is identical at 44% and the percentage identity between TvMGL2 and *P. putida* MGL1 and yeast CTL is 45% and 43% respectively. Amino acid sequence comparison of the two

*T. vaginalis* methionine γ-lyase homologues with the sequences of related sulphur amino acid metabolising enzymes from yeast and *E. coli* reveals that they have lower overall sequence identities and similarities than with the methionine γ-lyase of *P. putida* or cystathionine γ-lyase of *E. coli*. MGL1 has 35%, 37% and 30% identity to yeast *O*-acetyl homoserine sulphydrylase and *E. coli* cystathionine γ-synthase and cystathionine β-lyase respectively, with MGL2 having 38% identity to both yeast *O*-acetyl homoserine sulphydrylase and *E. coli* cystathionine γ-synthase and 30% identity to cystathionine β-lyase of *E. coli*.

### 4.2.14 Genomic organisation and expression of *mgl1* and *mgl2 T. vaginalis* methionine γ-lyase homologues.

The genomic organisation and expression of *mgl1* and *mgl2* was also investigated. Genomic DNA from *T. vaginalis* was digested with a variety of restriction enzymes and resolved by agarose gel electrophoresis prior to transfer to a Hybond N filter (Amersham) by the method of Southern (1975). The immobilised *T. vaginalis* genomic DNA was then subjected to analysis by hybridisation with specific *mgl1* and *mgl2* probes. The *mgl1* probe was made by digesting the $4_{100}$ plasmid with *Eco*RI and *Xho*I, which released a 1kb *Eco*RI/*Xho*I fragment along with a 300bp *Xho*I fragment. It was the larger 1kb fragment that was isolated from this double digest which was radioactively labelled using a random priming method and subsequently used for hybridisation to the *T. vaginalis* genomic DNA. Figure 4.23 illustrates the result of Southern blot analysis of *T. vaginalis* genomic DNA restricted with a variety of restriction enzymes and the *mgl1* specific *Eco*RI/*Xho*I fragment. Table 4.2 summarises

the sizes of the hybridising fragments after Southern analysis. Single hybridising bands for *Bam*HI, *Eco*RI, *Eco*RV, *Pst*I, *Sma*I and *Sph*I indicate that *mgl1* is at single copy in the *T. vaginalis* genome. Restriction of *T. vaginalis* genomic DNA with *Hin*cII and *Hin*dIII results in more than one hybridising band, which is due to the presence of that particular restriction enzyme site in the gene fragment used as the probe for the Southern hybridisation. However, there is more than one hybridising band present for the *T. vaginalis* DNA which was restricted with *Xho*I, this most probably arises as the result of a partial restriction digest of the *T. vaginalis* DNA with this particular enzyme.

Figure 4.24 illustrates the result of Southern blot hybridisation after differently restricted *T. vaginalis* genomic DNA was hybridised with a 800bp fragment of *mgl2*. The *mgl2* probe was made by digesting the $5_{100}$ plasmid with *Eco*RI and *Xho*I which released two fragments of 800bp and 400bp in size. The larger 800bp fragment was gel purified and radioactively labelled using a random priming method and used in hybridisation experiments with *T. vaginalis* genomic DNA. Hybridisation of the radioactively labelled *mgl2* probe to the *T. vaginalis* genomic DNA and the subsequent washing of the filters were performed at high stringency. As can be seen from Figure 4.24 single hybridising bands are obtained with the restriction enzymes *Bam*HI, *Eco*RI, *Hin*cII, *Hin*dIII, *Sma*I and *Sph*I, the sizes of which are summarised in Table 4.3. Such a result indicates that *mgl2* is also at single copy in the *T. vaginalis* genome, with the presence of more than one band with the restriction enzymes *Eco*RV and *Sal*I and being due to the presence of that particular restriction enzyme site within the 800bp *mgl2* probe. When the *Eco*RV restriction sites are considered for the 800bp *mgl2* probe one would expect hybridising fragments of >570bp, 70bp, 120bp and 421bp (See Figure 4.18). The

Southern blot data agrees well with the sizes of hybridising fragments expected, as two bands of 7kb and ~500bp were obtained, the smallest *Eco*RV bands are probably not detected on the Southern blot due to their small size. Once again there is more than one hybridising band present when *T. vaginalis* genomic DNA is restricted with *Xho*I which is probably explained by a partial restriction digest of the DNA with this particular enzyme.

It is also interesting to note from the Southern blots that *Eco*RI restricted *T. vaginalis* genomic DNA gives a 7.5kb hybridising fragment when probed with both the *mgl1* and *mgl2* gene fragments. This may indicate that both *mgl1* and *mgl2* genes fragment are linked. If indeed this is the case then this would suggest that *mgl1* and *mgl2* may have arisen via a gene duplication event. The genomic copies of *mgl1* and *mgl2* have subsequently been cloned by an inverse PCR approach by T. D Edlind, whilst on sabbatical to Dr. Mottram's laboratory. Genomic cloning and sequencing was achieved by *Cla*I digestion of *T. vaginalis* genomic DNA, which resulted in a 3.5 kb *Cla*I fragment being cloned for *mgl1* and a 2.6 kb *Cla*I fragment being cloned for *mgl2*. Sequencing of the two *Cla*I fragments for *mgl1* and *mgl2* has shown that the two genes are not tandemly arranged. (T. D. Edlind, personal communication).

### 4.2.15 Northern blot analysis of *T. vaginalis* total and poly[A]$^+$ RNA with *mgl1* and *mgl2* specific probes.

Expression of *mgl1* and *mgl2* was also investigated. Figure 4.25 shows Northern blot analysis of *T. vaginalis* total and poly[A]$^+$ RNA probed with the *mgl1* and *mgl2* gene fragments, in which the probe material was prepared in the same way as for the Southerns (see above). The two genes are transcribed to give identical size mRNAs of

1.3kb. The size of the transcripts agrees well with the size of the cDNAs isolated from the library and shows that the clones isolated were almost full length.

## 4.3 Discussion

The use of degenerate oligonucleotides designed against conserved regions of protein sequence, in PCRs, is a strategy that has successfully used for the isolation of homologous genes in parasitic protozoa and nematodes (Sakanari *et al.*, 1989). More specifically, cyclin dependent kinases have been isolated from *Trypanosoma brucei* using this approach (Mottram and Smith, 1995), as has a *rho* family gene from *E. histolytica* (Lohia and Samuelson, 1993). Several genes from *T. vaginalis* have also been isolated using this approach, some of these include P-glycoprotein, a 70 kDa cytosolic heat shock protein, β tubulin and the α subunit of succinyl CoA synthetase (all these proteins are referenced in Quon *et al.*, 1994) cysteine proteinase genes (Mallinson *et al.*, 1994) and adenosylhomocysteinase genes (Bagnara *et al.*, 1996).

Due to the success of the cloning of many genes from various parasitic protozoa including *T. vaginalis,* by the degenerate oligonucleotide/ PCR strategy, it was thought that such an approach may also be successful in obtaining *T. vaginalis* methionine γ-lyase gene homologues.

The sequence of the degenerate oligonucleotides used in this study were based on the sequences of human, rat and yeast cystathionine γ-lyase. This approach allowed the isolation of two genes, both of which have high sequence homology to cystathionine g-lyase from a variety of sources and to methionine γ-lyase which was isolated from

*Pseudomonas putida* in 1995 (Inoue *et al.*, 1995 and was sequence information that was not available when this project was initiated).

The reason for the success in isolating the *T. vaginalis* methionine γ-lyase homologues became clear when a comparison of the regions of homology used initially for the design of the degenerate oligonucleotides was performed using the actual protein sequences derived from the two isolated methionine γ-lyase gene homologues.

The region chosen for the design of the 5' degenerate oligonucleotide, a region that had a high degree of sequence homology between all of the different sequences for cystathionine γ-lyase has five out of the eight amino acid residues chosen for the design of the Cyst 5' oligonucleotide that are identical between the designed oligonucleotide and the actual amino acid sequence of the two genes that were isolated. The other three residues W151 and I152 and T156 encoded in the Cyst 5' oligonucleotide are conservative substitutions in *mgl1* and *mgl2* (See Figures 4.1a and 4.1b for reference). Secondly, the pyridoxal 5 phosphate binding region which was chosen for the design of the 3' degenerate oligonucleotide, has five out of seven identical amino acid residues in the sequences that are compared in Figures 4.1a and 4.1b.

There are a number of other points that raise themselves for discussion, when the predicted protein sequences of the two *T. vaginalis* methionine γ-lyase homologues are compared to the protein sequences of methionine γ-lyase from *P. putida*, to cystathionine γ-lyase from both yeast and human and also to other related pyridoxal 5'-phosphate linked enzymes from *E. coli* and yeast.

Firstly, the two *T. vaginalis* genes are only 69% identical to one another (Table 4.1)an inference that can be made from this comparison is that the two methionine γ-lyase cDNAs isolated from *T. vaginalis* are unlikely to be allelic, as they have a low degree of identity to one another.

Secondly, MGL1 has the same % identity to both methionine γ-lyase from *P. putida* and cystathionine γ-lyase from yeast (44%), whereas MGL2 has a slightly higher identity to methionine γ-lyase 1 from *P. putida* (45%) and a slightly lower identity to yeast cystathionine γ-lyase (43%). The two *T. vaginalis* methionine γ-lyase homologues also have significant percentage identities to other related pyridoxal 5'-phosphate linked enzymes involved in sulphur amino acid metabolism in yeast and *E. coli* (see Table 4.1). Thus, it is difficult to deduce, based on sequence comparison data what enzyme is encoded by the two *T. vaginalis*. The fundamental question that needs to be addressed is as follows:-Do the genes in *T. vaginalis* encode a methionine γ-lyase or do they encode a cystathionine γ-lyase or do they encode both enzyme activities?

In their 1995 paper Inoue *et al.*, report the primary structure of methionine γ-lyase from *P. putida*, as deduced from its nucleotide sequence. They show that methionine γ-lyase from *P. putida* has extensive homology with other known γ elimination and γ-replacement PLP dependent enzymes, such as cystathionine γ-lyase (Ono *et al.*, 1992), cystathionine γ synthase (Duchange *et al.*, 1983) and O-acetylhomoserine O-acetylserine sulphydrylase (Kerjan *et al.*, 1986) The percentage similarity of amino acid residues between the enzymes outlined above, *P. putida* methionine γ-lyase and the *T. vaginalis* homologues are at the same order of magnitude (38%-27%, see Table 4.1 for details) as

reported by Inoue *et al.*, 1995. Figure 4.26 shows an amino acid residue comparison between all of the PLP-linked enzymes mentioned above and cystathionine β-lyase from *E. coli* (Belfaiza *et al*, 1986) This figure illustrates the amino acid residues that are conserved between all of the sequences. The conservation of certain residues in all of the PLP-linked enzyme sequences intimate that these residues are important for maintaining the general structural or catalytic characteristics of γ-elimination enzymes. Recently, this has been confirmed by an analysis of the 3D structure of cystathionine β-lyase of *E. coli* which has recently been crystallised (Clausen *et al.*,1996). Cystathionine β-lyase, even though it catalyses β-elimination and replacement reactions has been attributed to the so called g class of PLP-linked enzymes as described by Alexander *et al.*, 1994. Points of interest that arise from the 3D structure of cystathionine β-lyase, which may be relevant to future determinations of the crystal structure of methionine γ-lyase from *T. vaginalis* are as follows: Firstly, the N-terminal domain of cystathionine β-lyase is composed of three α-helices and 1β-strand, this domain contributes to the tetramer formation of the protein and a part of its active site. The second domain of the protein harbours the cofactor, pyridoxal 5'-phosphate and the C-terminal domain is connected by a long α-helix to the PLP-binding domain and consists of four helices packed on a solvent accessible site of an anti parallel four stranded β-sheet. Specific residues that have been implicated in the catalytic function of cystathionine β-lyase are as follows: Arginine 59 anchors the distal carboxylate group of the cystathionine substrate, Alanine 87 forms a hydrogen bond with the second phosphate group of the PLP cofactor and finally Tyrosine 338 is one of three aromatic residues which may be involved in active site screening. The 3D structure of

cystathionine β-lyase will provide a useful base for any future molecular modelling with *T. vaginalis* methionine γ-lyase.

As can be seen the regions of homology are distributed throughout all the amino acid sequences, which suggests that the genes encoding the above proteins may have evolved from a common ancestor. This is a theory that has already been proposed by Belfaiza *et al.*, 1986 to explain similarities between the methionine biosynthetic enzymes of *E. coli*. It is interesting to note that the O-acetylhomoserine sulphydrylase from yeast has two distinct insertions in its sequence starting at positions D224 and D356, it is probable that these insertions contribute highly to the enzymatic specificity of this particular molecule, which involves the synthesis of homocysteine from OAH and a sulphur donor, such as hydrogen sulphide, this enzyme function may have arisen by an additional gene duplication event when compared to the other enzymes involved in transulphuration pathways.

Figure 4.26 also illustrates differences in amino acid residues between the different proteins, which one could imagine, arose through mutations in the common ancestral gene and therefore lead to the specialisation of the encoded proteins. When one looks more specifically at the amino acid sequences of the *T. vaginalis* methionine γ-lyase homologues, the *P. putida* methionine γ-lyase sequences and the sequences of cystathionine γ-lyase from yeast and human there are a number of points that raise themselves for discussion.

Firstly, as can be seen from Figure 4.22 and as outlined in the results section of this chapter there are residues in the sequences of the methionine γ-lyases from *T. vaginalis* and *P. putida* that are identical, for example Y29, Q30 and T31, the residues at the same positions in the yeast and human cystathionine γ-lyase are S29, L30 and S31. This comparison of the amino acid residues at this particular region in the molecules shows immediately that the hydrophobic tyrosine residue at position 29 in the methionine γ-lyases has changed to a hydrophilic serine residue in the cystathionine γ-lyases. The differences between the other residues in the two enzymes are more conservative substitutions with the glutamine at position 30 and the threonine at position 31 being substituted for leucine and serine residues respectively. These particular differences between the methionine γ-lyases of *T. vaginalis* and *P. putida* support the view that mutations in an ancestral gene could account for enzyme specialisation, with for example, the hydrophobic for hydrophilic residue substitution at position 30, being important for the catalytic breakdown of methionine or for maintaining a hydrophobic region in the tertiary structure of the protein. Such a view may be similarly applied to other differences or similarities in amino acid residues between the methionine γ-lyases of *T. vaginalis* and *P. putida* and the cystathionine γ-lyases of yeast and human.

Secondly, earlier studies by Nakayama *et al.*, 1988a and b show that methionine γ-lyase from *P. putida* contains a four cysteine residues in each subunit of which Cys 116 is catalytically important but not essential and is located close the PLP binding site of the enzyme. Cyanylation of Cys 116 resulted in substantial inactivation of methionine g-lyase with respect to both the αγ and αβ elimination reactions. The authors' sequence comparison with other various γ elimination and γ replacement pyridoxal 5' phosphate

dependent enzymes (mentioned above, Inoue *et al.*, 1995) show that this important cysteine residue is not conserved in these enzymes. The authors infer that it this unique cysteine residue in methionine γ-lyase of *P. putida* that makes it different from the other pyridoxal 5' phosphate sulphur amino acid metabolising enzymes to which it is compared. What is really interesting is that both of the *T. vaginalis* methionine γ-lyase homologues contain a cysteine residue at the same position (C113) and this cysteine residue is not conserved in the cystathionine γ-lyase genes of yeast or human. An inference from this is that the two genes isolated from *T. vaginalis* may well encode methionine γ-lyase and it is this essential cysteine residue that makes the molecule a methionine γ-lyase rather than a cystathionine γ-lyase. However, such a suggestion should be considered with caution as it is unlikely that a single amino acid residue difference at the active site/PLP binding region of the enzyme could wholly contribute to different substrate specificities between methionine γ-lyase and cystathionine γ-lyase, especially as there are several other amino acid residues that are unique to the methionine γ-lyases of *T. vaginalis* and *P. putida*.

Methionine γ-lyase in anaerobic bacteria such as *P. putida* and in anaerobic protists such as *T. vaginalis* may have evolved before cystathionine γ-lyase and indeed there is no cystathionine γ-lyase present in bacteria. Certainly, it has been shown that *T. vaginalis* is an ancient eukaryote, based on a number of phylogenetic studies (Sogin, 1993 and Doolittle *et al.*,1996 ) and that they branched early during eukaryote evolution. The phylogenetic position of *T. vaginalis* may explain why this ancient organism possesses methionine catabolising capabilities that are identical to anaerobic prokaryotes and why

more recently evolved organisms such as yeast and humans possess cystathionine γ-lyase rather than methionine γ-lyase.

Presence of a string of A residues at the 3' end of the cDNAs indicated that the cDNA was derived from a polyadenylated mRNA and was not a genomic DNA fragment. The$4_{100}$ cDNA had an insert of 1235bp, had a methionine start codon at its 5' end but had no sequence upstream of this start codon. The $5_{100}$ cDNA gene isolated, in contrast, was 1287bp in length but was missing a methionine start codon and therefore any upstream 5' sequence. The initial isolation and preliminary sequencing of the two cDNAs lead to the thought that $4_{100}$ possessed its true methionine start and that it was missing only a few nucleotides that would make up the 5'untranslated region (5'UTR) of the gene. Also, it was thought that $5_{100}$ was only missing the nucleotides that would comprise the methionine start site of the gene along with the 5'UTR. The belief that the two genes were probably almost full length was supported by the Northern blot data generated for the two genes. Northern blots showed that both *mgl1* and *mgl2* were being actively transcribed to transcript sizes of 1.3kb, a size that is only slightly larger than the insert sizes of the $4_{100}$ and $5_{100}$ cDNAs.

5' RACE-PCR was performed to confirm the methionine start site for the *mgl1* transcript and to determine the methionine codon for *mgl2* and to obtain the 5'UTR for transcripts for both genes, the 5' ends of the *mgl1* and *mgl2* transcripts were obtained by 5' RACE confirmed that the start codon present in the copy of *mgl1* cDNA isolated from the *T. vaginalis* library was indeed genuine. 5' RACE also revealed the methionine start codon of the *mgl2* transcript and confirmed that the clone isolated from the cDNA library was only twelve nucleotides of being full length (see Figure 4.18).

The 5' UTRs of *mgl1* and *mgl2* transcripts are thirteen and fourteen nucleotides respectively. The short 5' ends of *mgl1* and *mgl2* agrees with the short 5'UTR data that have been characteristically obtained for other *T. vaginalis* genes. Quon *et al.*, (1994) have characterised seven protein coding genes from *T. vaginalis* and have revealed the presence of a highly conserved DNA sequence motif immediately upstream of the coding region of these genes. They also found no typical TATA box was present at a position of 25-30 nucleotides upstream of the transcription start sites of these genes, as found in many other eukaryotic genes. Quon *et al.*, (1994) propose that the conserved motif in their seven protein coding genes acts as an initiator (Inr) element that is able to recruit RNA polymerase and associated transcription factors necessary for accurate transcription initiation. Such initiator elements are also found in mammalian genes that lack a traditional TATA motif and in such TATA-less promoters, the Inr element is critical in positioning RNA polymerase II (for review see Weis and Reinberg, 1992).

Outlined in Figure 4.27 are the conserved nucleotide sequences surrounding the transcription initiation site of the seven protein coding genes as characterised by Quon *et al*, 1994 and these are compared to the 5' UTRs of *mgl1*and *mgl2* transcripts as characterised by 5' RACE (see Figures 4.21 and 4.22).

As can be seen from Figure 4.27 the 5' UTRs characterised from transcripts derived from the genes *mgl1* and *mgl2,* even though characteristically short, appear to share only part of the consensus as drawn up by Quon *et al.*, 1994. The explanation for this may be that the genes isolated by Quon *et al.*, 1994 represent a bias in the type of proteins for which they are encoding. For example three of the seven proteins are derived from the hydrogenosome of the parasite and it may be that proteins present in this unusual

organelle possess a characteristic 5'UTR that are not found in other protein coding genes. Indeed support for this view is provided by the same group of workers in some of their later work. Quon *et al*, 1996 have characterised the 5' end of an RNA polymerase II gene from *T. vaginalis* and found that only 5 out of 13 bp match the consensus motif outlined above, also Bagnara *et al.*, 1996 comments on the breakdown of the consensus of the initiator region of *T. vaginalis* genes as more *T. vaginalis* genes are characterised.

The isolation and sequencing of DNA surrounding genomic copies of the two genes would confirm the site of initiation for *mgl1* and *mgl2* and would confirm the sequence obtained by 5'RACE. It would also allow characterisation of the upstream flanking sequence of the two genes and indeed confirm whether a typical TATA motif is present in these two methionine γ-lyase gene homologues from *T. vaginalis*. It is also interesting to note that short 5'UTRs are characteristic of other ancient parasitic anaerobic protists, namely *Entamoeba histolytica* (Edman *et al.*, 1987 and 1990 and Huber *et al.*, 1987 and 1988) and *Giardia lamblia* (Kirk-Mason *et al.*, 1989, Alonso and Peattie, 1992 and Ey *et al.*, 1993). Thus *T. vaginalis* and these other organisms can be used to study the molecular properties underlying transcription initiation in early diverging eukaryotes.

In conclusion, the data presented in this chapter indicate that two *T. vaginalis* methionine γ-lyase gene homologues have been isolated, they have extremely short 5' untranslated regions, are at single copy in the *T. vaginalis* genome and have a high degree of sequence identity and similarity to genes from a range of other organisms that are involved in transulphuration pathways.

**Figure 4.1a Multiple protein sequence alignment of cystathionine γ-lyase from human,** HsCGL (Lu *et al.*, 1992), **rat,** RnCGL (Erickson *et al.*, 1990) and **yeast,** CYS3 (Ono *et al.*, 1992). Regions of peptide sequence chosen for the design of degenerate oligonucleotides are indicated by the black boxes. Amino acid residue number for each sequence are indicated on the left and right-hand sides of the sequences. * indicate stop codons and (.) show gaps introduced to maximise the sequence alignment. The multiple sequence alignment was obtained by using the PILE-UP program of the University of Wisconsin Genetics Computer Group (GCG).

```
HsCGL     1 MQEKDASSQG FLPHFQHFAT QAIHVGQDPE QWTSRAVVPP ISLSTTFKQG 50
RnCGL     1                                          CCGAA HLLATTFKQD 15
ScCYS3    1          M TLQESDKFAT KAIHAGEHVD VHGS..VIEP ISLSTTFKQS 39

HsCGL    51 APGQHSG.FE YSRSGNPTRN CLEKAVAALD GAKYCLAFAS GLAATVTITH 99
RnCGL    16 SPGQSSG.FV YSRSGNPTRN CLEKAVAALD GAKHCLTFAR GLAATTTITH 64
ScCYS3   40 SPANPIGTYE YSRSQNPNRE NLERAVAALE NAQYGLAFSS GSATTATILQ 89

HsCGL   100 LLKAGDQIIC MDDVYGGTNR YFRQVASEFG LKISFVDCSK IKLLEAAITP 149
RnCGL    65 LLKAGDEVIC MDEVYGGTNR YFRRVASEFG LKISFVDCSK TKLLEAAITP 114
ScCYS3   90 SLPQGSHAVS IGDVYGGTHR YFTKVANAHG VETSFTN.DL LNDLPQLIKE 140

HsCGL   150 ETKLVWIETP TNPTQKVIDI EGCAHIVHKH G...DIILVV DNTFMSPYFQ 196
RnCGL   115 QTKLVWIETP TNPTLKLADI KACAQIVHKH K...DIILVV DNTFMSAYFQ 161
ScCYS3  141 NTKLVWIETP TNPTLKVTDI QKVADLIKKH AAGQDVILVV DNTFLSPYIS 190

HsCGL   197 RPLALGADIS MYSATKYMNG HSDVVMGLVS VNCESLHNRL RFLQNSLGAV 246
RnCGL   162 RPLALGADIC MCSATKYMNG HSDVVMGLVS VNSDDLNERL RFLQNSLGAV 211
ScCYS3  191 NPLNFGADIV VHSATKYING HSDVVLGVLA TNNKPLYERL QFLQNAIGAI 240

HsCGL   247 PSPIDCYLCN RGLKTLHVRM EKHFKNGMAV AQFLESN.PW VEKVIYPGLP 296
RnCGL   212 PSPFDCYLCC RGLKHCRSGW RNTFQDGMAV ARFLESN.PR VEKVIYPGLP 261
ScCYS3  241 PSPFDAWLTH RGLKTLHLRV RQAALSANKI AEFLAADKEN VVAVNYPGLK 290

HsCGL   297 SHPQHELVKR QCTGC..TGM VTFYIKGTLQ HAEIFLKNLK LFTLAESLGG 344
RnCGL   262 SHPQHELAKR SARAC..PGM VSFYIKGTLQ HAQVFLKNIK LFALAESLGG 309
ScCYS3  291 THPNYDVVLK QHRDALGGGM ISFRIKGGAE AASKFASSTR LFTLAESLGG 340

HsCGL   345 FESLAELPAI MTHASVLKND RDVLGISDTL IRLSVGLEDE EDLLEDLDQA 394
RnCGL   310 YESLAELPAI MTHASVPEKD RATLGISDTL IRLSVGLEDE KDLLEDLGQA 359
ScCYS3  341 IESLLEVPAV MTHGGIPKEA REASGVFDDL VRISVGIEDT DDLLEDIKQA 390

HsCGL   395 LKAAHPPSGI HS* 406
RnCGL   360 LKAAHP*       365
ScCYS3  391 LKQATN*        396
```

Cyst 5` degenerate oligonucleotide. 31 nucleotides, 3 inosines.

```
Amino acid residue                          V  W  I  E  T  P  T  N
Sense nucleotide sequence 5′ GCAAGCTTGTITGGATTGAGACICCIACGAA 3′
                             HindIII        C           A
                                            A           T
                                                        C
```

Cyst 3` degenerate oligonucleotide. 28 nucleotides, 2 inosines.

```
                                                  M
Amino acid residue                      A  T  K  Y  I  N  G
Sense nucleotide sequence          5′ GCTACIAAGTACATIAACGG 3′
                                      C     A  T
                                      G
                                      A

antisense nucleotide          5′ GCCTCGAGCCGTTIATGTACTTIGTAGC 3′
sequence                         XhoI         A  T     G
                                                       C
                                                       T
```

**Figure 4.1b Degenerate oligonucleotides designed to conserved regions of cystathionine γ-lyase from yeast, rat and human** (see Figure 4.1a). The complementary nucleotide sequence and encoded amino acid residues of the Cyst 3' degenerate oligonucleotide are shown below the antisense nucleotide sequence of this oligonucleotide. Inosine residues (I) were introduced at some positions of 4 fold degeneracy.

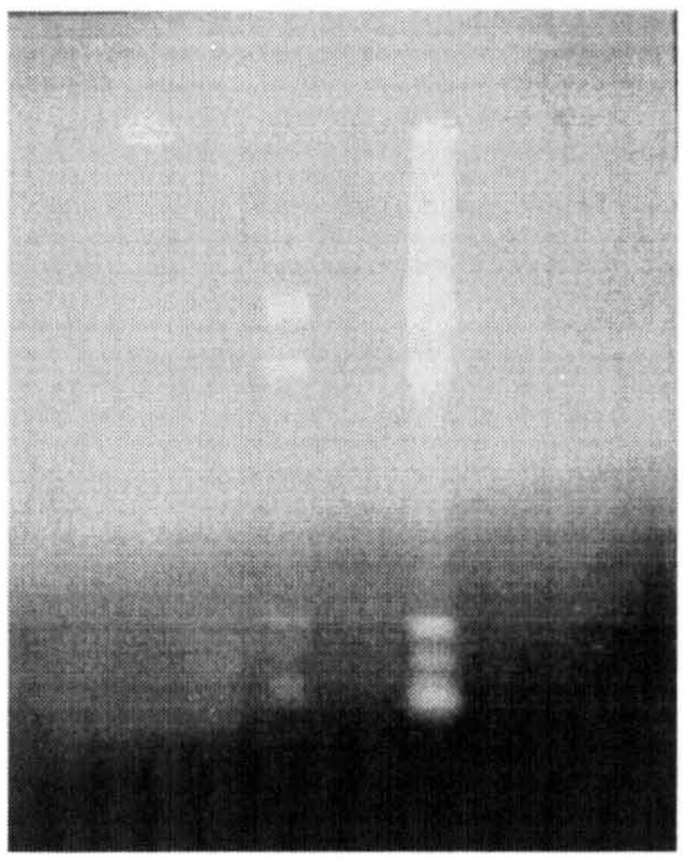



**Figure 4.2 Northern analysis of *T. vaginalis* total and poly [A]$^+$ RNA.**

1.5% non-denaturing agarose gel showing *T.vaginalis* total and poly[A]$^+$ RNA, stained with ethidium bromide and photographed under UV illumination.

Lane 1: 1μg *T. vaginalis* poly [A]$^+$ RNA

Lane 2: 1μg *T. vaginalis* total RNA

Lane 3: 4μg *T. vaginalis* total RNA

No markers were run on this gel

**Figure 4.3 PCR of *T. vaginalis* cDNA with degenerate primers designed to cystathionine γ-lyase.**

PCRs of *T. vaginalis* first strand cDNA or a control first strand cDNA using the degenerate oligonucleotides Cyst 5'and/or Cyst 3'were electrophoresed in a 2% TBE agarose gel, stained with ethidium bromide and photographed under UV illumination.

Lanes 1 and 8: 1kb ladder (Life Technologies)

Lane 2: Cyst 5' degenerate oligonucleotide and *T. vaginalis* first strand cDNA

Lane 3: Cyst 5' degenerate oligonucleotide and control reaction cDNA synthesis

Lane 4: Cyst 3' degenerate oligonucleotide and *T. vaginalis* first strand cDNA

Lane 5: Cyst 3' degenerate oligonucleotide and control reaction cDNA synthesis

Lane 6: Cyst 5' and Cyst 3' degenerate oligonucleotides and *T. vaginalis* first strand cDNA

Lane 7: Cyst 5' and Cyst 3' degenerate oligonucleotides and control reaction cDNA synthesis

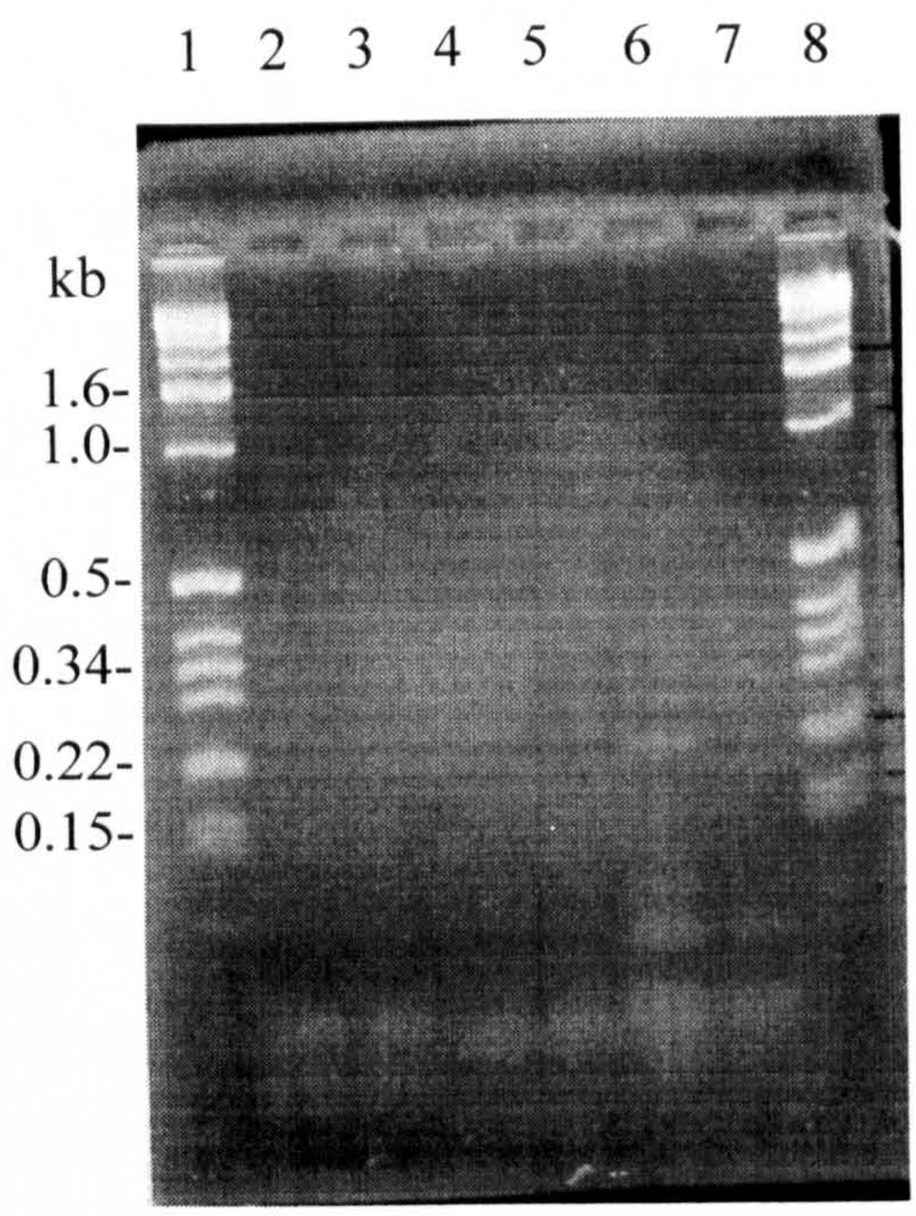

1 2 3 4 5 6 7 8
kb
1.6-
1.0-
0.5-
0.34-
0.22-
0.15-

**Figure 4.4 PCR of *T. vaginalis* cDNA.**

PCRs were electrophoresed in a 2% TBE agarose gel, stained with ethidium bromide and photographed under UV illumination.

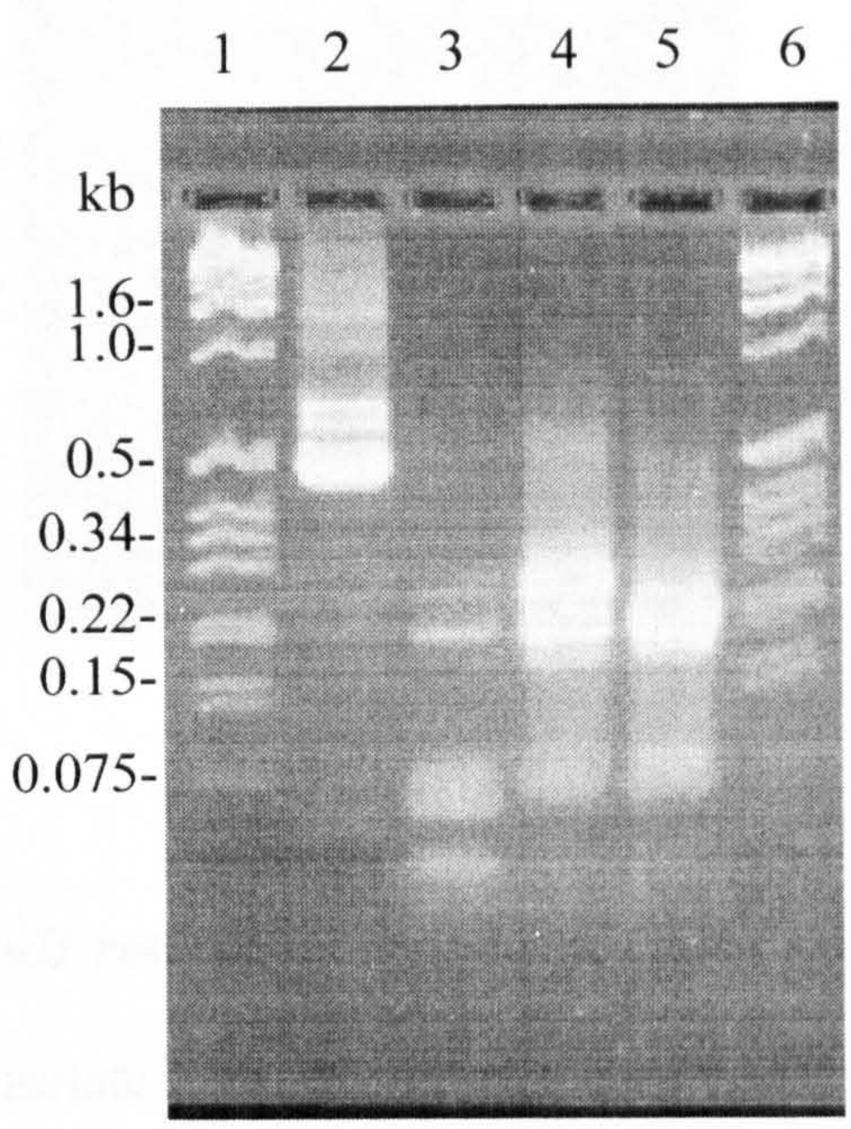
Lanes 1 and 6: 1kb ladder (Life Technologies)

Lane 2: Positive control PCR using *T. vaginalis* first strand cDNA and primers used for the isolation of TvCP1, a cysteine proteinase gene (Mallinson *et al.*, 1994).

Lane 3: *De novo* PCR using *T. vaginalis* first strand cDNA and Cyst 5' and Cyst 3' degenerate oligonucleotides

Lane 4: PCR using Cyst 5' and Cyst 3' degenerate oligonucleotides and gel purified upper band DNA from Figure 4.3, lane 4

Lane 5: PCR using Cyst 5' and Cyst 3' degenerate oligonucleotides and gel purified lower band DNA from Figure 4.3, lane 5


1 2 3 4 5 6
kb
1.6-
1.0-
0.5-
0.34-
0.22-
0.15-
0.075-

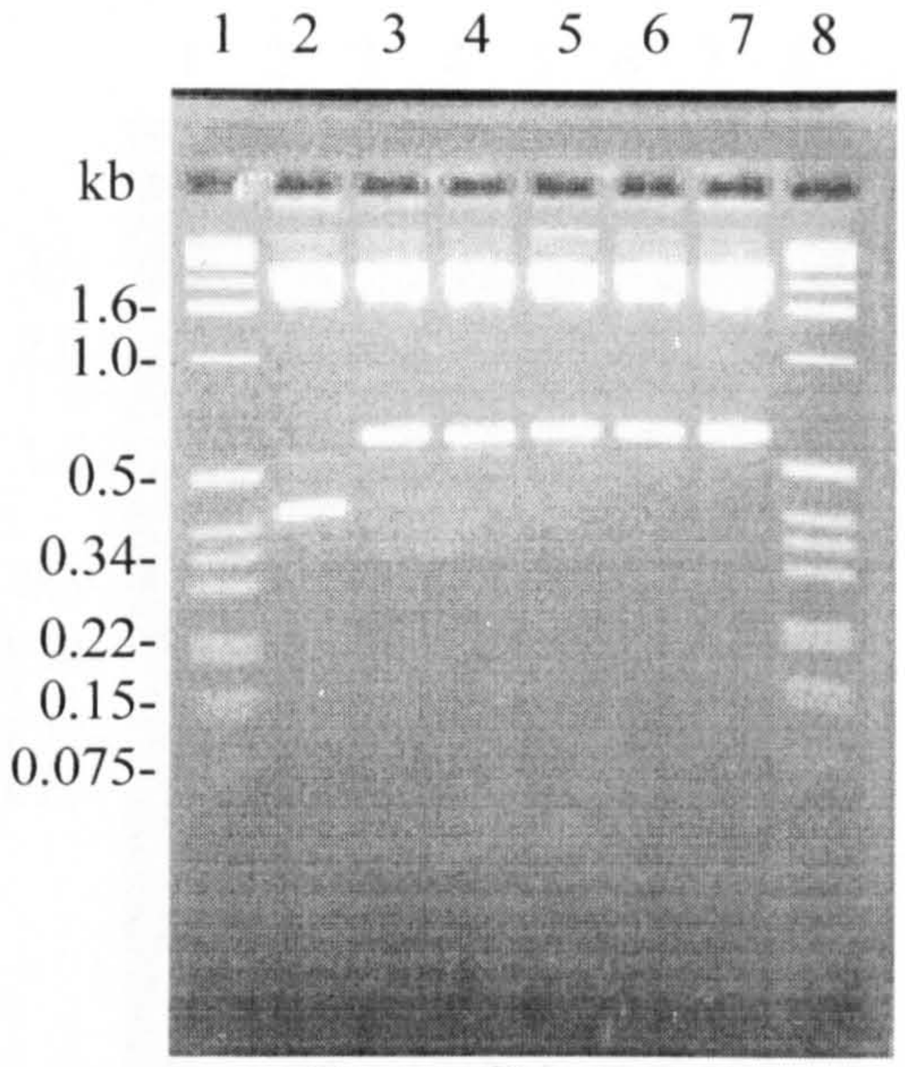



**Figure 4.5 *Pvu*II restriction analysis of Cysta clones .**

Plasmid DNA isolated from white XL1-Blue transformants was restricted with *Pvu*II and electrophoresed on a 2% TBE agarose gel, stained with EtBr and visualised under UV illumination.

Lanes 1 and 8: 1kb ladder (Life Technologies)
Lane 2: pBluescript (SK-) digested with *Pvu*II
Lane 3: Cysta 3 restricted with *Pvu*II
Lane 4: Cysta 4 restricted with *Pvu*II
Lane 5: Cysta 5 restricted with *Pvu*II
Lane 6: Cysta 6 restricted with *Pvu*II
Lane 7: Cysta 7 restricted with *Pvu*II

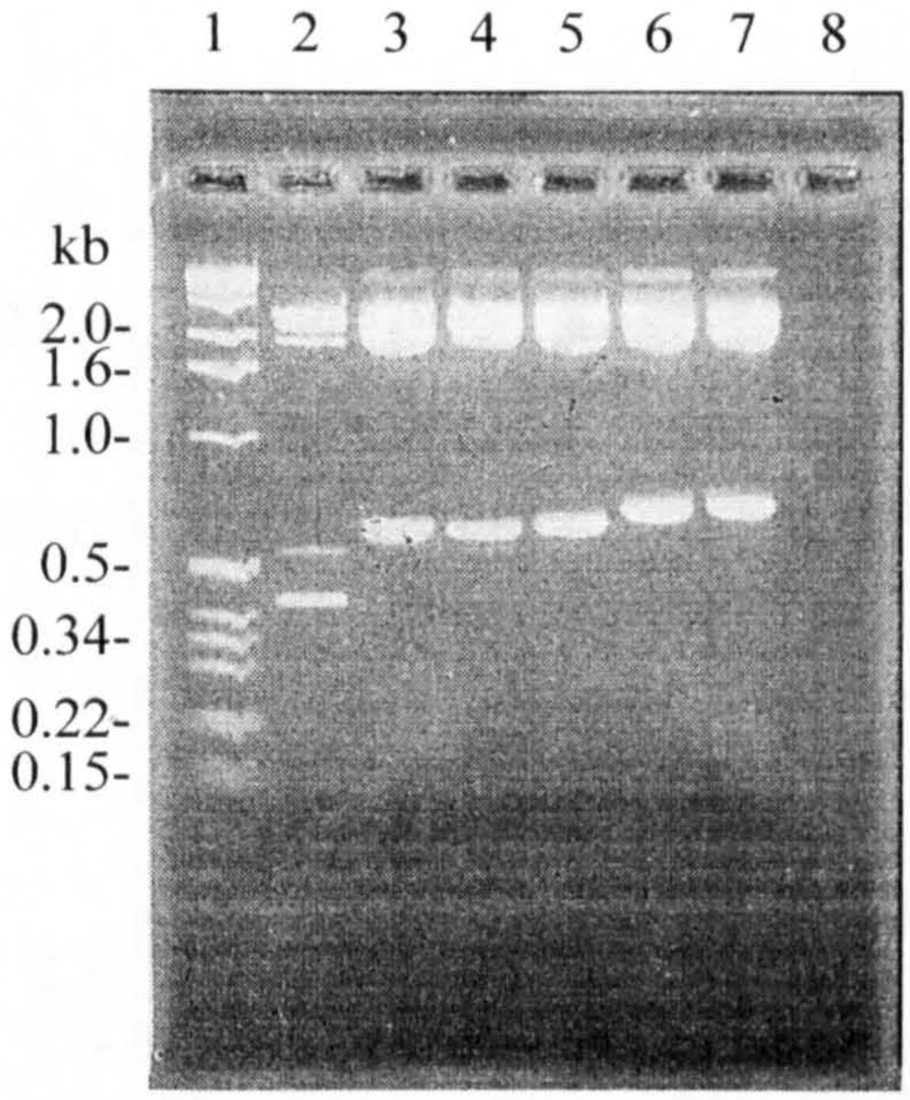



**Figure 4.6 *Pvu*II restriction analysis of Cysta clones.**

Plasmid DNA isolated from white XL1-Blue transformants was restricted and electrophoresed on a 1.5% TBE agarose gel, stained with EtBr and visualised under UV illumination.

Lane 1: 1kb ladder (Life Technologies)
Lane 2: pBluescript (SK-) digested with *Pvu*II
Lane 3: Cysta 9 restricted with *Pvu*II
Lane 4: Cysta 10 restricted with *Pvu*II
Lane 5: Cysta 11 restricted with *Pvu*II
Lane 6: Cysta 12 restricted with *Pvu*II
Lane 7: Cysta 13 restricted with *Pvu*II

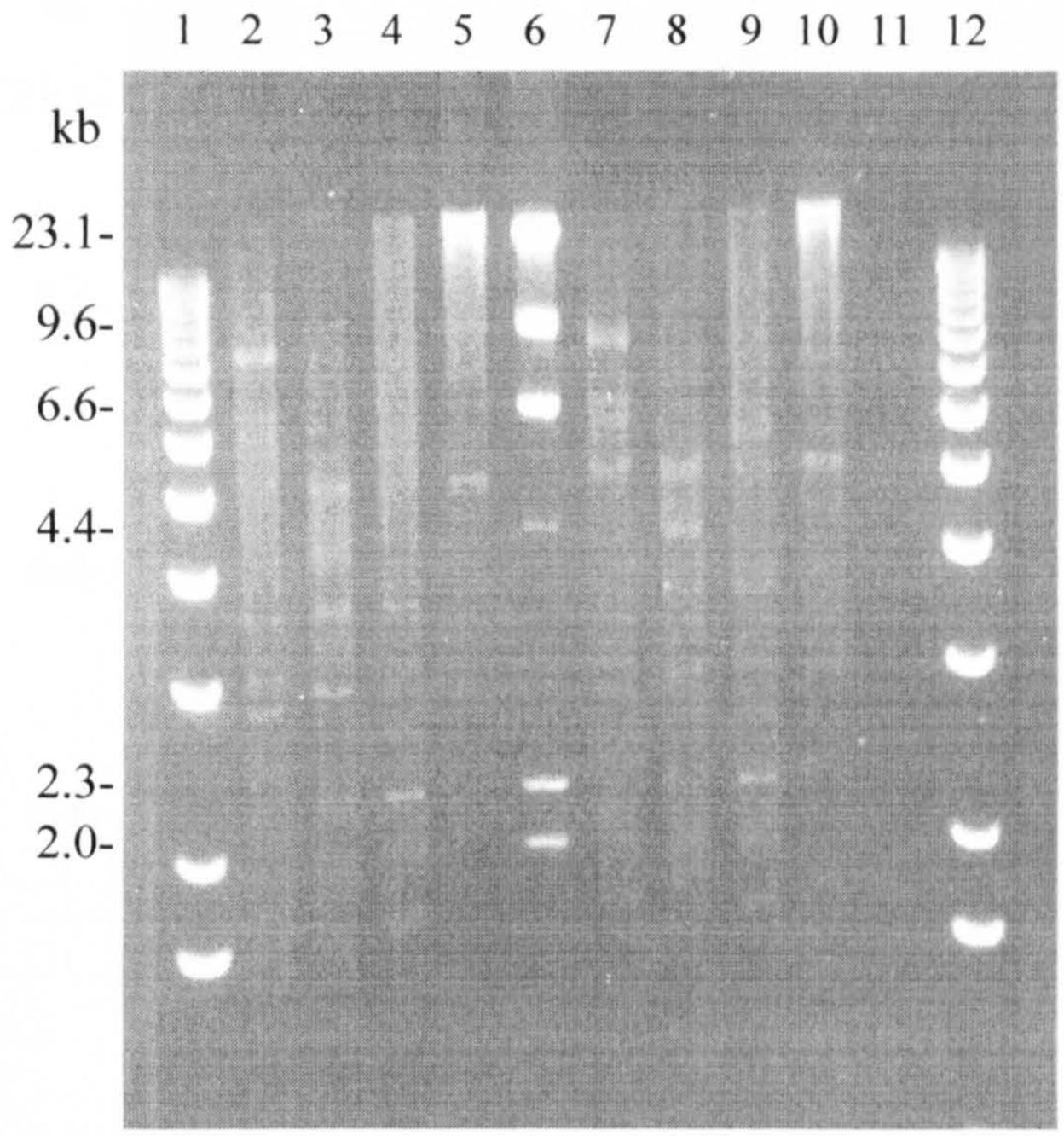



**Figure 4.7 *T. vaginalis* genomic DNA restrictions.**

Restricted *T. vaginalis* genomic DNA was electrophoresed on a 0.7% TBE agarose gel, stained with EtBr and visualised under UV illumination.

