Characterisation and Partial Purification of DNA Methylase from Pea (*Pisum sativum*)

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Abbreviations

The abbreviations used in this thesis are in agreement with the recommendations of the editors of the Biochemical Journal ((1990) *Biochem. J.* **265**, 1–21) with the following additions.

BSA	bovine serum albumin
DEAE cellulose	diethylaminoethyl cellulose
dH_2O	distilled H_2O
DTT	dithiothreitol
EDTA	ethylenediamine-tetra-acetic acid
EGTA	ethyleneglycol-bis-N,N'-tetra-acetic acid
kDa	kiloDalton
MOPS	(3-[N-morpholino]propane-sulphonic acid
PEG	polyethyleneglycol
PMSF	phenyl methyl sulphonyl fluoride
SAM	S–adenosyl–L–methionine
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	NNN'N'-tetramethylethylenediamine
TRIS	tris(hydroxymethyl)aminomethane
Tween 20	polyethylene sorbitan monolaureate

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Summary

Prior to the start of this project, pea DNA methylase had been partially purified from pea shoot tips by gel filtration. Only one species of the enzyme was identified (Yesufu *et al.*, 1991). In plants both CpG and CpNpG sites (where N is any base) are methylated and there is evidence that methylation of these sites might serve different functions (Hershkovitz *et al.*, 1990). It is therefore possible that there might be more than one DNA methylase in plants. Three DNA methyltransferases are known to exist in *E. coli* (Marinus, 1987), and although only one methylase gene has been identified in mice, possible post–translational modification has resulted in three closely related forms of the enzyme (Bestor *et al.*, 1988). Most methyltransferases recognize a single DNA sequence but in prokaryotes *Flavobacterium okeanokoites* displays different strand specificities while the *Bacillus subtillis* methyltransferase shows multiple sequence specificity (Gunthert and Trautner, 1984; Looney *et al.*, 1989; Sugisaki *et al.*, 1989). One of the aims of this project was to refine the purification procedure so that separate enzyme species if any could be identified.

Crude enzyme extract, E1, was shown to methylate synthetic DNA oligonucleotides representing CpG and CpNpG target sequences. A preference for hemimethylated over unmethylated substrate DNA was observed for both di– and trinucleotide target DNAs. DNA methylase was observed to have the highest affinity for hemimethylated CpNpG target DNA following competition studies and ligation of CpNpG DNA. The DNA–enzyme complex formed with this CpNpG substrate was observed to be salt resistant while DNA–enzyme complexes formed with the other substrates dissociated after varying intervals. This suggests that the enzyme forms a stable complex with hemimethylated trinucleotide DNA and is consistent with the idea that once bound to this substrate DNA it remains bound as suggested by competition studies and ligation of target DNA.

Partial purification of pea DNA methylase(s) was achieved by affinity chromatography involving elution from heparin Sepharose and Q Sepharose. Enzymic activity was assayed with hemimethylated trinucleotide and unmethylated dinucleotide synthetic oligonucleotides. The peak of enzyme activity was observed in different fractions when assayed with the two substrates. SDS–PAGE revealed bands of high molecular weight in all active fractions which were found to react with an antibody to a component of the wheat methylase enzyme. The large number of bands was considered to be due to degradation of the enzyme through proteolysis. The addition of further protease inhibitors such as benzamidine led to the loss of enzymic activity.

No distinct bands were visible on SDS–PAGE gels between fractions exhibiting different levels of methylation with different substrates. This is consistent with there being only one pea DNA methylase enzyme. The differences observed in relative activities with different substrates during purification might be due to different extents of proteolysis or to changes in conformation of the enzyme. Such changes might also explain the relative difference in activity with hemi– and unmethylated substrate DNA following heat treatment of extract E1 and purified samples and during early growth of pea shoots.

CHAPTER 1
Introduction

1.1 Occurrence and distribution of modified bases in DNA

The occurrence of methylated bases in DNA was first demonstrated almost 40 years ago (Hotchkiss, 1948; Wyatt, 1951; Dunn and Smith, 1958). The modified bases N^6–methyladenine, N^4–methylcytosine and 5–methylcytosine have been detected in prokaryotes, in certain green algae (Haltman *et al.*, 1978; Burton *et al.*, 1979) and in types of bacteriophage (Doerfler, 1983). The DNA of nematodes (Simpson *et al.*, 1986), Drosophila (Pollack *et al.*, 1984) and cellular slime moulds (Smith and Ratner, 1991) appear to contain no detectable methylated cytosine residues, while most species of fungi either lack 5–methylcytosine or have very low contents of this base (Magill and Magill 1989).