Lanes 1 and 12: 1kb ladder (Life Technologies)

Lane 6: λ *Hin*dIII marker (Life Technologies)

Lanes 2 and 7: *T. vaginalis* genomic DNA (2μg) digested with *Eco*RI

Lanes 3 and 8: *T. vaginalis* genomic DNA (2μg) digested with *Hin*cII

Lanes 4 and 9: *T. vaginalis* genomic DNA (2μg) digested with *Hin*dIII

Lanes 5 and 10: *T. vaginalis* genomic DNA (2μg) digested with *Xba*I

Size of markers to the left are λ*Hin*dIII (Lane 6)

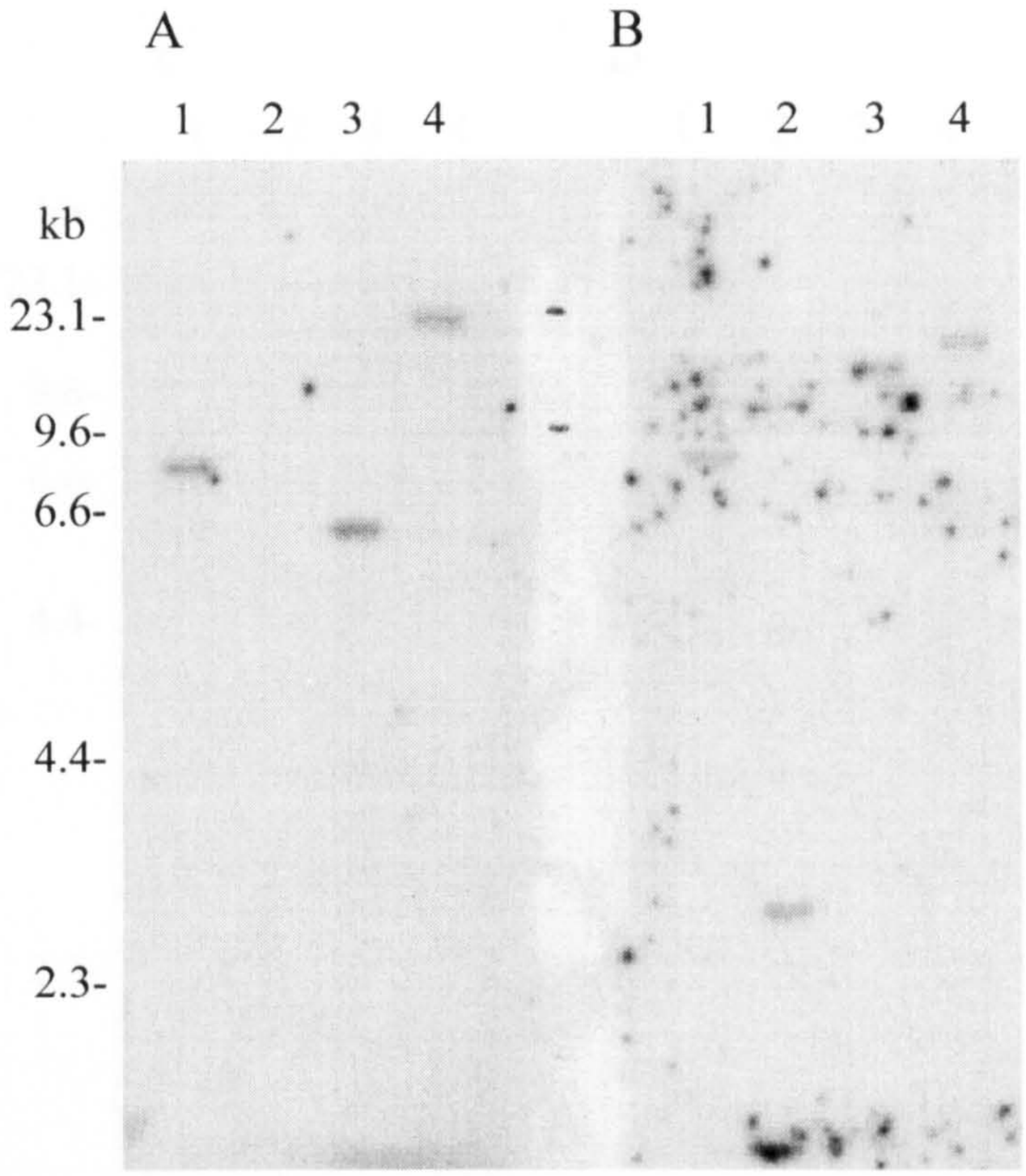



**Figure 4.8A/B Southern blot analysis of *T.vaginalis* genomic DNA with 200bp PCR generated fragment from Cysta 9** (Panel A) **and Cysta 8** (Panel B).

Digested *T. vaginalis* genomic DNA (2μg) was electrophoresed through a 0.7% TBE agarose gel, blotted to Hybond N (Amersham International plc), hybridised with the radiolabelled 200bp fragment from Cysta 9 or Cysta 8 and washed at high stringency prior to exposure to medical X-ray film.

Lane 1: *T. vaginalis* DNA digested with *Eco*RI
Lane 2: *T. vaginalis* DNA digested with *Hinc*II
Lane 3: *T. vaginalis* DNA digested with *Hin*dIII
Lane 4: *T. vaginalis* DNA digested with *Xba*I
Markers indicated on the left are λ*Hin*dIII (Life Technologies)

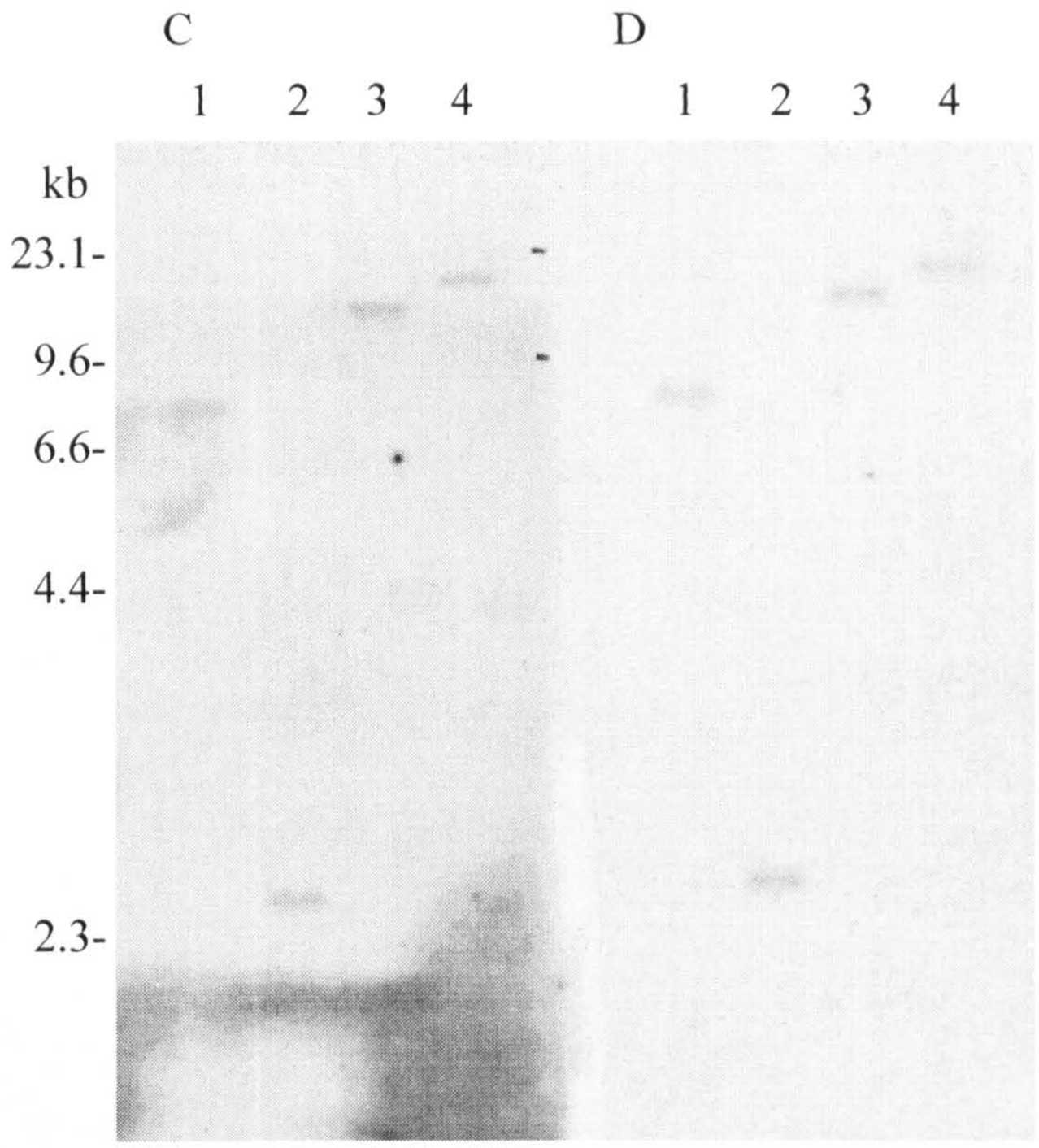



**Figure 4.8 C/D Southern blot analysis of *T.vaginalis* genomic DNA with 200bp PCR generated fragments from Cysta 14** (Panel C) **or a 200bp fragment fromCysta 16** (Panel D).

The digested *T.vaginalis* genomic DNA (2µg) was electrophoresed through a 0.7% TBE agarose gel, blotted to Hybond N (Amersham International plc), hybridised with the radiolabelled 200bp fragment from Cysta 14 or Cysta 16 and washed at high stringency prior to exposure to medical X-ray film.

Lane 1: *T. vaginalis* DNA digested with *Eco*RI

Lane 2: *T. vaginalis* DNA digested with *Hinc*II

Lane 3: *T. vaginalis* DNA digested with *Hin*dIII

Lane 4: *T. vaginalis* DNA digested with *Xba*I

Markers indicated on the left hand side are λ*Hin*dIII (Life Technologies)

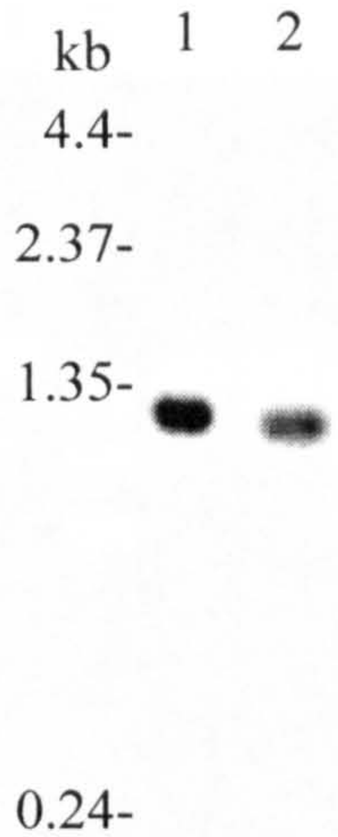



**Figure 4.9 Northern blot analysis of *T. vaginalis* total RNA and poly[A]$^+$ RNA hybridised with a 600bp *Pvu*II fragment of the Cysta 2 clone.**

*T. vaginalis* total and poly[A]$^+$ RNA were electrophoresed through a 1.5% agarose/MOPS/formaldehyde gel, blotted to Hybond N (Amersham International plc), hybridised with the 600bp *Pvu*II fragment from the Cysta 2 clone and washed at high stringency prior to exposure to medical X-ray film.

Lane 1: 10µg *T. vaginalis* total RNA

Lane 2: 1µg *T. vaginalis* poly[A]$^+$ RNA

9.5 kb- 0.24 kbRNA ladder (Life Technologies) are indicated on the left.

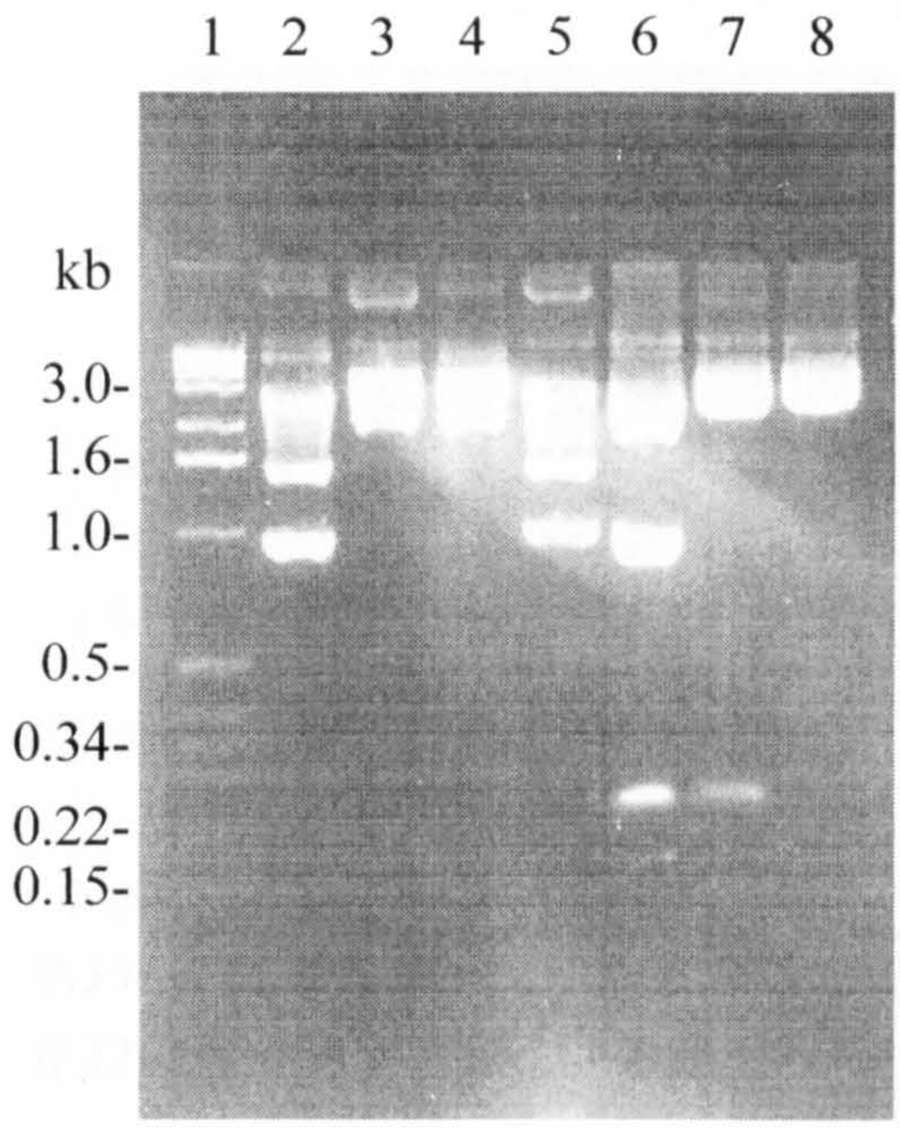



**Figure 4.10 Restriction enzyme analysis of $p1_{100}$ and $p4_{100}$, Cysta 2 hybridising plasmids.**

$p1_{100}$ and $p4_{100}$ DNA was restricted with various restriction enzymes as outlined below and electrophoresed in a 1.5% TBE agarose gel, stained with EtBr and visualised under UV illumination.

Lane 1: 1kb ladder (Life Technologies)

Lane 2: $p1_{100}$ digested with *Eco*RI and *Xho*I

Lane 3: $p1_{100}$ digested with *Xho*I

Lane 4: $p1_{100}$ digested with *Eco*RI

Lane 5: $p1_{100}$ digested with *Spe*I and *Kpn*I

Lane 6: $p\,4_{100}$ digested with *Eco*RI and *Xho*I

Lane 7: $p4_{100}$ digested with *Xho*I

Lane 8: $p4_{100}$ digested with *Eco*RI

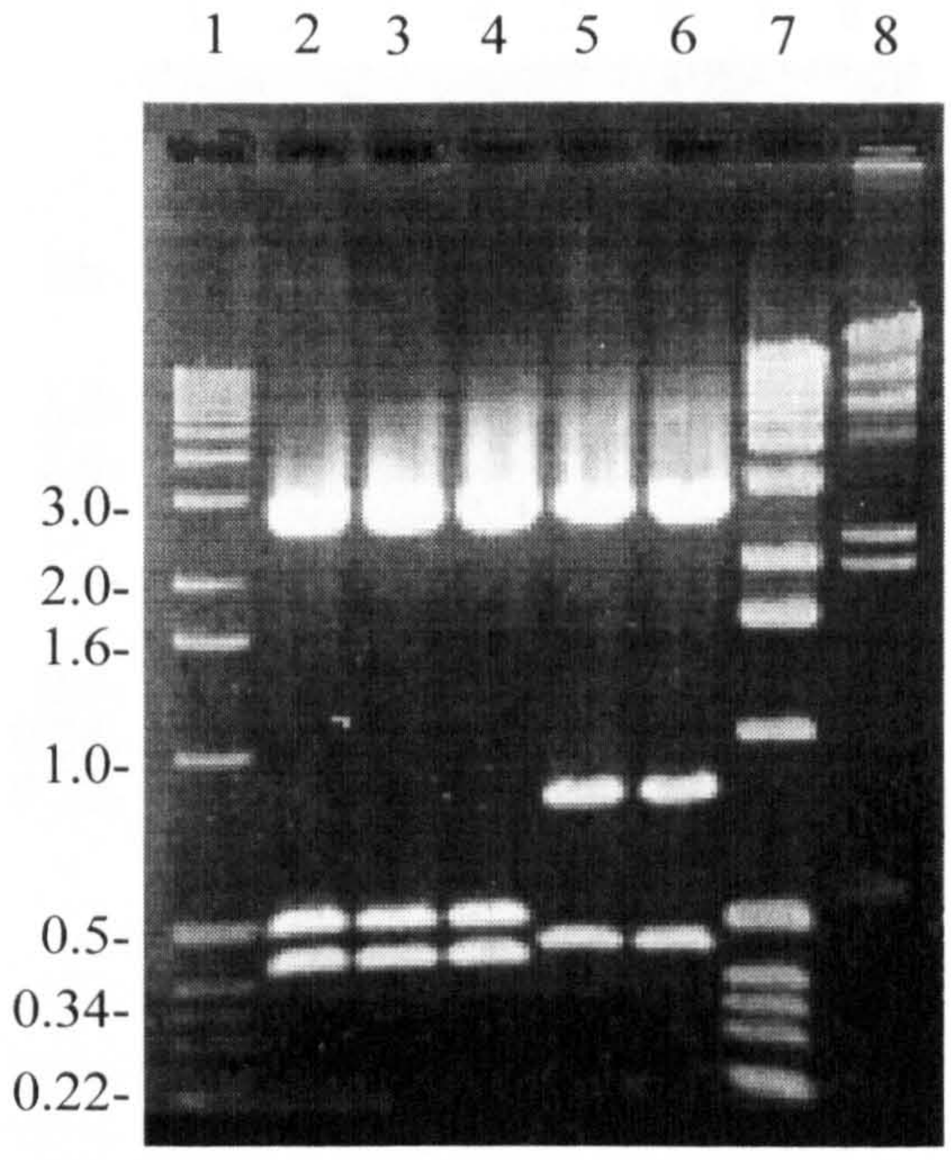



**Figure 4.11 Restriction enzyme analysis of a variety of Cysta 16 hybridising plasmids.**

Various plasmids were restricted with the enzymes outlined below and electrophoresed on a 1% TBE agarose gel, stained with EtBr and visualised under UV illumination.

Lanes 1 and 7: 1kb ladder (Life Technologies)
Lane 2: $1_{100a}$ plasmid digested with *Eco*RI and *Xho*I
Lane 3: $1_{100b}$ plasmid digested with *Eco*RI and *Xho*I
Lane 4: $3_{100}$ plasmid digested with *Eco*RI and *Xho*I
Lane 5: $5_{100}$ plasmid digested with *Eco*RI and *Xho*I
Lane 6: $6_{100}$ plasmid digested with *Eco*RI and *Xho*I
Lane 8: λ *Hin*dIII marker (Life Technologies)

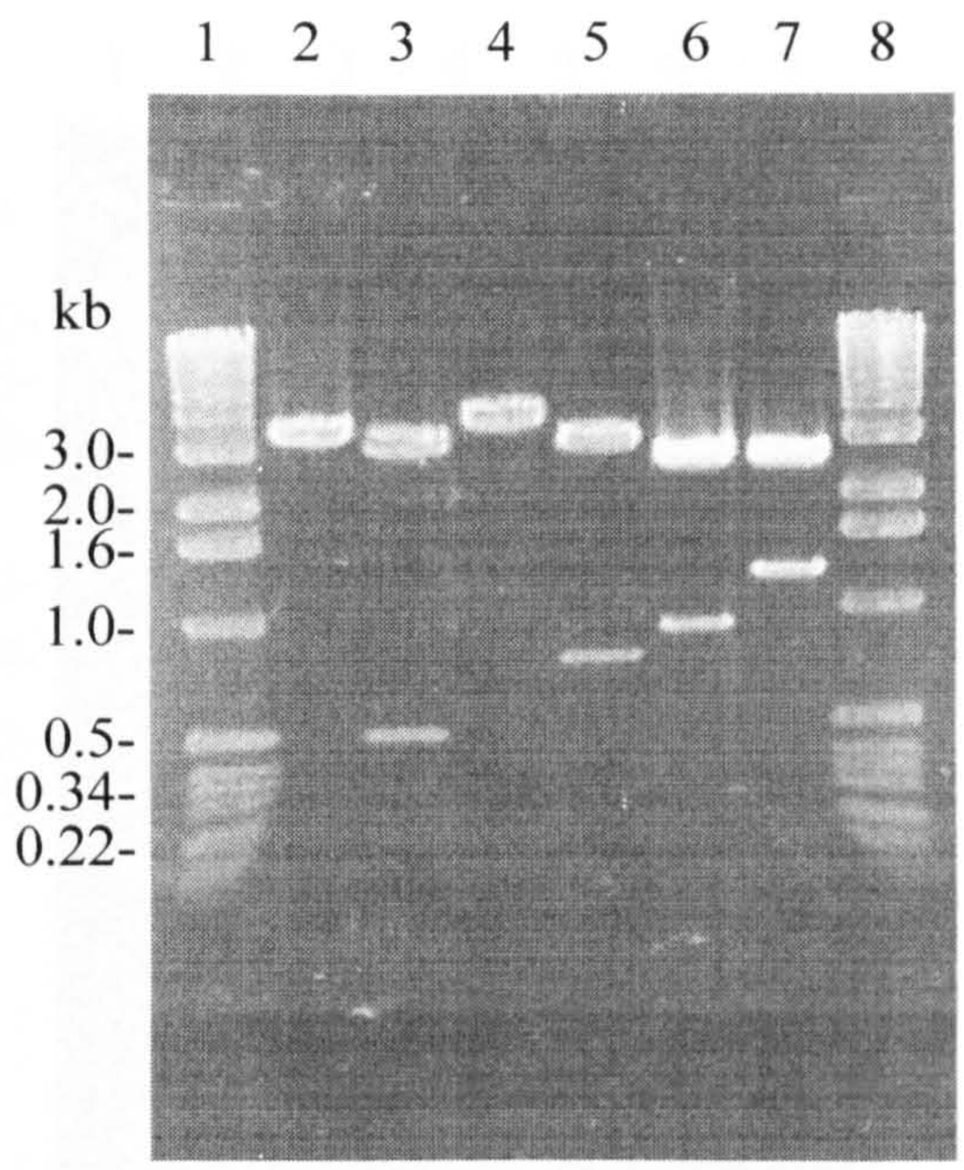



**Figure 4.12 Further restriction enzyme analysis of $p1_{100a}$ and $p5_{100}$, two Cysta 16 hybridising plasmids.**

$p1_{100a}$ and $p5_{100}$ were restricted with the enzymes outlined below and electrophoresed on a 1% TBE agarose gel, stained with EtBr and visualised under UV illumination.

Lanes 1 and 8: 1kb ladder (Life Technologies)
Lane 2: $p1_{100a}$ digested with *Xho*I
Lane 3: $p1_{100a}$ digested with *Eco*RI
Lane 4: $p5_{100}$ digested with *Xho*I
Lane 5: $p5_{100}$ digested with *Eco*RI
Lane 6: $p1_{100a}$ digested with *Spe*I and *Kpn*I
Lane 7: $p5_{100}$ digested with *Spe*I and *Kpn*I

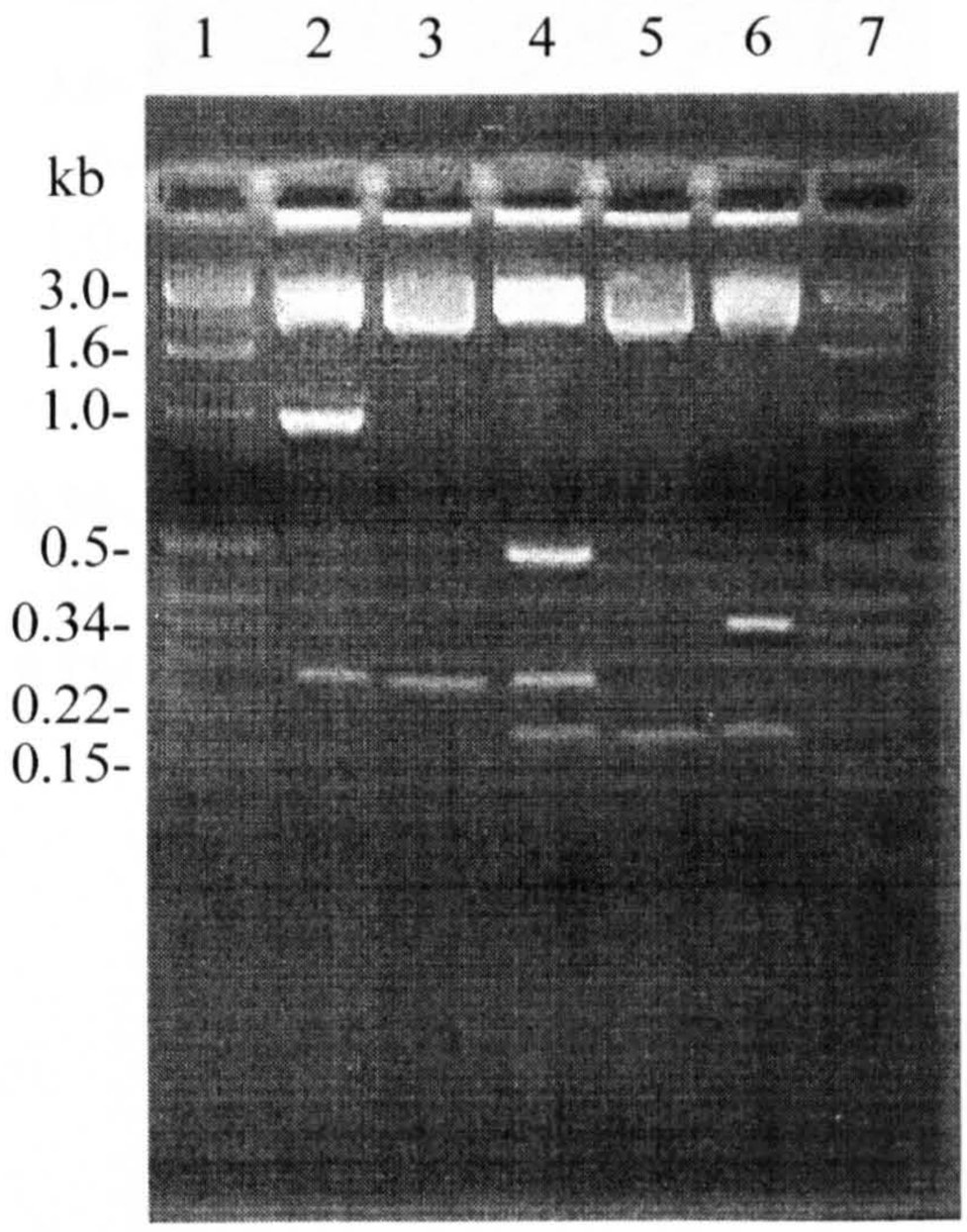



**Figure 4.13 Restriction enzyme digests of $p4_{100}$ cDNA.**

$p4_{100}$ was restricted with a variety of enzymes as outlined below and electrophoresed in a 2% TBE agarose gel, stained with EtBr and visualised under UV illumination. The double and single restriction enzyme digests of $p4_{100}$ were used to ascertain fragment sizes suitable for cloning and to draw up a preliminary restriction map of the cDNA contained in $p4_{100}$.

Lanes 1 and 7: 1kb ladder (Life Technologies)

Lane 2: $p4_{100}$ restricted with *Eco*RI and *Xho*I

Lane 3: $p4_{100}$ restricted with *Xho*I only

Lane 4: $p4_{100}$ restricted with *Eco*RV and *Xho*I

Lane 5: $p4_{100}$ restricted with *Eco*RV alone

Lane 6: $p4_{100}$ restricted with *Eco*RI and *Eco*RV

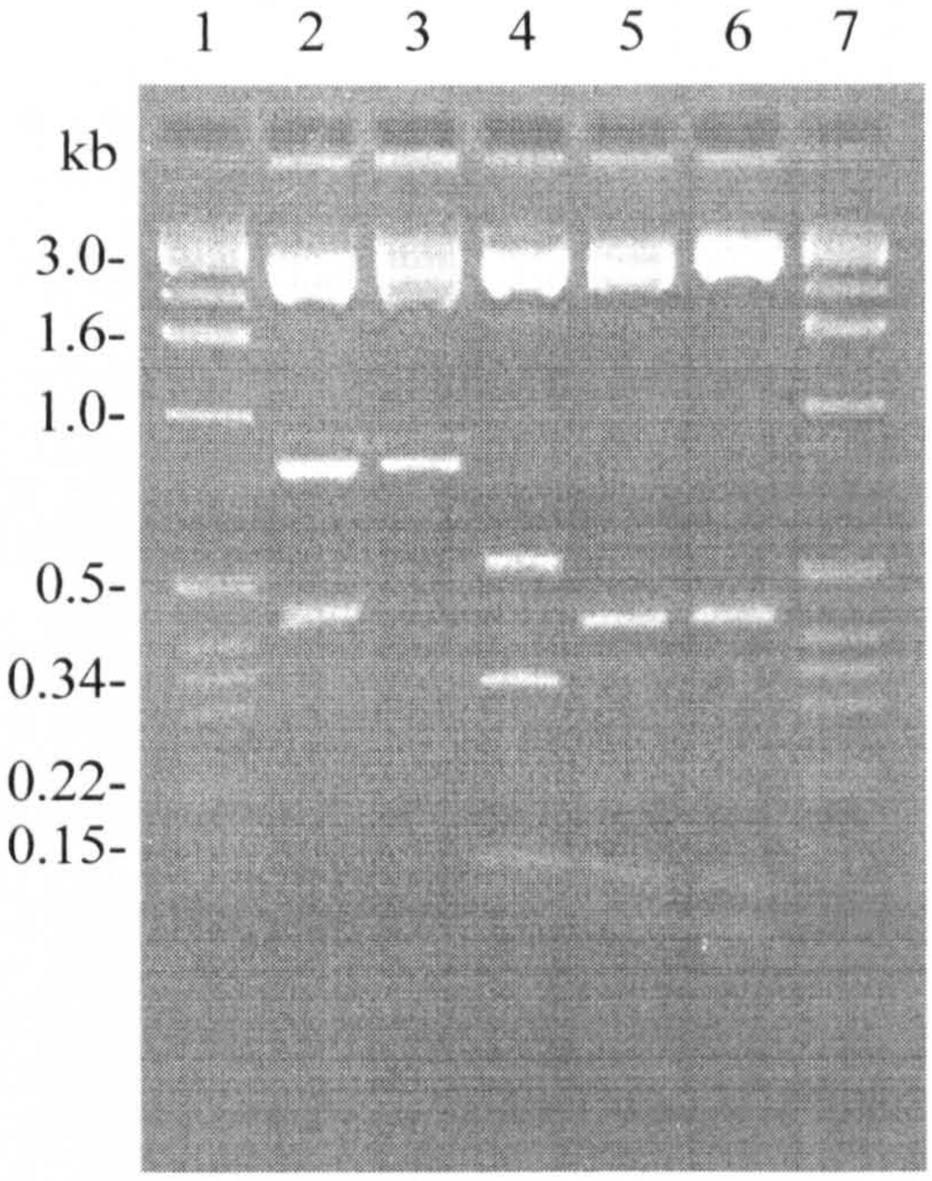



**Figure 4.14 Restriction enzyme digests of $p5_{100}$ cDNA.**

$p5_{100}$ was restricted with a variety of enzymes as outlined below and electrophoresed on a 2% TBE agarose gel, stained with EtBr and visualised under UV illumination. The double and single restriction enzyme digests of $p5_{100}$ were used to ascertain fragment sizes suitable for cloning and to draw up a preliminary restriction map of the cDNA contained in $p5_{100}$.

Lanes 1 and 7: 1kb ladder (Life Technologies)
Lane 2: $p5_{100}$ restricted with *Eco*RI and *Xho*I
Lane 3: $p5_{100}$ restricted with *Eco*RI only
Lane 4: $p5_{100}$ restricted with *Eco*RI and *Eco*RV
Lane 5: $p5_{100}$ restricted with *Eco*RV only
Lane 6: $p5_{100}$ restricted with *Eco*RV and *Xho*I

**Figure 4.15 Map and sequencing strategy for the *Tvmgl1* gene.**

The black box indicates the open reading frame and the black line the polylinker used in cloning the cDNA. The arrows indicate the extent and direction of sequencing. Relevant restriction sites used in creating subclones for sequencing are indicated. Sequence derived from subclones is indicated by black arrows. Sequence derived from internal oligonucleotides is indicated by dotted arrows.

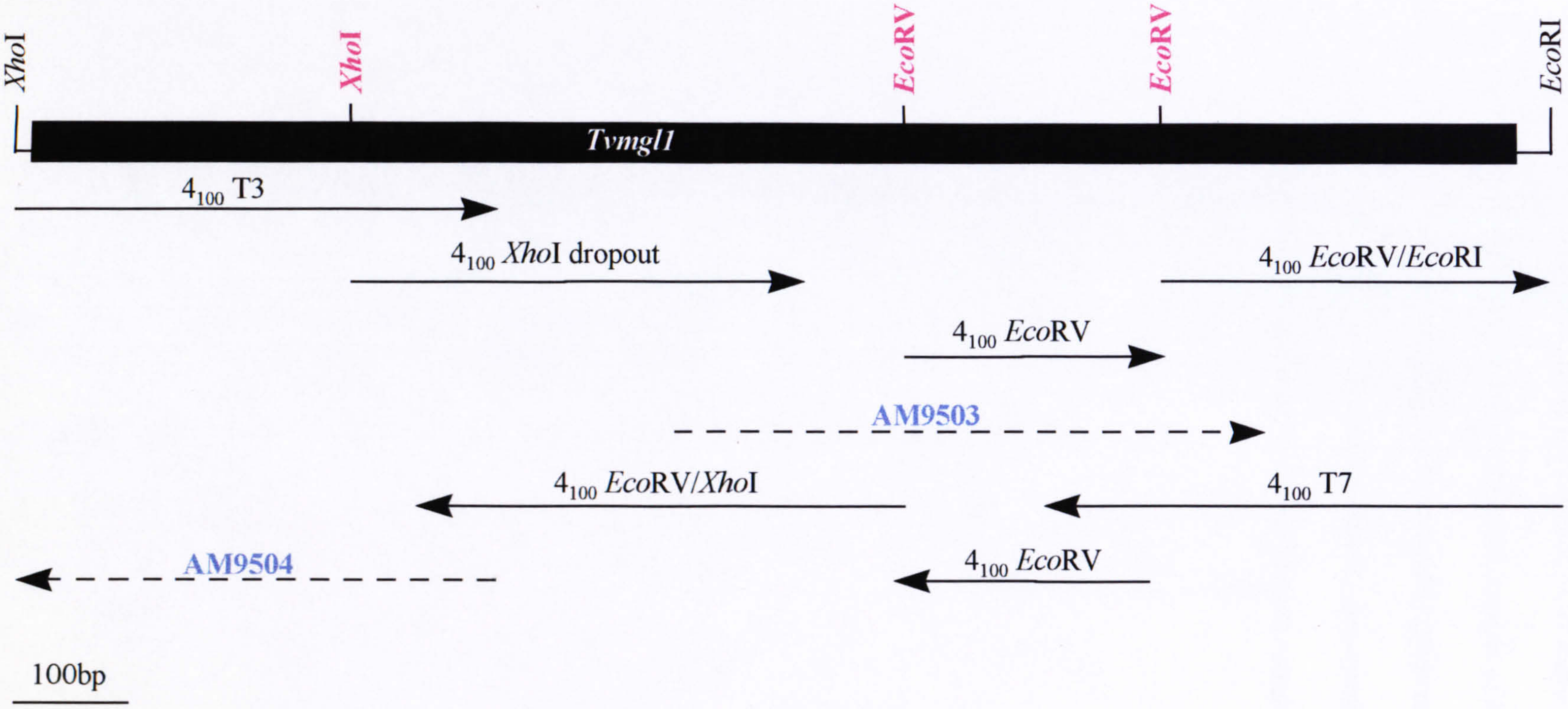

XhoI
XhoI
EcoRV
EcoRV
EcoRI
Tvmgl1
4100 T3
4100 XhoI dropout
4100 EcoRV/EcoRI
4100 EcoRV
AM9503
4100 EcoRV/XhoI
4100 T7
AM9504
4100 EcoRV
100bp

**Figure 4.16 Map and sequencing strategy for the *Tvmgl2* gene.**

The black box indicates the open reading frame and the black line the polylinker used in cloning the cDNA. The arrows indicate the extent and direction of sequencing. Relevant restriction sites used in creating subclones for sequencing are indicated. Sequence derived from subclones is indicated by black arrows. Sequence derived from internal oligonucleotides is indicated by dotted arrows.

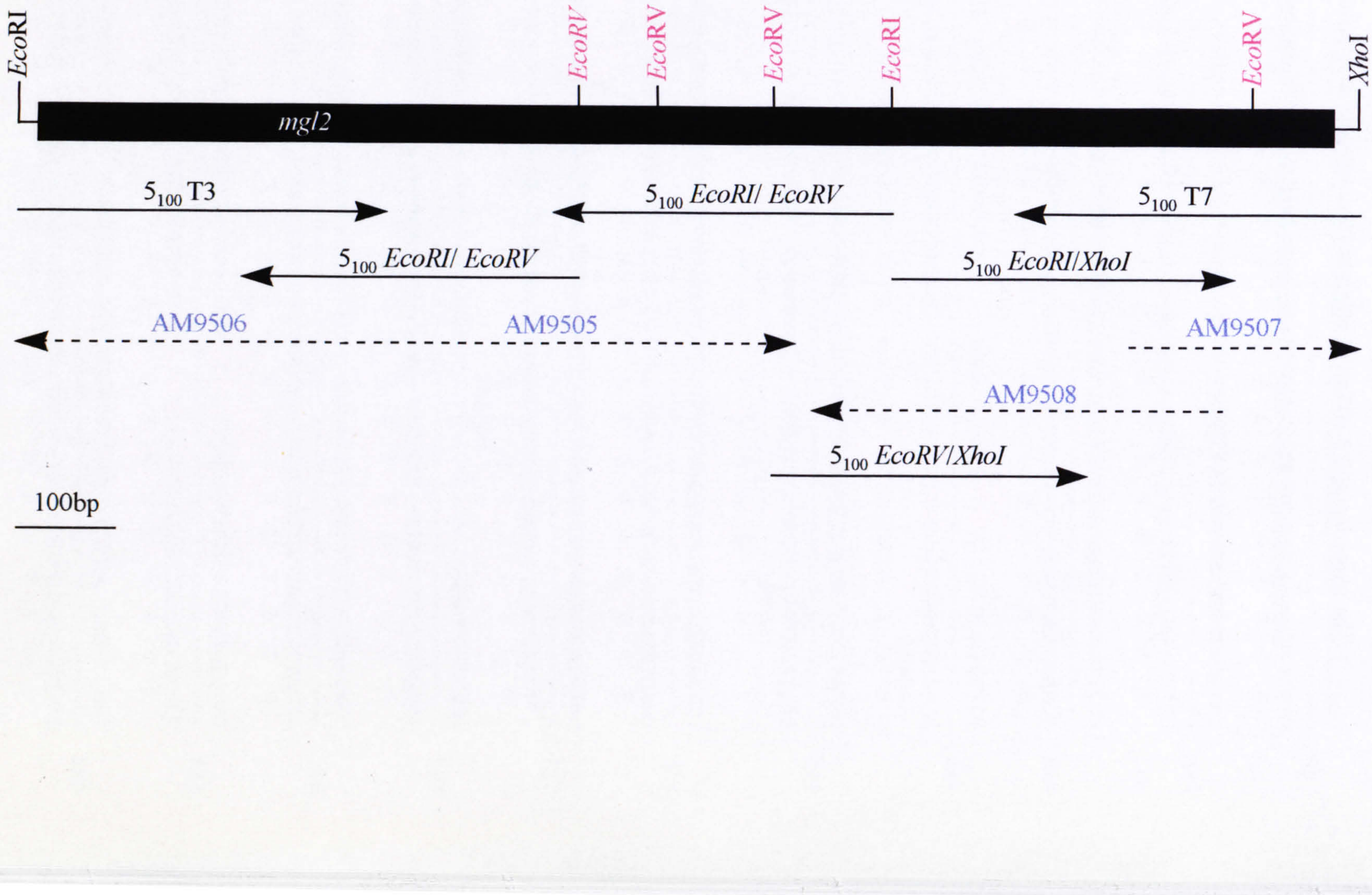

EcoRI
EcoRV
EcoRV
EcoRV
EcoRI
EcoRV
XhoI
mgl2
5100 T3
5100 EcoRI/ EcoRV
5100 T7
5100 EcoRI/ EcoRV
5100 EcoRI/XhoI
AM9506
AM9505
AM9507
AM9508
5100 EcoRV/XhoI
100bp

**Figure 4.17 Nucleotide and deduced amino acid sequence of *mgl1*.**

Key restriction sites used in subcloning experiments are indicated by pink text. The sequence and position of oligonucleotides used in sequencing and 5'-RACE are indicated by blue text. The sequence determined by the 5'-RACE experiments is indicated by red text.

```
                                                ATTTTTAGACAACATGTCTCACGAGAGA
                                          1     +---------+---------+----   28
                                                TAAAAATCTGTTGTACAGAGTGCTCTCT
                                                          M  S  H  E  R

    ATGACCCCAGCAACAGCATGCATCCATGCTAATCCACAGAAGGATCAGTTTGGAGCAGCC
29  -+---------+---------+---------+---------+---------+---------   88
    TACTGGGGTCGTTGTCGTACGTAGGTACGATTAGGTGTCTTCCTAGTCAAACCTCGTCGG
    M  T  P  A  T  A  C  I  H  A  N  P  Q  K  D  Q  F  G  A  A

    ATCCCACCAATCTACCAAACATCAACATTCGTTTTCGATAACTGCCAACAGGGTGGAAAC
89  -+---------+---------+---------+---------+---------+---------  148
    TAGGGTGGTTAGATGGTTTGTAGTTGTAAGCAAAAGCTATTGACGGTTGTCCCACCTTTG
    I  P  P  I  Y  Q  T  S  T  F  V  F  D  N  C  Q  Q  G  G  N

    AGATTCGCTGGTCAGGAATCCGGCTACATCTACACACGTCTCGGCAACCCAACAGTTTCA
149 -+---------+---------+---------+---------+---------+---------
    TCTAAGCGACCAGTCCTTAGGCCGATGTAGATGTGTGCAGAGCCGTTGGGTTGTCAAAGT  208
    R  F  A  G  Q  E  S  G  Y  I  Y  T  R  L  G  N  P  T  V  S
                                XhoI            SphI
    AACCTCGAAGGCAAGATCGCCTTCCTCGAGAAAACAGAAGCATGCGTTGCCACATCTTCT
209 --------+---------+---------+---------+---------+---------+---  268
    TTGGAGCTTCCGTTCTAGCGGAAGGAGCTCTTTTGTCTTCGTACGCAACGGTGTAGAAGA
                                                          AM9509
    N  L  E  G  K  I  A  F  L  E  K  T  E  A  C  V  A  T  S  S

    GGCATGGGTGCCATTGCTGCTACAGTTTTGACAATCCTCAAGGCCGGAGATCACTTAATC
269 ---------+---------+---------+---------+---------+---------+---  328
    CCGTACCCACGGTAACGACGATGTCAAAACTGTTAGGAGTTCCGGCCTCTAGTGAATTAG
                                                    AM9504
    G  M  G  A  I  A  A  T  V  L  T  I  L  K  A  G  D  H  L  I

    TCCGATGAGTGCCTTTATGGCTGCACACATGCTCTCTTTGAGCACGCATTGACAAAGTTC
329 -+---------+---------+---------+---------+---------+---------  388
    AGGCTACTCACGGAAATACCGACGTGTGTACGAGAGAAACTCGTGCGTAACTGTTTCAAG
    S  D  E  C  L  Y  G  C  T  H  A  L  F  E  H  A  L  T  K  F
             HincII
    GGCATCCAGGTCGACTTCATCAACACAGCCATCCCAGGCGAGGTCAAGAAGCACATGAAG
389 -+---------+---------+---------+---------+---------+---------  448
    CCGTAGGTCCAGCTGAAGTAGTTGTGTCGGTAGGGTCCGCTCCAGTTCTTCGTGTACTTC
    G  I  Q  V  D  F  I  N  T  A  I  P  G  E  V  K  K  H  M  K
                                                AM9503
    CCAAACACAAAGATTGTCTATTTCGAGACACCAGCCAACCCAACACTCAAGATCATCGAC
449 -+---------+---------+---------+---------+---------+---------  508
    GGTTTGTGTTTCTAACAGATAAAGCTCTGTGGTCGGTTGGGTTGTGAGTTCTAGTAGCTG
    P  N  T  K  I  V  Y  F  E  T  P  A  N  P  T  L  K  I  I  D

    ATGGAGCGCGTCTGCAAGGACGCCCACAGCCAGGAGGGCGTCTTAGTTATCGCCGATAAC
509 -+---------+---------+---------+---------+---------+---------  568
    TACCTCGCGCAGACGTTCCTGCGGGTGTCGGTCCTCCCGCAGAATCAATAGCGGCTATTG
    M  E  R  V  C  K  D  A  H  S  Q  E  G  V  L  V  I  A  D  N
                                HincII/SalI
    ACATTCTGCTCACCAATGATCACAAACCCAGTCGACTTTGGCGTCGATGTTGTTGTCCAC
569 -+---------+---------+---------+---------+---------+---------  628
    TGTAAGACGAGTGGTTACTAGTGTTTGGGTCAGCTGAAACCGCAGCTACAACAACAGGTG
    T  F  C  S  P  M  I  T  N  P  V  D  F  G  V  D  V  V  V  H

    TCTGCAACAAAGTACATCAACGGCCACACAGATGTCGTCGCTGGCCTTATCTGTGGCAAG
629 -+---------+---------+---------+---------+---------+---------  688
    AGACGTTGTTTCATGTAGTTGCCGGTGTGTCTACAGCAGCGACCGGAATAGACACCGTTC
    S  A  T  K  Y  I  N  G  H  T  D  V  V  A  G  L  I  C  G  K
```

*EcoRV*

```
      GCTGACCTCCTTCAACAGATTCGTATGGTTGGTATCAAGGATATCACAGGATCTGTTATC
689   -+---------+---------+---------+---------+---------+---------+--------  748
      CGACTGGAGGAAGTTGTCTAAGCATACCAACCATAGTTCCTATAGTGTCCTAGACAATAG
      A  D  L  L  Q  Q  I  R  M  V  G  I  K  D  I  T  G  S  V  I

      AGCCCACACGACGCTTGGCTCATCACACGTGGCCTCTCAACACTCAACATCAGAATGAAG
749   -+---------+---------+---------+---------+---------+---------+--------  808
      TCGGGTGTGCTGCGAACCGAGTAGTGTGCACCGGAGAGTTGTGAGTTGTAGTCTTACTTC
      S  P  H  D  A  W  L  I  T  R  G  L  S  T  L  N  I  R  M  K

      GCTGAGAGCGAGAACGCCATGAAGGTCGCTGAGTACCTCAAATCTCACCCAGCCGTTGAG
809   -+---------+---------+---------+---------+---------+---------+--------  868
      CGACTCTCGCTCTTGCGGTACTTCCAGCGACTCATGGAGTTTAGAGTGGGTCGGCAACTC
      A  E  S  E  N  A  M  K  V  A  E  Y  L  K  S  H  P  A  V  E
```

*EcoRV*

```
      AAGGTTTACTACCCAGGCTTCGAGGACCACGAGGGCCACGATATCGCTAAGAAGCAGATG
869   -+---------+---------+---------+---------+---------+---------+--------  928
      TTCCAAATGATGGGTCCGAAGCTCCTGGTGCTCCCGGTGCTATAGCGATTCTTCGTCTAC
      K  V  Y  Y  P  G  F  E  D  H  E  G  H  D  I  A  K  K  Q  M

      AGAATGTCGGGTTCAATGATCACATTCATCCTCAAGTCCGGCTTCGAAGGCGCTAAGAAG
929   -+---------+---------+---------+---------+---------+---------+--------  988
      TCTTACAGCCCAAGTTACTAGTGTAAGTAGGAGTTCAGGCCGAAGCTTCCGCGATTCTTC
      R  M  S  G  S  M  I  T  F  I  L  K  S  G  F  E  G  A  K  K
```

*HindIII*

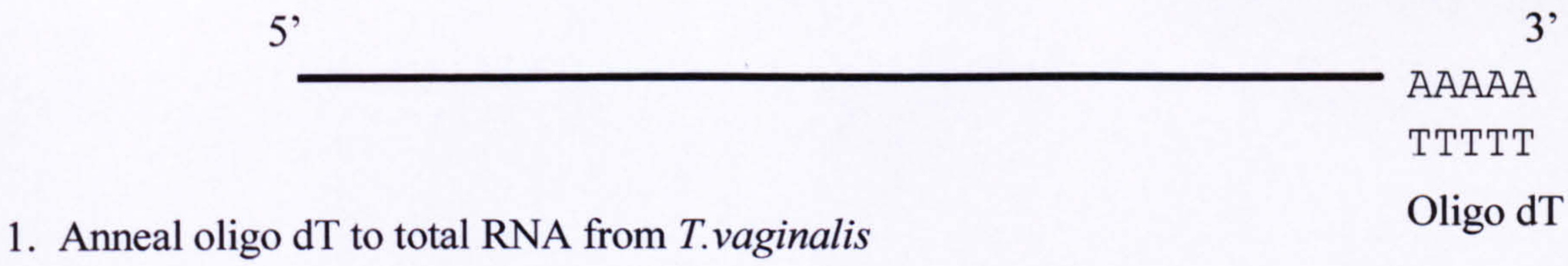
```
      CTCCTCGACAACCTCAAGCTTATCACACTTGCAGTTTCCCTTGGTGGCTGCGAGTCCCTC
989   -+---------+---------+---------+---------+---------+---------+--------  1048
      GAGGAGCTGTTGGAGTTCGAATAGTGTGAACGTCAAAGGGAACCACCGACGCTCAGGGAG
      L  L  D  N  L  K  L  I  T  L  A  V  S  L  G  G  C  E  S  L

      ATCCAGCACCCAGCTTCAATGACTCACGCTGTCGTTCCAAAGGAGGAGCGTGAGGCCGCT
1049  -+---------+---------+---------+---------+---------+---------+--------  1108
      TAGGTCGTGGGTCGAAGTTACTGAGTGCGACAGCAAGGTTTCCTCCTCGCACTCCGGCGA
      I  Q  H  P  A  S  M  T  H  A  V  V  P  K  E  E  R  E  A  A

      GGTATTACAGATGGCATGATCCGCCTTTCTGTCGGTATTGAAGATGCCGACGAACTCATC
1109  -+---------+---------+---------+---------+---------+---------+--------  1168
      CCATAATGTCTACCGTACTAGGCGGAAAGACAGCCATAACTTCTACGGCTGCTTGAGTAG
      G  I  T  D  G  M  I  R  L  S  V  G  I  E  D  A  D  E  L  I

      GCTGATTTCAAACAGGGCCTTGACGCTCTTTTATAA
1169  -+---------+---------+---------+----  1202
      CGACTAAAGTTTGTCCCGGAACTGCGAGAAAATATT
      A  D  F  K  Q  G  L  D  A  L  L
```

**Figure 4.18 Nucleotide and deduced amino acid sequence of *mgl2*.**

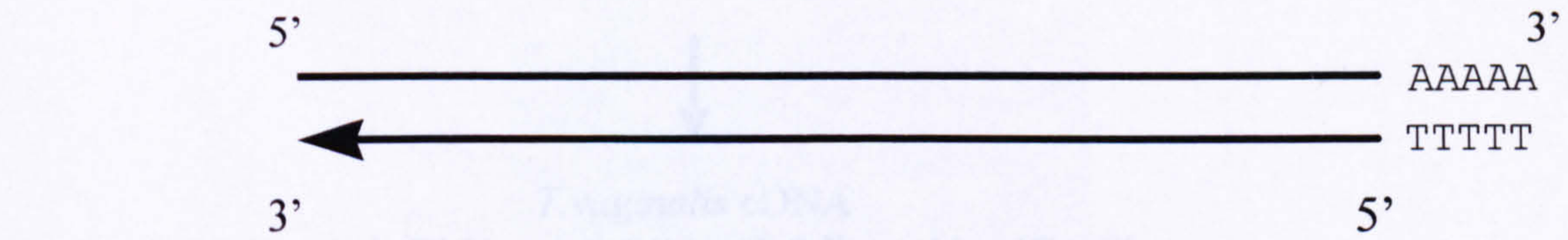
Key restriction sites used in subcloning experiments are indicated by pink text. The sequence and position of oligonucleotides used in sequencing and 5'-RACE are indicated by blue text. The sequence determined by the 5'-RACE experiments is indicated by red text.

```
                                                ACTTTATATAAAAGATGAGTGGCCAG
                                          1     ------------------------     27
                                                TGAAATATATTTTCTACTCACCGGTC
                                                              M  S  G  H

      GCTATCGACCCAACACATACAGACACACTTTCCATCCACGCCAACCCACAGAAGGATCAG
 28   ---------+---------+---------+---------+---------+---------+----------  87
      CGATAGCTGGGTTGTGTATGTCTGTGTGAAAGGTAGGTGCGGTTGGGTGTCTTCCTAGTC
      A  I  D  P  T  H  T  D  T  L  S  I  H  A  N  P  Q  K  D  Q

      TTCGGTGCTATTGTTGCTCCAATCTACCAAACATCCACCTTCCTCTTCGACAACTGCGAC
 88   ---------+---------+---------+---------+---------+---------+----------  147
      AAGCCACGATAACAACGAGGTTAGATGGTTTGTAGGTGGAAGGAGAAGCTGTTGACGCTG
      F  G  A  I  V  A  P  I  Y  Q  T  S  T  F  L  F  D  N  C  D

      CAGGGTGGTGCTCGTTTCGGTGGCAAGGAAGCCGGTTACATGTACACACGTATCGGTAAC
 148  --+---------+---------+---------+---------+---------+---------+--------  207
      GTCCCACCACGAGCAAAGCCACCGTTCCTTCGGCCAATGTACATGTGTGCATAGCCATTG
      Q  G  G  A  R  F  G  G  K  E  A  G  Y  M  Y  T  R  I  G  N
                                                            SphI
      CCAACAAACTCCGCACTCGAAGGCAAGATCGCCAAGCTCGAACACGCTGAGGCATGCGCT
 208  --+---------+---------+---------+---------+---------+---------+--------  267
      GGTTGTTTGAGGCGTGAGCTTCCGTTCTAGCGGTTCGAGCTTGTGCGACTCCGTACGCGA
      P  T  N  S  A  L  E  G  K  I  A  K  L  E  H  A  E  A  C  A
                           AM9505
      GCCACAGCTTCTGGCATGGGTGCTATTGCTGCTTCTGTCTGGACATTCCTCAAGGCCGGT
 268  --+---------+---------+---------+---------+---------+---------+--------  327
      CGGTGTCGAAGACCGTACCCACGATAACGACGAAGACAGACCTGTAAGGAGTTCCGGCCA
                           AM9506
      A  T  A  S  G  M  G  A  I  A  A  S  V  W  T  F  L  K  A  G

      GATCACCTTATCTCCGACGATTGCCTTTATGGCTGCACACACGCCCTCTTCGAGCATCAG
 328  --+---------+---------+---------+---------+---------+---------+--------  387
      CTAGTGGAATAGAGGCTGCTAACGGAAATACCGACGTGTGTGCGGGAGAAGCTCGTAGTC
      D  H  L  I  S  D  D  C  L  Y  G  C  T  H  A  L  F  E  H  Q

      CTCCGCAAGTTCGGCGTTGAAGTTGATTTCATCGACATGGCTGTCCCAGGAAACATTGAG
 388  --+---------+---------+---------+---------+---------+---------+--------  447
      GAGGCGTTCAAGCCGCAACTTCAACTAAAGTAGCTGTACCGACAGGGTCCTTTGTAACTC
      L  R  K  F  G  V  E  V  D  F  I  D  M  A  V  P  G  N  I  E

      AAGCACTTGAAGCCAAACACAAGAATCGTCTACTTCGAAACACCAGCTAACCCAACATTA
 448  --+---------+---------+---------+---------+---------+---------+--------  507
      TTCGTGAACTTCGGTTTGTGTTCTTAGCAGATGAAGCTTTGTGGTCGATTGGGTTGTAAT
      K  H  L  K  P  N  T  R  I  V  Y  F  E  T  P  A  N  P  T  L
                                                         EcoRV
      AAGGTTATCGACATCGAAGACGCCGTCAAGCAGGCCAGAAAGCAGAAGGATATCCTCGTT
 508  --+---------+---------+---------+---------+---------+---------+--------  567
      TTCCAATAGCTGTAGCTTCTGCGGCAGTTCGTCCGGTCTTTCGTCTTCCTATAGGAGCAA
      K  V  I  D  I  E  D  A  V  K  Q  A  R  K  Q  K  D  I  L  V
                                                               EcoRV
      ATCGTTGATAACACCTTCGCTTCACCAATTCTTACAAACCCACTCGACCTCGGTGTTGAT
 568  --+---------+---------+---------+---------+---------+---------+--------  627
      TAGCAACTATTGTGGAAGCGAAGTGGTTAAGAATGTTTGGGTGAGCTGGAGCCACAACTA
      I  V  D  N  T  F  A  S  P  I  L  T  N  P  L  D  L  G  V  D

      ATCGTCGTTCACTCCGCTACTAAGTACATCAATGGCCACACCGATGTTGTCGCCGGCCTT
 628  --+---------+---------+---------+---------+---------+---------+--------  687
      TAGCAGCAAGTGAGGCGATGATTCATGTAGTTACCGGTGTGGCTACAACAGCGGCCGGAA
      I  V  V  H  S  A  T  K  Y  I  N  G  H  T  D  V  V  A  G  L
                                                            EcoRV
      GTCTGCTCAAGAGCTGACATCATCGCTAAGGTCAAGTCCCAGGGTATCAAGGATATCACA
 688  --+---------+---------+---------+---------+---------+---------+--------  747
      CAGACGAGTTCTCGACTGTAGTAGCGATTCCAGTTCAGGGTCCCATAGTTCCTATAGTGT
      V  C  S  R  A  D  I  I  A  K  V  K  S  Q  G  I  K  D  I  T
```

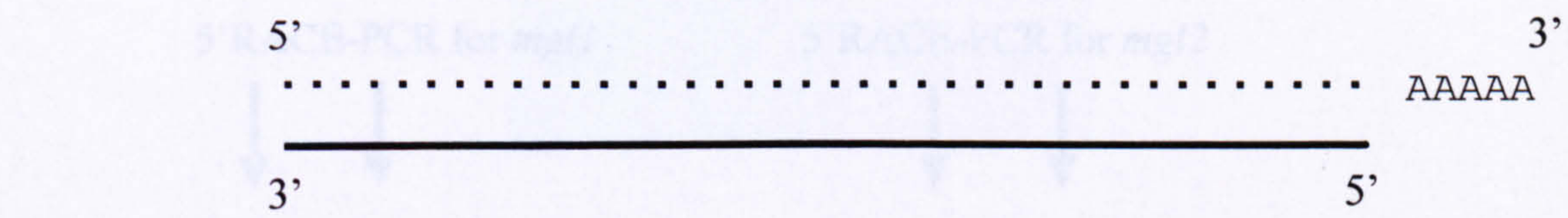
```
    GGCGCCATCATTTCCCCACACGACGCTTGGCTCATCACAAGAGGCACACTTACACTCGAT
748 --+---------+---------+---------+---------+---------+---------  807
    CCGCGGTAGTAAAGGGGTGTGCTGCGAACCGAGTAGTGTTCTCCGTGTGAATGTGAGCTA
    G  A  I  I  S  P  H  D  A  W  L  I  T  R  G  T  L  T  L  D
                                                 EcoRI
    ATGCGTGTCAAGCGCGCTGCCGAGAACGCTCAGAAGGTCGCTGAATTCCTCCATGAGCAC
808 --+---------+---------+---------+---------+---------+---------  867
    TACGCACAGTTCGCGCGACGGCTCTTGCGAGTCTTCCAGCGACTTAAGGAGGTACTCGTG
    M  R  V  K  R  A  A  E  N  A  Q  K  V  A  E  F  L  H  E  H

    AAGGCCGTCAAGAAGGTCTACTACCCAGGCCTTCCAGACCATCCAGGCCACGAAATCGCC
868 --+---------+---------+---------+---------+---------+---------  927
    TTCCGGCAGTTCTTCCAGATGATGGGTCCGGAAGGTCTGGTAGGTCCGGTGCTTTAGCGG
    K  A  V  K  K  V  Y  Y  P  G  L  P  D  H  P  G  H  E  I  A
          AM9507                                 HincII/SalI
    AAGAAGCAGATGAAGATGTTCGGCTCTATGATCGCATTCGATGTCGACGGATTAGAGAAG
928 --+---------+---------+---------+---------+---------+---------  987
    TTCTTCGTCTACTTCTACAAGCCGAGATACTAGCGTAAGCTACAGCTGCCTAATCTCTTC
    K  K  Q  M  K  M  F  G  S  M  I  A  F  D  V  D  G  L  E  K

    GCCAAGAAAGTCCTTGACAACTGCCACGTTGTTTCTCTCGCCGTTTCCCTCGGTGGTCCA
988 --+---------+---------+---------+---------+---------+---------  1047
    CGGTTCTTTCAGGAACTGTTGACGGTGCAACAAAGAGAGCGGCAAAGGGAGCCACCAGGT
              AM9508
    A  K  K  V  L  D  N  C  H  V  V  S  L  A  V  S  L  G  G  P

    GAATCCCTCATCCAGCACCCAGCTTCAATGACACACGCTGGTGTTCCAAAGGAGGAACGC
1048 --+---------+---------+---------+---------+---------+---------  1107
    CTTAGGGAGTAGGTCGTGGGTCGAAGTTACTGTGTGCGACCACAAGGTTTCCTCCTTGCG
    E  S  L  I  Q  H  P  A  S  M  T  H  A  G  V  P  K  E  E  R

    GAGGCTGCTGGCCTAACAGATAACCTCATCCGCCTCTCTGTTGGCTGTGAGAACGTTCAG
1108 --+---------+---------+---------+---------+---------+---------  1167
    CTCCGACGACCGGATTGTCTATTGGAGTAGGCGGAGAGACAACCGACACTCTTGCAAGTC
    E  A  A  G  L  T  D  N  L  I  R  L  S  V  G  C  E  N  V  Q
    EcoRV
    GATATCATCGACGACCTCAAGCAGGCTCTCGACTTAGTCCTC
1168 --+---------+---------+---------+----------  1209
    CTATAGTAGCTGCTGGAGTTCGTCCGAGAGCTGAATCAGGAG
    D  I  I  D  D  L  K  Q  A  L  D  L  V  L
```



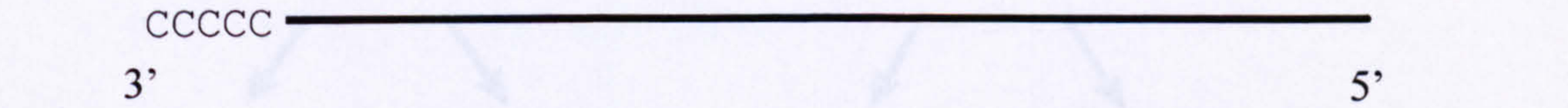

1. Anneal oligo dT to total RNA from *T.vaginalis*




2. Synthesise 1st strand cDNA using Superscript II reverse transcriptase




3. Degrade RNA using RNase H




4. Generate dC-tailed 1st strand cDNA using dCTP and terminal deoxynucleotide transferase


5. 1st round PCR: PCR amplify the dC-tailed 1st strand cDNA using the anchor primer and a gene specific primer for each gene (GSP1)

6. 2nd round PCR: PCR amplify the products from the 1st round PCR reaction using the anchor primer and a nested gene specific primer for each gene (GSP2)

**Figure 4.19 Summary of the 5' RACE methodology.**

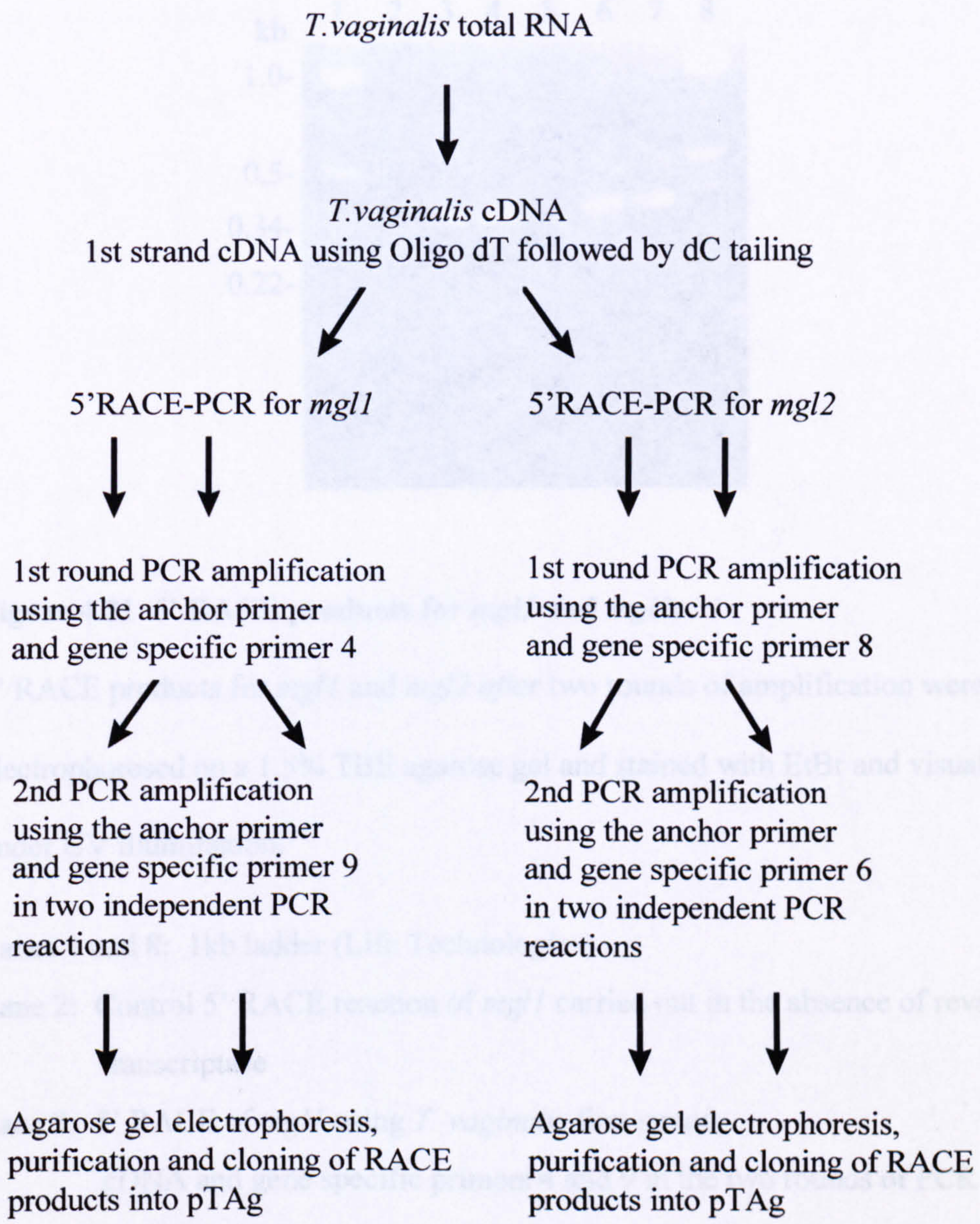



**Figure 4.20 A summary of the 5'RACE-PCR protocol carried out for the amplification of the 5' ends of *mgl1* and *mgl2*.**

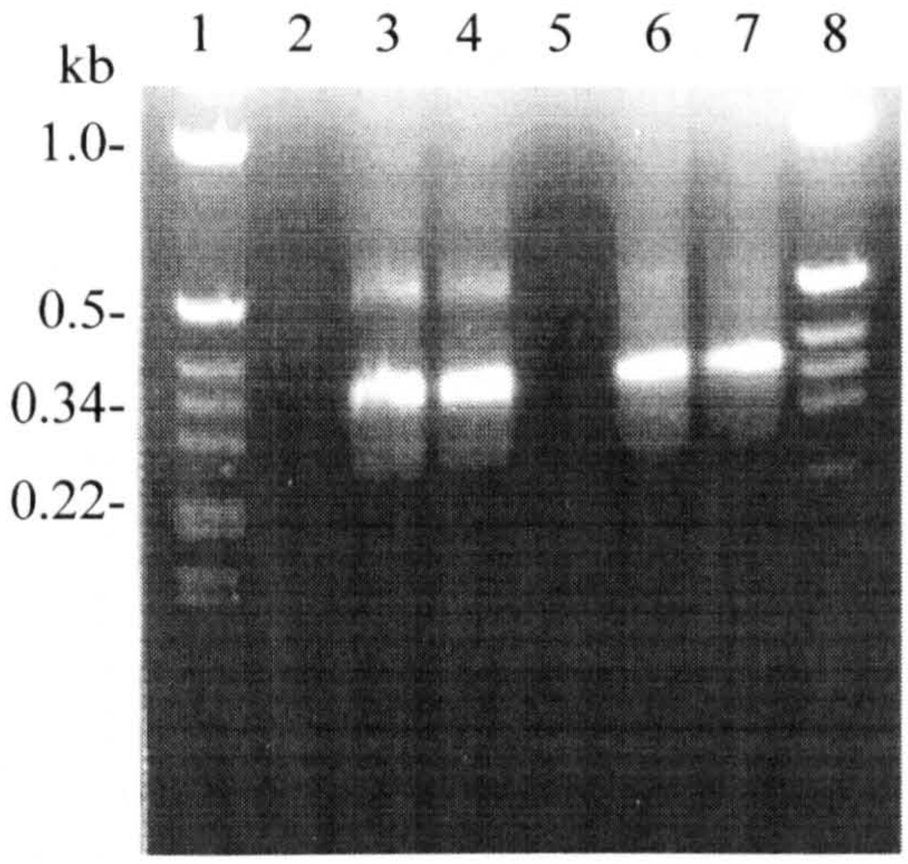



**Figure 4.21 5' RACE products for *mgl1* and *mgl2*.**

5' RACE products for *mgl1* and *mgl2 after* two rounds of amplification were electrophoresed on a 1.5% TBE agarose gel and stained with EtBr and visualised under UV illumination.

Lanes 1 and 8: 1kb ladder (Life Technologies)

Lane 2: Control 5' RACE reaction of *mgl1* carried out in the absence of reverse transcriptase

Lane 3: 5' RACE of *mgl1* using *T. vaginalis* first strand cDNA and gene specific primers 4 and 9 in the two rounds of PCR amplification

Lane 4: As lane 3 but an independent PCR reaction

Lane 5: Control 5' RACE of *mgl2* carried out in the absence of reverse transcriptase

Lane 6: 5' RACE of *mgl2* using *T. vaginalis* first strand cDNA and gene specific primers 8 and 6 in the two rounds of PCR amplification

Lane 7: As lane 6 but an independent PCR reaction

**Figure 4.22 Amino acid sequence alignment of methionine γ-lyases from *T. vaginalis* and *P. putida* and cystathionine γ-lyase from a variety of sources.**

Amino acid sequence alignment of *T. vaginalis* methionine γ-lyases, MGL1 and MGL2, this study, with two different methionine γ-lyases from *Pseudomonas putida* (PpMGL1, Inoue *et al.*, 1995 and PpMGL2, Hori *et al.*, 1996) and cystathionine γ-lyase from yeast (CYS3, Ono *et al.*, 1992) and human (HsCGL, Lu *et al.*, 1992). Each individual sequence is numbered accordingly. Residues that are conserved in all sequences are indicated by black boxes. Residues that are conserved in all four methionine γ-lyase sequences but are absent from the cystathionine γ-lyase sequences are indicated by a #. The proposed essential cysteine residue for the methionine γ-lyase is indicated by a * (.) indicate gaps introduced to maximise the sequence alignment. The multiple sequence alignment was obtained by using the PILE-UP program of the University of Wisconsin Genetics Computer Group (GCG).

```
                                                #         #        ###    #
TvMGL1     1           MS HERMTPA... TACIHANPQK DQFG.AAIPP IYQTSTFVFD    38
TvMGL2     1           MS GHAIDPTHTD TLSIHANPQK DQFG.AIVAP IYQTSTFLFD    41
PpMGL1     1           MH GSNKLPGFAT RAIHHGYDPQ DHGG.ALVPP VYQTATFTFP    41
PpMGL2     1           MR DSHNNTGFST RAIHHGYDPL SHGG.ALVPP VYQTATYAFP    41
ScCYS3     1            M TLQESDKFAT KAIHAGEHVD VHGS..VIEP ISLSTTFKQS    39
 HsCGL     1  MQEKDASSQG FLPHFQHFAT QAIHVGQDPE QWTSRAVVPP ISLSTTFKQG    50

                 #       #   #                                  # #       ##
TvMGL1    39   NCQQGGNRFA GQESGYIYTR LGNPTVSNLE GKIAFLEKTE ACVATSSGMG    88
TvMGL2    42   NCDQGGARFG GKEAGYMYTR IGNPTNSALE GKIAKLEHAE ACAATASGMG    91
PpMGL1    42   TVEYGAACFA GEQAGHFYSR ISNPTLNLLE ARMASLEGGE AGLALASGMG    91
PpMGL2    42   TVEYGAACFA GEEAGHFYSR ISNPTLALLE QRMASLEGGE AGLALASGMG    91
ScCYS3    40   SPANPIGTYE .......YSR SQNPNRENLE RAVAALENAQ YGLAFSSGSA    82
 HsCGL    51   APGQHSG.FE .......YSR SGNPTRNCLE KAVAALDGAK YCLAFASGLA    92

                #                    #  *  #    #
TvMGL1    89   AIAATVLTIL KAGDHLISDE CLYGCTHALF EHALTKFGIQ VDFINTAIPG   138
TvMGL2    92   AIAASVWTFL KAGDHLISDD CLYGCTHALF EHQLRKFGVE VDFIDMAVPG   141
PpMGL1    92   AITSTLWTLL RPGDEVLLGN TLYGCTFAFL HHGIGEFGVK LRHVDMADLQ   141
PpMGL2    92   AITSTLWTLL RPGDELIVGR TLYGCTFAFL HHGIGEFGVK IHHVDLNDAK   141
ScCYS3    83   T.TATILQSL PQGSHAVSIG DVYGGTHRYF TKVANAHGVE TSFTN.DLLN   130
 HsCGL    93   A.TVTITHLL KAGDQIICMD DVYGGTNRYF RQVASEFGLK ISFVDCSKIK   141

                          ##   #                                  #
TvMGL1   139   EVKKHMKPNT KIVYFETPAN PTLKIIDMER VCKDAHSQ.. .EGVLVIADN   185
TvMGL2   142   NIEKHLKPNT RIVYFETPAN PTLKVIDIED AVKQARKQ.. .KDILVIVDN   188
PpMGL1   142   ALEAAMTPAT RVIYFESPAN PNMHMADIAG VAKIARKH.. ..GATVVVDN   187
PpMGL2   142   ALKAAINSKT RMIYFETPAN PNMQLVDIAA VVEAVRGS.. ..DVLVVVDN   187
ScCYS3   131   DLPQLIKENT KLVWIETPTN PTLKVTDIQK VADLIKKHAA GQDVILVVDN   180
 HsCGL   142   LLEAAITPET KLVWIETPTN PTQKVIDIEG CAHIVHKHG. ..DIILVVDN   188

                                                          #
TvMGL1   186   TFCSPMITNP VDFGVDVVVH SATKYINGHT DVVAGLICGK ADLLQQIRMV   235
TvMGL2   189   TFASPILTNP LDLGVDIVVH SATKYINGHT DVVAGLVCSR ADIIAKVKSQ   238
PpMGL1   188   TYCTPYLQRP LELGADLVVH SATKYLSGHG DITAGIVVGS QALVDRIRLQ   237
PpMGL2   188   TYCTPYLQRP LELGADLVVH SATKYLSGHG DITAGLVVGR KALVDRIRLE   237
ScCYS3   181   TFLSPYISNP LNFGADIVVH SATKYINGHS DVVLGVLATN NKPLYE.RLQ   229
 HsCGL   189   TFMSPYFQRP LALGADISMY SATKYMNGHS DVVMGLVSVN CESLHN.RLR   237

                # ##  #       #
TvMGL1   236   GIKDITGSVI SPHDAWLITR GLSTLNIRMK AESENAMKVA EYLKSHPA.V   284
TvMGL2   239   GIKDITGAII SPHDAWLITR GTLTLDMRVK RAAENAQKVA EFLHEHKA.V   287
PpMGL1   238   GLKDMTGAVL SPHDAALLMR GIKTLNLRMD RHCANAQVLA EFLARQPQ.V   287
PpMGL2   238   GLKDMTGAAL SPHDAALLMR GIKTLALRMD RHCANALEVA QFLAGQPQ.V   287
ScCYS3   230   FLQNAIGAIP SPFDAWLTHR GLKTLHLRVR QAALSANKIA EFLAADKENV   279
 HsCGL   238   FLQNSLGAVP SPIDCYLCNR GLKTLHVRME KHFKNGMAVA QFLESN.PWV   286

                                  #    #
TvMGL1   285   EKVYYPGFED HEGHDIAKKQ MR..MSGSMI TFILKSGFEG AKKLLDNLKL   332
TvMGL2   288   KKVYYPGLPD HPGHEIAKKQ MK..MFGSMI AFDVD.GLEK AKKVLDNCHV   334
PpMGL1   288   ELIHYPGLAS FPQYTLARQQ MS..QPGGMI AFELKGGIGA GRRFMNALQL   334
PpMGL2   288   ELIHYPGLPS FAQYELAQRQ MR..LPGGMI AFELKGGIEA GRGFMNALQL   334
ScCYS3   280   VAVNYPGLKT HPNYDVVLKQ HRDALGGGMI SFRIKGGAEA ASKFASSTRL   329
 HsCGL   287   EKVIYPGLPS HPQHELVKRQ CTGC..TGMV TFYIKGTLQH AEIFLKNLKL   334

                #          ##  #          #
TvMGL1   333   ITLAVSLGGC ESLIQHPASM THAVVPKEER EAAGITDGMI RLSVGIEDAD   382
TvMGL2   335   VSLAVSLGGP ESLIQHPASM THAGVPKEER EAAGLTDNLI RLSVGCENVQ   384
PpMGL1   335   FSRAVSLGDA ESLAQHPASM THSSYTPEER AHYGISEGLV RLSVGLEDID   384
PpMGL2   335   FARAVSLGDA ESLAQHPASM THSSYTPQER AHHGISEGLV RLSVGLEDVE   384
ScCYS3   330   FTLAESLGGI ESLLEVPAVM THGGIPKEAR EASGVFDDLV RISVGIEDTD   379
 HsCGL   335   FTLAESLGGF ESLAELPAIM THASVLKNDR DVLGISDTLI RLSVGLEDEE   384

TvMGL1   383   ELIADFKQGL DALL                  396
TvMGL2   385   DIIDDLKQAL DLVL                  398
PpMGL1   385   DLLADVQQAL KASA                  398
PpMGL2   385   DLLADIELAL EACA                  398
ScCYS3   380   DLLEDIKQAL KQATN                 394
 HsCGL   385   DLLEDLDQAL KAAHPPSGIHS           405
```

**Figure 4.23 Southern blot analysis of *T. vaginalis* genomic DNA digested with various restriction endonucleases and hybridised with a *mgl1* gene specific fragment.**

The digested *T. vaginalis* genomic DNA (2µg) was electrophoresed through a 0.7% TBE agarose gel, blotted to Hybond N (Amersham International plc), hybridised with the 1kb *Eco*RI/*Xho*I *mgl1* gene fragment and washed at high stringency prior to exposure to medical X-ray film.

Lane 1: *T. vaginalis* genomic DNA digested with *Bam*HI

Lane 2: *T. vaginalis* genomic DNA digested with *Eco*RI

Lane 3: *T. vaginalis* genomic DNA digested with *Eco*RV

Lane 4: *T. vaginalis* genomic DNA digested with *Hin*cII

Lane 5: *T. vaginalis* genomic DNA digested with *Hin*dIII

Lane 6: *T. vaginalis* genomic DNA digested with *Pst*I

Lane 7: *T. vaginalis* genomic DNA digested with *Sal*I

Lane 8: *T. vaginalis* genomic DNA digested with *Sma*I

Lane 9: *T. vaginalis* genomic DNA digested with *Sph*I

Lane10: *T. vaginalis* genomic DNA digested with *Xho*I

λ*Hin*dIII markers are indicated on the left.

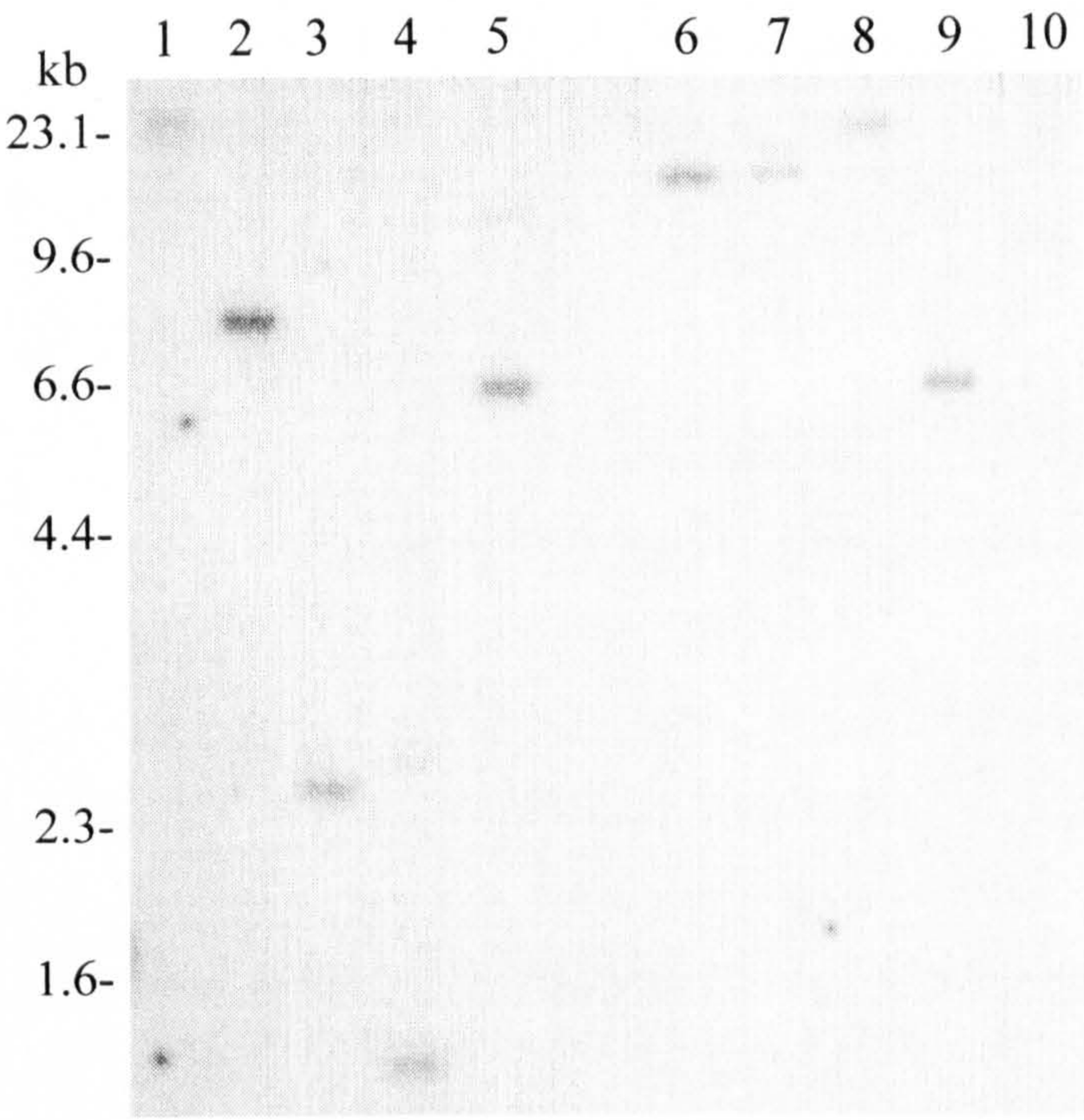

kb
1 2 3 4 5 6 7 8 9 10
23.1-
9.6-
6.6-
4.4-
2.3-
1.6-

**Figure 4.24 Southern blot analysis of *T. vaginalis* genomic DNA digested with various restriction endonucleases and hybridised with a *mgl2* gene specific fragment.**

The digested *T. vaginalis* genomic DNA (2μg) was electrophoresed through a 0.7% TBE agarose gel, blotted to Hybond N (Amersham International plc), hybridised with the 800bp *Eco*RI *mgl2* gene fragment and washed at high stringency prior to exposure to medical X-ray film.

Lane 1: *T. vaginalis* genomic DNA digested with *Bam*HI
Lane 2: *T. vaginalis* genomic DNA digested with *Eco*RI
Lane 3: *T. vaginalis* genomic DNA digested with *Eco*RV
Lane 4: *T. vaginalis* genomic DNA digested with *Hin*cII
Lane 5: *T. vaginalis* genomic DNA digested with *Hin*dIII
Lane 6: *T. vaginalis* genomic DNA digested with *Pst*I
Lane 7: *T. vaginalis* genomic DNA digested with *Sal*I
Lane 8: *T. vaginalis* genomic DNA digested with *Sma*I
Lane 9: *T. vaginalis* genomic DNA digested with *Sph*I
Lane10: *T. vaginalis* genomic DNA digested with *Xho*I
λ*Hin*dIII markers are indicated on the left.

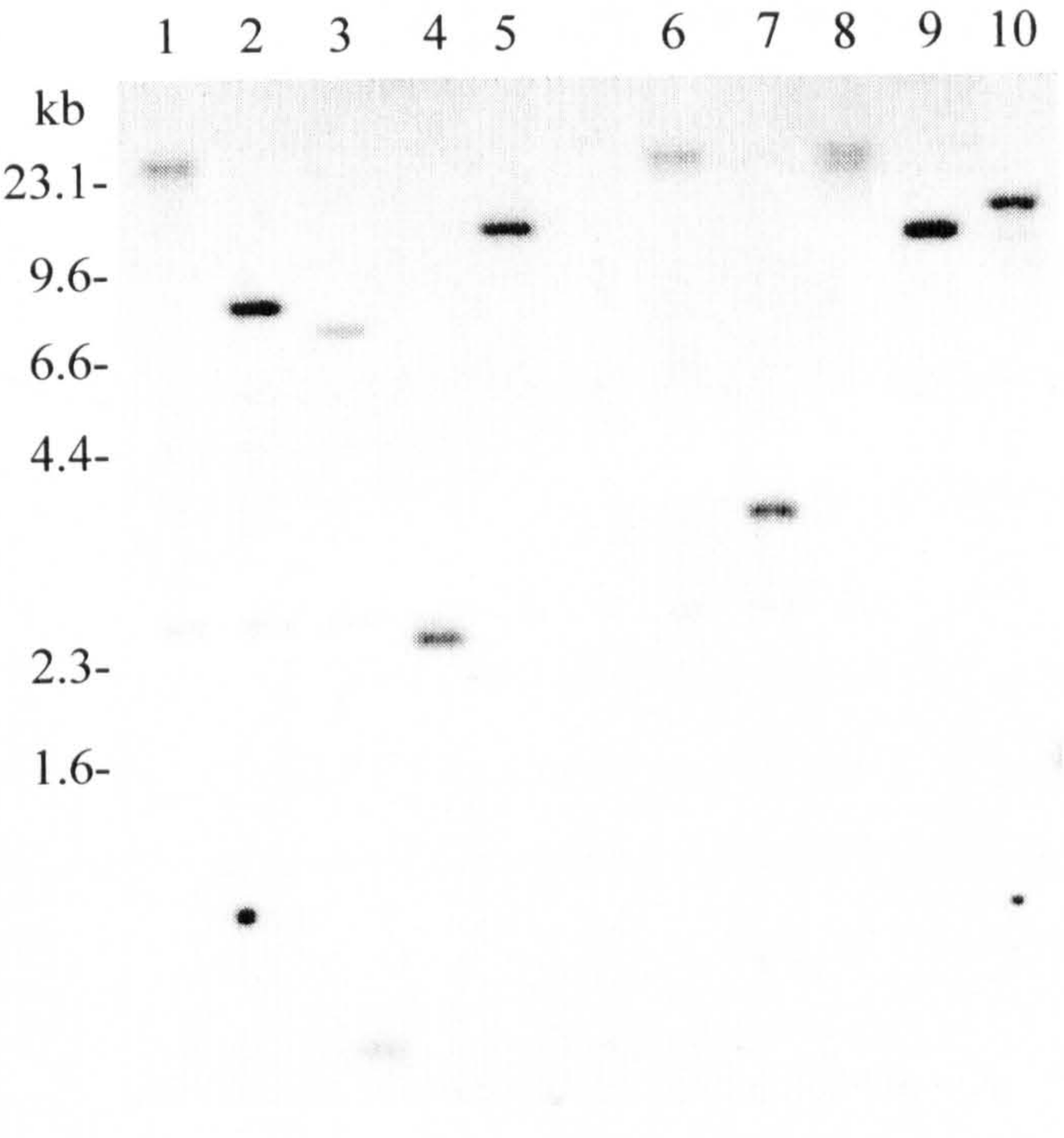

1 2 3 4 5 6 7 8 9 10
kb
23.1-
9.6-
6.6-
4.4-
2.3-
1.6-

**Figure 4.25 A/B Northern blot analysis of *T. vaginalis* total and poly [A]$^+$ RNA hybridised with the *mgl1* gene fragment (A) or the *mgl2* gene fragment (B).**

The *T. vaginalis* total and poly [A]$^+$ RNA were electrophoresed through a 1.5% agarose/MOPS/formaldehyde gel, blotted to Hybond N (Amersham International plc), hybridised with the 1kb *Eco*RI/*Xho*I *mgl1* gene fragment (A) or the 800bp *Eco*RI *mgl2* gene fragment (B) and washed at high stringency prior to exposure to medical X-ray film.

Lane 1: 10µg *T. vaginalis* total RNA

Lane 2: 1µg *T. vaginalis* poly[A]$^+$ RNA

9.5 kb-0.24 kb RNA ladder (Life Technologies) are indicated on the left.

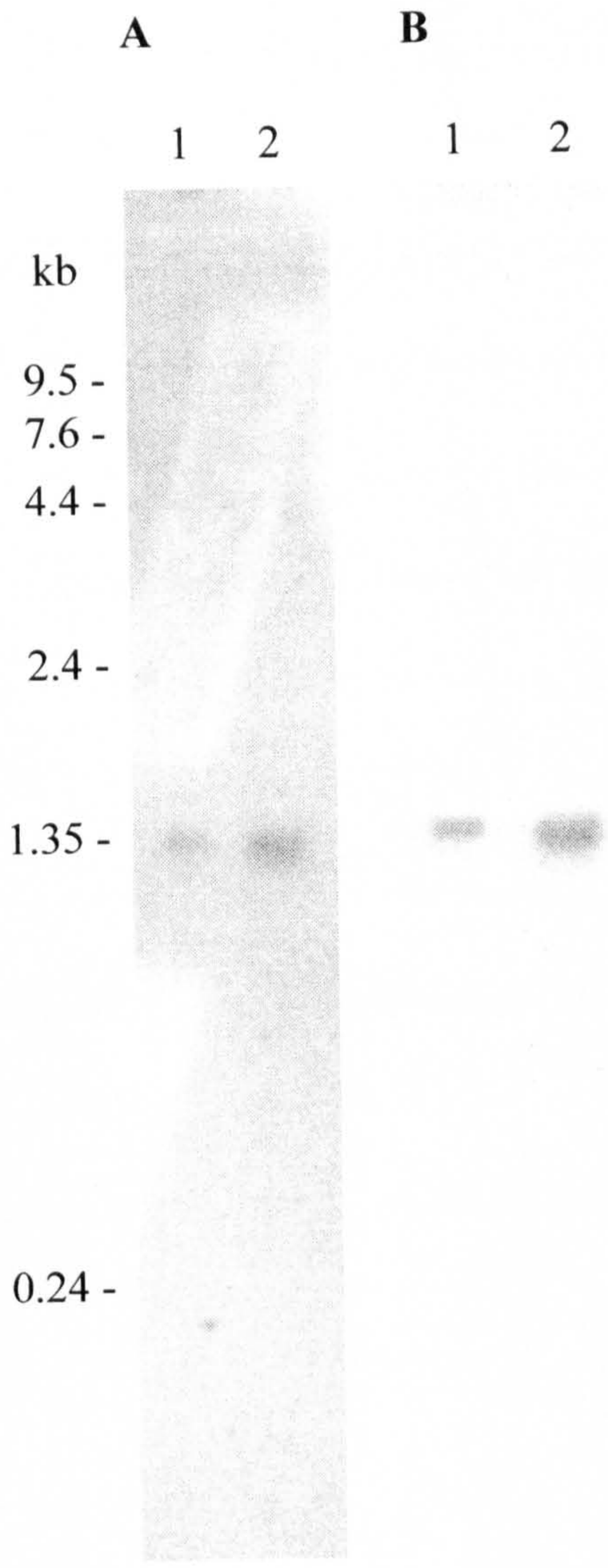

A
B
1
2
1
2
kb
9.5 -
7.6 -
4.4 -
2.4 -
1.35 -
0.24 -

**Figure 4.26 Amino acid sequence alignment of various enzymes involved in transulphuration pathways from a variety of organisms.**

Amino acid sequence alignment of *T. vaginalis* methionine γ-lyases, MGL1 and MGL2 (this study) with methionine γ-lyase from *Pseudomonas putida* (PpMGL, Inoue *et al.*,1995) and cystathionine γ-lyase from yeast (ScCYS3, Ono *et al.*, 1992) and human (HsCGL, Lu *et al.*, 1992), plus cystathionine γ synthase and cystathionine β-lyase from *E. coli* (EcMETB, Duchange *et al.*, 1983 and EcMETC, Belfaiza *et al.*, 1986. respectively) and O-acetylhomoserine sulphydrylase from yeast (ScMET25, Kerjan *et al.*, 1986) Each individual sequence is numbered accordingly. Residues that are conserved in all sequences are indicated by black boxes. (.) indicate gaps introduced to maximise the sequence alignment. The multiple sequence alignment was obtained by using the PILE-UP program of the University of Wisconsin Genetics Computer Group (GCG).

```
TvMGL1     1                MSHERMTPAT ACIHAN.PQK DQF..GAAIP PIYQTSTFVF  37
TvMGL2     1            MSG HAIDPTHTDT LSIHAN.PQK DQF..GAIVA PIYQTSTFLF  40
PpMGL1     1            MHG SNKLPGFATR AIHHGY.DPQ DHG..GALVP PVYQTATFTF  40
ScCYS3     1              M TLQESDKFAT KAIHAG.EHV D..VHGSVIE PISLSTTF..  36
HsCGL      1  MQEKDASSQG FLPHFQHFAT QAIHVG.QDP EQWTSRAVVP PISLSTTF..  47
EcMETB     1                  MTRKQAT IAVRSG.LND DE.QYGCVVP PIHLSSTYNF  35
EcMETC     1                MADKKLDT QLVNAG.RSK .KYTLGAVNS VIQRASSLVF  36
ScMET25    1                  MPSHFDT VQLHAGQENP GDNAHRSRAV PIYATTSYVF  37

TvMGL1    38  DNCQQGGNRF AGQESG.YIY TRLGNPTVSN LEGKIAFLEK TEACVATSSG  86
TvMGL2    41  DNCDQGGARF GGKEAG.YMY TRIGNPTNSA LEGKIAKLEH AEACAATASG  89
PpMGL1    41  PTVEYGAACF AGEQAG.HFY SRISNPTLNL LEARMASLEG GEAGLALASG  89
ScCYS3    37  ...KQSSP.. ANPIGT.YEY SRSQNPNREN LERAVAALEN AQYGLAFSSG  80
HsCGL     48  ...KQGAP.. GQHSG..FEY SRSGNPTRNC LEKAVAALDG AKYCLAFASG  90
EcMETB    36  TGF..... .NEPRA HDY SRRGNPTRDV VQRALAELEG GAGAVLTNTG  76
EcMETC    37  DSVEAKKHAT RNRANGELFY GRRGTLTHFS LQQAMCELEG GAGCVLFPCG  86
ScMET25   38  ENSKHGSQLF GLEVPG.YVY SRFQNPTSNV LEERIAALEG GAAALAVSSG  86

TvMGL1    87  MGAIAATVLT ILKAGDHLIS DECLYGCTHA LFEHALTKFG IQVDFINTAI  136
TvMGL2    90  MGAIAASVWT FLKAGDHLIS DDCLYGCTHA LFEHQLRKFG VEVDFIDMAV  139
PpMGL1    90  MGAITSTLWT LLRPGDEVLL GNTLYGCTFA FLHHGIGEFG VKLRHVDMAD  139
ScCYS3    81  S.ATTATILQ SLPQGSHAVS IGDVYGGTHR YFTKVANAHG VETSFTN.DL  128
HsCGL     91  L.AATVTITH LLKAGDQIIC MDDVYGGTNR YFRQVASEFG LKISFVDCSK  139
EcMETB    77  MSAIHLVTTV FLKPGDLLVA PHDCYGGSYR LFDSLAKRGC YRVLFVDQGD  126
EcMETC    87  AAAVANSILA FIEQGDHVLM TNTAYEPSQD FCSKILSKLG VTTSWFDPLI  136
ScMET25   87  QAAQTLAIQG LAHTGDNIVS TSYLYGGTYN QFKISFKRFG IEARFVEGDN  136

TvMGL1   137  PGEVKKHMKP NTKIVYFETP ANPTLKIIDM ERVCKDAHSQ ...EGVLVIA  183
TvMGL2   140  PGNIEKHLKP NTRIVYFETP ANPTLKVIDI EDAVKQARKQ ...KDILVIV  186
PpMGL1   140  LQALEAAMTP ATRVIYFESP ANPNMHMADI AGVAKIARKH ....GATVVV  185
ScCYS3   129  LNDLPQLIKE NTKLVWIETP TNPTLKVTDI QKVADLIKKH AAGQDVILVV  178
HsCGL    140  IKLLEAAITP ETKLVWIETP TNPTQKVIDI EGCAHIVHKH G...DIILVV  186
EcMETB   127  EQALRAALAE KPKLVLVESP SNPLLRVVDI AKICHLARE. ...VGAVSVV  172
EcMETC   137  GADIVKHLQP NTKIVFLESP GSITMEVHDV PAIVAAVRS. .VVPDAIIMI  184
ScMET25  137  PEEFEKVFDE RTKAVYLETI GNPKYNVPDF EKIVAIAHKH ....GIPVVV  182

TvMGL1   184  DNTF.CSPMI TNPVDFGVDV VVHSATKYIN GHTDVVAGLI C.........  223
TvMGL2   187  DNTF.ASPIL TNPLDLGVDI VVHSATKYIN GHTDVVAGLV C.........  226
PpMGL1   186  DNTY.CTPYL QRPLELGADL VVHSATKYLS GHGDITAGIV V.........  225
ScCYS3   179  DNTF.LSPYI SNPLNFGADI VVHSATKYIN GHSDVVLGVL A.........  218
HsCGL    187  DNTF.MSPYF QRPLALGADI SMYSATKYMN GHSDVVMGLV S.........  226
EcMETB   173  DNTF.LSPAL QNPLALGADL VLHSCTKYLN GHSDVVAGVV I.........  212
EcMETC   185  DNTW.AAGVL FKALDFGIDV SIQAATKYLV GHSDAMIGTA V.........  224
ScMET25  183  DNTFGAGGYF CQPIKYGADI VTHSATKWIG GHGTTIGGII VDSGKFPWKD  232

TvMGL1   224  .......... .......... ....GKADLL QQIRMVGIKD ITGSVISPHD  249
TvMGL2   227  .......... .......... ....SRADII AKVKSQGIKD ITGAIISPHD  252
PpMGL1   226  .......... .......... ....GSQALV DRIRLQGLKD MTGAVLSPHD  251
ScCYS3   219  .......... .......... ....TNNKPL YE.RLQFLQN AIGAIPSPFD  243
HsCGL    227  .......... .......... ....VNCESL HN.RLRFLQN SLGAVPSPID  251
EcMETB   213  .......... .......... ....AKDPDV V.TELAWWAN NIGVTGGAFD  237
EcMETC   225  .......... .......... ....CNARCW EQLRENAY.. LMGQMVDADT  248
ScMET25  233  YPEKFPQFSQ PAEGYHGTIY NEAYGNLAYI VHVRTELLRD .LGPLMNPFA  281

TvMGL1   250  AWLITRGLST LNIRMKAESE NAMKVAEYLK SHP.AVEKVY YPGFEDHEGH  298
TvMGL2   253  AWLITRGTLT LDMRVKRAAE NAQKVAEFLH EHK.AVKKVY YPGLPDHPGH  301
PpMGL1   252  AALLMRGIKT LNLRMDRHCA NAQVLAEFLA RQP.QVELIH YPGLASFPQY  300
ScCYS3   244  AWLTHRGLKT LHLRVRQAAL SANKIAEFLA ADKENVVAVN YPGLKTHPNY  293
HsCGL    252  CYLCNRGLKT LHVRMEKHFK NGMAVAQFLE SNPW.VEKVI YPGLPSHPQH  300
EcMETB   238  SYLLLRGLRT LVPRMELAQR NAQAIVKYLQ TQPL.VKKLY HPSLPENQGH  286
EcMETC   249  AYITSRGLRT LGVRLRQHHE SSLKVAEWLA EHP.QVARVN HPALPGSKGH  297
ScMET25  282  SFLLLQGVET LSLRAERHGE NALKLAKWLE QSPY.VSWVS YPGLASHSHH  330

TvMGL1   299  DIAKKQMR.. MSGSMITFIL KSGFEG.... ......AKKL LDNLKLITLA  336
TvMGL2   302  EIAKKQMK.. MFGSMIAFDV DGLEK..... ......AKKV LDNCHVVSLA  338
PpMGL1   301  TLARQQMS.. QPGGMIAFEL KGGIGA.... ......GRRF MNALQLFSRA  338
ScCYS3   294  DVVLKQHRDA LGGGMISFRI KGGAEA.... ......ASKF ASSTRLFTLA  333
HsCGL    301  ELVKRQCTGC T..GMVTFYI KGTLQH.... ......AEIF LKNLKLFTLA  338
EcMETB   287  EIAARQQKG. .FGAMLSFEL DGDEQT.... ......LRRF LGGLSLFTLA  324
EcMETC   298  EFWKRDFTGS SGLFSFVLKK KLNNEE.... ......LANY LDNFSLFSMA  337
ScMET25  331  ENAKKYLSNG .FGGVLSFGV KDLPNADKET DPFKLSGAQV VDNLKLASNL  379
```

```
 TvMGL1  337  VSLGGCESLI QHPASMTHAV VPKEEREAAG ITDGMIRLSV GIEDADELIA  386
 TvMGL2  339  VSLGGPESLI QHPASMTHAG VPKEEREAAG LTDNLIRLSV GCENVQDIID  388
 PpMGL1  339  VSLGDAESLA QHPASMTHSS YTPEERAHYG ISEGLVRLSV GLEDIDDLLA  388
 ScCYS3  334  ESLGGIESLL EVPAVMTHGG IPKEAREASG VFDDLVRISV GIEDTDDLLE  383
  HsCGL  339  ESLGGFESLA ELPAIMTHAS VLKNDRDVLG ISDTLIRLSV GLEDEEDLLE  388
 EcMETB  325  ESLGGVESLI SHAATMTHAG MAPEARAAAG ISETLLRIST GIEDGEDLIA  374
 EcMETC  338  YSWGGYESLI LANQPEHIAA IRPQGE..ID FSGTLIRLHI GLEDVDDLIA  385
ScMET25  380  ANVGDAKTLV IAPYFTTHKQ LNDKEKLASG VTKDLIRVSV GIEFIDDIIA  429

 TvMGL1  387  DFKQGLDALL          396
 TvMGL2  389  DLKQALDLVL          398
 PpMGL1  389  DVQQALKASA          398
 ScCYS3  384  DIKQALKQAT N        394
  HsCGL  389  DLDQALKAAH PPSGIHS  405
 EcMETB  375  DLENGFRAAN KG       386
 EcMETC  386  DLDAGFARIV          395
ScMET25  430  DFQQSFETVF AGQKP    444
```

| | |
|---|---|
| fd | TCACTTCTCTTTAGCGAATG |
| β-scs | TCACTTCACATTACAATG |
| α-scs | TCACTTCACATTAATG |
| chsp70 | TCATTTTTTAATAATG |
| Pgp1 | CCATTAATCATTAGTGATG |
| α-tub | TCACTCTTCATCATCAATG |
| β-tub | TCATTATTCACATG |
| *mgl1* | ATTTTTAGACAACATG |
| *mgl2* | ACTTTATATAAAAGATG |
| Consensus | TCAYTWYTCATTA |

**Figure 4.27 Conserved nucleotide sequence surrounding the transcription initiation sites of seven *T.vaginalis* protein coding genes, compared to the nucleotide sequence of *mgl1* and *mgl2* as determined by 5'RACE.** The 5' sequences immediately flanking the ATG translational start codon of the genes encoding the following proteins are shown: Ferredoxin (Fd), Johnson *et al.*, (1990), β-subunit succcinyl CoA synthetase (β-SCS), Lahti *et al.*, (1992), α-subunit succcinyl CoA synthetase (αSCS), cytosolic heat shock protein (cHSP70), P-glycoprotein, (PGP1), β-Tubulin, (βTUB), Quon *et al.*, (1994) and α-tubulin, (αTUB) Johnson *et al.*, (1990).

**Table 4.1 Percentage identities and percentage similarites (in brackets) of amino acid residues between various enzymes invloved in sulphur amino acid metabolism from a variety of different organisms.**

The percentage identities and similarities were determined using the GAP program of the University of Wisconsin Genetics Computer Group (GCG). The references of the sequences used are as follows: *T. vaginalis* methionine γ-lyases, MGL1 and MGL2 (this study), methionine γ-lyase from *Pseudomonas putida* (PpMGL, Inoue *et al.*, 1995) and cystathionine γ-lyase from yeast (ScCYS3, Ono *et al.*, 1992) and human (HsCGL, Lu *et al.*, 1992), plus cystathionine γ synthase and cystathionine β-lyase from *E. coli* (EcMETB, Duchange *et al.*, 1983 and EcMETC, Belfaiza *et al.*, 1986, respectively) plus O-acetylhomoserine sulphydrylase from yeast (ScMET25, Kerjan *et al.*, 1986)

| | *T. vaginalis* methionine γ-lyase 2 | *P. putida* methionine γ-lyase 1 | *P.putida* methionine γ-lyase 2 | *S. cerevisiae* cystathionine γ-lyase | *H. sapiens* cystathionine γ-lyase | *S. cerevisiae* O-acetyl homoserine sulphydrylase | *E. coli* cystathionine γ-synthase | *E. coli* cystathionine β-lyase |
|---|---|---|---|---|---|---|---|---|
| *T.vaginalis* methionine γ-lyase 1 | 69% (85%) | 44% (68%) | 44% (68%) | 44% (62%) | 42% (65%) | 35% (58%) | 37% (60%) | 30% (57%) |
| *T.vaginalis* methionine γ-lyase 2 | - | 45% (67%) | 44% (66%) | 43% (60%) | 43% (62%) | 38% (61%) | 38% (58%) | 30% (56%) |
| *P. putida* methionine γ-lyase 1 | | - | 81% (89%) | 40% (59%) | 45% (66%) | 36% (59%) | 38% (61%) | 27% (53%) |
| *P. putida* methionine γ-lyase 2 | | | - | 40% (58%) | 45% (63%) | 34% (58%) | 38% (60%) | 27% (54%) |
| *S.cerevisiae* cystathionine γ-lyase | | | | - | 52% (69%) | 39% (59%) | 39% (52%) | 29% (52%) |
| *H. sapiens* cystathionine γ-lyase | | | | | - | 36% (56%) | 42% (62%) | 30% (55%) |

| Restriction enzyme | Size of hybridising fragment (kb) |
|---|---|
| *Bam*HI | >23.1 |
| *Eco*RI | 7.5 |
| *Eco*RV | 2.4 |
| *Hinc*II | 2.5, 1.3 |
| *Hin*dIII | 15, 6.5 |
| *Pst*I | 20 |
| *Sal*I | >23.1, 20 |
| *Sma*I | >23.1 |
| *Sph*I | 6.5 |
| *Xho*I | >23.1, 14, 9 (very faint) |

**Table 4.2** A summary of the sizes of hybridising fragments obtained by Southern blot analysis after *T. vaginalis* genomic DNA was digested with various restriction enzymes and subsequently probed with a *mgl1*-specific probe, 1kb *Eco*RI/*Xho*I fragment of $p4_{100}$.

| Restriction enzyme | Size of hybridising fragment (kb) |
|---|---|
| *Bam*HI | >23.1 |
| *Eco*RI | 7.5 |
| *Eco*RV | 6.8, 0.5 |
| *Hin*cII | 2.5 |
| *Hin*dIII | 15 |
| *Pst*I | >23.1 |
| *Sal*I | >23.1 (faint) 3.5 |
| *Sma*I | >23.1 |
| *Sph*I | 14 |
| *Xho*I | 19, 13, 9 |

**Table 4.3** A summary of the sizes of hybridising fragments obtained by Southern blot analysis after *T. vaginalis* genomic DNA was digested with various restriction enzymes and subsequently probed with a *mgl2*-specific probe, 800bp *Eco*RI fragment of $p5_{100}$.

# CHAPTER 5: EXPRESSION, PURIFICATION AND BIOCHEMICAL CHARACTERISATION OF RECOMBINANT *T. VAGINALIS* METHIONINE γ-LYASE HOMOLOGUES.

## 5.1 Introduction.

Two gene homologues for methionine γ-lyase have been isolated from *T. vaginalis* which have a high level of identity to methionine γ–lyase from *P. putida* and to cystathionine γ-lyases from yeast and human.(Chapter 4). Amino acid residue comparisons of methionine γ-lyase from *P. putida* and cystathionine γ-lyase from yeast and human with the *T. vaginalis mgl1* and *mgl2* were unable to convincingly assign the *T. vaginalis* genes an identity. In order to address this problem the two genes were cloned into *E. coli* expression vectors in order to obtain recombinant proteins. These were biochemically characterised and lead to an enzyme activity being attributed to the products of the *T. vaginalis mgl1* and *mgl2* genes.

The QIAexpressionist system (Qiagen) was chosen for the expression and purification of *mgl1* and *mgl2*. They were cloned in to a QIAexpress pQE expression vector which encode a 6xHistidine tag at the N or C terminus of the expressed protein. The 6xHistidine tagged recombinant protein was then affinity purified using $Ni^{2+}$-NTA (nitrilo-tri-acetic acid) resin.

## 5.2 Results.

### 5.2.1 Cloning of *mgl1* into pQE60.

The *mgl1* cDNA $p4_{100}$ contained the complete open reading frame of the *mgl1* gene but was missing 13 nucleotides that made up the 5'UTR of the gene (See Chapter 4 for details). As the $p4_{100}$ cDNA of *mgl1* included the methionine start site it was decided to use a Type ATG (pQE60) vector to clone the complete open reading frame for *mgl1*. This would produce a recombinant protein with a 6xHistidine tag located at the C-terminus and utilises its authentic ATG codon. Cloning of the cDNA of $p4_{100}$ into a Type ATG construct also removes the authentic *mgl1* stop codon by mutating it and therefore expression the recombinant protein from the vector becomes dependent on the vector derived stop codon. Sequence analysis revealed that no restriction sites were available within $p4_{100}$ that would enable the direct cloning of *mgl1* into pQE60. In order to overcome these cloning problems, a PCR based cloning strategy was adopted to enable *mgl1* to be cloned into pQE60. Oligonucleotide primers were designed to the 5'and 3'ends of *mgl1* and included the restriction endonucleases *Nco*I and *Bgl*II respectively. The NcoI site at the 5' end of the construct serves to regenerate the AUG start codon. Through the PCR amplification process these two restriction sites, engineered onto the ends of the *mgl1* DNA, would facilitate cloning into *Nco*I and *Bgl*II restricted pQE vector. The nucleotide sequence of the two primers used in the PCR are outlined below:-

5' *Nco*I primer for *mgl1* PCR, for cloning into pQE60 (24nucleotides)

```
5′                              3′
CGCCATGGCTCACGAGAGAATGAC
  NcoI
```

3' *Bgl*II primer for *mgl1* PCR, for cloning into pQE60 (27nucleotides)

```
5′                                  3′
GCAGATCTTAAAAGAGCGTCAAGGCCC
  BglII
```

$p4_{100}$ was linearised using *Bam*HI, which served as the template for the PCR with the two oligonucleotides and *Pfu* polymerase, which has proof-reading activity . The following amplification protocol was employed. An initial denaturation step of 4 minutes at 94°C was followed by 30 cycles at 94°C for 1 minute, 42°C for 1 minute and 72°C for 1 minute. A final step of 5 minutes at 72°C was used to complete the extension. After PCR, contaminating nucleotides and polymerase were removed from the PCR product using Magic PCR Wizard preps. The cleaned up DNA was restricted with *Nco*I and *Bgl*II and ligated with *NcoI*/*Bgl*II restricted pQE60 and subsequently transformed into XL1-Blue cells. XL1-Blue were chosen in order to make the screening process easier. The cells into which the pQE plasmid are transformed for efficient protein expression, M15pREP4, contain an additional plasmid pREP4, which encodes the lac repressor and is required for the prevention of expression of the recombinant protein until IPTG is added to the cells.

In order to ascertain whether the cloning of the *mgl1* PCR product into pQE60 had been successful, restriction enzyme analysis of plasmid from transformed cells was performed. pQE60 plasmid was isolated from transformed XL1-Blue clones and restricted with *Nco*I and *Bgl*II, and electrophoresed in a 1% agarose gel (Figure 5.1, lanes 3-6). Lanes 3 and 5 contain a single band of 3.5kb which represents the plasmid pQE60 alone. Lanes 4 and 6, however, both contain two fragments of 3.5kb and 1.3kb and this indicated the cloning of the amplified 1.3kb fragment of *mgl1* into the pQE60 expression vector had been successful.

After the cloning of *mgl1* into pQE60, the intact plasmid was then transformed into M15pREP4. The protein expression clone for *mgl1* was named pQMGL1a (lane 4).

The nucleotide and predicted amino acid sequence of pQMGL1 is shown in Figure 5.2. Differences between pQMGL1 and *mgl1* are outlined by red and green text, which highlights the change in the second amino acid residue of pQMGL1 compared to *mgl1* and also shows the 6x Histidine tag that is present at the C-terminus of the recombinant protein.

### 5.2.2 Test induction and expression rMGL1 from pQMGL1.

Test expressions of *E. coli* containing pQMGL1a were carried out. Firstly, 100mls M15pREP4 cells containing pMGL1 were grown in LB amp/kan (100 μg and 25 μg/ml, respectively) at 37°C until the $OD_{600nm}$ reached 0.7-0.9 (~1.75 hours), the cells were induced with 2 mM IPTG and grown for a further 3 hours at 37°C. An experiment was then carried out to check the location and the solubility of the expressed protein, by following a protocol in the QIAexpressionist handbook (See QIAexpress handbook for details). The experiment was designed to find out whether the expressed protein was located in the pellet, periplasm or soluble fraction of the induced cells. SDS-PAGE analysis of the pellet, periplasmic and cytosolic fractions of the induced *E. coli* revealed the cellular location and solubility status of the expressed protein (Figure 5.3). A predominant protein was detected in the soluble fraction of the induced cells of 43 kDa (Figure 5.3, lane 4). The recombinant protein was the expected size to be produced when the insert of size of the plasmid is taken into consideration. There is a protein of the same size present in the pellet fraction of the induced cells along with some larger proteins (lane 5), but not in the same quantities as in the soluble fraction. There was no protein of the expected size present in the periplasmic fraction of the induced cells (lane 6).

### 5.2.3 Routine expression and purification of rMGL1

The expression and purification of recombinant MGL1 (rMGL1) was based on the results of the experiments outlined above, using 2 mM IPTG and isolating the protein from the soluble fraction of the induced cells. A 50 ml overnight culture of *E. coli* containing pQMGL1 was inoculated into 400 ml of LB broth containing 100μg/ml ampicillin and 25μg/ml kanamycin, which was grown for 1.75 hours at 37°C prior to induction with 2 mM IPTG, the cells grown for a further 2.25 hours before harvesting. The recombinant protein was released from the cells by sonication and separated from the bacterial cell debris by a subsequent centrifugation step prior to purification. The soluble fraction of the cells was filtered through a 0.22μM filter and loaded onto a 4ml $Ni^{2+}$-NTA column. The non-histidine tagged *E. coli* proteins were washed off using successive washes of sonication and wash buffer. The 6xHistidine tagged recombinant protein eluted from the column using a FPLC generated linear gradient of 0-500mM imidazole. Figure 5.4 shows a typical A280nm trace obtained from the purification of rMGL1 and summarises the FPLC protocol used to purify the 6xHistidine tagged protein. A large peak of absorbance was seen for the first 50 minutes of the purification of rMGL1, this was the large amount of *E. coli* proteins that do not bind to the $Ni^{2+}$-NTA column and are washed from the column by sonication buffer. There was also a second smaller peak observed at 100 minutes and this too represents *E. coli* proteins that are more hydrophobic, as they were eluted from the column by wash buffer that contained glycerol. Two peaks were eluted during the imidazole gradient. The first at 170-180 minutes and the second starting at 190 minutes. The large single peak that is eluted between 194 and 210 minutes had a maximum A280 nm absorption of 2AU and shows the rMGL1 being eluted specifically from the $Ni^{2+}$-NTA between 220 mM and

300 mM imidazole. Fractions shown to contain rMGL1, based on the A280 nm trace run were selected for analysis by SDS-PAGE (Figure 5.5)

The insoluble fraction of the induced pQMGL1 M15pREP4 cells, that was obtained after the sonication and centrifugation step is shown in Panel A, lane 1. The protein profile of the soluble induced fraction of M15pREP4 cells that was loaded onto the $Ni^{2+}$-NTA (Panel A, lane 2) shows a predominant band of ~43 kDa, which was the induced 6xHistidine tagged rMGL1. The immediate flow through fraction from the purification is shown in Panel A, lane 3 and SDS-PAGE analysis reveals that essentially all of the induced 6xHistidine tagged rMGL1 has bound to the $Ni^{2+}$-NTA column, with other *E. coli* proteins being eluted from the column in sonication buffer. Lanes 5 and 6 show a number of faintly wash buffer fractions that were eluted from the column, there are staining bands of molecular weights ranging between 45 and 66 kDa were detected in Panel A, lanes 5 and 6. Proteins eluted from the $Ni^{2+}$-NTA column between 174 and 180 minutes of the purification run are shown in Panel A, lanes 8-10. The proteins present in these fractions have various molecular weights and are different from the proteins previously eluted from the column using sonication and wash buffer. The fractions that were collected at 194-210 minutes into the protein purification, Figure 5.5B, lanes 2-10. These fractions contain the 6xHistidine tagged rMGL1 protein that was eluted from the column between 220 mM and 300 mM imidazole and which made up the main peak that was eluted from the column during the running of the imidazole gradient. Two main proteins were eluted from the column, one ~43kDa, which is the size of recombinant protein expected to be produced when the size of the *mgl1* open reading frame is taken into consideration, the second protein was of higher molecular weight, greater than 66 kDa. (Panel B, lanes 2-10). The rMGL1 samples were only

heated to 37°C in the presence of Laemmli sample buffer so as to prevent the hydrolysis of acid labile bonds that exist between the recombinant protein and the imidazole buffer.

To investigate whether the higher molecular weight protein present in the fractions eluted from the column was a multimeric complex being formed by the rMGL1 molecules, the imidazole used to elute the recombinant protein from the column was dialysed away and the sample subjected to SDS-PAGE analysis after being boiled with Laemmli sample buffer (Figure 5.6). rMGL1 was heated to 37°C in the presence of imidazole buffer. Two main bands of protein, one of greater than 66 kDa in size and the other of ~43 kDa were detected and this was the same as outlined in Figure 5.5, Panel B, lanes 2-10. When rMGL1 was boiled in the presence of Laemmli sample buffer in the absence of imidazole buffer, one main protein of ~43 kDa was detected.(Figure 5.6, lane 2). There was no evidence of higher molecular bands. Thus rMGL1 forms multimeric complexes under certain conditions.

A typical purification of rMGL1 from 450 ml of *E. coli* containing pQMGL1 yielded ~15 mg of the recombinant protein.

### 5.2.4 Cloning of the *mgl2* into pQE30.

The $p5_{100}$ cDNA was missing 26 nucleotides which made up the 5' end of the gene, including the AUG start codon. The presence of suitable restriction sites present in the $p5_{100}$ cDNA allowed the cloning of *mgl2* directly into a Type IV pQE expression construct. The Type IV construct places the 6xHistidine tag at the N-terminus of the protein. $p5_{100}$ was restricted with *Bam*HI and *Xho*I and the 1.3 kb insert was gel purified and ligated with *Bam*HI and *Sal*I restricted pQE30 and transformed into XL1-Blue cells. Mini-prep DNA from individual transformants was tested for the presence of insert by

of restriction enzyme analysis. pQE30 alone was restricted with *Pst*I (Figure 5.7, lane 2) and *Bam*HI and *Hin*dIII (Figure 5.7, lane 3), showed a single band of ~3.5 kb, which is the size of the vector. Restriction analysis of plasmid isolated from two individual transformants that was restricted with *Bam*HI and *Hin*dIII revealed two different size fragments (Figure 5.7, lanes 5 and 6). The 3.5kb band is the vector and the 1.3kb band, which was the expected size of the insert of p5100 ligated into the expression vector.

The expression vector construct for *mgl2* was named pQMGL2 and the nucleotide and predicted amino acid sequence of the insert of *mgl2* contained in this plasmid is shown in Figure 5.8. As can be seen the 6xHistidine is located at the N terminus of the protein but due to nature in which the *mgl2* insert from $p5_{100}$ was cloned into pQE30 there are an additional 8 amino acid residues derived from pBluescript located at the N terminus of the protein, this vector derived sequence is shown by blue text in Figure 5.8.

### 5.2.5 Test induction and expression of rMGL2 from pQMGL2.

Test expressions were also carried out for pQMGL2 in the same way as for pQMGL1. SDS-PAGE analysis of the various fractions obtained for the induced *E. coli* cells containing pQMGL2 is shown in Figure 5.9. The protein profile of uninduced and induced bacterial cells are shown in Figure 5.9, lanes 1 and 2 respectively. The protein constituents of the soluble fraction of the induced cells are shown in Figure 5.9, lane 4, there are a number of proteins of different molecular weights, with a predominant band at ~43 kDa, the expected size of rMGL2. The insoluble fraction of the induced cells (Figure 5.9, lane5), shows a predominant band of 43 kDa along with other proteins of different molecular weights. No protein bands are visible in the periplasmic fraction of the induced cells (Figure 5.9, lane 6). It would appear results that the 6xHistidine

tagged rMGL2 is present both in the soluble and insoluble fractions of the induced *E. coli* cells. Based on the results of the above experiment it was decided to reduce the concentration of IPTG that was being used to initiate rMGL2 expression ten fold, this change was decided as it was most likely that the expression of rMGL2 was too great initially and that expressed protein was being produced in very large quantities that was causing probably two thirds of the protein to become insoluble. A lower concentration of IPTG would encourage a more steady production of the recombinant protein over time and allow a larger quantity of the protein to remain in the soluble fraction of the induced cells, which could be purified more easily than if the protein was to become insoluble.

### 5.2.6 Routine expression and purification of rMGL2.

Based on the experiment outlined above it was decided that the expression of rMGL2 would identical to the protocol used for the expression of rMGL1, with the exception that the cells would be induced with 0.2 mM IPTG.

Figure 5.10 shows the A280 nm profile obtained from a typical purification of rMGL2. The profile is very similar to the one obtained for the purification of rMGL1. There is a large flow through and wash peak (>2AU) that is obtained when the $Ni^{2+}$-NTA is washed with sonication buffer during the first 50 minutes of the protein purification. There is another small peak at 100 minutes into the purification, which were *E. coli* proteins that are eluted from the column in the presence of wash buffer, that contains glycerol. Between 180 and 195 minutes of the purification there is a large peak (>2AU) that is eluted from the column, this is the 6xHistidine tagged protein that is displaced from the column by imidazole.

Figure 5.11A/B shows SDS-PAGE analysis of certain fractions that were collected during the purification of rMGL2 for which the A280 nm profile is shown in Figure 5.10. Figure 5.11, Panel A, lane 1 shows the protein constituents of the insoluble fraction of the induced *E. coli* cells obtained after the sonication and centrifugation step of the purification of rMGL2. There are many proteins that make up the insoluble fraction of the cells but there is no induced protein of ~43 kDa present in this fraction of the cells, which indicates that the reduction in the concentration of IPTG used to induce the cells was successful in preventing the rMGL2 going into the insoluble fraction of the bacteria. The protein profile of the soluble fraction of the induced *E. coli* (Figure 5.11A, lane 2), shows a predominant band of ~43 kDa, which is the 6xHistidine tagged rMGL2, along with other soluble bacterial proteins of various molecular weights. The immediate flow through fraction from the column (Figure 5.11A, lane 3),shows that the 6xHistidine tagged rMGL2 has bound to the $Ni^{2+}$-NTA, with other non tagged *E. coli* proteins being eluted from the column with sonication buffer. The *E. coli* proteins eluted from the $Ni^{2+}$-NTA column when washed with sonication buffer (Figure 5.11A, lane 5), show a few faintly staining bands of various molecular weights. The bacterial proteins that were eluted from the column with wash buffer (Figure 5.11A, lane 7), due to the faintness of the staining of the bands in this fraction they are not visible in Figure 5.11, but there were several bands of different molecular weights present in this fraction. rMGL2 that was eluted from the $Ni^{2+}$-NTA column by imidazole between 178 and 194 minutes of the purification run is shown in Figure 5.11B, lanes 1-4, 6-10 As can be seen from all lanes, especially lanes 2-4 and 6-8 (which are fractions taken from the middle of the peak eluted by imidazole), there is a predominant protein band of ~43 kDa with a small amount of a another band of >66 kDa in size. As with rMGL1 the higher

molecular weight band is a multimeric complex of rMGL2, which in the absence of imidazole and boiled in the presence of Laemmli sample buffer is reduced to the 43 kDa monomer. The amount of multimeric complex formed by rMGL2 is not nearly as great as that formed by rMGL1.

A typical purification of rMGL2 from 450 ml of induced *E. coli* culture typically yielded ~20mg of protein.

Preliminary enzyme assays investigated the catabolism of homocysteine, one of the substrates broken down by methionine γ-lyase from both *T. vaginalis* and *P. putida* and they revealed that both of the recombinant proteins produced from the two *T. vaginalis* methionine γ-lyase gene homologues were able to break down this substrate. This was a very exciting finding as enzymatically active recombinant *T. vaginalis* methionine γ-lyase provided the material with which to fully biochemically characterise the two methionine γ-lyase gene homologues and therefore assign an enzyme activity to the two genes that had been isolated.

### 5.2.7 Stabilisation studies of rMGL1 and rMGL2.

Good quantities of both rMGL1 and rMGL2 (~15-20mg/ purification run) could be obtained from a single protein purification, which would enable extensive biochemical characterisation of the two recombinant proteins. A study was undertaken to determine the conditions under which optimum enzyme activity could be maintained over a number of weeks. Based on stabilisation conditions that had been used for native methionine γ-lyase (Lockwood and Coombs, 1991) and for enzyme stabilisation in general, a number of different conditions were investigated, for their ability to stabilise

the two recombinant enzymes. Tables 5.1 and 5.2 summarise the results of the stabilisation experiments for rMGL1 and rMGL2, respectively.

Before the two purified recombinant proteins were combined with the different enzyme stabilisation solutions (A-F) they were treated in one of three ways. 1. Recombinant protein that was eluted from the $Ni^{2+}$-NTA column was combined immediately with the different enzyme stabilisation solutions (rMGL1 or rMGL2 + Imidazole). 2. Recombinant proteins eluted from the column were dialysed overnight against 0.1M sodium phosphate buffer pH 7.5 and 20% (v/v) glycerol, 15 μM DTT and 20 μM PLP, in order to remove the imidazole that was present (rMGL1 or rMGL2 -Salt). 3. Purified proteins were dialysed overnight against 0.1M sodium phosphate buffer pH 7.5 with the same additions as in 2. but which also contained 300mM sodium chloride (rMGL1 or rMGL2 +Salt). The results presented in Tables 5.1 and 5.2 basically show the same overall trends with regards to the stability of the recombinant proteins under the variety of conditions. Firstly it appears that the replacement of the imidazole, in which the proteins are eluted, with the dialysis buffer containing sodium chloride, glycerol, DTT and PLP increases the stability of the enzyme. Thus, whereas rMGL1 retained 47% of its homocysteine desulphurase activity after storage in buffer that contained imidazole for 14 days, 96% of the original activity was retained over the same time when the enzyme was in the stabilisation buffer containing 300mM sodium chloride. For rMGL2 the same trend is observed: 47% homocysteine desulphurase activity was retained in the presence of the buffer with imidazole whereas 91% activity was retained under the same storage condition but after dialysis overnight with buffer containing sodium chloride. For nearly all stabilisation conditions used, the trend was similar for both rMGL1 and rMGL2. As for the addition of reducing agents and PLP on the maintenance of homocysteine desulphurase activity of the two recombinant proteins, it can be seen from

Tables 5.1 and 5.2 that 100% activity was retained by both of the proteins over 14 days subsequent to being dialysed against buffer containing sodium chloride then being combined with stabilisation buffer F and stored at -20°C. Thus, due to the excellent stabilisation of enzymatic activity of the two recombinant proteins under these conditions outlined above, it was decided they would be adopted as the standard stabilisation conditions for rMGL1 and rMGL2. These enzyme stabilisation experiments were also useful in that they revealed the time period over which the enzyme activity was retained. It was important to know this before the onset of detailed biochemical characterisations. Further analyses were carried out on the stored enzyme in stabilisation buffer F at -20°C, no loss in homocysteine desulphurase activity was found, after the two recombinant proteins were stored over a period of three months.

### 5.2.8 Determination of pH optima for homocysteine breakdown by rMGL1 and rMGL2.

The pH optima were also determined for the breakdown of homocysteine by rMGL1 and rMGL2. Such experiments were carried out so that the pH at which the enzymatic activities were conducted could be standardised. As can be seen from Figures 5.12 and 5.13, the pH optimum for both rMGL1 and rMGL2 appears to be pH 6.5. Imidazole buffer was used as it covered the expected pH range of the two recombinant proteins and preliminary pH optimum experiments had indicated that homocysteine breakdown by the two recombinants was elevated in the presence of imidazole buffer. Thus, all future enzyme assays were conducted at pH6.5 in imidazole buffer, also the pH of the dialysis buffer and enzyme stabilisation buffer used at the end of the purification of the two recombinant proteins were changed to pH 6.5. The pH optimum was not determined for the other substrates used by the two recombinant proteins.

## 5.2.9 Biochemical characterisation of rMGL1 and rMGL2.

### 5.2.9.1 Substrate specificities of rMGL1 and rMGL2.

A number of biochemical characterisations were carried out for the two recombinant proteins in order to determine the likely physiological roles of the two enzymes. Firstly, the breakdown of a number of key substrates by rMGL1 and rMGL2 was investigated. The substrates selected were from those that had been used to characterise native methionine γ-lyase from *T. vaginalis*. Table 5.3 summarises the specific activities obtained for rMGL1 and rMGL2 with a range of substrates. The specific activities were calculated using values obtained for the production of the α-keto acid from each substrate. As can be seen from Table 5.3, the two recombinant proteins had a very high specific activity for homocysteine-64 μmol/min/mg and 54 μmol/min/mg for rMGL1 and rMGL2 respectively. The two recombinant proteins were also able to break down methionine, with the value of 8.4 μmol/min/mg being obtained for rMGL1 and 1.8 μmol/min/mg being obtained for rMGL2. rMGL1 and rMGL2 also have activity towards O-acetyl-L-serine and cysteine, with the specific activities calculated for O-acetyl-L-serine being very similar for the two proteins and the activity for the breakdown of cysteine being nearly two-fold higher for rMGL1 than for rMGL2. The activity toward S-adenosyl-methionine of the two proteins is also very low. Neither recombinant protein showed activity towards cystathionine. This suggests that *mgl1* and *mgl2* are truly methionine γ-lyases and probably not cystathionine γ-lyases. The substrate specificity data presented in Table 5.3 would support this view, as the substrates broken down by rMGL1 and rMGL2 are also characteristically broken down

by methionine γ-lyase from anaerobic bacteria such as *P. putida* and *Aeromonas species* (Esaki and Soda, 1987).

### 5.2.9.2 Determination of kinetic parameters for rMGL1 and rMGL2.

Kinetic parameters for rMGL1 and rMGL2 were also determined when homocysteine, methionine and cysteine were substrates for the two proteins. Figures 5.14 to 5.19 show typical saturation curves obtained for homocysteine, methionine and cysteine for the two recombinant proteins. The saturation curves determined for homocysteine and cysteine used the hydrogen sulphide trapping assay, whereas the methionine saturation curves were determined using the colourimetric reagent, MBTH. As can be seen from Figures 5.14 and 5.15 rMGL1 displayed hyperbolic kinetics with respect to homocysteine and methionine. However, the saturation curve for cysteine with rMGL1 is sigmoidal (Figure 5.16), which suggests that co-operativity may exist between the subunits of rMGL1 when cysteine is used as a substrate and that regulatory modulators may be able to act on rMGL1. For rMGL2 it is interesting to note that Michaelis-Menten kinetics are presented for methionine as a substrate (Figure 5.18). Sigmoidal kinetics, however, are presented for both homocysteine and cysteine (Figures 5.17 and 5.19 respectively), which may suggest the possibility of allosteric regulation of rMGL2 by some unknown effector. Table 5.4 summarises the Km and Vmax data obtained for rMGL1 and rMGL2 from a number of experiments with the three substrates outlined above. As can be seen from Table 5.4, the Km value obtained when methionine is the substrate for rMGL1 is low when compared to the values obtained for homocysteine and cysteine, 0.65mM for methionine compared to 4.7 mM for homocysteine and 6.1 mM for cysteine. Comparison of the Km values obtained for rMGL1 for the three substrates to the values obtained for methionine with rMGL2 reveal that the MGL2 Km values are

higher in all cases. There is a significant difference in the Km value obtained for methionine when rMGL2 is compared to rMGL1, 10.6 mM compared to 0.65 mM. The Km values for homocysteine and cysteine with rMGL2 are approximately four-fold greater than the values obtained for the same substrates with rMGL1. Comparison of the Vmax values for both proteins with the three different substrates reveal that for both homocysteine and cysteine the Vmax values for the two proteins are very similar. However, the Vmax value calculated for methionine with rMGL1 is two fold higher than the equivalent value calculated for rMGL2.

#### 5.2.9.3 The effect of exogenous thiol on homocysteine breakdown by rMGL1 and rMGL2.

The breakdown of homocysteine by rMGL1 and rMGL2 was assayed in two ways. Firstly, by monitoring the production of hydrogen sulphide in a continuous assay, in which the trapping agent lead acetate was used. This was converted to lead sulphide, the production of which can be monitored at 360 nm (Thong and Coombs, 1985) Secondly, the production of α-ketobutyrate which used the reagent 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) which reacts with α-keto acids to form azines which absorb maximally at 320nm (Soda, 1968) It was found that the calculated specific activities for the breakdown of homocysteine by rMGL1 and rMGL2 differed depending on which assay system was used. As can be seen from Table 5.5, the specific activity calculated for the breakdown of homocysteine by rMGL1 using the $H_2S$-trapping system was 246 μmol/min/mg protein compared with 49 μmol/min/mg protein when the production of α-ketobutyrate was investigated. Similarly, the specific activities calculated for rMGL2 were 323 μmol/min/mg protein for the $H_2S$ detection

compared to 50μ mol/min/mg protein for the α-keto acid detection. This was surprising as the γ-elimination of homocysteine catalysed by methionine γ-lyase gives equimolar quantities of the three products, α-ketobutyrate, hydrogen sulphide and ammonia (see Figure 5.20). As elevated levels of hydrogen sulphide were produced by both recombinant proteins compared with α-keto acid production it was thought that an exchange/replacement reaction could be occurring. As is detailed in Figure 5.20 this type of reaction is able to occur when exogenous thiol (eg. β-mercaptoethanol) is added along with homocysteine and other sulphur-containing amino acids. The elimination/exchange reaction results in more thiol being liberated from the substrate and a decrease in the amount of α-keto acid formed. However, it was not likely that rMGL1 and rMGL2 with regards to homocysteine breakdown, as there was no exogenous thiol added to the assays. However, to investigate this further assays were carried out in the presence β-mercaptoethanol. The results of this experiment are also given in Table 5.5. For both proteins, there was a decrease in the amount of α-ketobutyrate produced by the two proteins compared with the equivalent assay without exogenous thiol (2.6 and 13.3 μmol/min/mg protein for rMGL1 and rMGL2, respectively), but there is also a decrease in the amount of thiol produced from homocysteine by rMGL1 (152 μmol/min/mg protein) and the amount of hydrogen sulphide produced from the breakdown of homocysteine by rMGL2 is basically the same as for the control experiment (326 μmol/min/mg protein). These results appear to indicate that some sort of exchange reaction was taking place as judged by the decrease in the amount of α-keto acid, but an increase in the amount of thiol liberated was not occurring. Indeed it appeared for rMGL1 that the exogenous thiol may be inhibiting the

exchange reaction. It could be that some thiol other than hydrogen sulphide is being produced, that is not able to be detected by the lead acetate of the trapping assay.

### 5.2.9.4 SDS-PAGE analysis of rMGL1 and rMGL2.

Further biochemical analyses of the two recombinant proteins included SDS-PAGE. PAGE analysis of the two recombinant proteins on large acrylamide gels revealed differences in size between rMGL1 and rMGL2. As can be seen from Figure 5.21 the rMGL1 protein is 44 kDa in size, whereas the rMGL2 protein is of slightly higher molecular mass at 47 kDa. It must be remembered that the 6xHistidine tagged rMGL2 protein is expected to be of higher molecular weight when compared to rMGL1, due to the way in which *mgl2* was cloned into pQE30. rMGL2 has eight additional amino acid residues that are derived from pBluescript (see Figure 5.8).

### 5.2.9.5 Gel filtration chromatography of rMGL1 and rMGL2.

The native molecular weights of the two recombinant proteins were investigated by gel filtration chromatography. A Superose 12 column was used for the native molecular weight determinations. The calibrated gel filtration column revealed that the native molecular mass of rMGL1 was > 440 kDa in size. The protein was eluted from the gel filtration column after 23 minutes compared to the 27 minutes that it took for ferritin to be eluted, which is 440 kDa, thus one is able to conclude that rMGL1 is larger than ferritin. This result was really not that surprising when the characteristics of rMGL1 in not fully reduced conditions on Laemmli gels are taken into consideration. It has already been shown earlier in this chapter that rMGL1 has a tendency to form high molecular weight complexes which can be dissociated by boiling in the presence of Laemmli sample buffer, after the removal of imidazole (Figure 5.6). The result from the

gel filtration experiment appear to indicate that high molecular weight complexes are formed by rMGL1, which do not to affect enzyme activity. The native molecular of rMGL2 as determined by gel filtration chromatography was ~158 kDa. The protein was eluted from the column at exactly the same time as aldolase, which has a molecular mass of 158 kDa. The size of rMGL2 would appear to indicate that this protein is forming a tetrameric structure, which is characteristic of other methionine γ-lyases isolated to date.

### 5.2.9.10 Native gel electrophoresis of rMGL1 and rMGL2.

Native gel electrophoresis was also carried out for the two recombinant proteins, Figure 5.22 shows the result of this experiment. Lanes 1 and 2 show Coomassie Blue R250 staining of rMGL1 and rMGL2, respectively. The mobility of rMGL2 was slower than that of rMGL1, consistent with the difference in molecular weight of the two proteins. Figure 5.22, Lanes 3 and 4 show the homocysteine desulphurase activity of the two proteins and as can be seen the homocysteine catabolising capabilities of the two proteins are coincident with the Coomassie stained proteins. Finally lanes 5 and 6 show the effect of the inhibitor propargylglycine on the homocysteine desulphurase activity of the two recombinant proteins. The activity of the two proteins was completely inhibited by a 10 mM concentration of this inhibitor.

### 5.2.9.11 Western blot analysis of *T. vaginalis* homogenates using antibodies raised against rMGL1 and rMGL2.

Polyclonal antibodies were raised against the two recombinant proteins and they were subsequently used in Western blot analyses with *T. vaginalis* homogenates, pellet and supernatant fractions. Figure 5.23 shows the result of the Western blots using antisera

against rMGL1 and rMGL2. Figure 5.23, Panel A, lanes 6 and 7 show the reactivity of the anti rMGL1 antiserum with rMGL1 and rMGL2, respectively. A single band of ~43 kDa is detected with the anti rMGL1 and the rMGL1 protein, a slightly larger size band is detected with the same antibody and rMGL2. A single band of ~43 kDa was detected in a *T. vaginalis* homogenate (panel A, lane 8) and soluble fraction (Panel A, lane 10), there is no reactivity of the anti-rMGL1 serum and a pellet fraction from *T. vaginalis* (panel A, lane 9). Figure 5.23 Panel A lanes 1-5 show the result of Western blot analysis when the same samples as described above were reacted with the appropriate serum. There was no reactivity of the samples with the pre-immune serum. Figure 5.23, Panel B, lanes 6 and 7 show the reactivity of the anti rMGL2 antiserum with purified rMGL1 and rMGL2 respectively. There was no reactivity of the anti rMGL2 antibody and purified rMGL1 (Lane 6), whereas the same antiserum recognises a protein of ~43 kDa when reacted with rMGL2. A single band of ~43 kDa is detected in a *T. vaginalis* homogenate (Panel B, lane 8) and soluble fraction (Panel B, lane 10), there is no reactivity of the anti-rMGL1 antiserum and a pellet fraction from *T. vaginalis* (panel B lane 9). Panel B lanes 1-5 show the result of Western blot analysis in which the same samples as described above are reacted with pre-immune anti-rMGL2 antiserum, as can be seen there was no reactivity of the samples with the pre-immune serum.

Western blot analysis with lysates of M15pREP4 cells with the antisera raised against rMGL1 and rMGL2 revealed that no proteins in the bacterial cell lysate were recognised by the two antisera.

## 5.3 Discussion.

The cloning of the two *T. vaginalis* methionine γ-lyase homologues, *mgl1* and *mgl2* into protein expression vectors for the production of recombinant proteins was successful. The production and subsequent purification of recombinant proteins using the pQE vector and $Ni^{2+}$-NTA system has been used widely in a number of biological disciplines, for example in characterisation of HIV proteins (Certa *et al.*, 1986), the characterisation of an IFN-γ receptor (Fountoulakis, 1989) and for the production of large mammalian proteins (Traunecker, 1991). Also, in parasitology research, the system has been used for the production and subsequent characterisation of a number of proteins including, *Leishmania mexicana* cell cycle regulatory protein homologues (Mottram and Grant, 1996). The system has also been used for the characterisation of hydrogenosomal proteins from the anaerobic fungus *Neocallimastix frontalis* (Brondijk *et al.*, 1996).

Fortuitously, both of the recombinant proteins produced from the *T. vaginalis* genes were enzymatically active and this property of the recombinants opened up a vista of biochemical characterisations that could be performed on the two proteins. The various biochemical characterisations performed for the two recombinant proteins raise a number of points for discussion.

An important feature to note from this table is that neither protein possesses the ability to metabolise cystathionine (Table 5.3). This finding provides important information on the possible identities of the two *T. vaginalis* genes. It would appear, from the inability of rMGL1 and rMGL2 to breakdown cystathionine, as well as the ability by the two proteins to catabolise a number of key substrates, that have been associated with methionine γ-lyases especially from bacterial sources such as *P. putida* (Esaki and Soda, 1987), that the two genes isolated from *T. vaginalis* are indeed methionine γ-lyase

homologues. The breakdown of homocysteine, cysteine, O-acetyl L-serine and S-adenosylmethionine are at very similar levels for rMGL1 and rMGL2. However, the breakdown of methionine by rMGL1 is approximately 4 fold higher than for rMGL2. In terms of substrate specificity there are no striking differences between the two proteins, even though at the amino acid level the proteins are only 69% identical. However, the kinetic parameters calculated for certain substrates with the two proteins may intimate more subtle differences in enzyme function.

Comparison of the substrate specificities of the two recombinant proteins are compared to purified native methionine γ-lyase from *T. vaginalis* provides further data for the identity of the two isolated *T. vaginalis* genes. The substrate specificities of rMGL1, rMGL2 and native MGL from *T. vaginalis* are shown in Table 5.6, the activities are expressed relative to methionine breakdown by each protein. The breakdown of all substrates relative to methionine by rMGL2 are greater than the equivalent values for rMGL1. The relative value for the breakdown of homocysteine by native MGL is most similar to the relative breakdown of this substrate by rMGL1 (903 and 764 relative activities, respectively), whereas the breakdown of cysteine by native MGL is most similar to the relative value obtained for the breakdown of this substrate by rMGL2 (99 and 77 relative activities respectively). This result may possibly be explained by the fact that the native MGL purified from *T. vaginalis* may have been a mixture of proteins encoded by *mgl1* and *mgl2*. Table 5.6 also reveals distinct differences between the relative activities of rMGL1 and 2 and the native MGL, notably native MGL has huge relative activities for O-acetyl L-serine (945) and S-adenosylmethionine (830). In the case of O-acetyl L-serine the relative activity is greater than that obtained for homocysteine. The explanation I propose for this result is that the native MGL from *T.*

*vaginalis* was not purified to homogeneity. Support for this explanation comes from attempted purifications of the native enzyme by the published method of Lockwood and Coombs, (1991) as presented in Chapter 3 of this thesis. Purifications of the native enzyme by the published methods failed to produce the molecule in a homogeneous state, there were numerous contaminating proteins still present in the native MGL preparation even after three chromatographic column steps. Evidence also comes from immunoprecipitation data presented in Chapter 3 of this thesis, in that antibodies raised to supposedly pure MGL from *T. vaginalis* immunoprecipitated many proteins from metabolically labelled *T. vaginalis* proteins. I suggest, therefore, that the high activities toward O-acetyl-L-serine and S-adenosyl-methionine that are seen with native MGL are due to contaminating proteins that were 'copurified' with the native MGL. Further support for this view is provided by the fact that during the purification of cystathionine γ-lyase from yeast it was found that O-acetyl-L-serine/O-acetyl L-homoserine sulphydrylase (OAS/OAH sulphydrylase) was a contaminating activity of the γ-elimination enzyme (Ono *et al.*, 1993).

The substrate specificities obtained for the two *T. vaginalis* recombinant proteins also agree well with published data for methionine γ-lyases characterised from other, mainly anaerobic bacteria, sources, thus, providing further evidence that they are methionine γ-lyase homologues  Methionine γ-lyase isolated from *P. putida* and *Aeromonas* breakdown methionine, homocysteine and cysteine at very similar relative activities to the two *T. vaginalis* recombinant proteins (Nakayama *et al.*, 1984, Tanaka *et al.*, 1985 and Esaki and Soda, 1987). Similar specific activities were also obtained for purified

methionine γ-lyase from *Clostridium sporogenes* (Kreis and Hession, 1973) when compared to rMGL1 and rMGL2.

Secondly, it appears that both rMGL1 and rMGL2 display Michaelis-Menten kinetics with methionine as the substrate. The Km determined for rMGL1 with methionine as the substrate was 16-fold lower than the equivalent Km calculated for rMGL2, this result intimates that rMGL1 has a greater affinity for methionine than rMGL2 and that rMGL1 may be responsible for the authentic methionine catabolising activity in the parasite. Also the ratio of specific activities for MGL1 (Table 5.6) are most similar to the native enzyme from *T. vaginalis*.

Native methionine γ-lyase from *P. putida* had a calculated Km value for methionine of 1 mM (Esaki and Soda, 1987) and recombinant methionine γ-lyase from *P. putida* also had a Km of 1 mM for methionine (Hori *et al.*, 1996), so the Km value calculated for methionine for rMGL1 is, therefore, in the same range as the Km values calculated for the bacterial enzyme. It is interesting to note that sigmoidal saturation curves were obtained with cysteine as the substrate for both rMGL1 and rMGL2 and for homocysteine with rMGL2. As mentioned in the results such results indicate that there may be co-operativity between the subunits of these enzymes when cysteine or homocysteine, in the case of rMGL2, is used as the substrate. There may be some activator that is able to bind to these enzymes and exert some type of metabolic regulation. It would be interesting to see what effect an exogenous thiol had on the saturation curves of cysteine for both of the proteins and on the homocysteine saturation curve of rMGL2. It may be that thiol concentrations in the cell are able to regulate the cysteine and homocysteine-catabolising capabilities of the native enzymes. This idea

arises from thoughts about the functional significance of the homocysteine, methionine and cysteine breakdown in *T. vaginalis*. One idea is that methionine γ-lyase is functioning to maintain the redox balance of the cell. As thiols are produced from the breakdown of homocysteine, methionine and cysteine, it may be that the enzymes that produce these compounds are able to regulate their activity by binding thiols. The functional significance of methionine γ-lyase in trichomonads will be further addressed in Chapter 6 of this thesis.

Another point that must be mentioned in this discussion is the discrepancy that appears to exist between the specific activities calculated for the breakdown of homocysteine by rMGL1 and rMGL2, when the assays for hydrogen sulphide and α-ketobutyrate were used. It was thought that the γ-exchange/replacement reaction that is known to occur with sulphur amino acids and exogenous thiol may have been responsible for this discrepancy, however, this was thought not to be the case (see Table 5.5). The explanation, therefore, that I give for this discrepancy is simply the difference in sensitivity of the two assay methods that were used. Indeed the unequal production of thiol and α-ketobutyrate from homocysteine by *T. vaginalis* lysates has been reported by Thong, 1987, but without comment.

Thirdly, *mgl1* and *mgl2* genes encode proteins with predicted molecular mass of 42.9 kDa and 43.1 kDa respectively and SDS-PAGE analysis showed that rMGL1 is approximately 43kDa, whereas, rMGL2 is slightly larger than predicted because of extra amino acids that are derived from pBluescript. The sizes of rMGL1 and rMGL2 agree well with molecular size data published for the methionine γ-lyases from bacterial

sources. Methionine γ-lyase from *P. putida* has a subunit size of 43 kDa whereas *Aeromonas sp* methionine γ-lyase has a subunit size of 41 kDa (Tanaka *et al.*,1985 and Esaki and Soda, 1987).

The native molecular masses of rMGL1 and rMGL2 were determined as >440 kDa and 158 kDa, respectively. The native molecular mass calculated for rMGL2 is indicative of a homotetrameric structure, in which the active enzyme is composed of four identical monomer subunits each of 43 kDa. The calculation of the molecular weight of rMGL2 is in good agreement with the native molecular weights of the *P. putida* and *Aeromonas sp* enzyme. The *P. putida* enzyme has a molecular mass of 165 kDa and the *Aeromonas* enzyme a native molecular weight of 149 kDa. (Tanaka *et al.*, 1985). The rMGL1 native molecular mass is considerably larger than the expected size when the subunit molecular mass is taken into consideration. It is thought that this protein aggregates to form higher molecular weight complexes which are still enzymatically active. It is probable that that the high molecular weight complex is composed of homotetramers that have aggregated together. One could imagine that the tertiary structure of rMGL1 is such that surface located amino acid residues encourage subunit aggregation above the normal tetrameric structure. Further experiments that would favour the dissociation of the multimeric complexes of rMGL1 may reveal the true native molecular weight of this protein.

Fourthly, antibodies raised against rMGL1 and rMGL2 were used to investigate the expression of the two proteins in *T. vaginalis* homogenates, soluble and pellet fractions. Western blot analysis using the two antibodies revealed that a ~43 kDa protein, in both cases, expressed in homogenates and more specifically in the soluble fraction of *T.*

*vaginalis* lysates. This result ties up with Northern blot data from Chapter 4 and shows that the *mgl1* and *mgl2* genes are being actively transcribed and translated in *T. vaginalis*.

It should be remembered that rMGL2 was missing four N terminal amino acids of the open reading frame (these were characterised by 5'-RACE as outlined in Chapter 4 of this thesis). Also the protein had an additional eight amino acids that were derived from pBluescript as a result of the cloning of *mgl2* into the pQE30 vector. As rMGL2 is enzymatically active and has many biochemical characteristics that are similar to the same enzyme from bacterial sources, it is unlikely that the absence and presence of certain amino acid residues as outlined above is having a great effect on the biochemical characteristics of this recombinant protein. However, one could never be completely confident that the missing/additional residues were not important for enzyme function without cloning and expressing the full length *mgl2*. If time had permitted, this would have been carried out.

To end this discussion there are several ideas that I want to propose based on the results presented in this Chapter. It appears that the two methionine γ-lyase homologues isolated from *T. vaginalis*, which are 68% identical at the amino acid residue level, are in terms of a number of biochemical characteristics, quite similar. The exceptions to this appear to be the kinetic parameters calculated for methionine and that saturation curves for homocysteine and cysteine for rMGL2 and cysteine for rMGL1 exhibit allosteric kinetics. Also the native molecular mass of rMGL1 is considerably larger than that for rMGL2.

One may ask the question: Why have two genes that are only 69% identical but whose products are biochemically very similar? I feel that there are a number of possible answers to this question. Firstly, it may be that the possession of two genes by *T. vaginalis* that give products with very similar biochemical characteristics may be an insurance policy adopted by the organism. If the gene product has an essential function in the parasite, then the organism may have safeguarded against damage to one of the genes by having a back-up copy. Certainly, *T. vaginalis* has several copies of β-tubulin genes (Katiyar and Edlind, 1994). Secondly, it may be that I haven't discovered the crucial substrate that would be able to distinguish the two gene products. I tend to believe, however, that this is not the case, as a fairly rigorous biochemical characterisation of the two recombinant proteins has taken place, in which the majority of the classic substrates used by methionine γ-lyases have been tested with rMGL1 and rMGL2. Thirdly, it may be possible that the two gene products may combine in the parasite to give a heterotetramer that possesses different substrate specificities to the homotetramers of rMGL1 and rMGL2. Finally, it may be that the two recombinant proteins have different locations in the parasite, where they perform slightly different functions, indeed this theory may be supported to some extent by immunolocalisation data using intact *T. vaginalis* and the MGL1 and MGL2 antisera presented in the next chapter of this thesis.

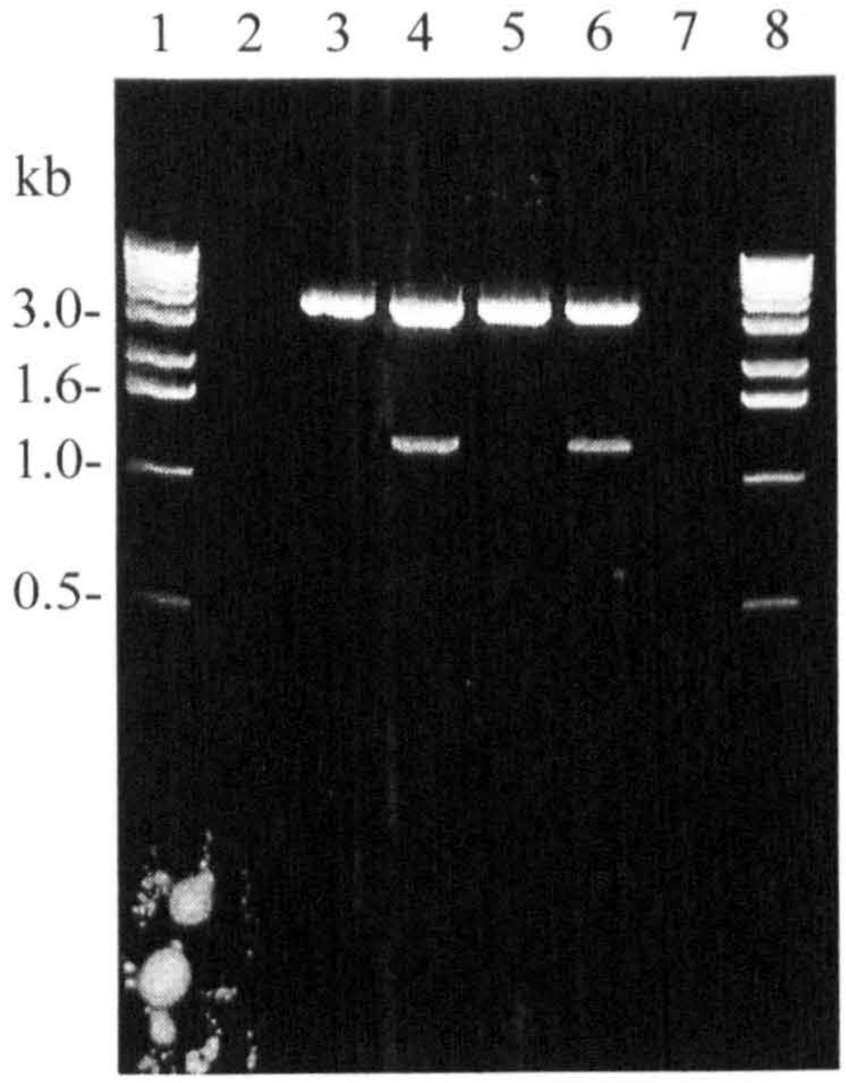



**Figure 5.1 Restriction analysis of pQMGL1 clones.**

Plasmid DNA isolated from individual XL1-Blue transformants was restricted with *Nco*I and *Bgl*II and electrophoresed on a 1%TBE agarose gel, stained with EtBr and visualised under UV illumination.

Lanes 1 and 8: 1kb ladder (Life Technologies)

Lane 3: pQMGL1a restricted with *Nco*I and *Bgl*II

Lane 4: pQMGL1b restricted with *Nco*I and *Bgl*II

Lane 5: pQMGL1c restricted with *Nco*I and *Bgl*II

Lane 6: pQMGL1a restricted with *Nco*I and *Bgl*II

**Figure 5.2 Nucleotide and deduced amino acid sequence of Histidine-tagged MGL1.**

The clone used in this study was pQMGL1b. Changes in *mgl1* sequence in pQE60 compared to *mgl1* in pBluescript at the 5' end are indicated in red text. The 6-Histidine tag is indicated in green text and other sequence encoded by pQE60 is shown in blue text.

```
   1  CCATGGCTCACGAGAGAATGACCCCAGCAACAGCATGCATCCATGCTAATCCACAGAAGG    60
   1    M  A  H  E  R  M  T  P  A  T  A  C  I  H  A  N  P  Q  K  D    20

  61  ATCAGTTTGGAGCAGCCATCCCACCAATCTACCAAACATCAACATTCGTTTTCGATAACT   120
  21    Q  F  G  A  A  I  P  P  I  Y  Q  T  S  T  F  V  F  D  N  C    40

 121  GCCAACAGGGTGGAAACAGATTCGCTGGTCAGGAATCCGGCTACATCTACACACGTCTCG   180
  41    Q  Q  G  G  N  R  F  A  G  Q  E  S  G  Y  I  Y  T  R  L  G    60

 181  GCAACCCAACAGTTTCAAACCTCGAAGGCAAGATCGCCTTCCTCGAGAAAACAGAAGCAT   240
  61    N  P  T  V  S  N  L  E  G  K  I  A  F  L  E  K  T  E  A  C    80

 241  GCGTTGCCACATCTTCTGGCATGGGTGCCATTGCTGCTACAGTTTTGACAATCCTCAAGG   300
  81    V  A  T  S  S  G  M  G  A  I  A  A  T  V  L  T  I  L  K  A   100

 301  CCGGAGATCACTTAATCTCCGATGAGTGCCTTTATGGCTGCACACATGCTCTCTTTGAGC   360
 101    G  D  H  L  I  S  D  E  C  L  Y  G  C  T  H  A  L  F  E  H   120

 361  ACGCATTGACAAAGTTCGGCATCCAGGTCGACTTCATCAACACAGCCATCCCAGGCGAGG   420
 121    A  L  T  K  F  G  I  Q  V  D  F  I  N  T  A  I  P  G  E  V   140

 421  TCAAGAAGCACATGAAGCCAAACACAAAGATTGTCTATTTCGAGACACCAGCCAACCCAA   480
 141    K  K  H  M  K  P  N  T  K  I  V  Y  F  E  T  P  A  N  P  T   160

 481  CACTCAAGATCATCGACATGGAGCGCGTCTGCAAGGACGCCCACAGCCAGGAGGGCGTCT   540
 161    L  K  I  I  D  M  E  R  V  C  K  D  A  H  S  Q  E  G  V  L   180

 541  TAGTTATCGCCGATAACACATTCTGCTCACCAATGATCACAAACCCAGTCGACTTTGGCG   600
 181    V  I  A  D  N  T  F  C  S  P  M  I  T  N  P  V  D  F  G  V   200

 601  TCGATGTTGTTGTCCACTCTGCAACAAAGTACATCAACGGCCACACAGATGTCGTCGCTG   660
 201    D  V  V  V  H  S  A  T  K  Y  I  N  G  H  T  D  V  V  A  G   220

 661  GCCTTATCTGTGGCAAGGCTGACCTCCTTCAACAGATTCGTATGGTTGGTATCAAGGATA   720
 221    L  I  C  G  K  A  D  L  L  Q  Q  I  R  M  V  G  I  K  D  I   240

 721  TCACAGGATCTGTTATCAGCCCACACGACGCTTGGCTCATCACACGTGGCCTCTCAACAC   780
 241    T  G  S  V  I  S  P  H  D  A  W  L  I  T  R  G  L  S  T  L   260

 781  TCAACATCAGAATGAAGGCTGAGAGCGAGAACGCCATGAAGGTCGCTGAGTACCTCAAAT   840
 261    N  I  R  M  K  A  E  S  E  N  A  M  K  V  A  E  Y  L  K  S   280

 841  CTCACCCAGCCGTTGAGAAGGTTTACTACCCAGGCTTCGAGGACCACGAGGGCCACGATA   900
 281    H  P  A  V  E  K  V  Y  Y  P  G  F  E  D  H  E  G  H  D  I   300

 901  TCGCTAAGAAGCAGATGAGAATGTCGGGTTCAATGATCACATTCATCCTCAAGTCCGGCT   960
 301    A  K  K  Q  M  R  M  S  G  S  M  I  T  F  I  L  K  S  G  F   320

 961  TCGAAGGCGCTAAGAAGCTCCTCGACAACCTCAAGCTTATCACACTTGCAGTTTCCCTTG  1020
 321    E  G  A  K  K  L  L  D  N  L  K  L  I  T  L  A  V  S  L  G   340

1021  GTGGCTGCGAGTCCCTCATCCAGCACCCAGCTTCAATGACTCACGCTGTCGTTCCAAAGG  1080
 341    G  C  E  S  L  I  Q  H  P  A  S  M  T  H  A  V  V  P  K  E   360

1081  AGGAGCGTGAGGCCGCTGGTATTACAGATGGCATGATCCGCCTTTCTGTCGGTATTGAAG  1140
 361    E  R  E  A  A  G  I  T  D  G  M  I  R  L  S  V  G  I  E  D   380

1141  ATGCCGACGAACTCATCGCTGATTTCAAACAGGGCCTTGACGCTCTTTTAAGATCTCATC  1200
 381    A  D  E  L  I  A  D  F  K  Q  G  L  D  A  L  L  R  S  H  H   400

1201  ACCATCACCATCACTAAGCTTAATTAGCTGAG                              1232
 401    H  H  H  H                                                   410
```

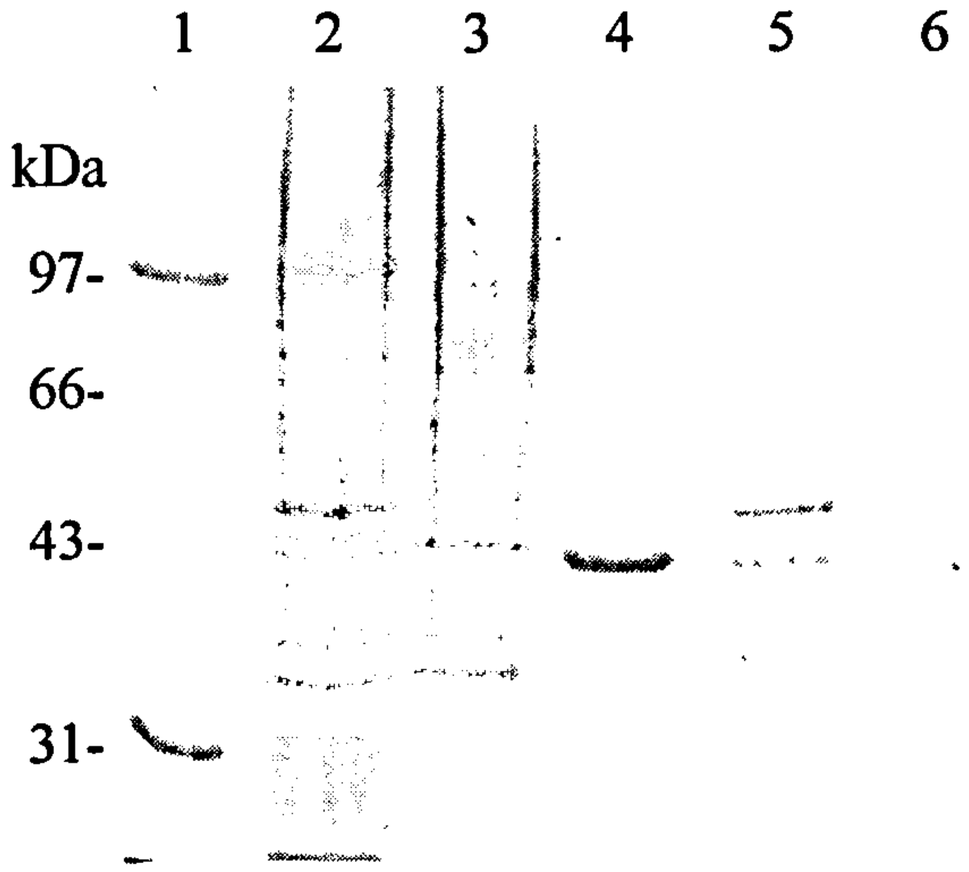



**Figure 5.3 SDS-PAGE showing the location and solubility of the 6xHistidine tagged rMGL1 in M15pREP4 cells.**

Different fractions of the bacterial cells were electrophoresed on a 10% acrylamide gel and stained with Coomassie Blue R250.

Lane 1: Molecular weight markers (Biorad)
Lane 2: Uninduced whole cell homogenate of M15pREP4
Lane 3: Induced whole cell homogenate of M15pREP4
Lane 4: Soluble fraction of induced M15pREP4 cells
Lane 5: Insoluble fraction of induced M15pREP4 cells
Lane 6: Periplasmic fraction of induced M15pREP4 cells

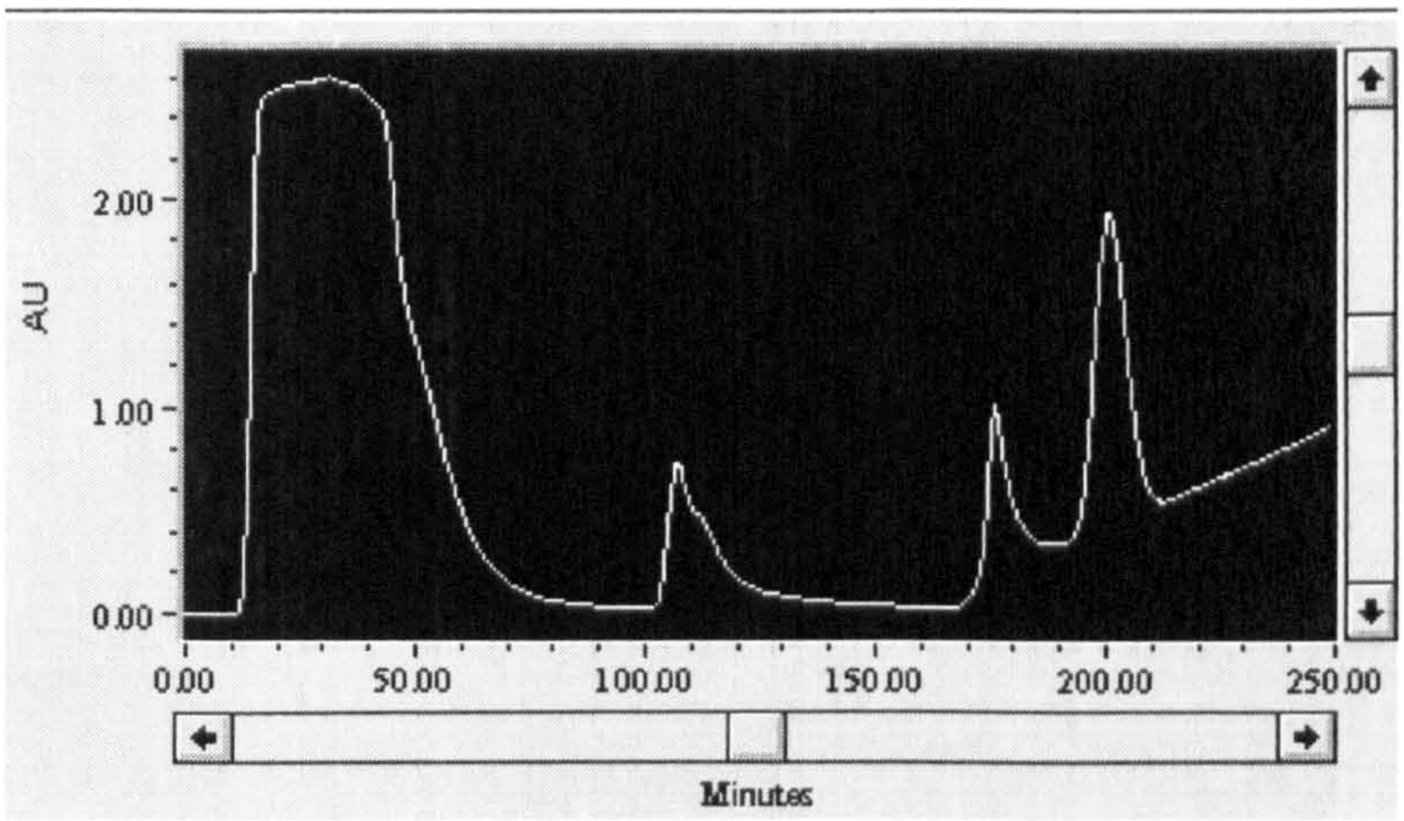



**Figure 5.4 $A_{280nm}$ trace obtained from the purification of rMGL1.**

The FPLC generated programme for the purification of rMGL1 is outlined below.

0-30 minutes: Sample application at 0.2ml/min in sonication buffer
(50mM Sodium phosphate buffer pH8.0, 300mM NaCl)
30-90 minutes: Column wash with sonication buffer(as above) at 0.5ml/min
90-150 minutes: Column wash with wash buffer (50mM Sodium phosphate buffer pH6.0
300mM NaCl, 10% glycerol) at 0.5ml/min
150-250 minutes: Linear imidazole gradient 0-500mM in wash buffer (as above)
at 0.5 ml/min
2 minute fractions (1ml) were collected over the imidazole gradient

**Figure 5.5A/B SDS-PAGE of various fractions from a purification of rMGL1.**

Samples were boiled in Laemmli buffer for five minutes prior to application to a 12% acrylamide gel, samples containing imidazole were only heated to 37°C prior to application to the acrylamide gel, after the electrophoretic run the gels were stained with Coomassie Blue R250.

**Panel A**

Lane 1: Insoluble pellet fraction of induced M15pREP4 and pQMGL1

Lane 2: Induced soluble fraction of M15pREP4and pQMGL1 which was loaded onto the $Ni^{2+}$-NTA column

Lane 3: Flow through fraction of induced M15pREP4 from the $Ni^{2+}$-NTA column

Lane 4: Molecular weight markers, SDS 7 (Sigma)

Lane 5: Wash buffer fraction at 100 minutes

Lane 6: Wash buffer fraction at 110 minutes

Lane 7: Imidazole gradient fraction at 174 minutes

Lane 8: Imidazole gradient fraction at 176 minutes

Lane 9: Imidazole gradient fraction at 178 minutes

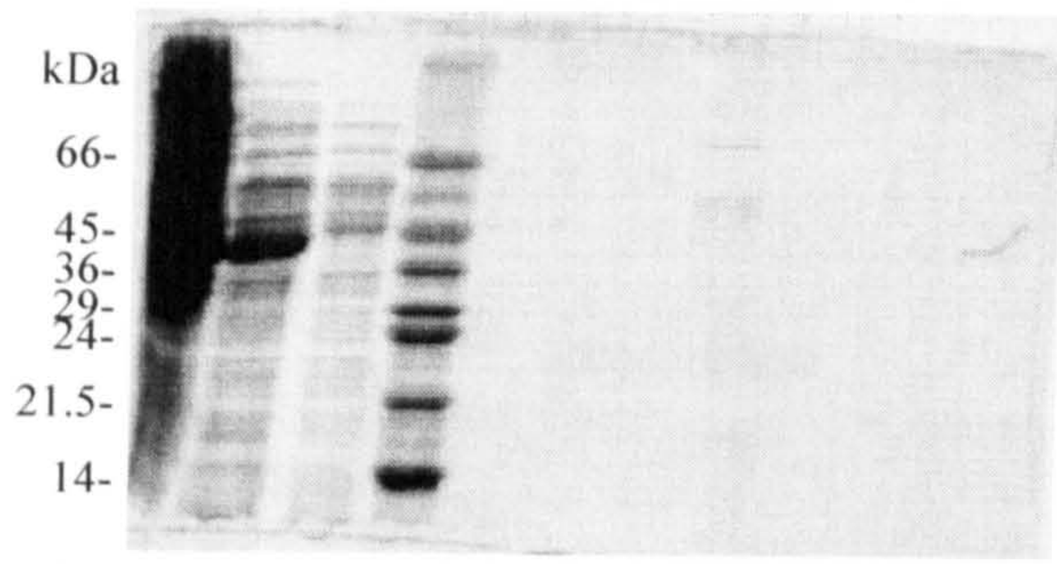
Lane 10: Imidazole gradient fraction at 180 minutes

**Panel B**

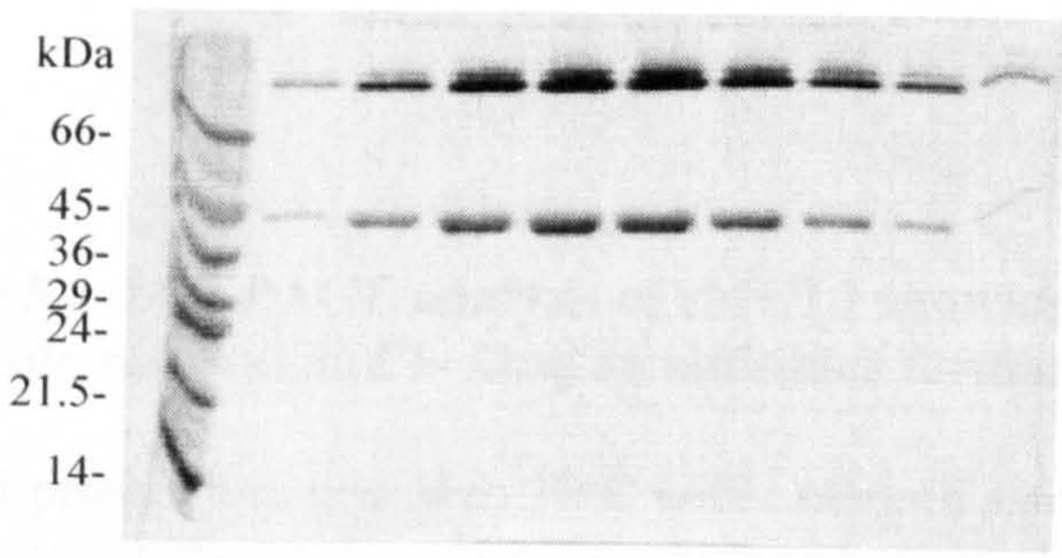
Lane 1: Molecular weight markers, SDS 7 (Sigma)

Lanes 2-10: Protein eluted from the $Ni^{2+}$-NTA column by imidazole between 194 and 210 minutes of the purification

A


1 2 3 4 5 6 7 8 9 10
kDa
66-
45-
36-
29-
24-
21.5-
14-

B


1 2 3 4 5 6 7 8 9 10
kDa
66-
45-
36-
29-
24-
21.5-
14-

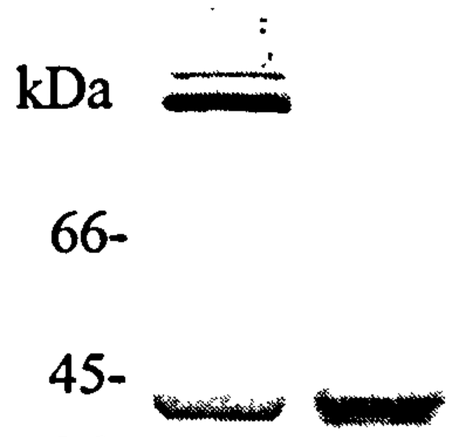



**Figure 5.6 SDS-PAGE analysis of rMGL1 showing the effects of imidazole removal and boiling on multimer formation.**

rMGL1 protein was heated to 37°C with Laemmli sample buffer and applied directly to a 12% acrylamide gel or, dialysed overnight against sodium phophate buffer pH 6.5 and 300mM sodium chloride and boiled in the presence of Laemmli buffer prior to application. After the electrophoretic run the gel was stained with Coomassie Blue R250.

Lane 1: rMGL1 in the presence of imidazole buffer

and heated to only 37°C with Laemmli reducing sample buffer

Lane 2: rMGL1 in the absence of imidazole buffer and boiled in Laemmli reducing sample buffer

SDS 7 (Sigma) molecular weight markers are shown on the left

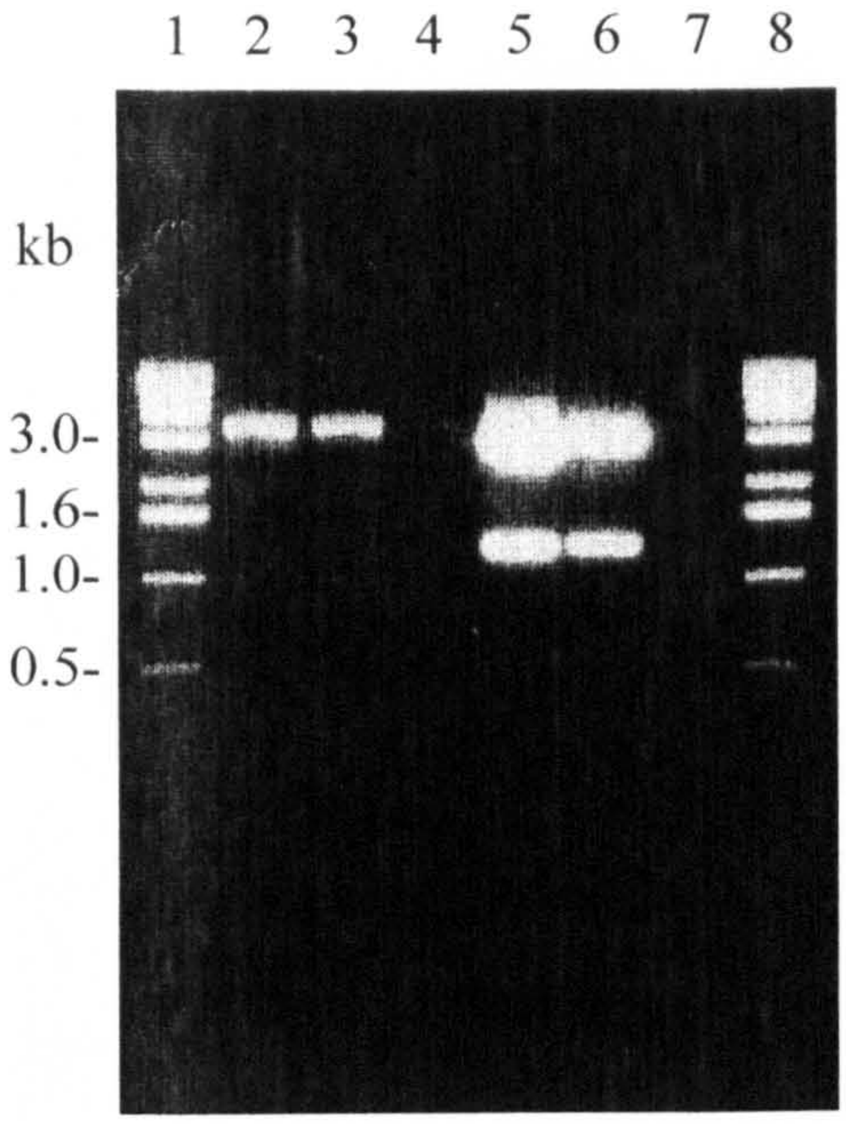



**Figure 5.7 Restriction analysis of pQMGL2 clones.**

pQE30 and plasmid DNA isolated from individual XL1-Blue transformants was restricted with *Bam*HI and *Hin*dIII and electrophoresed in a 1% TBE agarose gel, stained with EtBr

and visualised under UV illumination.

Lanes 1 and 8: 1kb ladder (Life Technologies)
Lane 2: pQE30 restricted with *Pst*I
Lane 3: pQE30 restricted with *Bam*HI and *Hin*dIII
Lane 5: pQMGL2a restricted with *Bam*HI and *Hin*dIII
Lane 6: pQMGL2b restricted with *Bam*HI and *Hin*dIII

**Figure 5.8 Nucleotide sequence and deduced amino acid sequence of Histidine-tagged pQMGL2.**

The clone used in this study was pQMGL2b. The 6-Histidine tag is indicated in green text and other pBluescript derived sequence is shown in blue text.

```
   1  ATGAGAGGATCGCATCACCATCACCATCACGGATCCCCCGGGCTGCAGGAATTCGGCACG    60
   1  M  R  G  S  H  H  H  H  H  H  G  S  P  G  L  Q  E  F  G  T     20

  61  AGCGCTATCGACCCAACACATACAGACACACTTTCCATCCACGCCAACCCACAGAAGGAT   120
  21  S  A  I  D  P  T  H  T  D  T  L  S  I  H  A  N  P  Q  K  D     40

 121  CAGTTCGGTGCTATTGTTGCTCCAATCTACCAAACATCCACCTTCCTCTTCGACAACTGC   180
  41  Q  F  G  A  I  V  A  P  I  Y  Q  T  S  T  F  L  F  D  N  C     60

 181  GACCAGGGTGGTGCTCGTTTCGGTGGCAAGGAAGCCGGTTACATGTACACACGTATCGGT   240
  61  D  Q  G  G  A  R  F  G  G  K  E  A  G  Y  M  Y  T  R  I  G     80

 241  AACCCAACAAACTCCGCACTCGAAGGCAAGATCGCCAAGCTCGAACACGCTGAGGCATGC   300
  81  N  P  T  N  S  A  L  E  G  K  I  A  K  L  E  H  A  E  A  C    100

 301  GCTGCCACAGCTTCTGGCATGGGTGCTATTGCTGCTTCTGTCTGGACATTCCTCAAGGCC   360
 101  A  A  T  A  S  G  M  G  A  I  A  A  S  V  W  T  F  L  K  A    120

 361  GGTGATCACCTTATCTCCGACGATTGCCTTTATGGCTGCACACACGCCCTCTTCGAGCAT   420
 121  G  D  H  L  I  S  D  D  C  L  Y  G  C  T  H  A  L  F  E  H    140

 421  CAGCTCCGCAAGTTCGGCGTTGAAGTTGATTTCATCGACATGGCTGTCCCAGGAAACATT   480
 141  Q  L  R  K  F  G  V  E  V  D  F  I  D  M  A  V  P  G  N  I    160

 481  GAGAAGCACTTGAAGCCAAACACAAGAATCGTCTACTTCGAAACACCAGCTAACCCAACA   540
 161  E  K  H  L  K  P  N  T  R  I  V  Y  F  E  T  P  A  N  P  T    180

 541  TTAAAGGTTATCGACATCGAAGACGCCGTCAAGCAGGCCAGAAAGCAGAAGGATATCCTC   600
 181  L  K  V  I  D  I  E  D  A  V  K  Q  A  R  K  Q  K  D  I  L    200

 601  GTTATCGTTGATAACACCTTCGCTTCACCAATTCTTACAAACCCACTCGACCTCGGTGTT   660
 201  V  I  V  D  N  T  F  A  S  P  I  L  T  N  P  L  D  L  G  V    220

 661  GATATCGTCGTTCACTCCGCTACTAAGTACATCAATGGCCACACCGATGTTGTCGCCGGC   720
 221  D  I  V  V  H  S  A  T  K  Y  I  N  G  H  T  D  V  V  A  G    240

 721  CTTGTCTGCTCAAGAGCTGACATCATCGCTAAGGTCAAGTCCCAGGGTATCAAGGATATC   780
 241  L  V  C  S  R  A  D  I  I  A  K  V  K  S  Q  G  I  K  D  I    260

 781  ACAGGCGCCATCATTTCCCCACACGACGCTTGGCTCATCACAAGAGGCACACTTACACTC   840
 261  T  G  A  I  I  S  P  H  D  A  W  L  I  T  R  G  T  L  T  L    280

 841  GATATGCGTGTCAAGCGCGCTGCCGAGAACGCTCAGAAGGTCGCTGAATTCCTCCATGAG   900
 281  D  M  R  V  K  R  A  A  E  N  A  Q  K  V  A  E  F  L  H  E    300

 901  CACAAGGCCGTCAAGAAGGTCTACTACCCAGGCCTTCCAGACCATCCAGGCCACGAAATC   960
 301  H  K  A  V  K  K  V  Y  Y  P  G  L  P  D  H  P  G  H  E  I    320

 961  GCCAAGAAGCAGATGAAGATGTTCGGCTcTATgATCGCATTCGATGTCGACGGATTAGAG  1020
 321  A  K  K  Q  M  K  M  F  G  S  M  I  A  F  D  V  D  G  L  E    340

1021  AAGGCCAAGAAAGTCCTTGACaACTGCCACGTTGTTTCTCTCGCCGTTTCCCTCGGTGGT  1080
 341  K  A  K  K  V  L  D  N  C  H  V  V  S  L  A  V  S  L  G  G    360

1081  CCAGAATCCCTCATCCAGCACCCAGCTTCAATGACACACGCTGGTGTTCCAAAGGAGGAA  1140
 361  P  E  S  L  I  Q  H  P  A  S  M  T  H  A  G  V  P  K  E  E    380

1141  CGCGAGGCTGCTGGCCTAACAGATAACCTCATCCGCCTCTCTGTTGGCTGTGAGAACGTT  1200
 381  R  E  A  A  G  L  T  D  N  L  I  R  L  S  V  G  C  E  N  V    400

1201  CAGGATATCATCGACGACCTCAAGCAGGCTCTCGACTTAGTCCTCTAAATTTAACTTTCG  1260
 401  Q  D  I  I  D  D  L  K  Q  A  L  D  L  V  L                  420

1261  AATTTCAGTAATAAAATCCTAGATATCTTCCCCCCCCCAAAAAAAAAAAAAAAAAAAAAA  1320

1321  AAAAAAAAAAAAAAAAAAAA  1341
```

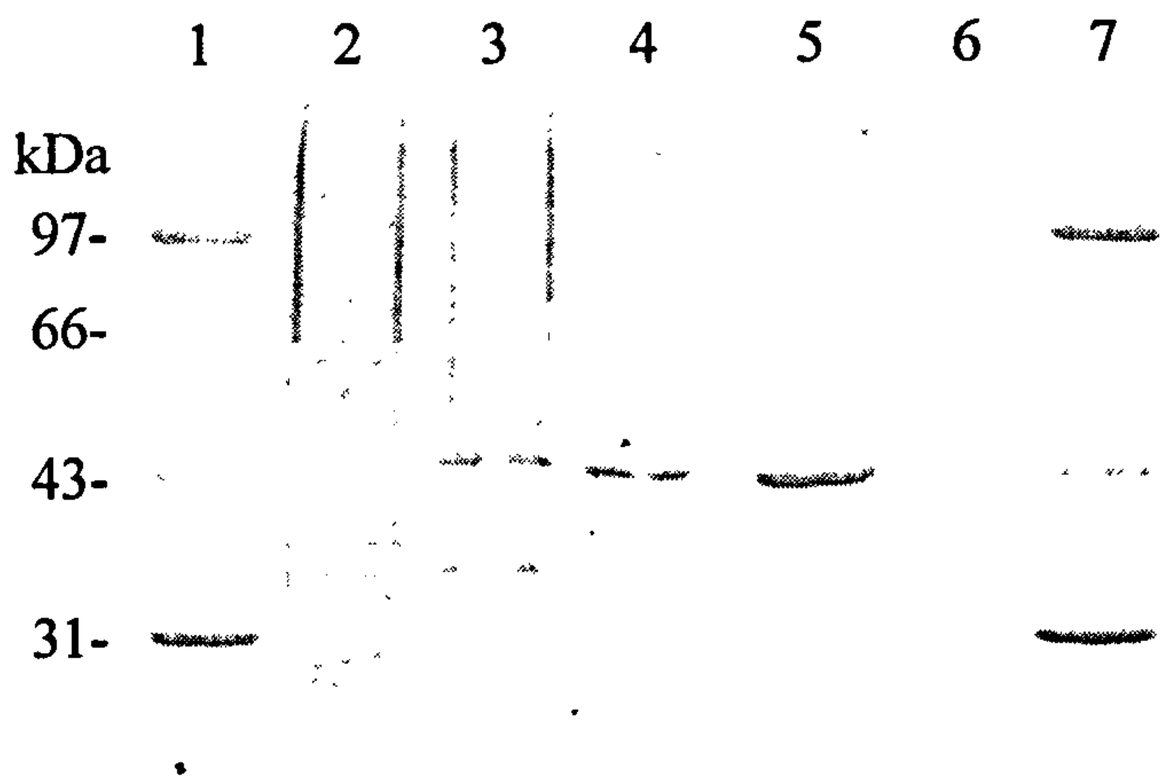



**Figure 5.9 SDS-PAGE showing the location and solubility the 6xHistidine tagged rMGL2 in M15pREP4 cells.**

Different fractions of the bacterial cells were electrophoresed on a 10% acrylamide gel and stained with Coomassie Blue R250.

Lanes 1 and 7: Molecular weight markers (Biorad)
Lane 2: Uninduced whole cell homogenate of M15pREP4 and pQMGL2a
Lane 3: Induced whole cell homogenate of M15pREP4 and pQMGL2a
Lane 4: Soluble fraction of induced M15pREP4 cells
Lane 5: Insoluble fraction of induced M15pREP4 cells
Lane 6: Periplasmic fraction of induced M15pREP4 cells

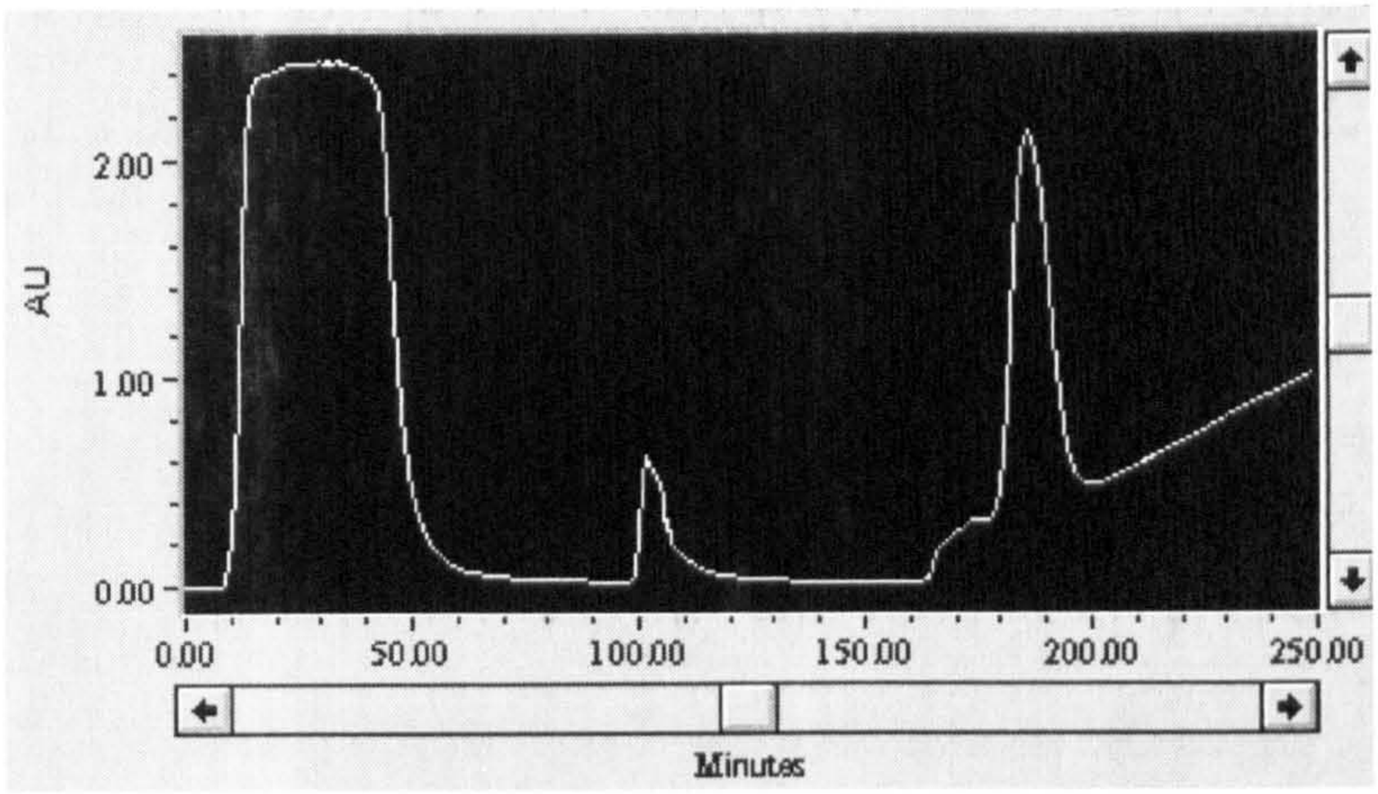



**Figure 5.10 $A_{280nm}$ trace obtained from the purification of rMGL2.**

The FPLC generated programme for the purification of rMGL2 is outlined below

0-30 minutes: Sample application at 0.2ml/min in sonication buffer
(50mM Sodium phosphate buffer pH8.0, 300mM NaCl)
30-90 minutes: Column wash with sonication buffer(as above) at 0.5ml/min
90-150 minutes: Column wash with wash buffer (50mM sodium phosphate buffer pH6.0, 300mM NaCl, 10% glycerol) at 0.5ml/min
150-250 minutes: Linear imidazole gradient 0-500mM in wash buffer (as above) at 0.5 ml/min
2 minute fractions (1ml) were collected over the imidazole gradient

**Figure 5.11 A/B SDS-PAGE of various fractions from a purification of rMGL2.**

Samples were boiled in Laemmli buffer for five minutes prior to application to a 12% acrylamide gel, samples containing imidazole were only heated to 37°C prior to application to the acrylamide gel. After electrophoresis the gels were stained with Coomassie Blue R250.

**Panel A**

Lane 1: Insoluble pellet fraction of induced M15pREP4 and pQMGL2

Lane 2: Induced soluble fraction of M15pREP4 and pQMGL2 loaded onto the $Ni^{2+}$-NTA column

Lane 3: Flow through fraction of induced M15pREP4 from the $Ni^{2+}$-NTA column

Lane 4: Molecular weight markers, SDS 7 (Sigma)

Lane 5: Sonication buffer wash fraction at 40 minutes

Lane 6: Sonication buffer wash fraction at 80 minutes

Lane 7: Wash buffer fraction at 100 minutes

Lane 8: Wash buffer fraction at 140 minutes

Lane 9: Imidazole gradient fraction at 196 minutes

Lane 10: Imidazole gradient fraction at 198 minutes

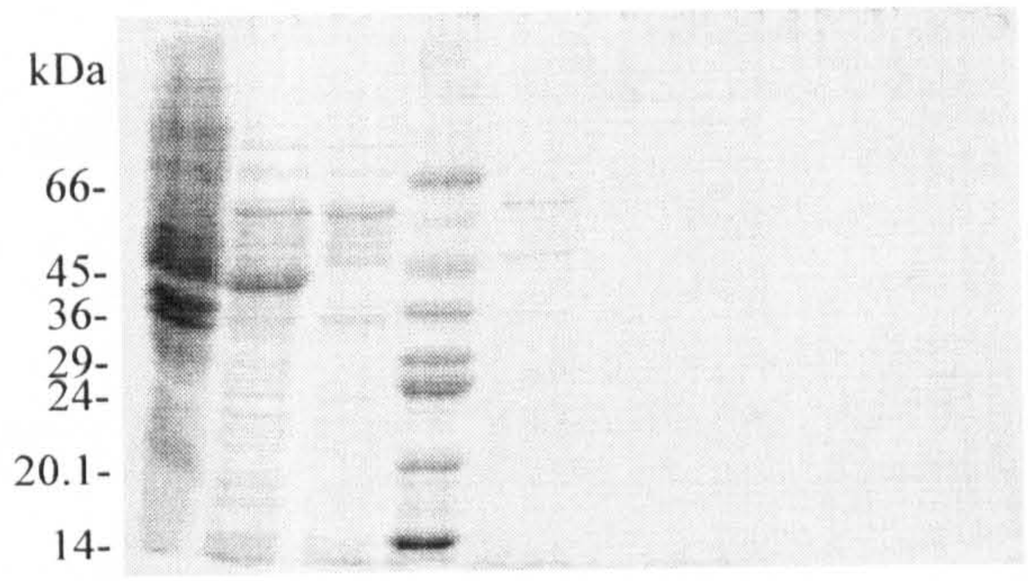
**Panel B**

Lanes 1-4: Protein eluted from the $Ni^{2+}$-NTA column by imidazole between 178-186 minutes of the purification

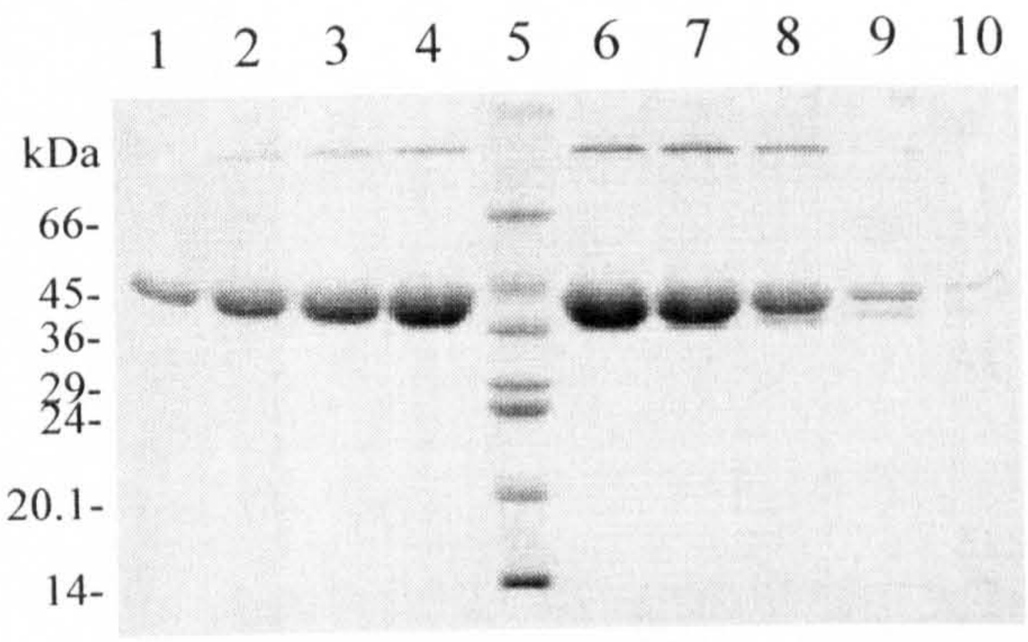
Lane 5: Molecular weight markers, SDS 7 (Sigma)

Lanes 6-10: Protein eluted from the $Ni^{2+}$-NTA column by imidazole between 188-194 minutes of the purification

A


1 2 3 4 5 6 7 8 9 10
kDa
66-
45-
36-
29-
24-
20.1-
14-

B


1 2 3 4 5 6 7 8 9 10
kDa
66-
45-
36-
29-
24-
20.1-
14-

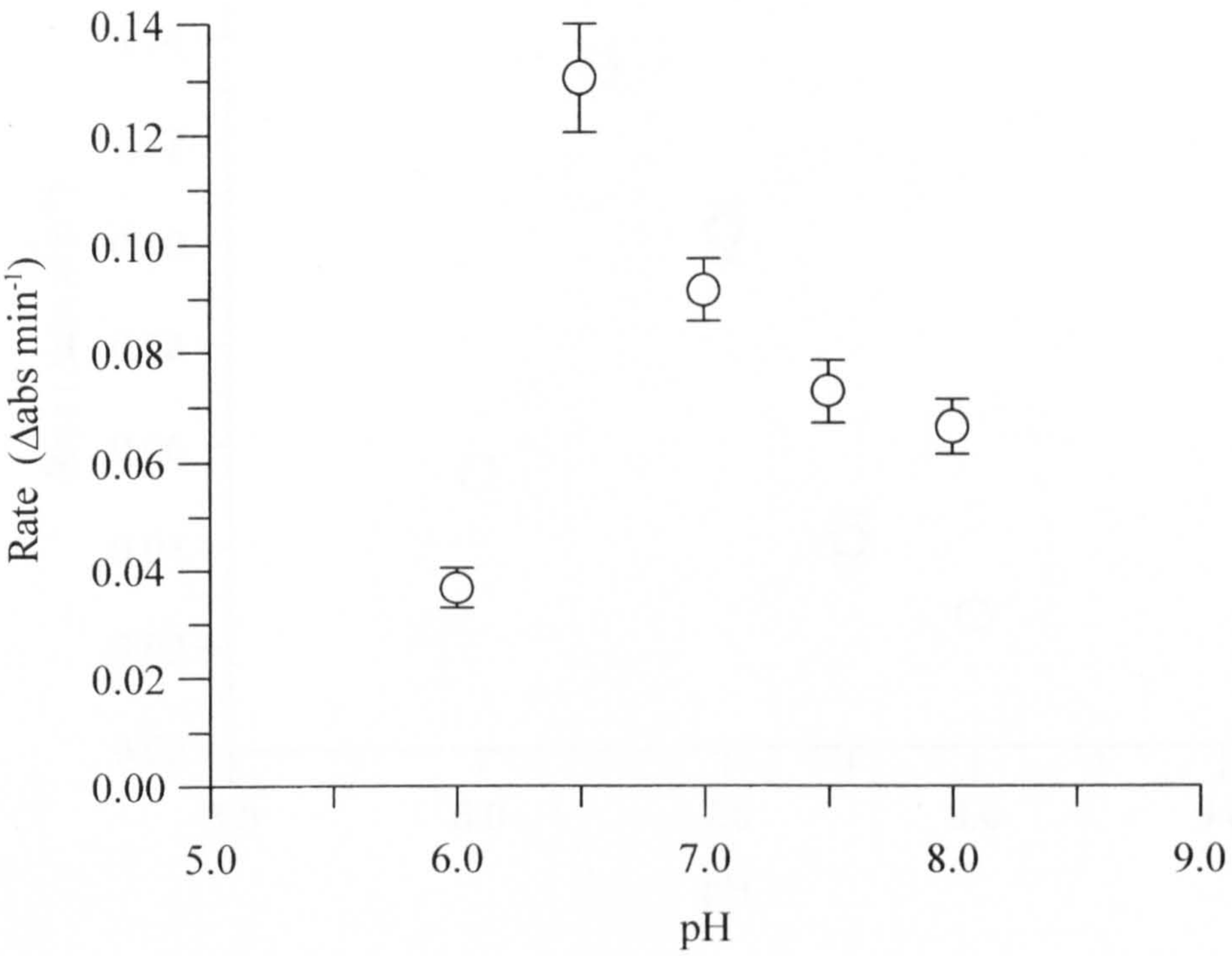



**Figure 5.12 pH optimum for homocysteine breakdown by rMGL1 using imidazole buffer.** Data points are means with the error bars representing the S.D. (n=3).

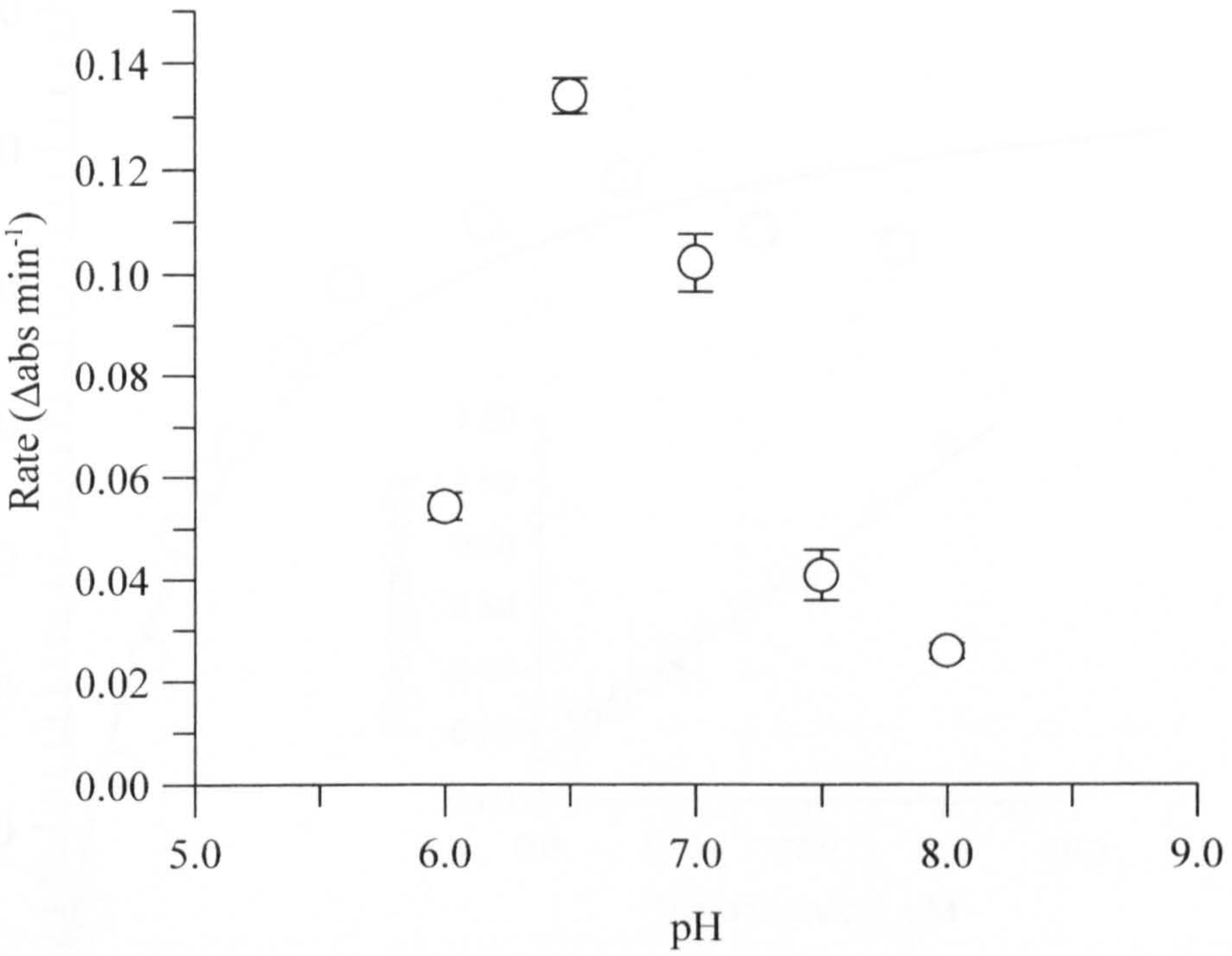



**Figure 5.13 pH optimum for homocysteine breakdown by rMGL2 using imidazole buffer.** Data points are means with the error bars representing the S.D. (n=3)

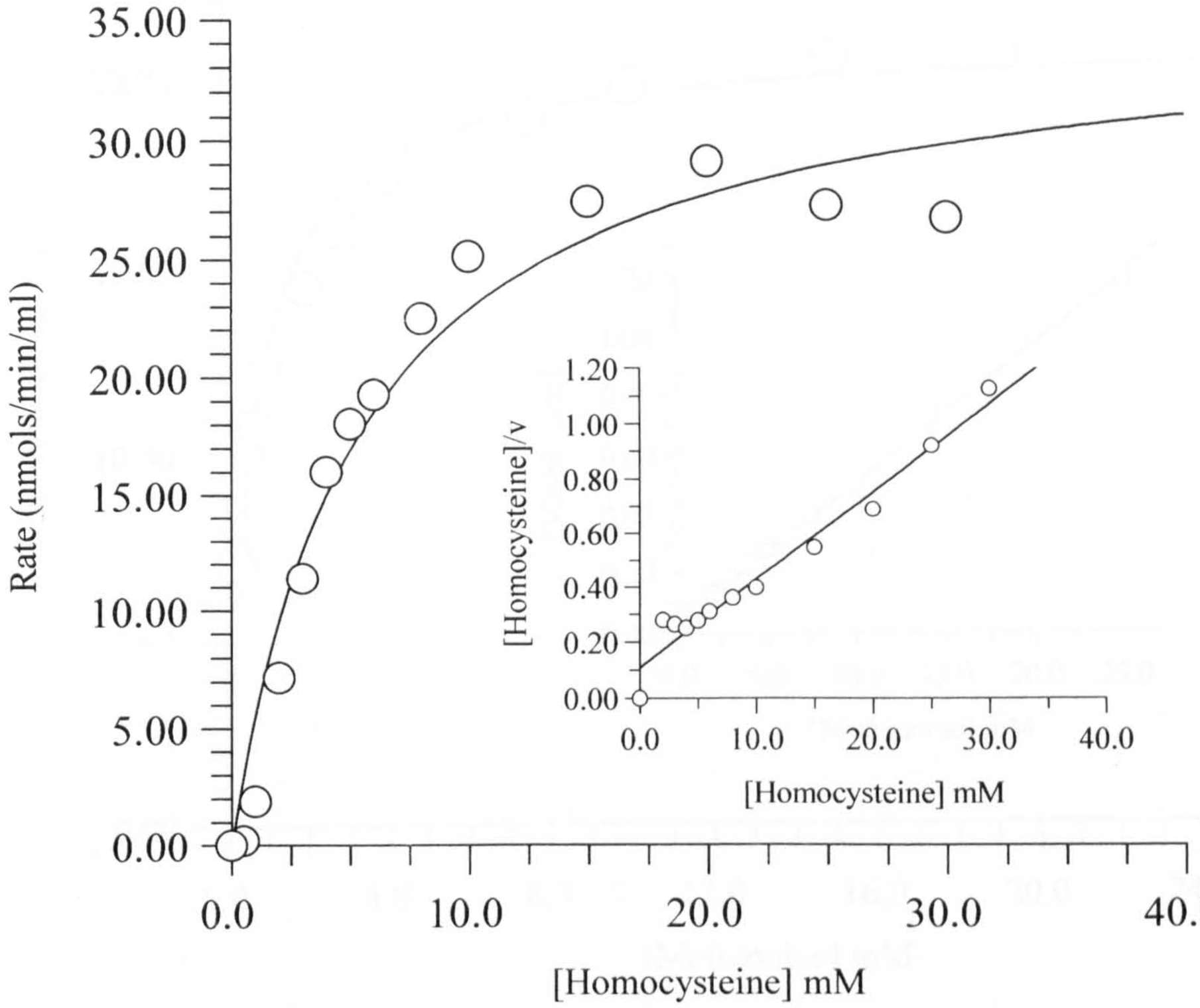



**Figure 5.14 A representative homocysteine saturation curve of rMGL1 and Hanes plot of data** (insert). The activity associated with a fixed quantity of recombinant protein was assayed at different concentrations of homocysteine. The kinetic parameters for this experiment were: Km, 5.4 mM and the Vmax, 35 nmol/min/ml.

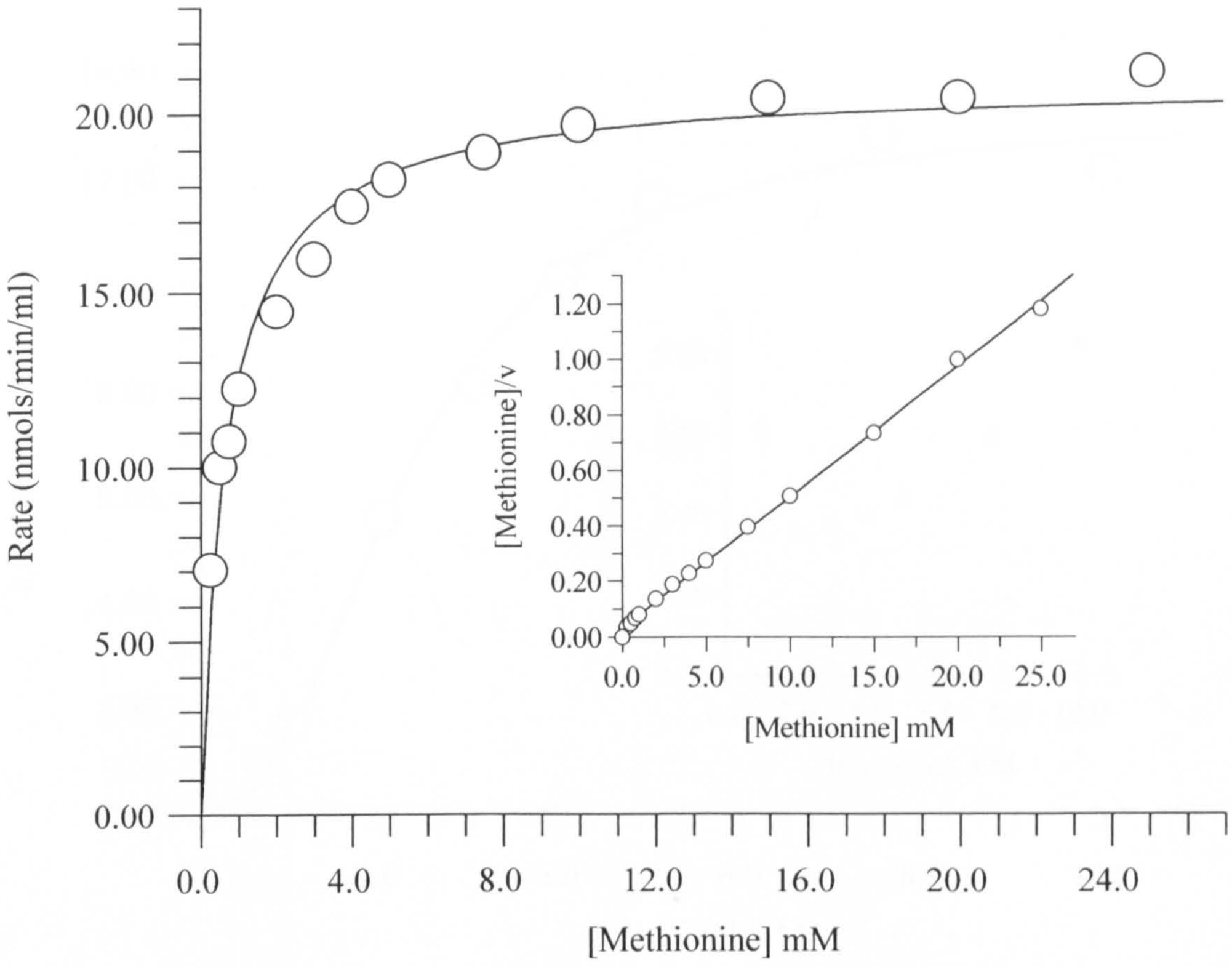



**Figure 5.15 A representative methionine saturation curve of rMGL1 and Hanes plot of data** (insert). The activity of a fixed quantity of recombinant protein was assayed at different concentrations of methionine. The kinetic parameters for this experiment were: Km, 0.66mM and the Vmax, 20.8 nmol/min/ml

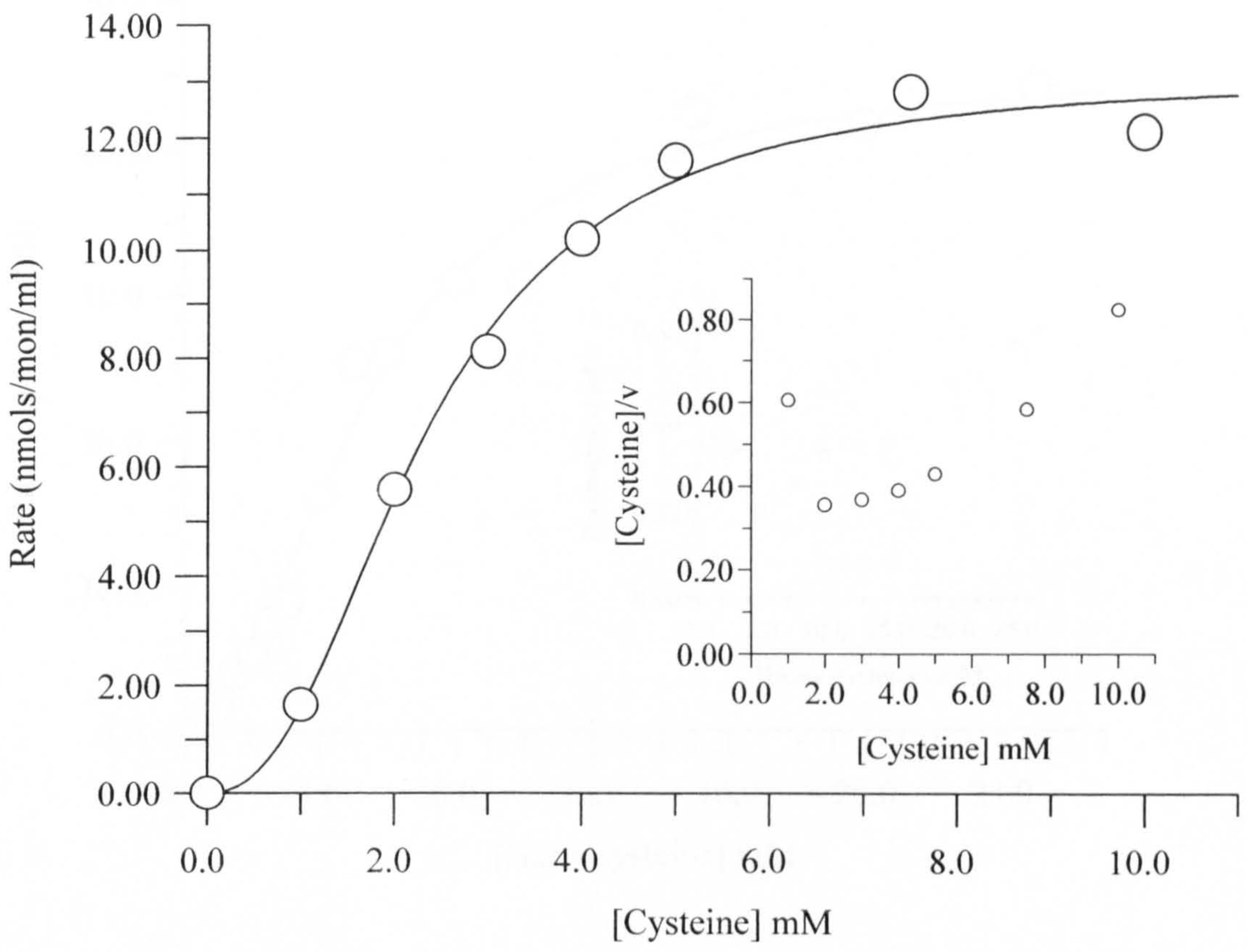



**Figure 5.16 A representative cysteine saturation curve of rMGL1 and Hanes plot (insert).** The activity of a fixed quantity of recombinant protein was assayed at different concentrations of cysteine. The kinetic parameters for this experiment were: Km 7.2mM, Vmax, 13.1 nmol/min/ml and Hill Coefficient 2.4

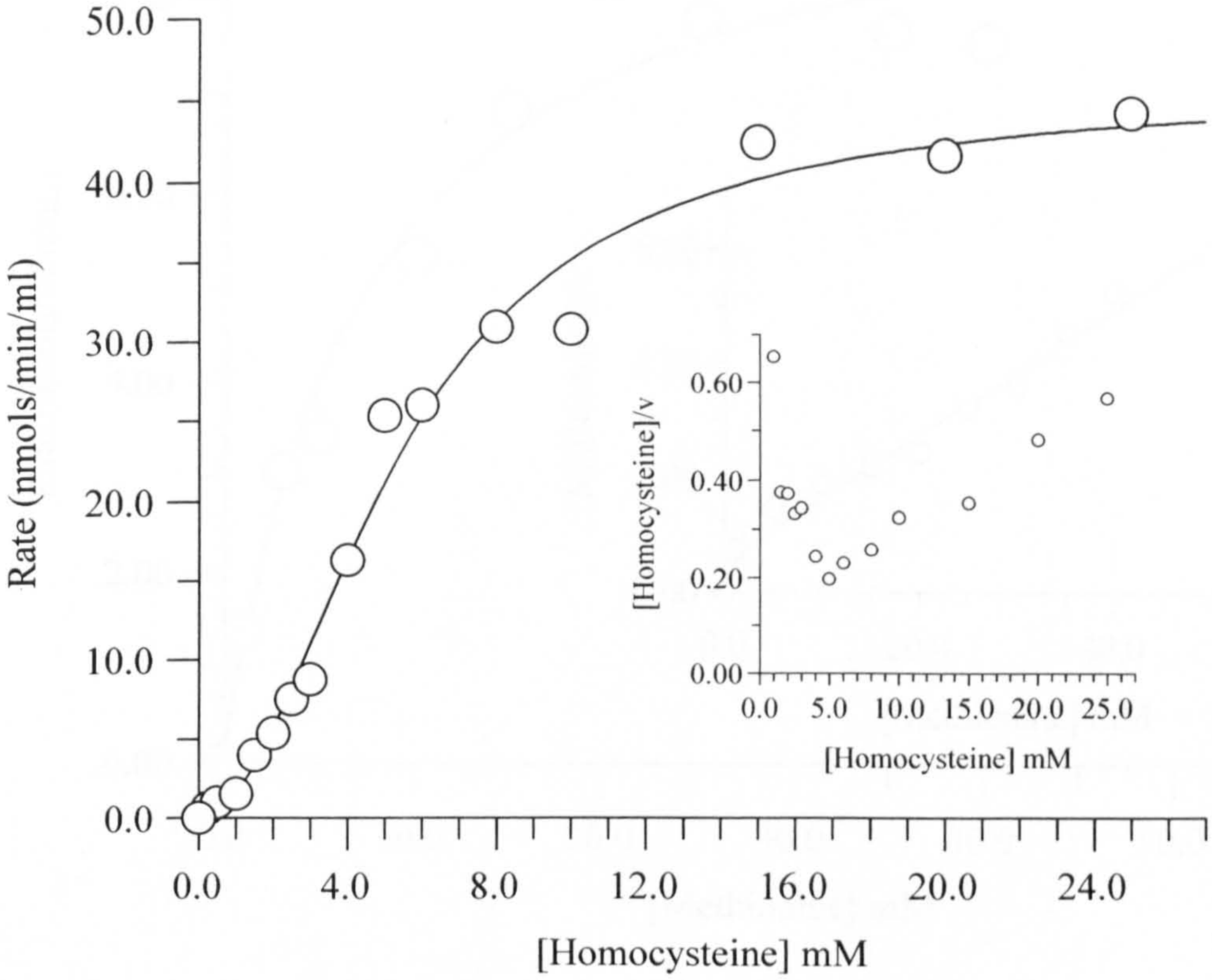



**Figure 5.17 A representative homocysteine saturation curve of rMGL2 and Hanes plot of data (insert).** The activity of a fixed quantity of recombinant protein was assayed at different concentrations of homocysteine. The kinetic parameters of this experiment were: Km27.1mM, Vmax, 45.3 nmol/min/ml and Hill coefficient 1.9

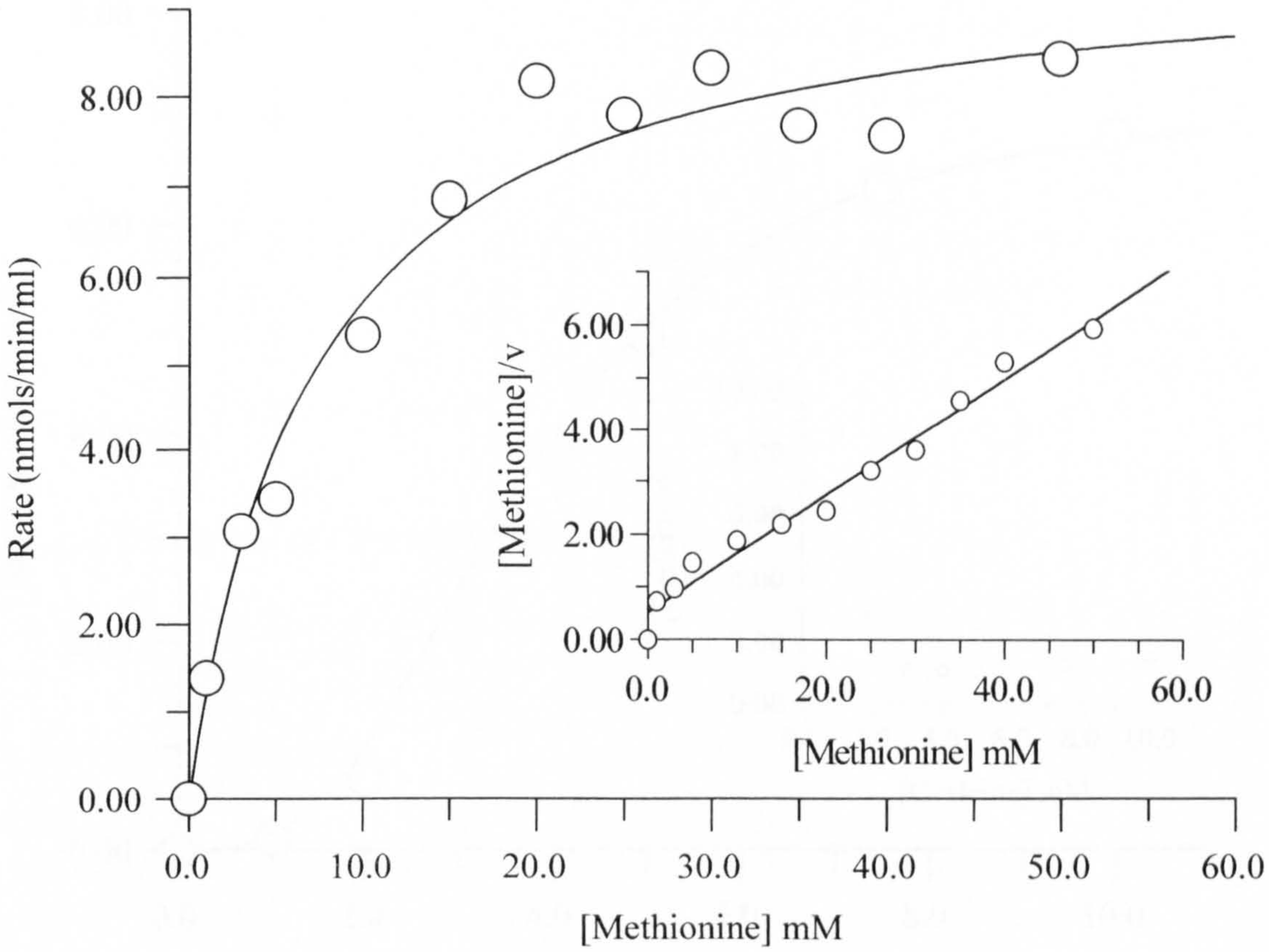



**Figure 5.18 A representative methionine saturation curve of rMGL2 and Hanes plot of data (insert).** The activity of a fixed quantity of recombinant protein was assayed at different concentrations of methionine. The kinetic parameters for this experiment were: Km, 7.0mM and the Vmax, 9.7 nmol/min/ml

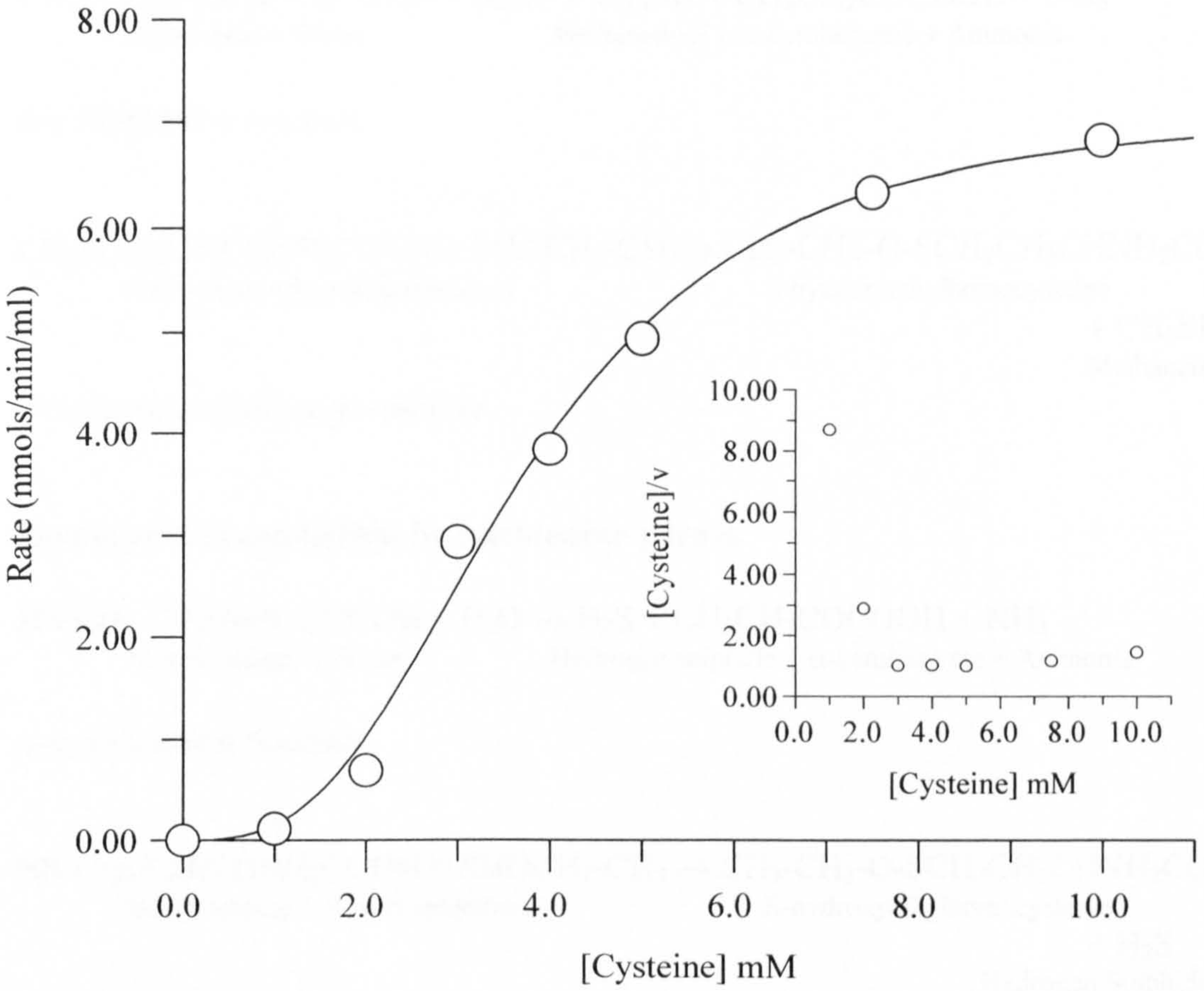



**Figure 5.19 A representative cysteine saturation curve of rMGL2 and Hanes plot of the data (insert).** The activity of a fixed quantity of recombinant protein was assayed at different concentrations of cysteine. The kinetic parameters for this experiment were: Km, 44.6mM, Vmax, 7.1 nmol/min/ml and Hill coefficient 2.9

**Methionine catabolism by methionine γ-lyase**

$CH_3SCH_2CH_2CHNH_2COOH + H_2O \rightarrow CH_3SH + CH_3CH_2COCOOH + NH_3$

Methionine + Water → Methanethiol + α-ketobutyrate + Ammonia

α-γ elimination reaction

$CH_3SCH_2CH_2CHNH_2COOH + SHOCH_2\text{-}CH_3 \rightarrow CH_3\text{-}CH2\text{-}O\text{-}SCH_2CH_2CHNH_2COOH + CH_3SH$

Methionine + β-mercaptoethanol → S-hydroxyethylhomocysteine + Methanethiol

γ-replacement/exchange reaction

**Homocysteine catabolism by methionine γ-lyase**

$HS\text{-}CH_2\text{-}CH_2CHNH_2COOH + H_2O \rightarrow H_2S + CH_3CH_2COCOOH + NH_3$

Homocysteine +. Water → Hydrogen sulphide + α-ketobutyrate + Ammonia

α-γ elimination reaction

$HS\text{-}CH_2\text{-}CH_2CHNH_2COOH + SHOCH_2\text{-}CH_3 \rightarrow CH_3\text{-}CH_2\text{-}O\text{-}SCH_2CH_2CHNH_2COOH + H_2S$

Homocysteine + β-mercaptoethanol → S-hydroxyethylhomocysteine + Hydrogen Sulphide

γ-replacement/exchange reaction

**Cysteine catabolism by methionine γ-lyase**

$SH\text{-}CH_2CHNH_2COOH + H_2O \rightarrow H_2S + CH_3COCOOH + NH_3$

Cysteine + Water → Hydrogen sulphide + Pyruvate + Ammonia

α-β elimination

$SH\text{-}CH_2CHNH_2COOH + SHOCH_2\text{-}CH_3 \rightarrow CH_3\text{-}CH_2\text{-}O\text{-}SCH_2CHNH_2COOH + H_2S$

Cysteine + β-mercaptoethanol → S-hydroxymethylhomocysteine + Hydrogen Sulphide

β-replacement/exchange reaction

**Figure 5.20 The reactions catalysed by methionine γ-lyase with the substrates methionine, homocysteine and cysteine.**

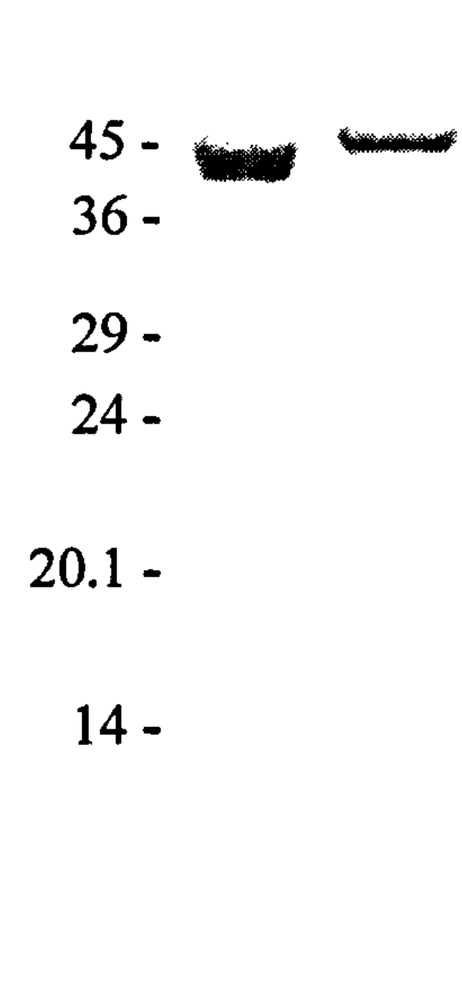



**Figure 5.21 SDS-PAGE analysis of purified rMGL1 and rMGL2.**

Purified rMGL1 and rMGL2 was dialysed overnight against 0.1M sodium phosphate buffer pH6.5 and 300 mM sodium chloride, boiled with Laemmli reducing sample buffer prior to application on a 12% acrylamide gel.

Lane 1: Purified rMGL1

Lane 2: Purified rMGL2

Molecular weight size markers (Sigma SDS 7) are indicated on the left

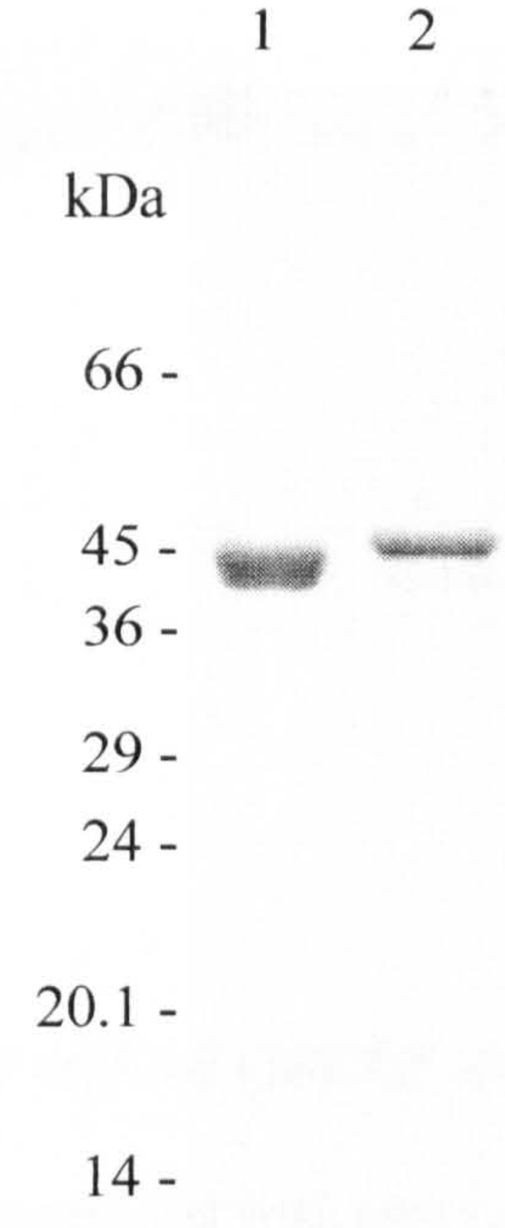



**Figure 5.21 SDS-PAGE analysis of purified rMGL1 and rMGL2.**

Purified rMGL1 and rMGL2 was dialysed overnight against 0.1M sodium phosphate buffer pH6.5 and 300 mM sodium chloride, boiled with Laemmli reducing sample buffer prior to application on a 12% acrylamide gel.

Lane 1: Purified rMGL1

Lane 2: Purified rMGL2

Molecular weight size markers (Sigma SDS 7) are indicated on the left

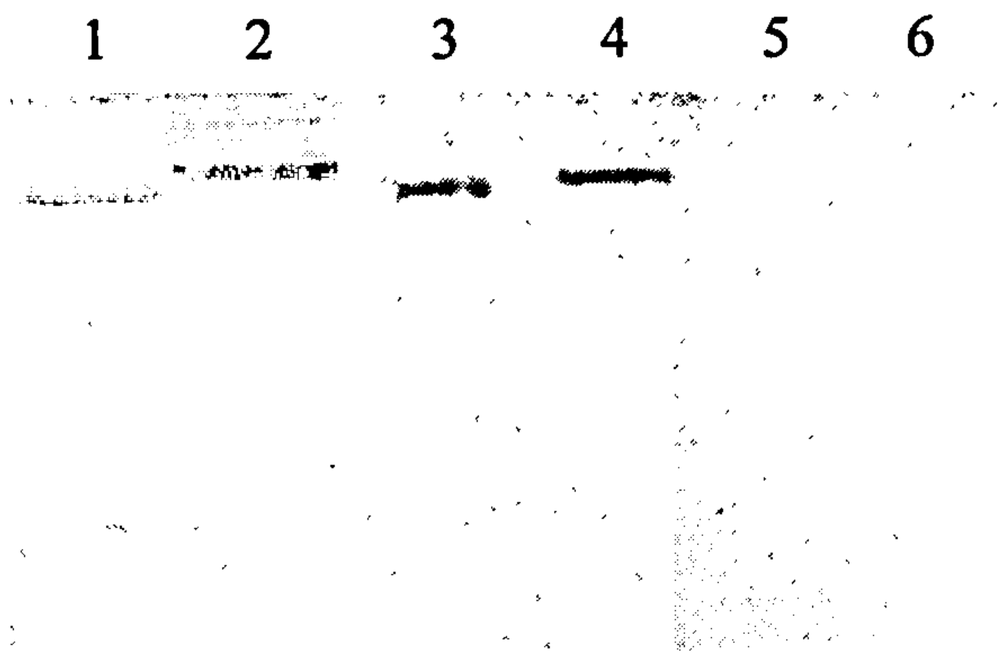



**Figure 5.22 Native PAGE of rMGL1 and rMGL2.**

rMGL1 and rMGL2 combined with non reducing Laemmli sample buffer was applied to a non-denaturing 12% acrylamide gel and after electrophoresis placed in homocysteine desulphurase activity stain.

Lane 1: Coomassie Blue R250 stained rMGL1

Lane 2: Coomassie Blue R250 stained rMGL2

Lane 3: Homocysteine desulphurase staining of rMGL1

Lane 4: Homocysteine desulphurase staining of rMGL2

Lane 5: Homocysteine desulphurase staining of rMGL1 after incubation of the gel slice in 10 mM propargylglycine

Lane 6: Homocysteine desulphurase staining of rMGL2 after incubation of the gel slice in 10 mM propargylglycine

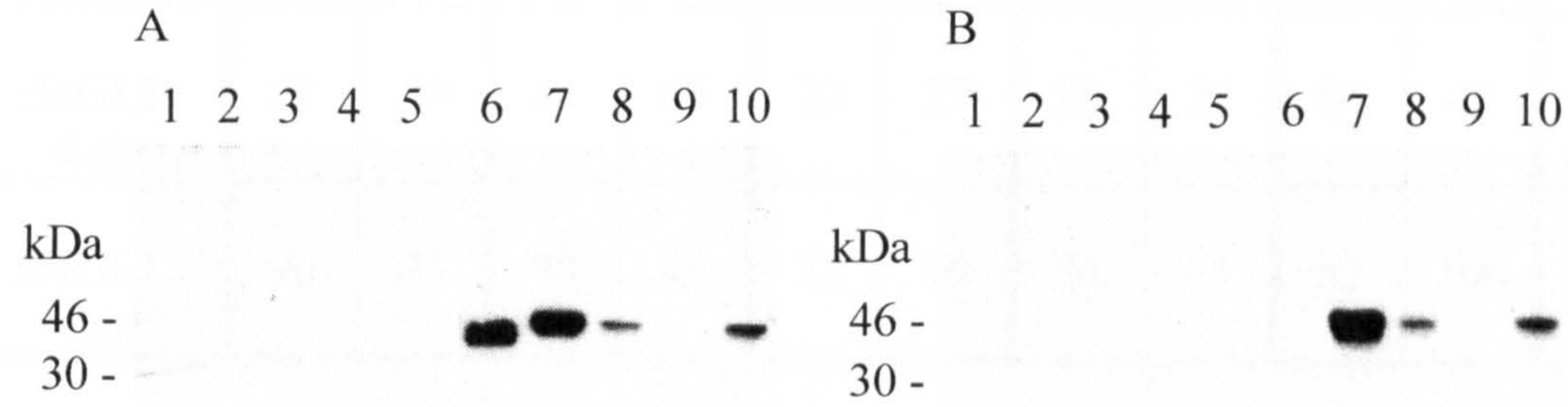



**Figure 5.23 Western blot analysis of rMGL1 and rMGL2 and *T. vaginalis* homogenates, pellet and supernatant fractions.**

**Panel A**

Lane 1: rMGL1 probed with preimmune serum

Lane 2: rMGL2 probed with preimmune serum

Lane 3: *T. vaginalis* homogenate probed with preimmune antiserum

Lane 4: *T. vaginalis* pellet fraction probed with preimmune antiserum

Lane 5: *T. vaginalis* soluble fraction probed with preimmune antiserum

Lanes 6-10: Loaded with the same samples as above but probed with immune serum raised against rMGL1

**Panel B**

Lanes 1-5: Loaded with the same samples as in Panel A, lanes 1-5 but probed with preimmune serum

Lanes 6-10: Loaded with the same samples as in Panel A, lanes 1-5 but probed with serum raised against rMGL2

| | A | | B | | C | | D | | E | | F | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | -20C | -70C | -20C | -70C | -20C | -70C | -20C | -70C | -20C | -70C | -20C | -70C |
| rMGL1 +Imidazole | 47 | 44 | 59 | 54 | 54 | 66 | 58 | 64 | 76 | 58 | 65 | 43 |
| rMGL1 -Salt | 63 | 39 | 64 | 65 | 72 | 27 | 89 | 27 | 94 | 45 | 74 | 29 |
| rMGL1 +Salt | 96 | 43 | 98 | 46 | 72 | 70 | 94 | 65 | 82 | 100 | 100 | 93 |

**Table 5.1 Stabilisation studies of enzymatic activity of rMGL1.**

Homocysteine desulphurase activities of rMGL after 14 days, expressed as a percentage of the homocysteine desulphurase activity of freshly purified recombinant protein.

rMGL1 + Imidazole, rMGL1 combined with stabilisation buffers A-F immediately after elution from the Ni2+-NTA column.

rMGL1 -Salt, rMGL1 combined with stabilisation buffers A-F after dialysis against sodium phosphate buffer without sodium chloride

rMGL1 +Salt, rMGL1 combined with stabilisation buffers A-F after dialysis against sodium phosphate buffer with sodium chloride

The different conditions and final concentration of enzyme stabilisation buffer components used were:

A: 40% glycerol in 0.1M sodium phosphate buffer pH7.5

B: 40% glycerol in 0.1M sodium phosphate buffer pH7.5 and 15 μM βmercaptoethanol

C: 40% glycerol in 0.1M sodium phosphate buffer pH7.5 and 15 μM DTT

D: 40% glycerol in 0.1M sodium phosphate buffer pH7.5 and 20 μM PLP

E: 40% glycerol in 0.1M sodium phosphate buffer pH7.5, 15 μM β-mercaptoethanol and 20 μM PLP

F: 40% glycerol in 0.1M sodium phosphate buffer pH7.5, 15 μM DTT and 20 μM PLP

| | A | | B | | C | | D | | E | | F | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | -20C | -70C | -20C | -70C | -20C | -70C | -20C | -70C | -20C | -70C | -20C | -70C |
| rMGL2 +Imidazole | 47 | 60 | 38 | 73 | 34 | 87 | 34 | 88 | 50 | 51 | 67 | 59 |
| rMGL2 -Salt | 88 | 68 | 60 | 50 | 85 | 74 | 50 | 72 | 72 | 44 | 72 | 59 |
| rMGL2 +Salt | 91 | 81 | 85 | 89 | 58 | 96 | 77 | 89 | 71 | 85 | 100 | 91 |

**Table 5.2 Stabilisation studies of enzymatic activity of rMGL2.**

Homocysteine desulphurase activities of rMGL2 after 14 days, expressed as a percentage of the homocysteine desulphurase activity of freshly purified recombinant protein. rMGL2 + Imidazole, rMGL2 combined with stabilisation buffers A-F immediately after elution from the Ni2+-NTA column.

rMGL1 -Salt, rMGL2 combined with stabilisation buffers A-F after dialysis against sodium phosphate buffer without sodium chloride

rMGL1 +Salt, rMGL2 combined with stabilisation buffers A-F after dialysis against sodium phosphate buffer with sodium chloride

The different conditions and final concentration of enzyme stabilisation buffer components used were:

A: 40% glycerol in 0.1M sodium phosphate buffer pH7.5

B: 40% glycerol in 0.1M sodium phosphate buffer pH7.5 and 15 μM βmercaptoethanol

C: 40% glycerol in 0.1M sodium phosphate buffer pH7.5 and 15 μM DTT

D: 40% glycerol in 0.1M sodium phosphate buffer pH7.5 and 20 μM PLP

E: 40% glycerol in 0.1M sodium phosphate buffer pH7.5, 15 μM β-mercaptoethanol and 20 μM PLP

F: 40% glycerol in 0.1M sodium phosphate buffer pH7.5, 15 μM DTT and 20 μM PLP

| Substrate | rMGL1 | rMGL2 |
|---|---|---|
| Homocysteine | 64.2±16.0 (12) | 53.9±0.11 (12) |
| Methionine | 8.4±1.1 (7) | 1.8±0.09 (8) |
| Cysteine | 2.5±0.54 (5) | 1.4±0.76 (6) |
| O-acetyl-L-serine | 2.7±0.11 (11) | 2.5±0.43 (11) |
| S-adenosyl-L-methionine | 0.14±0.01 (8) | 0.04±0.01 (8) |
| Cystathionine | ND<0.001 (12) | ND<0.001 (12) |

**Table 5.3 Summary of the specific activities obtained for the *T. vaginalis* recombinant proteins rMGL1 and rMGL2 with various substrates.**

The data are the enzyme activities in μmol/min/mg protein and are Means ± S.D., n in parentheses. N.D., not detectable

| | rMGL1 | | rMGL2 | |
|---|---|---|---|---|
| | Km | Vmax | Km | Vmax |
| Homocysteine | 4.7±0.8 mM (3) | 28.6±7.4 nmols/min/ml (3) | 23.7±3.0 mM (3) | 31.3±12.3 nmols/min/ml (3) |
| Methionine | 0.65±0.02 mM (3) | 17.0±3.4 nmols/min/ml (3) | 10.6±4.0 mM (4) | 8.7±1.3 nmols/min/ml (4) |
| Cysteine | 6.1±1.1 mM (3) | 11.8±1.1 nmols/min/ml (3) | 25.7±16.5 mM (3) | 7.4±0.2 nmols/min/ml (3) |

**Table 5.4 Summary table of $K_m$s and $V_{maxs}$ obtained for the *T. vaginalis* recombinant proteins rMGL1 and rMGL2.**

The substrates homocysteine, methionine and cysteine were used in the kinetic determinations.

The data are means ± S.D., n in parentheses

| | rMGL1 | rMGL2 |
|---|---|---|
| Homocysteine $H_2S$ detection | 246±3.2 (8) | 323±19.8 (8) |
| Homocysteine and 10mM β-mercaptoethanol $H_2S$ detection | 152±15.0 (8) | 326±24.0 (8) |
| Homocysteine α-keto-acid detection | 49.2±3.6 (8) | 50.6±2.0 (8) |
| Homocysteine and 10mM β-mercaptoethanol α-keto-acid detection | 2.6±1.2 (8) | 13.3±2.4 (8) |

**Table 5.5 Effect of β-mercaptoethanol on homocysteine desulphurase activity of rMGL1 and rMGL2.**

Summary table of specific activites for rMGL1 and rMGL2 when homocysteine is used as a substrate with and without β-mercaptoethanol in the assay. Two different assay methods were used to determine the breakdown of homocysteine. Enzyme activities μmol min/mg are means ± S.D., n in parentheses

| Substrate | rMGL1 | rMGL2 | native MGL |
|---|---|---|---|
| Methionine | 100 | 100 | 100 |
| Homocysteine | 764 | 2994 | 903 |
| Cysteine | 30 | 77 | 99 |
| O-acetyl L-serine | 32 | 139 | 945 |
| S-adenosylmethionine | 1.7 | 2.2 | 830 |
| Cystathionine | ND | ND | ND |

**Table 5.6 Substrate specificity of recombinant MGLs compared to native MGL from *T. vaginalis*.**

Results are expressed as the activity relative to that towards methionine, when assaying for the production of α-keto acids. Data for native MGL are taken from Lockwood and Coombs, (1991)

N.D., not detectable.

# CHAPTER 6: INVESTIGATIONS INTO THE FUNCTIONS OF METHIONINE γ-LYASE AND OTHER SULPHUR AMINO ACID-METABOLISING ENZYMES IN *T. VAGINALIS* AND OTHER TRICHOMONADS.

## 6.1 Introduction.

The results from molecular and biochemical characterisation of methionine γ-lyase from *T. vaginalis* has been presented in the previous two chapters of this thesis. Questions, however, remain unanswered with regards to the function of this unusual enzyme in the *T. vaginalis*. Two main ideas have been proposed for the purpose of an enzyme that is able to catabolise homocysteine and methionine at a high rate (Thong *et al.*, 1987)

The first is that the α-keto acids produced from the breakdown of several sulphur-containing amino acids by *T. vaginalis* are catabolised via energy generating pathways similar to the breakdown of pyruvate. Support for this proposal comes from the finding that pyruvate: ferredoxin oxidoreductase, the enzyme that catalyses the conversion of pyruvate to acetyl CoA, will also catalyse the conversion of α-ketobutyrate to propionyl CoA (Williams *et al.*, 1987).

The second idea is that methionine γ-lyase along with serine sulphydrase, another sulphur amino acid-catabolising enzyme of *T. vaginalis* (Thong and Coombs, 1985), are involved in maintaining a reducing environment in the cell. Indeed, it has been suggested that *Entamoeba histolytica* and *Giardia lamblia*, as well as *T. vaginalis*, require thiols (particularly cysteine) to counter the toxic effects of oxygen (Gillin *et al.*,

1984 and Fairlamb, 1989). It may be that in *T. vaginalis*, methionine γ-lyase and serine sulphydrase are themselves involved in countering the toxic effects of oxygen.

The work presented in this chapter details some experiments carried out to investigate the possibility of methionine γ-lyase and serine sulphydrase being involved in maintaining the redox balance of the cell. The homocysteine-catabolising capabilities of *T. vaginalis*, *Tritrichomonas foetus* and *Trichomitus augusta* were investigated. As has been outlined in previous chapters, it appears that methionine γ-lyase is also responsible for the breakdown of homocysteine, particularly in *T. vaginalis*. Therefore, in this chapter the enzyme names homocysteine desulphurase and methionine γ-lyase are interchanged. Homocysteine desulphurase activity reflects the ability by methionine γ-lyase to breakdown homocysteine.

This chapter also describes some work carried out using the polyclonal antibodies that were raised against recombinant MGL1 and MGL2, in localisation studies of methionine γ-lyase in *T. vaginalis*, *T. foetus* and *T. augusta*.

## 6.1 Results.

### 6.2.1 Effects of propargylglycine on methionine γ-lyase and serine sulphydrase activities of *T. vaginalis*.

Firstly, *T. vaginalis* was grown in the presence of a range of propargylglycine concentrations, a known inhibitor of γ-elimination enzymes (Abeles and Walsh, 1973). See 2.1.1.2 for details of *T. vaginalis* cultivation. The addition of propargylglycine to cultures of *T. vaginalis* did not affect parasite growth. As can be seen from Table 6.1,

homocysteine desulphurase activity in *T. vaginalis* was inhibited at the lowest concentration of propargylglycine whilst serine sulphydrase activity was elevated in comparison to the control. When the concentration of propargylglycine was increased there was an accompanying increase in the specific activity of serine sulphydrase. It must be noted, however, that the activity of serine sulphydrase when *T. vaginalis* was grown in 1 mM propargylglycine was lower than when grown with 100 μM of the inhibitor. Homocysteine desulphurase activity remained inhibited.

These results were in agreement with those presented by Thong and Coombs, 1987. This experiment was only conducted once, which may raise questions as to the reliability of the data. The same experiment, however, has been repeated by two parasitology students in their honours projects and the same general trend observed, which gives me confidence that the results obtained are real.

### 6.2.2 Effects of exogenous cysteine, serine and propargylglycine on the methionine γ-lyase and serine sulphydrase activities of *T. vaginalis*.

Table 6.2 shows the result of an experiment when *T. vaginalis* was grown in the presence of various concentrations of propargylglycine in the medium and supplemented with 10 mM cysteine or 10 mM serine. When *T. vaginalis* was grown in medium supplemented with cysteine alone and in the presence of the inhibitor plus cysteine, the elevation of serine sulphydrase did not occur. Also homocysteine desulphurase activity was not completely abolished. However, when the cells were grown in additional serine, the parasites responded as they did when grown in the presence of propargylglycine alone.

### 6.2.3 Methionine γ-lyase activities in *Tritrichomonas foetus* and *Tritrichomonas augusta.*

The activity of methionine γ-lyase was also investigated in two other species of trichomonad, *T. foetus* and *T. augusta.* Table 6.3 shows the results obtained when homocysteine catabolism was investigated in the two different species of trichomonad. As can be seen from Table 6.3, no homocysteine catabolising activity could be detected in homogenates of *T. foetus* or *T. augusta.*

### 6.2.4 Western blot analysis of *T. foetus* and *T. augusta* homogenates, soluble and pellet fractions with the anti-rMGL1 and anti-rMGL2 sera.

Enzymatic activity was not detected for the breakdown of homocysteine by *T. foetus* or *T. augusta.* It was decided to investigate the of enzyme homologues in lysates of the two parasites by using the two antibodies that had been raised to recombinant proteins encoded by the two methionine γ-lyase homologues that had been isolated from *T. vaginalis.* Figure 6.1 shows Western blot analysis of homogenates, pellet and soluble fractions of the two parasites with the anti-rMGL1 and anti-rMGL2 sera.

As can be seen from Panel A, lanes 1 and 3 there was a tiny amount of reactivity of the *T. foetus* soluble fraction and homogenate with the pre-immune rMGL1 serum, with a protein of ~68 kDa being recognised. Panel A, lanes 4-6, also show some reactivity with the *T. augusta* samples and the rMGL1 pre-immune serum. There were a number of proteins recognised by the pre-immune serum in the *T. augusta* homogenate that range in size from ~96-35 kDa, two proteins of ~68 kDa and ~35 kDa were recognised by the same serum in the *T. augusta* soluble fraction.

Panel B shows the results of Western blotting of identical samples as in Panel A, but using the immune rMGL1 serum. As can be seen from Panel B, lanes 1 and 3, there was a small amount of reactivity in the *T. foetus* soluble and homogenate fractions, with a protein of >96 kDa being recognised in the soluble fraction (Panel B, lane 1) and several proteins of between 68->96 kDa being recognised in the *T. foetus* homogenate (Panel B, lane 3). No proteins were recognised in the *T. foetus* pellet fraction (Panel B, lane 2) by the immune rMGL1 sera. Panel B, lanes 4-6 show the reactivity of *T. augusta* proteins with the immune rMGL1 sera. As can be seen from Panel B, lane 4, there are some high molecular weight bands of >96 kDa that were recognised with the immune rMGL1 serum, that were not recognised with the pre-immune serum. Panel B, lane 6 shows that several proteins of the *T. augusta* homogenate are recognised by the immune rMGL1 serum that are not recognised by the pre-immune serum. For example, the protein of ~35 kDa recognised with the pre-immune rMGL1 serum is more strongly recognised with the immune rMGL1 serum. Proteins >43 kDa->96 kDa were also recognised by the immune rMGL1 serum but not the pre-immune serum.

Panel C, lanes 1-3 show the reactivity of the pre-immune rMGL2 serum with *T. foetus* soluble and pellet fractions and homogenates, respectively. There was only a slight reactivity of two proteins of >96 kDa and ~68 kDa in the *T. foetus* homogenate (Panel C, lane 3). Panel B, lanes 4-6 show the reactivity of the same fractions but from *T. augusta*. There was only a small amount of reactivity of proteins of ~68 kDa and ~75 kDa and *T. augusta* soluble fraction with the pre-immune rMGL2 serum (Panel C, lane 4).

Panel D, lanes 1 and 3 show the reactivity of *T. foetus* soluble fraction and homogenate with the immune rMGL2 serum. As can be seen from lanes 1 and 3, there were many bands of >43kDa recognised by the rMGL2 immune serum. There were no proteins recognised in the pellet fraction of *T. foetus* with the immune rMGL2 antibody (Panel D, lane 2). Panel D, lanes 4 and 6, show the reactivity of *T. augusta* soluble fraction and homogenates with the immune rMGL2 serum, as can be seen from lanes 4 and 6 there were several proteins ranging from ~50kDa to >96kDa recognised be the immune rMGL2 serum. There was a single protein of >96kDa recognised by the immune rMGL2 serum in the pellet fraction of *T. augusta* (Panel D, lane 5).

### 6.2.5 Immunolocalisation of methionine γ-lyase homologues in intact *T. vaginalis*, *T. foetus* and *T. augusta*.

The results of the Western blots for *T. vaginalis* (Chapter 5), *T. foetus* and *T. augusta* homogenates, pellet and supernatant fractions with the two antisera that had been raised against rMGL1 and rMGL2 prompted immunolocalisation experiments in the intact parasites.

Figure 6.2 shows the results of immunofluorescence with intact *T. vaginalis* and the polyclonal antibodies raised against rMGL1 and rMGL2. Figure 6.2, Panel A, shows DAPI nuclear counter staining of fixed *T. vaginalis* under UV epifluorescence. Panel B shows immunolabelling of *T. vaginalis* with the polyclonal anti-rMGL1 serum under UV epifluorescence. As can be seen from Panel B there appears to be a very specific labelling of the nucleus of *T. vaginalis* with the rMGL1 antibody. There also appears to

be a general labelling of the cytoplasm of the cell with the antibody, but it is not as intense as the immunostaining of the nucleus of the parasite.

Figure 6.2, Panel C shows DAPI nuclear counter staining of fixed *T. vaginalis* under UV epifluorescence. Panel D shows immunolabelling of *T. vaginalis* with the polyclonal anti-rMGL2 serum under UV epifluorescence. As can be seen from Panel D there appears to be a very specific labelling of the nucleus of *T. vaginalis* with the rMGL2 antibody. There also appears to be a general less intense labelling of the cytoplasm of the cell with the antibody.

Figure 6.3 shows the results of immunofluorescence with intact *T. foetus* and the polyclonal antibodies raised against rMGL and rMGL2. Figure 6.3, panel A shows DAPI nuclear counter staining of fixed *T. foetus* under UV epifluorescence. Panel B shows immunolabelling of *T. foetus* with the polyclonal anti rMGL1 serum under UV epifluorescence. As can be seen from Panel B, there appears to be a very specific labelling of a structure anterior to the nucleus in this parasite, as well as a more general staining of the cytoplasm of these cells.

Figure 6.3, Panel C shows DAPI nuclear counter staining of fixed *T. foetus* under UV epifluorescence. Panel D shows immunolabelling of *T. foetus* with the polyclonal anti-rMGL2 serum under UV epifluorescence. As with the rMGL1 serum and *T. foetus* there was specific staining of a structure anterior to the nucleus and general staining of the cytoplasm of the parasites with the rMGL2 antiserum.

Figure 6.4, Panel A, shows DAPI nuclear counter staining of fixed *T. augusta* under UV epifluorescence. There is no figure showing the immunostaining of these parasites with the rMGL1 antiserum, but it can be seen from Panel A that there is a structure anterior of the nucleus that is visible on the DAPI filter and this structure was recognised by the rMGL1 antibody.

Figure 6.4, Panel B, shows DAPI nuclear counter staining of fixed *T. augusta* under UV epifluorescence. Panel C shows immunostaining with the rMGL2 antiserum can be seen there is a structure anterior to the nucleus that is specifically labelled with the antibody, as was the result with *T. foetus*.

## 6.3 Discussion.

The work presented in this chapter indicates that methionine γ-lyase and serine sulphydrase of *T. vaginalis* may have complementary functions, as has been previously suggested by Thong *et al.*, 1987. It was found that methionine γ-lyase was inhibited by 1 μM propargylglycine whilst, serine sulphydrase activity was increasingly elevated up to 100 μM of inhibitor. The further increase in serine sulphydrase activity that is observed even when methionine γ-lyase activity is inhibited can probably be explained by other pyridoxal 5'-phosphate enzymes present in *T. vaginalis* being inhibited. For example, cystathionine γ-lyase which is reported to exist in *T. vaginalis* (Thong and Coombs, 1985) is a pyridoxal 5'-phosphate linked enzyme. The enzyme in other systems is reported to be inhibited by propargylglycine (Abeles and Walsh, 1973). The products of cystathionine breakdown by cystathionine γ-lyase are cysteine, α-ketobutyrate and ammonia. It may be that other enzymes present in *T. vaginalis* have a

function in maintaining the redox balance of the cell or providing thiols to keep surface proteins of the parasite reduced, and that these enzymes have a similar sensitivity to propargylglycine (ie. cystathionine γ-lyase). Thus the effects of propargylglycine on methionine γ-lyase and serine sulphydrase may be a cumulative effect of the inhibitor on several enzymes in *T. vaginalis* that are involved in redox balance of the cell. It would be interesting therefore to assay, for example, cystathionine γ-lyase of *T. vaginalis* when the parasites were grown in different concentrations of propargylglycine.

Results obtained for the experiment in which *T. vaginalis* was grown in the presence of 10 mM cysteine are indicative of serine sulphydrase of *T. vaginalis* operating in the direction of cysteine synthesis. The elevation of serine sulphydrase activity brought about by the inhibition of methionine γ-lyase by propargylglycine does not need to be balanced as there is already sufficient cysteine in the environment of the cell to keep conditions reduced. It is interesting to note that in the presence of cysteine and various concentrations of propargylglycine the activity of homocysteine desulphurase was not completely abolished. It is probable that cysteine is present in the active site of methionine γ-lyase, and this is why a residual activity is seen. It should also be noted that at high concentrations of propargylglycine (1 mM), the activity of serine sulphydrase also decreases. The likely reason for this is that at high concentrations of propargylglycine the activity of serine sulphydrase the activity of this enzyme is also being inhibited. I feel this result probably reflects the different sensitivities of methionine γ-lyase and serine sulphydrase to propargylglycine. The determination of $K_i$s for methionine γ-lyase and serine sulphydrase of *T. vaginalis* with propargylglycine is an experiment that would need to be carried out to support this hypothesis.

Further evidence for methionine γ-lyase being important for production of thiols which may be important in protecting the parasite from the toxic effects of oxygen is provided by Lloyd *et al.*, 1991, who has shown that methanethiol, a reaction product from the catabolism of methionine, can be auto-oxidised to dimethyldisulphide in *T. vaginalis*.

The importance of cysteine and other reducing agents in the biology of *T. vaginalis* was questioned by the findings presented by Paget and Lloyd, 1989. Their data indicated that oxygen was less toxic to *T. vaginalis* in the absence of reducing agents. However, cysteine in particular may have role in the generation, maintenance and/ or protection of thiol groups on the surface of the parasite (Gillin *et al.*, 1984). Indeed Paget and Lloyd, (1989) showed that *T. vaginalis* grown in 6 mM cysteine and 1 mM ascorbate had a rough surface and a shape varying from pyriform to spherical, whereas, *T. vaginalis* grown without an excess of reducing agents had smooth surfaces and uniform shapes. Paget and Lloyd stated that such structural changes in *T. vaginalis* in the presence of reducing agents could be explained if cysteine was a major component of a structural surface protein of *T. vaginalis*. Gillin *et al.*, 1984 had previously showed that *T. vaginalis* was killed by incubation with non-penetrative thiol blocking reagents, which is indicative of thiols being present at the surface of the parasite. Indeed, it may be that the production of cysteine in *T. vaginalis* is important in adherence of the parasite to the vaginal epithelial cells and their binding of host macromolecules (Graves and Gardner, 1993), both of which would aid the survival of *T. vaginalis* in its human host.

The results presented on the immunofluorescence of intact *T. vaginalis*, *T. foetus* and *T. augusta* with the two polyclonal antibodies raised to rMGL1 and rMGL2 are very

interesting. There is a very specific localisation of both antisera to the nuclei in *T. vaginalis* and a general localisation in the cytoplasm of the cells. Western blot analysis of *T. vaginalis* homogenates and pellet and soluble fractions (Chapter 5) indicated that both rMGL1 and rMGL2 were expressed as a 43 kDa protein in the soluble fraction of the cells. Also subcellular localisation studies of methionine γ-lyase showed that the majority of the protein was found in the non-sedimentable fraction of the parasite (Lockwood and Coombs,1991). The explanation that I give for the strong reactivity of both polyclonal antibodies with intact *T. vaginalis* is simply a cross reactivity with an epitope in the nucleus of these parasites, which is very similar to the methionine γ-lyase homologues to which the antibodies were raised.

Similarly, the results of immunolocalisation with the rMGL1 and rMGL2 antisera and *T. foetus* and *T. augusta* are very interesting. Both parasites with both antisera show a specific immunolocalisation to a structure which is anterior to the nucleus. The results of the Western blots of *T. foetus* and *T. augusta* homogenates, pellet and supernatant fractions showed that there was no specific recognition of a single protein, as there was for *T. vaginalis*. The reactivity of the two antisera with intact *T. foetus* and *T. augusta,* as with *T. vaginalis* is thought to be a cross reactivity of the antibodies with similar epitopes to the protein to which the antibodies were originally raised. It is thought that the structure being recognised by the anti-rMGL1 and anti-rMGL2 sera was the Golgi apparatus. Recently Diaz *et al.*, (1996) have isolated the Golgi complex from *T. foetus* and have carried out ultrastructural studies for this parasite which revealed the position of this organelle in the parasite.

Confirmation of the structure of *T. foetus* and *T. augusta* being recognised by the two anti-sera raised against the two recombinant methionine γ-lyases of *T. vaginalis*, as the Golgi apparatus, could be achieved through by using a variety of approaches. Firstly, subcellular fractionation of *T. foetus* and *T. augusta* could be carried out to enrich for Golgi apparatus of these parasites, followed by immunostaining with the two anti-sera raised against the recombinant methionine γ-lyase. Immuno-electron microscopy of the two parasites and the two anti-sera could be carried out. Thirdly, a molecular probe specific to Golgi apparatus could be used in an attempt to counterstain the organelle along with immunostaining using the two antisera.

**Figure 6.1 A/B/C/D Western blot analysis of *T. foetus* and *T. augusta* supernatant, pellet and soluble fractions with anti-rMGL1 and -rMGL2 sera.**

Panel A

Lane 1: *T. foetus* soluble fraction

Lane 2: *T. foetus* pellet fraction

Lane 3: *T. foetus* homogenate

Lane 4: *T. augusta* soluble fraction

Lane 5: *T. augusta* pellet fraction

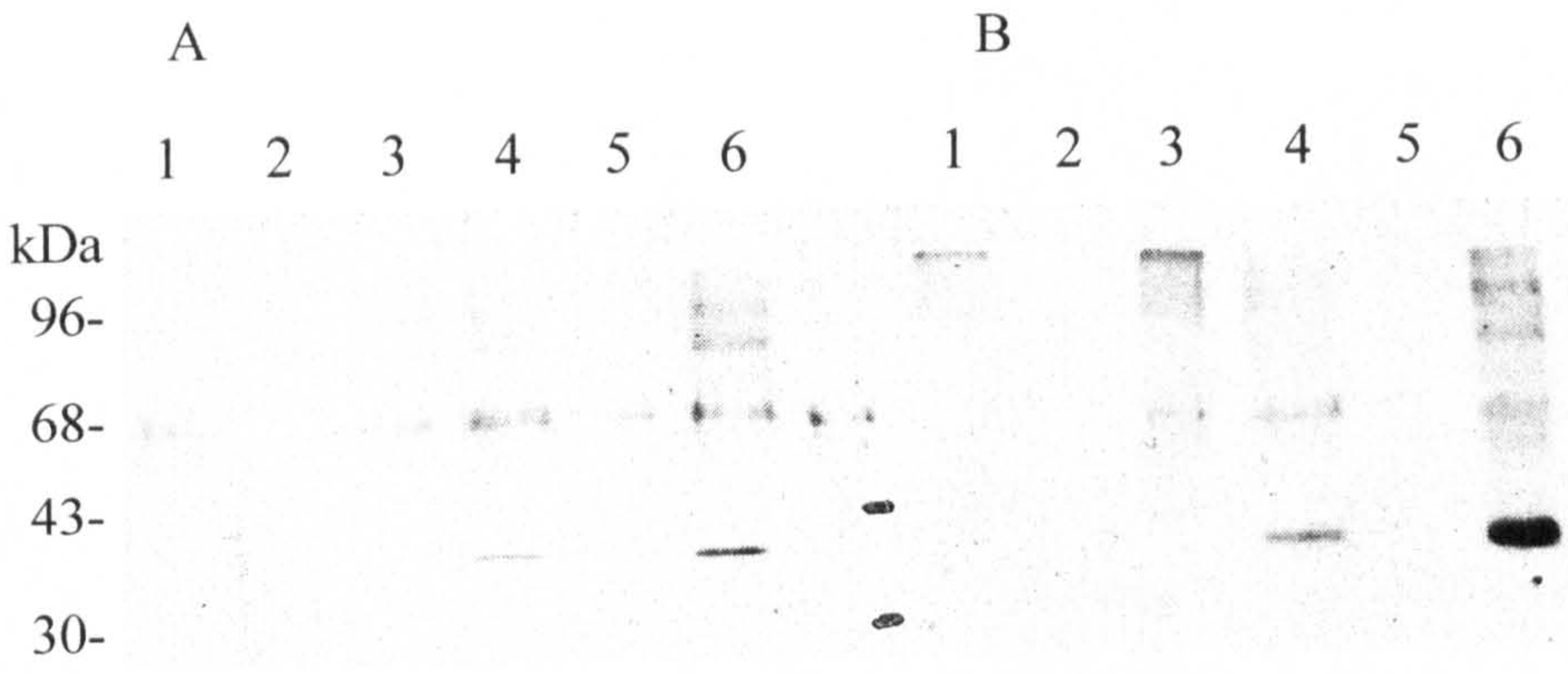
Lane 6: *T. augusta* homogenate

Probed with pre-immune rMGL1 sera

Panel B
Lanes as above, probed with immune rMGL1

Panel C
Lanes as above, probed with pre-immune rMGL2

Panel D
Lanes as above, probed with immune rMGL2


A
B
1 2 3 4 5 6
1 2 3 4 5 6
kDa
96-
68-
43-
30-

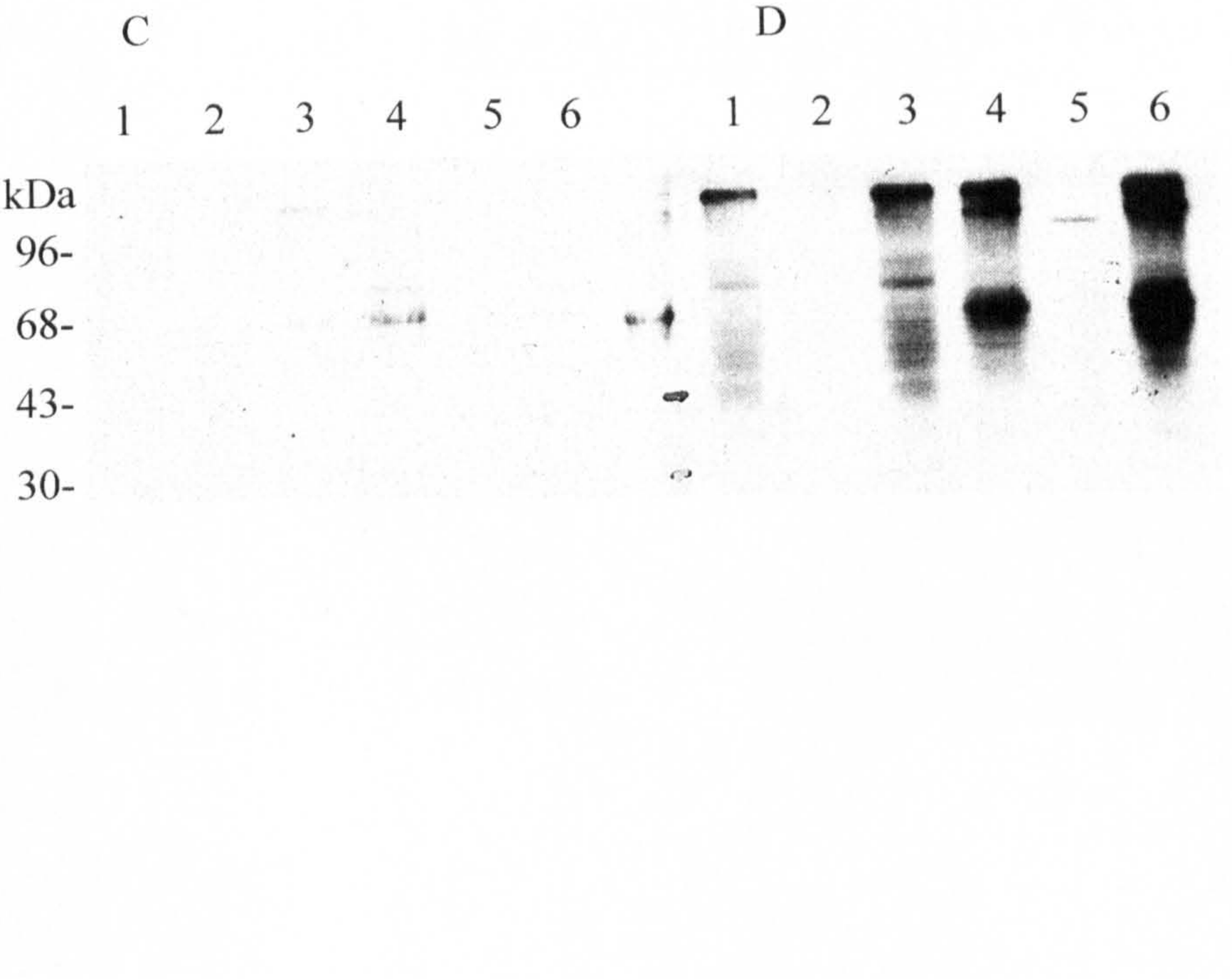

C
D
1 2 3 4 5 6
1 2 3 4 5 6
kDa
96-
68-
43-
30-

**Figure 6.2 A/B/C/D Light micrographs showing specificity of immunolabelling of *T. vaginalis* with polyclonal antisera raised against rMGL1 and rMGL2.**

Panel A: *T. vaginalis* under UV epifluorescence showing DAPI nuclear counterstain (x63 magnification).

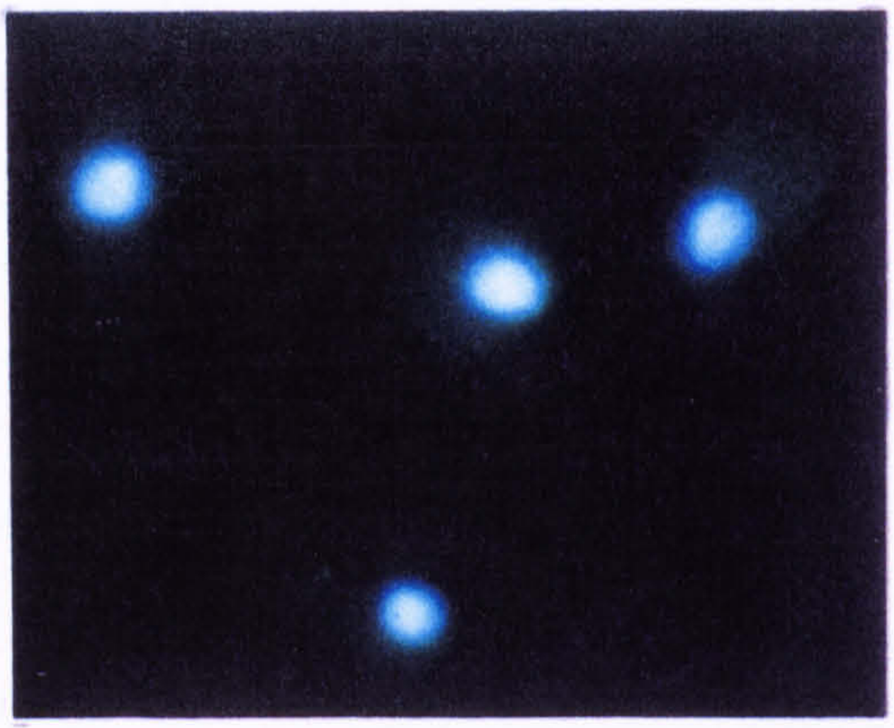
Panel B: *T. vaginalis* under UV epifluorescence showing fluorescent labelling with anti-rMGL1 serum.

Panel C: As Panel A.

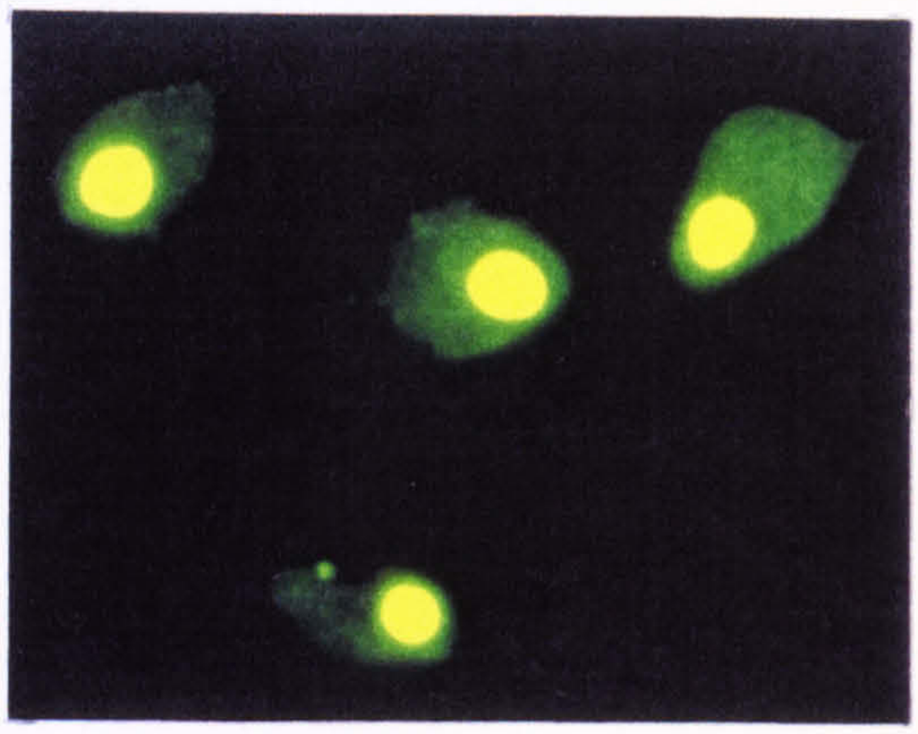
Panel D: As Panel B but with anti-rMGL2 serum.

Scale bar: 10 μm.

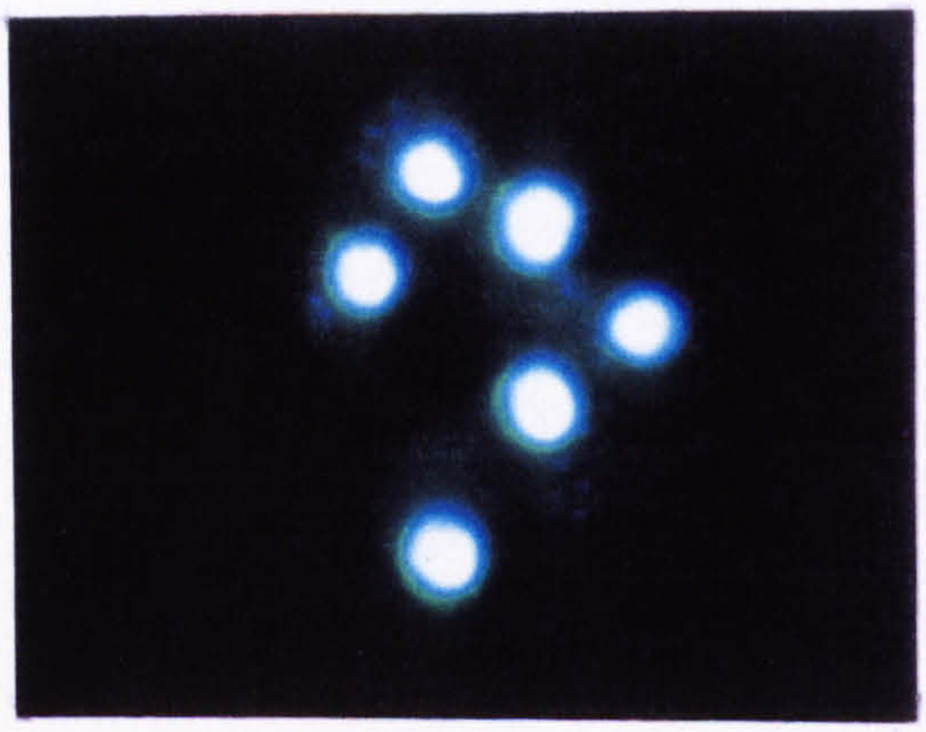
A



B



C



D

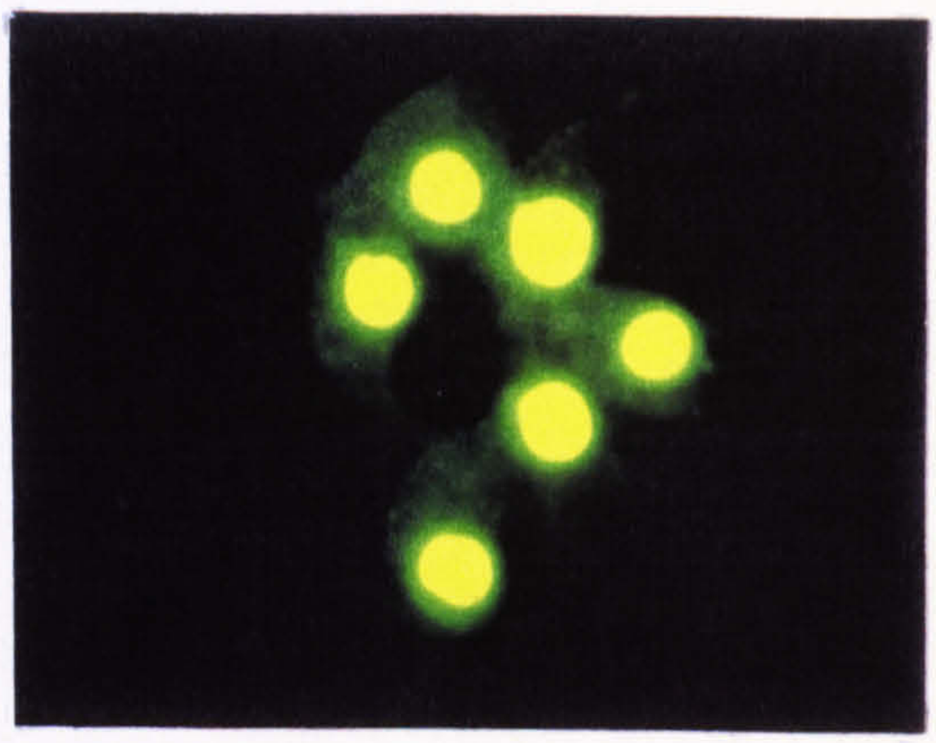


**Figure 6.3 A/B/C/D Light micrographs showing specificity of immunolabelling of *T. foetus* with polyclonal antisera raised against rMGL1 and rMGL2.**

Panel A: *T. foetus* under UV epifluorescence showing DAPI nuclear counterstain (x63 magnification).

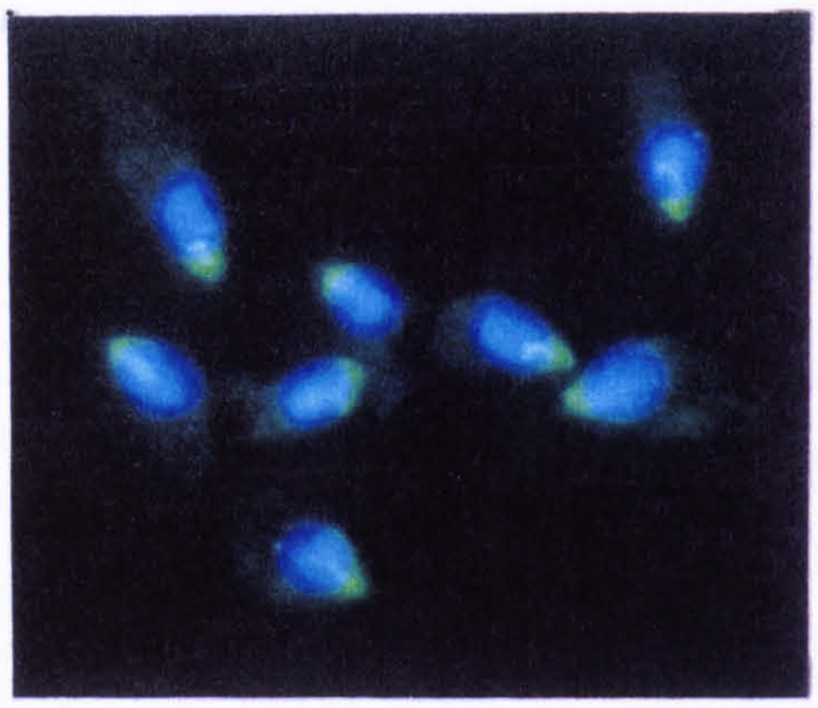
Panel B: *T. foetus* under UV epifluorescence showing fluorescent labelling with anti-rMGL1 serum.

Panel C: As Panel A.

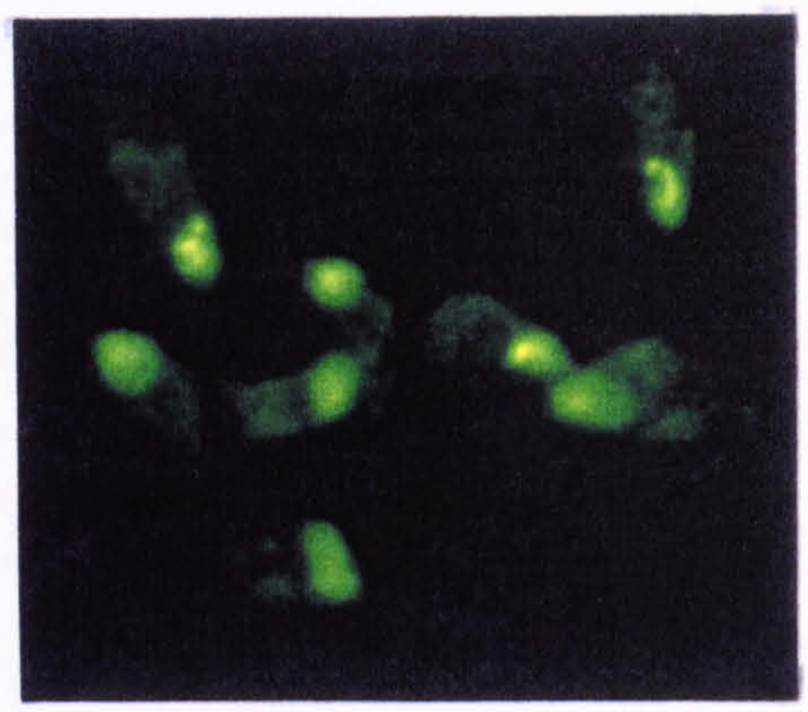
Panel D: As Panel B but with anti-rMGL2 serum.

Scale bar: 10 μm.

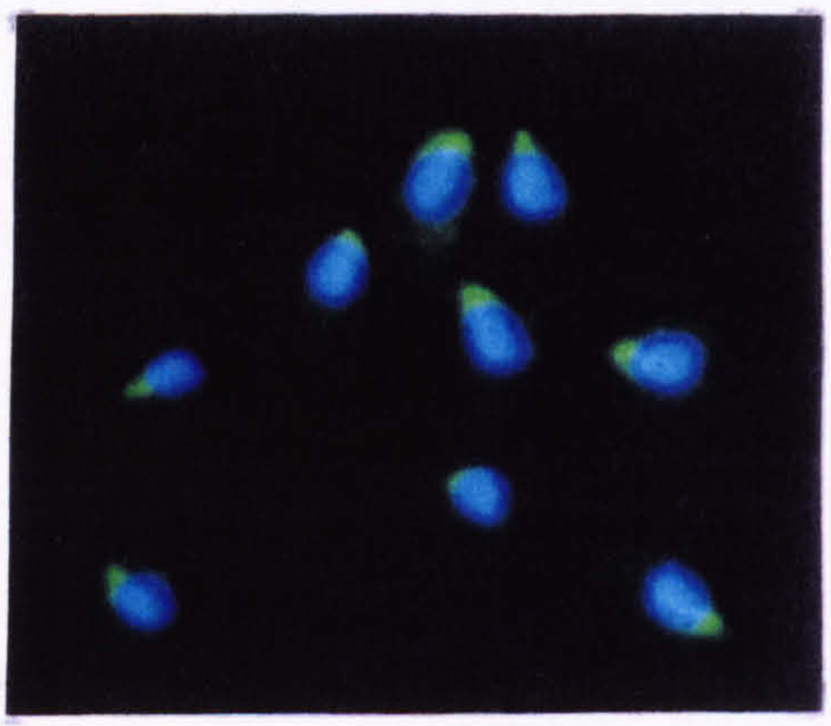
A



B



C



D

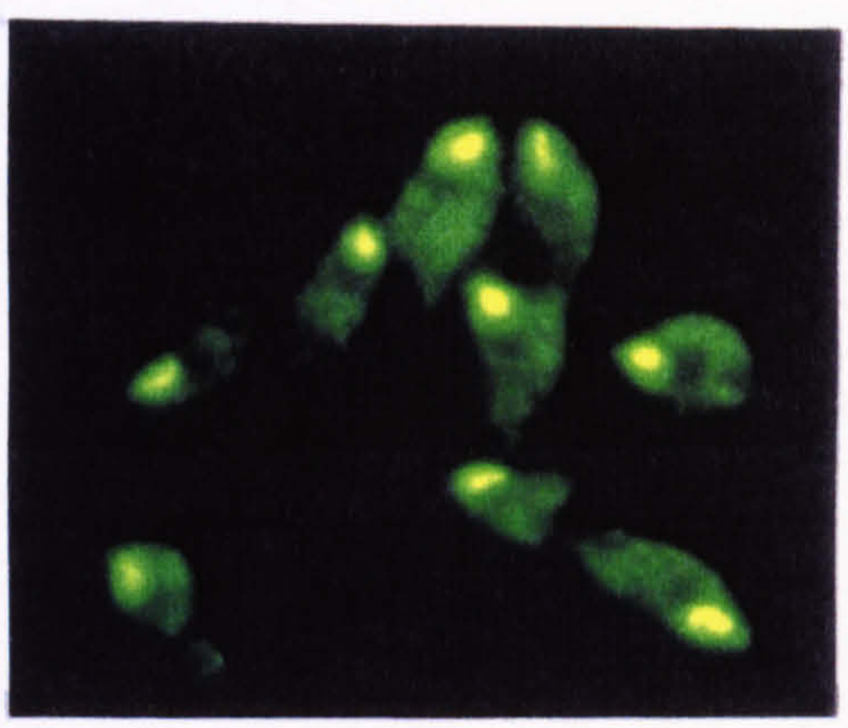


**Figure 6.4 A/B/C/D Light micrographs showing specificity of immunolabelling of *T. augusta* with polyclonal antisera raised against rMGL1 and rMGL2.**

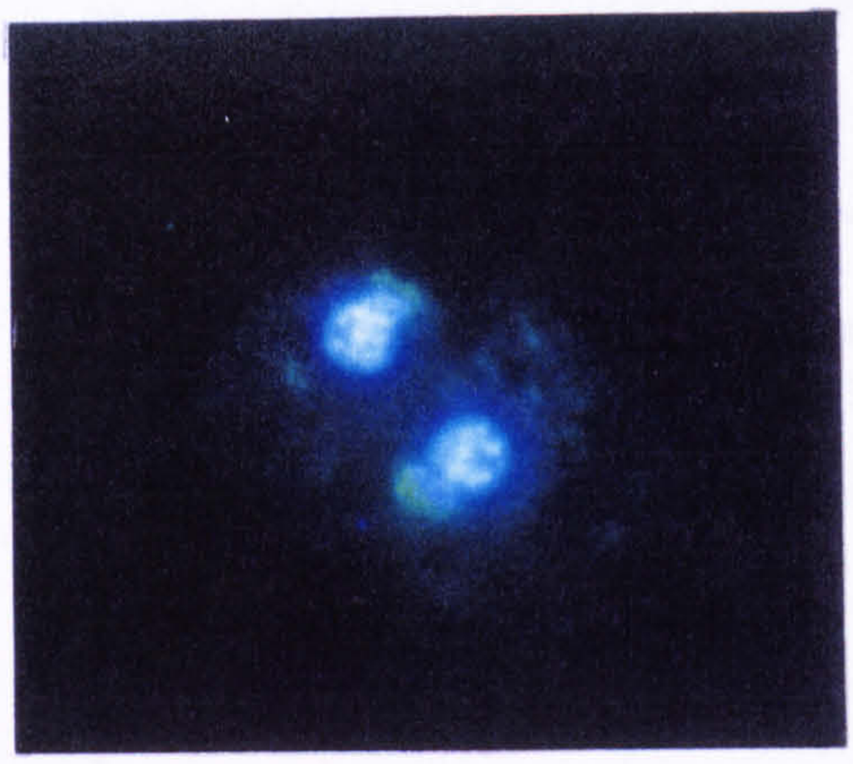
Panel A: *T. augusta* under UV epifluorescence showing DAPI nuclear counterstain (x63 magnification)

Panel B: As Panel A

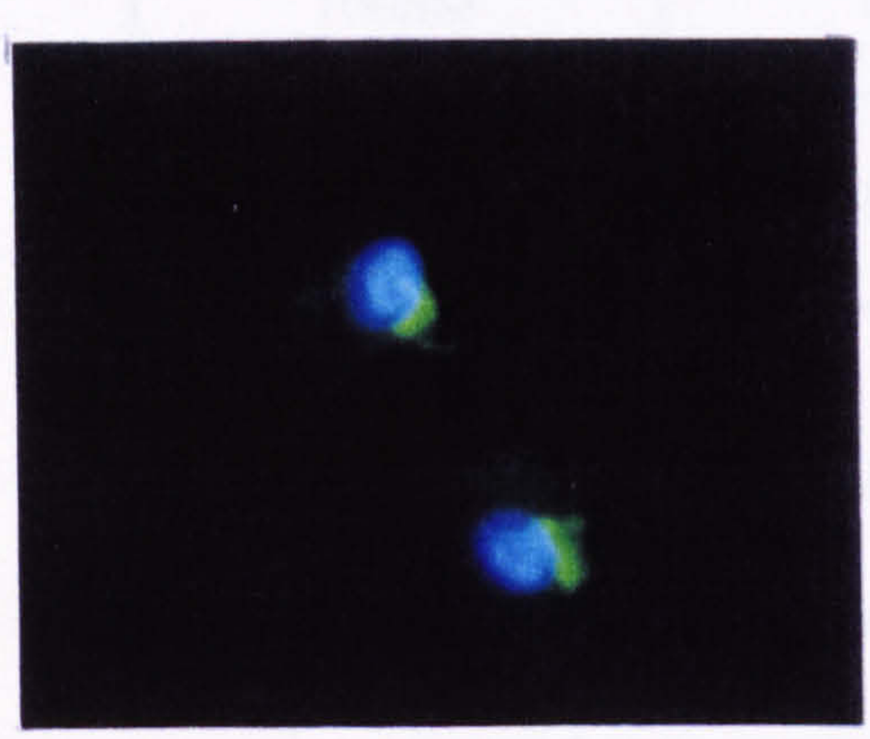
Panel C: *T. augusta* under UV epifluorescence showing fluorescent labelling with anti-rMGL2 serum

Scale bar: 10 μm

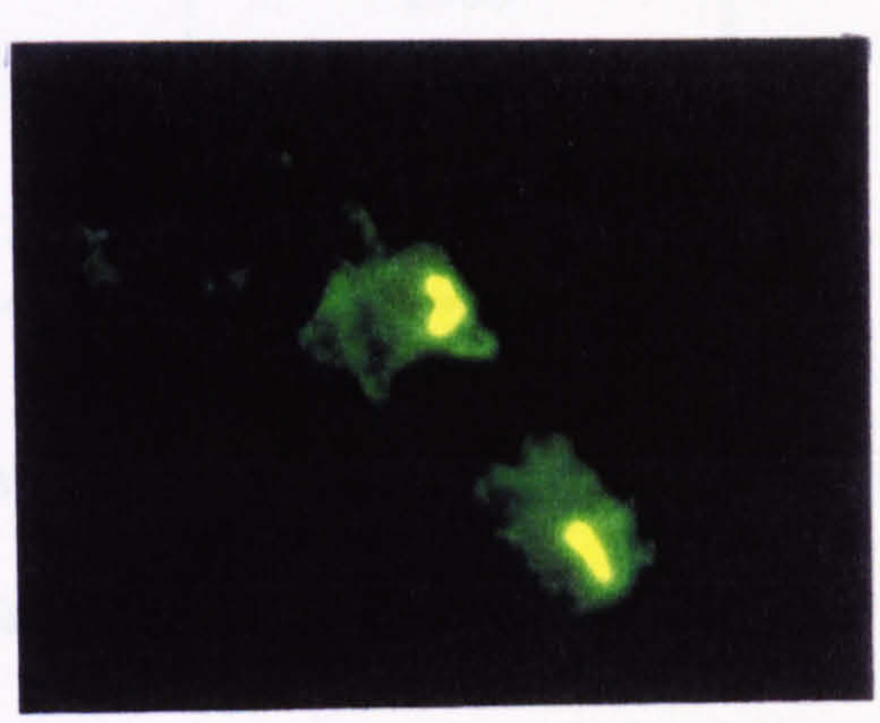
A



B



C



| | *T. vaginalis* | |
|---|---|---|
| **[Propargylglycine]** | **Homocysteine desulphurase** | **Serine sulphydrase** |
| Control | 139 | 217 |
| 1μM | ND<3 | 468 |
| 10μM | ND<3 | 1390 |
| 100μM | ND<3 | 2580 |
| 1mM | ND<3 | 1790 |

**Table 6.1: Homocysteine desulphurase and serine sulphydrase activities in supernatant fractions of *T. vaginalis* grown in the presence of various concentrations of propargylglycine.**

This experiment was only carried out once.

Units: nmol/min/mg protein).

ND: not detectable.

| | 10 mM cysteine | | 10 mM serine | |
|---|---|---|---|---|
| **[Propargylglycine]** | **Homocysteine desulphurase** | **Serine sulphydrase** | **Homocysteine desulphurase** | **Serine sulphydrase** |
| Control<br>No inhibitor<br>No amino acid | 230 | 506 | 112 | 200 |
| Amino acid only | 212 | 8.2 | 83 | 206 |
| 1μM | 21 | 4.1 | - | - |
| 10μM | 4.1 | 5.3 | ND<3 | 1420 |
| 100μM | 3.8 | 3.7 | ND<3 | 1620 |
| 1mM | 5.1 | 3.4 | ND<3 | 1440 |

**Table 6.2: Homocysteine desulphurase and serine sulphydrase activities in supernatant fractions of *T. vaginalis* when incubated with various concentrations of propargylglycine and supplemented with either cysteine or serine.**

This experiment was only carried out once.

Units: nmol/min /protein·

ND: not detectable

| | *T. foetus* | *T. augusta* |
|---|---|---|
| Homocysteine desulphurase | ND<1 (3) | ND<2 (3) |

**Table 6.3 Homocysteine desulphurase activity in *T. foetus* and *T. augusta* supernatant fractions.**

ND: not detectable

Units: nmol/min/mg protein·

n in parentheses

# CHAPTER 7: GENERAL DISCUSSION.

Two methionine γ-lyase gene homologues have been isolated and biochemically characterised from *T. vaginalis* . Molecular characterisation of the two genes have revealed that they have extremely short 5' untranslated regions, are at single copy in the *T. vaginalis* genome, are transcribed to give 1.3 kb poly $[A]^+$ RNAs and have a high degree of sequence identity and similarity to genes from a range of other organisms that are involved in transulphuration pathways. The two methionine γ-lyase gene homologues were cloned into expression vectors and recombinant protein produced. Biochemical characterisation of the two recombinant proteins revealed that both of the gene products were able to catabolise methionine and a range of other sulphur-containing amino acids. Interestingly, the two *T. vaginalis* methionine γ-lyase homologues were not able to breakdown cystathionine.

The essentiality of the two gene products to the parasite is currently only an area for speculation. However, this is a question that could be realistically addressed in future research. The isolation of the genomic copies of the two genes for *mgl1* and *mgl2* by T. D. Edlind (personal communication) now provides the opportunity for knock-out vectors to be constructed, which would allow the targeted gene disruption of the *mgl1* and *mgl2* loci by integration of an antibiotic resistance marker. This is an approach that has been used to test the essentiality of cysteine proteinase genes in *Leishmania mexicana* (Mottram *et al.*, 1996). Discovery of the essentiality of the two *T. vaginalis* methionine γ-lyase gene homologues is, of course, dependent on a stable transfection system being developed for *T. vaginalis*. Such a transfection system would probably be based on that used for *L. mexicana* gene disruption as described above.

Another question that should be addressed is, does *T. vaginalis* really possess a cystathionine γ-lyase? There has been only one report of the breakdown of cystathionine by *T. vaginalis* (Thong and Coombs, 1985a) and this particular enzyme has received no further attention until this study. As has been presented in Chapter 4, the degenerate oligonucleotides designed for the isolation of methionine γ-lyase gene homologues from *T. vaginalis* were based on cystathionine γ-lyase from a number of sources. Initially, when the two gene homologues were isolated from *T. vaginalis*, both of which had a similar level of amino acid sequence identity to methionine γ-lyase from *P. putida* and to cystathionine γ-lyase from *S. cerevisiae*, it was thought that maybe one of the genes would encode the methionine catabolising capability of the parasite and the other the cystathionine catabolising capability. However, the production and biochemical characterisation of the two recombinant proteins for the methionine γ-lyase gene homologues revealed that this was not the case. There needs to be confirmation of cystathionine catabolising capability in *T. vaginalis*.

The production of recombinant proteins for the two *T. vaginalis* methionine γ-lyase gene homologues opens a vista of future research in this area of sulphur-amino acid metabolism in this parasite. The natural progression of research in this area, in my view, would be the crystallographic characterisation of these two methionine γ-lyase gene homologues. Such an approach would allow the characterisation of the 3D structure of the enzymes, this could also include the molecular modelling specific of inhibitors to the active site of the enzymes.

Site directed mutagenesis of key amino acid residues in the two recombinant proteins is another option for future research. This is a study that has already been initiated by Dr. John Walker, using a PCR based site directed mutagenesis system. Dr. Walker has mutated the Cysteine 113 residue in *mgl1* and *mgl2*, the residue that has been implicated in the active site/pyridoxal 5-phosphate binding-region of the *P. putida* methionine γ-lyase. The data obtained for mutant rMGL1 correlate well with the results of the chemical modification of the *P. putida* methionine γ-lyase (Nakayama *et al.*, 1988), with the α γ-elimination activity being reduced to ~8% of the original and the α β-elimination being reduced to ~40%. The data obtained for mutant rMGL2 shows a reduction in the α γ-elimination activity to 22% of the original, whilst the α β-elimination activity is elevated to 180% of the original (personal communication, Dr J Walker). Interestingly, the mutation of Cysteine 113 to a glycine residue does not confer the ability, by the two proteins, to break down cystathionine.

Sulphur-amino acid metabolism in *T. vaginalis* is an area which has great research potential. There are many more avenues that can be opened to investigation. In particular, it would be interesting to confirm whether there is a functional transulphuration pathway in *T. vaginalis*. Cystathionine β-synthase, the first enzyme of the transulphuration sequence, has not been looked at in *T. vaginalis*. However, the presence of activated serine sulphydrase activity in *T. vaginalis* indicates that the capacity to produce cystathionine from homocysteine and serine probably does exist in the parasite. Also, as mentioned above, there needs to be confirmation of a cystathionine catabolising capability in the parasite. The presence of other enzymes involved in sulphur amino acid metabolism could be confirmed by future research, for

example, does cystathionine γ-synthase, cystathionine β-lyase and/or O-acetyl homoserine/O-acetyl serine sulphydrylase exist in *T. vaginalis*? Also the function, as significance to the parasite of enzymes involved in sulphur amino acid metabolism in *T. vaginalis* could be addressed further.

Finally, one should remember that all of the work conducted in this study was based on *T. vaginalis* that was obtained via *in vitro* cultivation. Whilst methionine γ-lyase is at high activity in *in vitro* cultivated parasite material, the role of the enzyme *in vivo* may become all the more important. One must consider that the conditions in the urinogenital tract are considerably different from those found a bottle of MDM. It is probable that the availability of carbohydrate sources in the host are limited and that proteinaceous substrates, available for proteolytic degradation by *T. vaginalis* proteinases, are much more abundant. Amino acids, in particular sulphur amino acids, which may result from the breakdown of keratin containing proteins in the urinogenital tract of the host, may become more important with regards to the provision of energy to the parasite. As well as the provision of energy resulting from the breakdown of sulphur containing and other amino acids, the thiols that are also generated from sulphur amino acid catabolism may be important for the maintenance of a *T. vaginalis* infection in the host.

## REFERENCES.


Abeles R. H. and Walsh, C. T. (1973) Acetylenic Enzyme inactivators: Inactivation of γ-cystathionase , in vitro and in vivo by propargylglycine. *Journal of the American Chemical Society* **95**, 6124-6125.

Ackers, J. P., Lumsden, W. H. R., Catterall, R. D. and Coyle, R. (1975) Antitrichomonal antibodies in the vaginal secretions of women infected with *T. vaginalis*. *British Journal of Venereal Disease* **51**, 319-323.

Ackers, J. P., (1985) Immunologic aspects of human trichomoniasis. In: B. M., Honiberg, ed. *Trichomonads Parasitic in Humans*. New York: Springer Verlag. pp36-52.

Alderete, J. F., Lehker, M. W. and Arroyo, R. (1995) The mechanisms and molecules involved in cytoadherence and pathogenesis of *Trichomonas vaginalis*. *Parasitology Today* **11**, 70-74.

Alexander, F. W., Sandmeier, E., Mehta, P., K. and Christen, P. (1994) Evolutionary relationships among pyridoxal 5'-phosphate dependent enzymes. Regio specific α, β and γ families. *European Journal of Biochemistry* **219**, 959-960.

Alonso, R. A. and Peattie, D. A. (1992) Nucleotide sequence of a second alpha giardin gene and molecular analysis of the alpha giardin genes and transcripts in *Giardia lamblia*. *Molecular and Biochemical Parasitology* **50**, 95-104.

Arroyo, R. and Alderete, J. F. (1989) *T. vaginalis* surface proteinase activity is necessary for parasite adherence to epithelial cells. *Infection and Immunity* **57**, 2991-2997.

Bagnara, A. S., Tucker, V. E., Minotto, L., Howes, E., R., Gyung-Ae, K., Edwards, M., R. and Dawes I., W. (1996) Molecular characterisation of adenosylhomocysteinase from *T. vaginalis*. *Molecular and Biochemical Parasitology* **81**, 1-11.

Beard, C. M., Noller, K. L., and O' Fallow, W. M. (1979) Lack of evidence of cancer due to use of metronidazole. *New England Journal of Medicine* **301**, 519-522.

Beck, J. T. and Wang C. C. (1993) The hypoxanthine-guanine-xanthine phophoribosyltransferase from *T. foetus* has unique properties. *Molecular and Biochemical Parasitology* **60**, 187-194.

Belfaiza J., Parsot, C., Martel, A., De La Tour, C., Margarita, D., Cohen, G. N. annd Saint-Girons, I. (1986) Evolution in biosynthetic pathways: Two enzymes catalyzing consecutive steps in methionine biosynthesis originate form a common ancestor and possess a similar regulatory region. *Proceedings of the National Academy of Sciences, United States of America* **83**, 867-871.

Bergmeyer, H. U. (1965) Methods of Enzymatic Analysis, Volume 2 (3rd Ed). New York. pp574-579. Academic Press.

Borst, P., Fase-Fowler, F., Frasch A. C. C., Hoeijmakers J. H. J. and Wiejers, P. J. (1980) Characterisation of DNA from *Trypanosoma brucei* and related trypanosomes by restriction endonuclease digestion. *Molecular and Biochemical Parasitology* **1**, 221-246.

Brondijk, T. H. C., Durand, R., van der Giezen, M., Gottschal, J. C., Prins, R. A and Fevre, M. (1996) scsB, a cDNA encoding the hydrogenosomal β subunit of succinyl-CoA synthetase from the anaerobic fungus *Neocallimastix frontalis*. *Molecular and General Genetics* **253**, 315-323.

Brul, S., Lawers, A. and Vogels, G. D. (1994) Hydrogenosomes: Ubiquitous organelles in anaerobic proteozoa and anaerobic fungi. In: Micro-organisms in ruminant nutrition. (Prins, R. A. and Stewart, C. S.(Eds)), pp209-221. Nottingham University Press.

Brush, A. W. and Paulus, H. (1971) O-acetylhomoserine sulphydrylase from *Bacillus subtilus*. *Biochemical and Biophysical Research Communications* **45**, 735-741.

Brush, A. W. and Paulus, H. (1973) O-acetylhomoserine sulphydrylase from *Brevibacterium flavum*. *Federation Proceedings* **32**, 63.

Brzywczy, J. and Paszewski, A. (1993) Role of O- acetylhomoserine sulphydrylase in sulphur amino acid synthesis in various yeasts. *Yeast* **9**, 1335-13342.

Bui, E. T. N., Bradley, P. J. and Johnson, P. J. (1996) A common evolutionary origin for mitochondria and hydrogenosomes. *Proceedings of the National Academy of Sciences, United States of America* **93**, 9651-9656.

Bui, E. T. N. and Johnson, P. J (1996) Identification and characterisation of [Fe]-hydrogenase in the hydrogenosome of *Trichomonas vaginalis*. *Molecular and Biochemical Parasitology* **76**, 305-310.

Burch, T. A., Reis, C. W. and Reardon, L. V. (1959) Epidemiologic studies on human trichomoniasis. *American Journal of Tropical Medicine and Hygiene* **8**, 312-318.

Cavalier-Smith, T. (1987) The simultaneous symbiotic origin of mitochondria, chloroplasts and microbodies. *Annals New York Academy of Sciences* **503**, 55-72.

Cavalier-Smith, T. (1993) Kingdom protozoa and its 18 phyla. *Microbiological Reviews* **57**, 953-994.

Certa, U., Bannworth, W., Stuber, D., Gentz, R., Lanzer, M. and Mous, J. (1986) Subregions of a conserve of a part of the HIV gp41 transmembrane protein are

differentially recognised by the antibodies of infected individuals. *EMBO Journal* **5**, 3051-3056.

Champney, W. S., Chittum, H. S. and Samuels, R. (1992) Ribosomes from trichomonad protozoa have prokaryotic characteristics. *International Journal of Biochemistry* **24**, 1125-1133.

Chen K. C. S., Amsel, R., Eschenbach, D. A. and Holmes, K. K. (1982) Biochemical Diagnosis of Vaginitis: Determination of diamines in vaginal fluid. *The Journal of Infectious Diseases* **145**, 337-345.

Clausen, T., Huber, R., Laber, B., Pohlenz, H. D. and Messerschmidt, A. (1996) Crystal structure of the pyridoxal 5'-phosphate dependent cystathionine β-lyase from *Escherichia coli* at 1.83Å. *Journal of Molecular Biology* **262**, 202-224.

Cook, P. F., Tai. C. H., Hwang, C. C., Woehl, E. U., Dunn, M. F. and Schnackerz, K. D. (1996) Substitution of pyridoxal 5'-phosphate in the O-acetylserine sulphydrylase from *Salmonella typhimurium* by cofactor analogues provides a test of the mechanism proposed for the formation of the α-aminoacrylate intermediate. *Journal of Biological Chemistry*. **271**, 25842-25849.

Coombs, G. H. and Muller, M. (1995) In Marr, J. J. and Muller, M. (Eds) *Biochemistry and Molecular Biology of Parasites*. Energy metabolism in Anaerobic protozoa. pp407-417.

Cooper, A. J. (1983) Biochemistry of sulphur containing amino acids. *Annual Reveiw of Biochemistry*. **52**, 187-222.

Corliss, J. C. (1994) An interim utilitarian (user friendly) hierarchical classification and characterisation of the protists. *American Protozoologist* **33**, 1-51.

Coyne, M., Brasseur, P. and Baller, J.T. (1975) Detection and characterisation of serum antritrichomonal antibodies in urogenital trichomoniasis. *Journal of Clinical Microbiology*. **21**, 588-592.

Dawson, R. M. C., Elliot, D. C. and Elliot, W. H. (1989) Data for biochemical research (3rd edition). Oxford University Press.

Demes, P., Gombosova, A., Valent, M. (1988) Differential susceptibility of fresh *T. vaginalis* isolates to complement in menstrual blood and cervical mucous. *Genitourinary medicine* **64**, 176-179.

Diamond, L. (1957) The establishment of various trichomonads of animals and man in axenic culture. *Journal of Parasitology* **43**, 488-490.

Diaz, J. A. M., Monteiro-Leal, L., H. and De Souza, W. (1996) *Tritrichomonas foetus*: Isolation and characterisation of the Golgi complex. *Experimental Parasitology*, **83**, 174-183.

Donne, A. (1896) Animalcules observes dans maitres purulentes et le produit des secretion des organes genitaux de l'homme et de la femme. *C. R. Academy of Sciences III* **3**, 385-386.

Doolittle, R. F., Feng, D. F., Tsang, S., Cho, G. and Little, E. (1996) Determining divergence times of the major kingdoms of living organisms with a protein clock. *Science, Washington* **271**, 470-476.

Dore E., Birago, C., Frontali, P. A. and Battaglia, P. A. (1980) Kinetic complexity and repetitivity of *Plasmodiun berghei* DNA. *Molecular and Biochemical Parasitology* **1**, 199-208.

Droux, M., Ravanel, S. and Douce, R. (1995) Methionine biosynthesis in higher plants. Purification and Characterisation of cystathionine β-lyase from spinach chloroplasts. *Archives of Biochemistry and Biophysics.* **316**, 585-595.

Duchange, N., Zakin, M. M., Ferrara, P., Saint-Girons, I, Park, I., Tran, S.V., Py, M. C. and Cohen, G. N. (1983) Structure of the metJBLF cluster in *E. coli* K12. Sequence of the *metB* structural gene and the 5' and 3' flanking regions of the *metBL* operon. *Journal of Biological Chemistry* **258**, 14868-14871.

Dwivedi, C. M., Ragin, R. C. and Uren J. R. (1982) Cloning, purification and characterisation of β-cystathionase from *E. coli. Biochemistry* **21**, 3064-3069.

Edman, U., Meza, I. And Agabian, N. (1987) Genomic and cDNA actin sequences from a virulent strain of *Entamoeba histolytica. Proceedings of the National Academy of Sciences, United States of America,* **84**, 3024-3028.

Edwards, M. R. (1989) Alanine is a major end product if metabolism by *Giardia lamblia. Molecular and Biochemical Parasitology* **37**, 19-26.

Edwards, M. R. (1992) Arginine metabolism during the culture of *Giardia intestinalis. Molecular and Biochemical Parasitology* **53**, 97-104.

Erickson. P. F., Maxwell, I. H., Su, L. J., Baumann, M. and Glode, L. M. (1990) Sequence of cDNA for rat cystathionine γ-lyase and comparison of deduced amino acid sequence with related *Escherichia coli* enzymes. *Biochemical Journal* **269**, 335-340.

Esaki, N. and Soda, K. (1987) L-methionine γ-lyase from *Pseudomonas putida* and *Aeromonas. Methods in Enzymology.* **143**, 459-465.

Ey, P. L., Khanna, K., Manning, P. A. and Mayrhofer, G. (1993) A gene encoding a 69 kilodalton major surface protein of *Giardia intestinalis* trophozoites. *Molecular and Biochemical Parasitology* **58**, 247-258.

Fairlamb, A. (1989) Novel biochemical pathways in parasitic protozoa. *Parasitology* 99, S93-S112.

Ferris, D. G., Hendrich, J., Payne, P. M., Getts, A., Rassekh, R., Mathis, D. and Litaker, M. S. (1996) Office based diagnosis of vaginitis. *The Journal of Family Practice* **41**, 575-581.

Flavin, M. and Slaughter, C. (1964a) Cystathionine cleavage enzymes of Neurospora. *Journal of Biological Chemistry* **239**, 2212-2219.

Flavin, M. and Segal, A. (1964) Purification and properties of the cystathionine γ cleavage enzyme of Neurospora. *Journal of Biological Chemistry* **239**, 2220-2227.

Flavin, M. (1975) Methionine biosynthesis (Chapter 11). In *Metabolic Pathways* (3rd Ed), Metabolism of Sulphur Compounds, Volume 7. (Greenberg, D. M. (Ed)), pp 457-501, Academic Press.

Fountoulakis, M. (1989) Structure and membrane topology of the high-affinity receptor for human IFN-gamma: Requirements for binding IFN gamma. *Journal of Immunology*, 143, 3266-3276.

Francioli, P., Shio, H., Roberts, R. B. and Muller, M., (1983) Phagocytosis and killing of *Neisseria gonorrhoeae* by *T. vaginalis*. *Journal of Infectious Disease*. **147**, 87-94.

Gallai, Z. and Sylvester, L. (1966) The present status of urinogenital trichomoniasis. A general review of the literature. *Applied Therapeutics* **8**, 773-778.

Garber, G. E., Lemchuk-Faver, L. T. and Bowie, W. R. (1989) Isolation of a cell detachment factor of *T. vaginalis*. *Journal of Clinical Microbiology*. **27**, 1548-1553

Gentry-Weeks, C. R., Spokes, J. and Thompson, J. (1995) β cystathionase of *Bordetella avium*. *Journal of Biological Chemistry* **270**, 7695-7702.

Germot, A., Phillipe, H. and Le Guyader, H. (1996) Presence of a mitochondrial-type 70kDa HSP in *Trichomonas vaginalis* suggests a very early mitochondrial endosymbiosis in eukaryotes. *Proceedings of the National Academy of Sciences, United States of America* **93**, 14614-14617.

Gillin, F. D., Reiner, D., S. Levy, R. B. and Henkart, P. A. (1984) Thiol groups on the surface of anaerobic parasitic protozoa. *Molecular and Biochemical Parasitology*. **13**, 1-12.

Giovanelli, J. and Mudd, S. H. (1971) Transulphuration of higher plants. Partial purification and properties of β-cystathionase of spinach. *Biochimica Biophysica Acta* **227**, 654-660.

Giovanelli, J. (1987) Sulphur amino acids in plants: An overview. *Methods in Enzymology*. **143**, 438-452.

Gorrell, T. E. (1985) Effect of culture medium iron content on the biochemical composition and metabolism of *T. vaginalis in vitro*. *Journal of Bacteriology*. **161**, 1228-1230.

Graves, A., and Gardner, W. A. (1993) Pathogenicity of *T. vaginalis*. *Clinical Obstetrics and Gynaecology* **36**, 145-152.

Gunderson, J., Hinkle, G. and Liepe, D. (1995) Phylogeny of Trichomonads inferred from small-subunit rRNA sequences. *Journal of Eukaryotic Microbiology* **42**, 411-415.

Hall, D. I. And Smith I. K. (1983) Partial purification and characterisation of cysteine lyase form cabbage (*Brassica oleracea* var. capita). *Plant Physiology* **77**, 87-92.

Hamamoto, A. and Mazelis, M. (1983) The C-S lyase of higher plants. Isolation and properties of homogeneous cysteine lyase form broccoli (*Brassica oleracea* var. Botrytis). *Plant Physiology* **80**, 702-706.

Harlow, E. and Lane, D. (1988) Antibodies: A laboratory manual. Cold Spring Harbor Press.

Hasseltine, P. (1942) Transmission studies of *Trichomonas vaginalis*. *Journal of Parasitology* **8**, 51-56.

Heine, P. and McGregor, J. A. (1993) *Trichomonas vaginalis*: A re-emerging pathogen. *Clinical Obstetrics and Gynaecology* **36**, 137-144.

Heyworth, P. G., Gutteridge, W. E. and Ginger, C. D. (1982) Purine metabolism in *T. vaginalis*. *FEBS Letters* **141**,106-110.

Hollander, D. H. and Leggett, N. C. (1985) Vitamin $B_{12}$ requirement for the growth of *T. vaginalis in vitro*. *Journal of Parasitology* **71**, 683-684.

Honigberg, B. M. (1989) Trichomonads of importance in humans. In: Krier J., P. (ed) *Parasitic protozoa*, Vol 2. pp 275-454, Orlando Academic Press.

Hori, H., Takabayashi, K., Orvi, L., Carson, D. A. and Nobori, T. (1996) Gene cloning and characterisation of *Pseudomonas putida* L-methionine-α-deamino-γ-mercaptomethane lyase. *Cancer Research* **56**, 2116-2122.

Horner, D. S., Hirt, R. P., Kilvington, S., Lloyd, D. and Embley, T. M. (1996) Molecular data suggest an early aquisition of the mitochondrion endosymbiont. *Proceedings of the Royal Society B-Biological Sciences* **263**, 1053-1059.

Huber, M. (1987) *Entamoeba histolytica*: Cloning and characterisation of actin cDNA. *Molecular and Biochemical Parasitology* **24**, 227-235.

Huber, M., Garfinkel, L., Gitler, C., Murelman, D., Revel, M. and Rozenblatt, S. (1988) Nucleotide sequence analysis of an *Entamoeba histolytica* ferredoxin gene. *Molecular and Biochemical Parasitology* **31**, 27-34.

Huggins, G. R. and Petri, G. (1981) Vaginal odours and secretions. *Clinical Obstetrics and Gynaecology*. **24**, 355-377.

Inoue, H., Inagaki, K., Sugimoto, M., Esaki, N., Soda, K. and Tanaka, H. (1995) Structural analysis of the L- methionine γ-lyase gene from *Pseudomonas putida*. *Journal of Biochemistry (Tokyo)* **117**, 1120-1127.

Ito, S,. Nakamura, T. and Eguchi, Y. (1975) Exchange reactions catalysed by methioninase from *Pseudomonas putida*. *Journal of Biochemistry* (Tokoyo). **78**,1105-1107.

Jaroszewicz, L. and Malyszko, E. (1965) Amino transferase activity of strains of the genus *Trichomonas*. *Experimental and Medical Microbiology* **17**,48-51.

Johnson, P. J., d'Oliveira, C. E., Gorrel. T. E. and Muller, M. (1990) Molecular analysis of the hydrogenosomal ferredoxin of the anaerobic protist *Trichomonas vaginalis*. *Proceedings of the National Academy of Sciences, United States of America* **87**, 6097-6101.

Johnson, P. J., Lahti, C. J. and Bradley, P. J. (1993) Biogenesis of the hydrogenosome: An unusual organelle found in the anaerobic protist *Trichomonas vaginalis*. *Journal of Parasitology* **79**, 664-670.

Johnson, P. J., Schuck, B. L. and Delagdillo, M. G. (1994) Analysis of a single domain P-glycoprotein-like gene in the early -diverging protist *Trichomonas vaginalis*. *Molecular and Biochemical Parasitology* **66**, 127-137.

Juliano, C., Monaco, G., Bandiera, P.(1987) Action of anticytoskeletal compounds of *in vitro* cytopathic effects, phagocytosis, and adhesiveness of *Trichomonas vaginalis* *Genitourinary Medicine* **63**, 256-263

Kallio, R. E. and Larson, A. D. (1955) A symposium on amino acid metabolism (McElroy, W. D. and Glass, H. B., eds.). pp 616-634, John Hopkins Press, Baltimore.

Katiyar, S. K. and Edlind, T. D. (1994) β-tubulin genes of *Trichomonas vaginalis*. *Molecular and Biochemical Parasitology* **64**, 33-42.

Keeling, P. J. and Doolittle, W. F. (1995) Concerted evolution in protists-recent homogenisation of a polyubiquitin gene in *Trichomonas vaginalis*. *Journal of Molecular Evolution* **41**, 556-562.

Kerjan, P., Cherest, H. and Surdin-Kejan, Y. (1986) Nucleotide sequence of the *Saccharomyces cerevisiae* MET 25 gene. *Nucleic Acids Research* **14**, 7861-7871.

Kim, J. S. and Leustek, T. (1996) Cloning and analysis of the gene for cystathionine γ-synthase from *Arabidopsis thaliana*. *Plant Molecular Biology and Biochemistry* **32**, 1117-1124.

Kirk-Mason K. E., Turner, M. J. and Chakraborty, P. R. (1989) evidence for unusually short tubulin mRNA leaders and characterisation of tubulin genes in *Giardia lamblia*. *Molecular and Biochemical Parasitology* **36**, 87-100.

Knodler, L. A., Edwards, M.R. and Schofield, P. J. (1994) The intracellular amino acid pools of *Giardia intestinalis, Trichomonas vaginalis and Crithidia lucilie*. *Experimental Parasitology* **79**, 117-125.

Kraus, J. P. and Rosenberg, L. E. (1983) Cystathionine β-synthase from human liver: Improved purification scheme and additional characterisation of the enzyme in crude and pure form. *Archives of Biochemistry and Biophysics* **222**, 44-52.

Kreft, B. D., Townsend, A., Pohlenz, H. D. and Laber, B (1994) Purification and properties of cystathionine γ-synthase from wheat. *Plant Physiology*. **104**, 1215-1220.

Kreft, B. D., Townsend, A., Pohlenz, H. D. and Laber, B. (1994) Purification and properties of cystathionine γ-synthase from wheat (*Triticum aestivum* L.). *Plant Physiology* **104**, 1215-1220.

Krieger, J. (1981) Urologic aspects of trichomoniasis. *Investigations of Urology*. **18**, 411-417

Kreiger, J. N. and Rein, M. F. (1982) Zinc sensitivity of *T. vaginalis*: *In vitro* studies and clinical implications. *Journal of Infectious Diseases*, **146**, 341-345

Kreis, W. and Hession, C. (1973) Isolation and purification of L-methionine α-deamino γ-mercaptomethane-lyase (L-methioninase) from *Clostridium sporogenes*. *Cancer Research*. **33**, 1862-1865.

Kukner, S., Ergin, T., Cicek N., Ugar M., Yesilyurt, H. and Gokmen. O. (1996) Treatment of vaginitis. *International Journal of Gynaecology and Obstetrics* **52**, 43-47.

Laber, B., Clausen, T., Huber, R., Messerschmidt, A., Egner, U., Muller-Fahrnow, A. and Pohlenz. H. D. (1996) Cloning, purification and crystallisation of *Escherichia coli* cystathionine β-lyase. *FEBS Letters* **379**, 94-96.

Laemmli, U., K. (1971) Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature*, **227**, 680-685.

Lahti, C. J. and Johnson, P. J. (1991) *Trichomonas vaginalis* hydrogenosomal proteins are synthesised in free polyribosomes and may undergo processing upon maturation. *Molecular and Biochemical Parasitology* **46**, 307-310.

Lahti, C. J., d'Oliveira, C. E. and Johnson, P. J. (1992) β-succinyl coenzyme A synthetase from Trichomonas vaginalis is a soluble hydrogenosomal protein with an amino terminal sequence that resembles mitochondrial presequences. *Journal of Bacteriology* **174**, 6822-6830.

Lahti, C. J., Bradley, P. J. and Johnson, P. J. (1994) Molecular characterisation *of T. vaginalis* hydrogenosomal succinyl CoA synthetase. *Molecular and Biochemical Parasitology* **66**, 309-318.

Lawrence, F. and Robert-Gero, M. (1991) S-adenosylmethionine metabolism in parasitic protozoa. In: *Biochemical Protozoology.* (Coombs, G. H. and North, M. J.(Eds.)), pp 436-449. Taylor and Francis, London.

Leatherbarrow, R. J. (1992) *Grafit version 3.0.* Erithacus Software Ltd. Staines, U. K.

Lehker, M. W., Arroyo, R. and Alderete, J. F. (1991) The regulation by iron of the synthesis of adhesins and cytoadherence levels in the protozoan *T. vaginalis. Journal of Experimental Medicine.* **174**, 311-318.

Leipe, D. D., Gunderson, J. H., Nerad, T. A. and Sogin, M. L. (1993) Small subunit rRNA of *Hexamita inflata* and the quest for the first branch in the eukaryotic tree. *Molecular and Biochemical Parasitology* **59**, 41-48.

Linstead, D. and Cranshaw, M. (1983) The pathway of arginine catabolism in the parasitic flagellate *Trichomonas vaginalis. Molecular and Biochemical Parasitology* **8**, 241-252.

Livengood, C. H., Lossick, J. G. (1991) Resolution of resistant vaginal trichomoniasis associated with the use of intravaginal nonoxynol-9. *Obstetrics and Gynaecology* 7(2), 955-957

Lloyd, D., Lauritsen, F. R., and Degn, H. (1991) The parasitic flagellates *T. vaginalis* and *T. foetus* produce indole and dimethyldisulphide: direct characterisation by membrane inlet tandem mass spectrometry. *Journal of General Microbiology.* **137**, 1743-1747.

Lockwood, B. C. and Coombs, G. H. (1989) The catabolism of amino acids by *Trichomonas vaginalis.* In Lloyd, D., Coombs, G. H. and Paget, T. A. (Eds) *Biochemistry and Molecular Biology of Anaerobic Protozoa.* pp 93-111, London: Harwood.

Lockwood, B. C. and Coombs, G. H. (1991a) Purification and characterisation of methionine γ-lyase from *Trichomonas vaginalis. Biochemical Journal.* **279**, 675-682.

Lockwood, B. C. and Coombs, G. H. (1991b) Amino acid catabolism in anaerobic protists. In Coombs, G. H. and North, M. J. (eds) *Biochemical Protozoology*, pp. 113-122.

Loh. Y., Elliot, J. F., Cwirla, S., Lanier, L. L. and Davies, M. M. (1989) Rapid Amplification of cDNA Ends (RACE) *Science* **243**, 217.

Lohia, A. and Samuelson, J. (1993) Molecular cloning of a rho family gene of *Entamoeba histolytica. Molecular and Biochemical Parasitology* **58**, 177-180.

Lossick, J. G. (1982) Treatment of *Trichomonas vaginalis* infections. *Rev Infectious Diseases*. **4** (suppl), 5801-5818

Lossick, J. G. (1989) Epidemiology of urinogenital trichomoniasis. In Honinberg, B., M. (Ed) *Trichomonads parasitic in humans.* pp 311-323. New York: Springer Verlag

Lossick, J. G. (1990) Therapy of urinogenital trichomoniasis. In Honingberg, B., M. (Ed) *Trichomonads parasitic in humans.* pp 324-341. New York: Springer Verlag

Lowe, P. N. and Rowe, A. F. (1985) Aspartate: 2-oxoglutarate aminotransferase from *Trichomonas vaginalis*, identity of aspartate aminotransferase and aromatic amino acid transferase. *Biochemical Journal* **232**, 689-695.

Lowe, P. N. and Rowe, A. F. (1986a) Aminotransferase activities in *Trichomonas vaginalis. Molecular and Biochemical Parasitology* **21**, 65-74.

Lowe, P. N. and Rowe, A. F. (1986b) Aspartate: 2-oxoglutarate aminotransferase from *Trichomonas vaginalis*: Comparison with pig heart cytoplasmic enzyme. *Comparative Biochemistry and Physiology* **85B**, 93-99.

Lowe, P. N. and Rowe, A. F (1987) Probing the active site of aspartate 2-oxoglutarate amino transferases from Trichomonas vaginalis and pig heart cytoplasm using substrate analogues. *Comparative Biochemistry and Physiology* **88B**, 223-227.

Lu, Y., O' Dowd, B. F., Orrego, H. and Israel, Y. (1992) Cloning and nucleotide sequence of human liver cDNA encoding for cystathionine γ-lyase. *Biochemical and Biophysical Research Communications* **189**, 749-758.

Mallinson, D. J., Lockwood, B. C., Coombs, G. H. and North, M. J. (1994) Identification and molecular cloning of four cysteine proteinase genes from the pathogenic protozoon *Trichomonas vaginalis. Microbiology* **140**, 2725-2735.

Marinets, A., Muller, M., Johnson, P. J., Kulda, J., Scheiner, O., Wiederman, G. and Duchene, M. (1996) The sequence and organisation of the core histone H3 and H4 genes in the early branching amitochondriate protist *Trichomonas vaginalis. Journal of Molecular Evolution* **43**, 563-571.

Martel, A., de la Tour, B. and Le Goffic, F. (1987) Pyridoxal 5'-phosphate binding site of *Escherichia coli* β cystathionase and cystathionine γ-synthase, comparison of their sequences. *Biochemical and Biophysical Research Communications* **147**, 565-571.

Mason, P. R. and Forman, L. (1982) Polymorphonuclear cell chemotaxis to secretions of pathogenic and non-pathogenic *T. vaginalis*. *Journal of Parasitology*, **68**, 457-462.

Matsuo, Y and Greenberg, D. (1959a) A crystalline enzyme that cleaves homoserine and cystathionine. Coenzyme resolution, activators and inhibitors. *Journal of Biological Chemistry* **234**, 507-515.

Matsuo, Y and Greenberg, D. (1959b) A crystalline enzyme that cleaves homoserine and cystathionine. Mechanism of action, reversibility and substrate specificity. *Journal of Biological Chemistry* **234**, 516-519.

McLellan, R., Spence, M. R., Brockman, M., Rattel, L. and Smith, J. L. (1982) The clinical diagnosis of trichomoniasis. *Obstetrics and Gynaecology* **60**, 30-34

Merricks, D. L. and Salsbury, R. L. (1974) Involvement of vitamin B6 in the dethiomethylation of methionine by rumen micro-organisms. *Applied Microbiology* **28**, 106-111.

Meysick, K. C., Dimock, K. and Garber, G. E. (1996) Molecular characterisation and expression of a N-acetylneuraminate lyase gene from *Trichomonas vaginalis*. *Molecular and Biochemical Parasitology* **76**, 289-292.

Miwantani, T., Omukai, Y. and Nakada, D. (1954) Enzymatic cleavage of methionine and homocysteine by bacteria. *Medical Journal of Osaka University* **5**, 347-352.

Mottram, J. C and Smith, G. (1995) A family of trypanosome cdc2-related protein kinases. *Gene* **162**, 147-152.

Mottram, J. C. and Grant, K. M. (1996) *Leishmania mexicana* $p12^{cks1}$, a homologue of fission yeast $p13^{suc1}$, associates with a stage-regulated histone H1 kinase. *Biochemical Journal* **316**, 833-839.

Mottram, J. C., Souza, A. E., Hutchison, J. E., Carter, R., Frame, M. J. and Coombs, G. H. (1996) Evidence from disruption of the *lmcpb* gene array of *Leishmania mexicana* that cysteine proteinases are virulence factors. *Proceedings of the National Academy of Sciences, United States of America* **93**, 6008-6013.

Muller, M. (1980) The hydrogenosome. *Symposium of the Society for General Microbiology* **30**, 127-142.

Muller, M. (1983) Mode of action of metronidazole on anaerobic bacterial and protozoa. *Surgery* **93**, 165-170.

Muller, M. (1988) Energy metabolism of protozoa without mitochondria. *Annual Review of Microbiology* **42**, 465-488.

Muller, M. (1989) Biochemistry of *Trichomonas vaginalis*. In Honigberg, B. M. (Ed.) *Trichomonads parasitic in Humans*, pp53-83, New York: Springer Verlag.

Muller, M. (1993) The hydrogenosome. *Journal of General Microbiology* **139**, 2879-2889.

Muresu, R., Rubino, S., Rizzu, P., Baldini, A., Colombo, M. and Cappuccinelli, P. (1994) A new method for identification for *Trichomonas vaginalis* by fluorescent DNA *in situ* hybridisation. *Journal of Clinical Microbiology* **32**, 1018-1022.

Murooka, Y., Seto, K. and Harada, T. (1970) O-alkylhomoserine synthesis from O-acetylhomoserine and alcohol. *Biochemical Biophysical Research Communications* **41**, 407-414.

Nakagawa, H. and Kimura, H. (1968) Purification and properties of cystathionine synthetase from rat liver: Separation from of cystathionine synthetase from serine dehydratase. *Biochemical and Biophysical Research Communications* **32**, 208-214.

Nakayama, K., Kase, H. and Harada, T. (1969) O-acetyl homoserine is used in the synthesis of cystathionine in Arthrobacter paraffineus. *Agricultural Biological Chemistry*. **33**, 1664-1665.

Nakayama, T., Esaki, N., Sugie, K. Beresov, T. T., Tanaka, H. and Soda, K. (1984) Purification of L-methionine γ-lyase from *Pseudomonas putida*. *Analytical Biochemistry* **138**, 421-424.

Nakayama, T., Esaki, N., Lee, W. J., Tanaka, I., Tanaka, H. and Soda, K. (1984) Purification and properties of L-methionine γ-lyase from *Aeromonas sp*. *Agricultural Biological Chemistry* **48**, 2367-2369.

Nakayama, T., Esaki, N., Tanaka, H. and Soda, K (1988a) Specific labelling of the essential cysteine residue of L-methionine γ-lyase with a cofactor analogue, N-(Bromoacetyl)pyridoxamine phosphate. *Biochemistry*. **27**, 1587-1591.

Nakayama, T., Esaki, N., Tanaka, H. and Soda, K (1988b) Chemical modification of cysteine residues of L-methionine γ-lyase. *Agricultural Biological Chemistry*. 52, 177-183.

Nagasawa, T. and Yamada, H. (1987) O- acetylserine sulphydrylase sulphydrylase from *Bacillus sphaericus*. *Methods in Enzymology* **143**, 474-478.

Narcisi, E. M. and Secor, W. E. (1996) In vitro effect of tinidazole and furazolidone in metronidazole resistant *Trichomonas vaginalis*. *Antimicrobial Agents and Chemotherapy* **40**, 1121-1125.

Nock, L. P. and Mazelis, M. (1986) The C-S lyases of higher plants. Preparation and properties of alliin lyase from garlic (*Allium sativum). Archives of Biochemistry and Biophysics* **249**, 27-33.

Nock, L. P. and Mazelis, M. (1987) The C-S lyases of higher plants. Direct comparison of the physical properties of homogeneous alliin lyase form garlic (*Allium sativum*) and onion (*Allium cepa*). *Plant Physiology* **85**, 1079-1083.

North, M. J. (1991) Proteinases of trichomonads and *Giardia*. In Coombs, G. H. and North, M. J. (eds) *Biochemical Protozoology*, pp. 234-244, Taylor and Francis, London.

Ono, B. I., Tanaka, K., Naito, K., Heike, C., Shinoda, S., Yamamoto, S., Ohmori, S., Oshima, T. and Toh-E, A. (1992) Cloning and characterisation of the CYS3 gene of *Saccharomyces cerevisiae*. *Journal of Bacteriology* **174**, 3339-3347.

Ono, B.I., Kijima, K., Inoue, T., Miyoshi, S. I., Matsuda, A. and Shinoda, S. (1994) Purification and properties of *Saccharomyces cerevisiae* cystathionine β-synthase. *Yeast* **10**, 333-339.

Paces, J., Urbankova, V. and Urbanek, P. (1992) Cloning and characterisation of repetitive DNA sequence specific for *Trichomonas vaginalis*. *Molecular and Biochemical Parasitology* **54**,247-256.

Paget, T. A. and Lloyd, D. (1991) The effects of reducing agents on the metabolism of *Trichomonas vaginalis* C1 NIH. In Lloyd, D., Coombs, G. H. and Paget, T. A. (Eds) *Biochemistry and Molecular Biology of Anaerobic Protozoa*. pp123-136, London: Harwood.

Paszewski, A. and Grabski, J. (1974) Regulation of S-amino acids biosynthesis in *Aspergillus nidulans*. Role of cysteine and/ or homocysteine as regulatory effectors. *Molecular and General Genetics* **132**, 307-320.

Patterson, D. J. and Sogin, M. L. (1993) Eukaryotic origins and protistan diversity. In The origin and evolution of the cell. (Hartman, H. and Matsuno, K. (Eds)). Singapore, World Scientific. pp13-46.

Pheifer, T. A., Forsyth, P. S., Durkee, M. A., Pollock, H. M. and Holmes, K. K. (1978) Nonspecific vaginitis: Role for haemophilus vaginalis and treatment with metranidazole. *New England Journal of Medicine* **298**, 1429-1434.

Piotrowska, M., Kruszewska, A. and Paszewski, A. (1980) Homocysteine synthesis in *Neurospora crassa*. *Acta Biochimica Poland* **27**, 395-403.

Quon, D. V. K., Delgadillo, M. G., Khachi, A., Smale, S. T. and Johnson, P. J. (1994) Similarity between a ubiquitous promoter element in an ancient eukaryote and mammalian initiator elements. *Proceedings of the National Academy of Sciences, United States of America* **91**, 4579-4583.

Quon, D. V. K., Delgadillo, M. G. and Johnson, P. J. (1996) Transcription in the early diverging eukaryote *Trichomonas vaginalis*. An unusual RNA polymerase II and a-amanitin resistant transcription of protein coding genes. *Journal of Molecular Evolution* **43**, 253-262.

Ravanel, S., Droux, M. and Douce, R. (1995) Methionine biosynthesis in higher plants. Purification and characterisation of cystathionine γ-synthase from spinach chloroplasts. *Archives of Biochemistry and Biophysics* **316**, 572-584.

Ravanel, S., Ruffet, M. L. and Douce, R. (1995) Cloning of *Arabidopsis thaliana* cDNA encoding cystathionine β-lyase by functional complementation in *Escherichia coli*. *Plant Molecular Biology* **29**, 875-882.

Ravanel, S., Job, D. and Douce, R. (1996) Purification and properties of cystathionine β lyase from *Arabidopsis thaliana* overexpressed in *Escherichia coli*. *Biochemical Journal* **320**, 383-392.

Read, C P. (1957) Comparative studies on the physiology of trichomonal protozoa. *Journal of Parasitology* **43**, 385-394.

Rein, M. F. and Chepel, T. A. (1975) Trichomoniasis, candidiasis and the minor venereal diseases. *Clinical Obstetrics and Gynaecology* **18**, 73-88.

Rein, M. F. and Muller M. (1989) *T. vaginalis* and trichomonaisis. In Holmes, K., K., Mardt, P., A. and Sparling, A., F. (eds) *Sexually Transmitted Diseases*. pp 481-492. New York: McGraw Hill

Rein, M. F. (1990) Clinical manifestations of urogenital trichomaniasis in women. In Honingberg, B., M. (ed) *Trichomonads parasitic in humans*. pp 225-234. New York: Springer Verlag

Riley, D. E., Samadpour, M. and Kreiger, J. N. (1991) Detection of variable DNA repeats in diverse eukaryotic microorganisms by a single set of PCR primers. *Journal of Clinical Microbiology* **29**, 2746-2751.

Riley, D. E. and Kreiger, J. N. (1996) Kinetics of killing or growth of *Trichomonas vaginalis* in the presence of aminoglycosides , neomycin and geneticin (G418). *International Journal of Antimicrobial Agents* **7**, 257-259.

Roger, A. J., Clark, C. G. and Doolittle, W. F. (1996) A possible mitochondrial gene in the early branching amitochondriate protist *Trichomonas vaginalis*. *Proceedings of the National Academy of Sciences, United States of America* **93**, 14618-14622.

Roitman, I., Heyworth, P. G. and Gutteridge, W. E. (1978) Lipid synthesis by *T. vaginalis*. *Annals of Tropical and Medical Parasitology* **172**, 583-585.

Rolland, N., Ruffet, M. L., Job, Douce, R. and Droux, M. (1996) Spinach chloroplast O-acetylserine (thiol)-lyase exhibits two catalytically non-equivalent pyridoxal -

5'phosphate-containing active sites. *European Journal of Biological Chemistry* **236**, 272-282.

Rosa, F. W., Baum, C., Shaw, M. (1987) Pregnancy outcomes after first trimester vaginitis drug therapy. *Obstetrics and Gynaecology* **69**, 751-755

Rowe, A. F. and Lowe, P. N. (1986) Modulation of amino acid and 2-oxo-acid pools in *T. vaginalis* by aspartate aminotransferase inhibitors. *Molecular and Biochemical Parasitology* **21**, 17-24.

Rubino, S., Muresa, R., Rappelli, P. (1991) Molecular probe for identification of *T. vaginalis* DNA. *Journal of Clinical Microbiology* **29**, 702-706

Sakanari, J. A., Staunton, C. E., Eakin, A. E., Craik, C. S. and McKerrow, J. H. (1989) Serine proteases from nematode and proteozoan parasites: Isolation of sequence homologues using generic molecular probes. *Proceedings of the National Academy of Sciences, United States of America* **86**, 4863-4867.

Sambrook, J.. Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning, a laboratory manual (2nd ed.). Cold Spring Harbor Laboratory Press.

Schnackerz, K. D. and Cook, P. F. (1995) Resolution of pyridoxal 5'-phosphate from O-acetylserine sulphydrylase from *Salmonella typhimurium* and reconstitution of apoenzyme with cofactor and cofactor analogues as a probe of the cofactor binding site. *Archives of Biochemistry and Biophysics* **324**, 71-77.

Schnell, J. G. (1974) The incidence of vaginal *candida* and *trichomonas* infections and treatment of *Trichomonas vaginalis* with clotrimazole. *Postgradraduate Medical Journal*. **50** (suppl 1), 81-83.

Schofield, P. J., Upcroft, C. and Upcroft, P. J. (1990) The arginine dihydrolase pathway is present in *Giardia intestinalis*. *International Journal of Parasitology* **20**, 697-699.

Schofield, P. J. Upcroft, C. and Upcroft, P. J. (1992) The pathway of arginine catabolism in *Giardia intestinalis*. *Molecular and Biochemical Parasitology* **51**, 29-36.

Short, J. M., Fernandez, J. M., Sorge, J. A. and Huse, W. D. (1988) λZAP: A bacteriophage λ expression vector with *in vivo* excision properties. *Nucleic Acids Research* **16**, 7583-7600.

Smale, S. T. and Baltimore (1989) The 'initiator' as a transcription control element. *Cell* **57**, 103-113.

Soda, K. (1968) Micro-determination of D-amino acids and D-amino oxidase activity with 3-methyl-2-benzothiazolone hydrazone hydrochloride. *Analytical Biochemistry* **25**, 228-235.

Soda, K. (1987) Microbial sulphur amino acids: An overview. *Methods in Enzymology.* **143**, 452-459

Sogin, M. L. (1989) Evolution of Eukaryotic Micro-organisms and their small subunit ribosomal RNAs. *American Zoologist* **29**, 487-499.

Southern, E., M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503.

Tanaka, H., Esaki, N., Yamamoto, T. and Soda, K. (1976) Purification and properties of methioninase from *Pseudomonas ovalis. FEBS Letters* **66**, 307-311.

Tanaka, H., Esaki, N. and Soda, K. (1977) Properties of L-methionine γ-lyase from *Pseudomonas ovalis. Biochemistry* **16**, 100-106.

Tanaka, H., Esaki, N. and Soda, K. (1985) A versatile bacterial enzyme L-methionine γ-lyase. *Enzyme Microbial Technology* **7**, 530-537.

Thomason, J. L., Wilcoski, L. M., McLaughlin, C. A. (1984) *Trichomoniasis. Clinical Microbiological Newsletters* **8**, 9-12.

Thomason, J. L. and Gelbart, S. M. (1989) *T. vaginalis. Obstetrics and Gynaecology* **74**, 536-541

Thomason, J. L., Gelbart, S. M., Sobun, J. F., Schulen, M. B. (1988) Comparison of four methods to detect *Trichomonas. Clinical Microbiology* **26**, 1869-1870.

Thong, K-W. and Coombs, G. H. (1985a) Homocysteine desulphurase activity in trichomonads. *IRCS Medical Science* **13**, 493-494.

Thong, K-W. and Coombs, G. H. (1985b) L-serine sulphydrase activity in trichomonads. *IRCS Medical Science* **13**, 495-496.

Thong, K-W., Coombs, G. H .and Sanderson, B. E. (1985c) S-adenosylhomocysteine hydrolase activity in *Trichomonas vaginalis* and other trichomonads. *Molecular and Biochemical Parasitology* **17**, 35-44.

Thong, K. W. (1987) The biochemistry of sulphur-containing amino acids in trichomonads. PhD thesis, University of Glasgow.

Thong, K-W. and Coombs, G. H. (1987a) *Trichomonas* species: Homocysteine desulphurase and serine sulphydrase activities. *Experimental Parasitology* **63**, 143-151.

Thong, K-W., Coombs, G. H. and Sanderson, B. E. (1987b) L-methionine metabolism in trichomonads. *Molecular and Biochemical Parasitology* **73**, 193-198.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences, United States of America* **76**, 4350-3454.

Traunecker, A., Oliveri, F. and Karjalainen (1991) Myeloma based expression system for the production of large mammalian proteins. *Trends in Biotechnology* **9**, 109-113.

Turner, A. C. and Lushbaugh, W. B. (1988) *Trichomonas vaginalis*. Characterisation of its glutamate dehydrogenase. *Experimental Parasitology* **67**, 47-53.

Viscogliosi, E., Durieux, I., Delgado- Viscogliosi, P., Bayle, D. and Dive, D. (1996) Phylogenetic implication of iron-containing superoxide dismutase genes from trichomonad species. *Molecular and Biochemical Parasitology* **80**, 209-214.

Walker, J. and Barrett, J. (1991) Cystathionine β-synthase and γ-cystathionase in helminths. *Parasitology Research* **77**, 709-713.

Wang, A. L. and Wang, C. C. (1985) Isolation and characterisation of DNA from *Tritrichomonas foetus* and *Trichomonas vaginalis*. *Molecular and Biochemical Parasitology* **14**, 323-335.

Wang, C. L. and Cleng, H. W. (1984) Salvage of pyrimidine nucleotides by *T. vaginalis*. *Molecular and Biochemical Parasitology* **10**, 171-184.

Washtien, W. and Abeles, R. H. (1977) Mechanism of inactivation of γ-cystathionase by the acetylenic substrate analogue propargylglycine. *Biochemistry* **16**, 2485-2491.

Weis, L. and Reinberg, D. (1992) Transcription by RNA polymerase II: Initiator-directed formation of transcription competent complexes. *FASEB Journal* **6**, 3300-3309.

Weston, T. E. T. and Nicol, C. S. (1963) Natural history of trichomonal infection in males. *British Journal of Venereal Diseases* **39**, 251-257.

Whately, F. R. (1981) The establishment of mitochondria: *Paracoccus* and *Rhodopseudomonas*. *Annals of the New York Academy of Sciences* **361**, 330-340.

Whittington, J. M. (1951) The survival of *Trichomonas vaginalis* at various temperatures below 37°C. *Journal of Hygiene* **49**, 400-409.

Whittington, J. M. (1957) Epidemiology of infections with Trichomonas vaginalis in the light of improved diagnostic methods. *American Journal of Tropical Medicine and Hygiene* **8**, 312-318.

Wiesdanger, S. and Nisman, B. (1953) Methionine γ-lyase form *Clostridium sp.* C. R. *Hebd. Seances Acad Sci.* **237**, 764-765..

Williams. K., Lowe, P.N. and Leadlay, P. F. (1987) Purification and characterisation of pyruvate: ferredoxin oxidoreductase from the anaerobic protozoon *Trichomonas vaginalis. Biochemical Journal* **246**, 529-536.

Witkin, S. S., Inglis, S. R. and Polaneczky, M. (1996) Detection of *Chlamydia trachomatis* and *Trichomonas vaginalis* by PCR and introital specimens from pregnant women. *American Journal of Obstetrics and Gynaecology* **175**, 165-167.

Wolner-Hanssen, P., Krieger, J. N., Hillier, S. and Holmes, K. K. (1989) Clinical Manifestations of vaginal trichomoniasis. *The Journal of the American Medical Association* **261**, 571-576.

Won, T. and Mazelis, M. (1989) The C-S lyases of higher plants. Purification and characterisation of homogeneous alliin lyase of leek (*Allium porrum*) *Physiologica Planta* **77**, 87-92.

Wyman, A. and Paulus, H. (1975) Homocysteine synthesis in *Bacillus polymyxa. Journal of Biological Chemistry* **50**, 3897-3903.

Yamagata, S. (1971) Homocysteine synthesis in various yeasts. *Journal of Biochemistry* **70**, 1035-1045.

Yamagata, S., Takeshima, K. and Naiki, N. (1975) O-acetylserine and O-acetylhomoserine sulphydrylase of yeast, studies with methionine auxotrophs. *Journal of Biochemistry* **77**, 1029-1036.

Yamagata, S. (1984) O-acetylhomoserine sulphydrylase of the fission yeast *Schizosaccharomyces pombe*: partial purification, characterisation and its probable role in homocysteine biosynthesis. *Journal of Biochemistry* **96**, 1511-1523.

Yamagata, S. (1987) O-acetylhomoserine sulphydrylase from *Schizosaccharomyces pombe. Methods in Enzymology.* **143**, 465-469.

Yamagata, S. (1987) O-acetylserine/O-acetylhomoserine sulphydrylase from *Saccharomyces cerevisiae. Methods in Enzymology.* **143**, 469-483.

Yamagata, S. (1989) Roles of O-acetyl homoserine sulphydrylase in micro-organisms. *Biochimie* **71**, 1125-1137.

Yamagata, S. (1993) Cloning and bacterial expression of the CYS3 gene encoding cystathionine γ-lyase of *Saccharomyces cerevisiae* and the physicochemical and enzymatic properties of the protein. *Journal of Bacteriology* **175**, 4800-4808.

Yarlett, N. (1988) Polyamine biosynthesis and inhibition of *Trichomonas vaginalis. Parasitology Today* **4**, 357-360.

Yule, A., Gellan, M. C., Oriel, J. D. and Packers, J. P. (1987) Detection of *T. vaginalis* antigen in women by enzyme immunoassay. *Journal of Clinical Pathology* **40**, 566-568.

Zuo, X., Lockwood, B. C. and Coombs, G. H. (1994) Amino acid consumption by *T. vaginalis*. *Microbiology* **141**, 2637-2642.

GLASGOW UNIVERSITY LIBRARY