In vertebrates, 5–methylcytosine appears to be the major modified nucleotide with 3–6% of cytosines methylated in the dinucleotide sequence CpG. Methylated cytosine residues are also found in higher plant DNA in addition to N^6–methyladenine (Vanyushin *et al.*, 1988). In higher plants there is additional methylation of cytosine residues contained in the trinucleotide sequence CpNpG, where N represents any base (Gruenbaum *et al.*, 1981). Belanger and Hepburn (1990) have proposed that the CpG and CpNpG methylation systems in plants evolved at the same time. In plant nuclear DNA up to 30% of all cytosines are methylated (Wagner and Capesius, 1981). Plant cells contain a unique class of DNA containing organelles, the plastids which include chloroplasts. Methylated DNA has been detected in maize mesophyll chloroplasts, tomato chromoplasts and sycamore amyloplasts (Kobayashi *et al.*, 1990) as well as in chloroplasts of the green algae *Chlamydomonas reinhardtii* (Sagar and Lane, 1972) and more recently in pea where about 0.5% of cytosine residues are methylated in chloroplast DNA (Ohta *et al.*, 1991). In both vertebrates and plants there is a deficiency in the number of CpG sequences as compared to the number expected from a random distribution (Adams *et al.*, 1987; Boudraa and Perrin, 1987). The incidence of the CpG dinucleotide in mammalian DNA is 1/5 th of that expected in bulk DNA and between 60–90% of

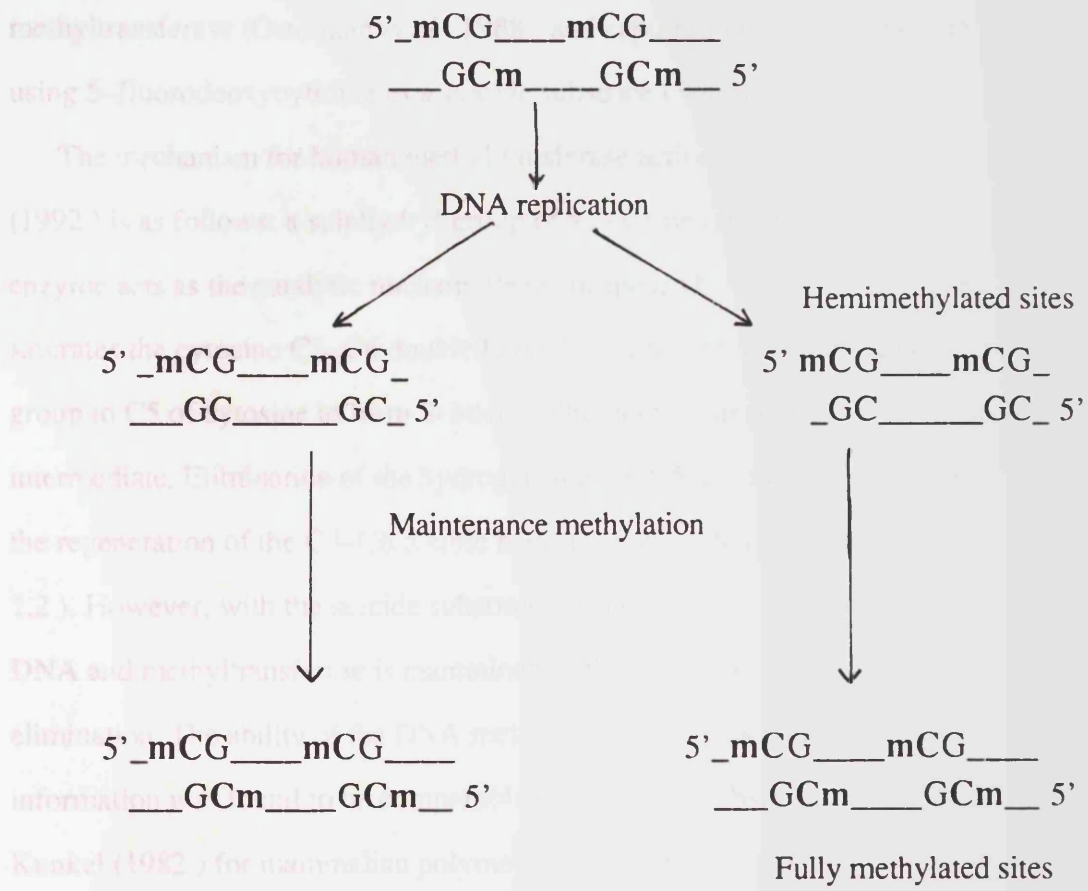
these CpGs are methylated (Bird, 1986). However, there are regions of 0.5–2 kb within the genome where CpG residues are abundant. These regions are termed CpG or *Hpa* II tiny fragments (HTF) islands and are associated with house-keeping genes. They are unmethylated in all tissues so far tested including the germ line. Only on the inactive X–chromosome have these regions been found to be methylated. Clusters of unmethylated CpG and CpNpG sites resembling CpG islands have been identified in maize, tobacco, wheat (Antequera and Bird, 1988) and tomato (Messeguer *et al.*, 1991). In maize some of these islands are associated with genes (Antequera and Bird, 1988).

1.2 Maintenance and *de novo* methylation

Patterns of DNA methylation are inherited and maintained over many cell generations (Wigler *et al.*, 1981) by a process known as maintenance methylation. This is achieved by the action of the enzyme DNA methyltransferase which uses S–adenosyl methionine as methyl donor. The semi-conservative nature of DNA replication results in the parental strand remaining methylated while the newly synthesized daughter strand is unmethylated (Figure 1.1). In an early post replicative step (Burdon and Adams, 1969) this hemimethylated DNA is recognized by DNA methyltransferase which, using the parental strand as a template, symmetrically imposes methyl groups on the newly synthesized strand. This mechanism was first proposed by Holliday and Pugh (1975) and Riggs (1975).

De novo methylation involves the transfer of methyl groups to previously unmethylated DNA and in doing so generates new patterns of methylation. During embryogenesis there is active *de novo* methylation which is thought to be important in the control of development and will be discussed further in section 1.5.3.

Figure 1.1 **Inheritance of DNA methylation patterns**



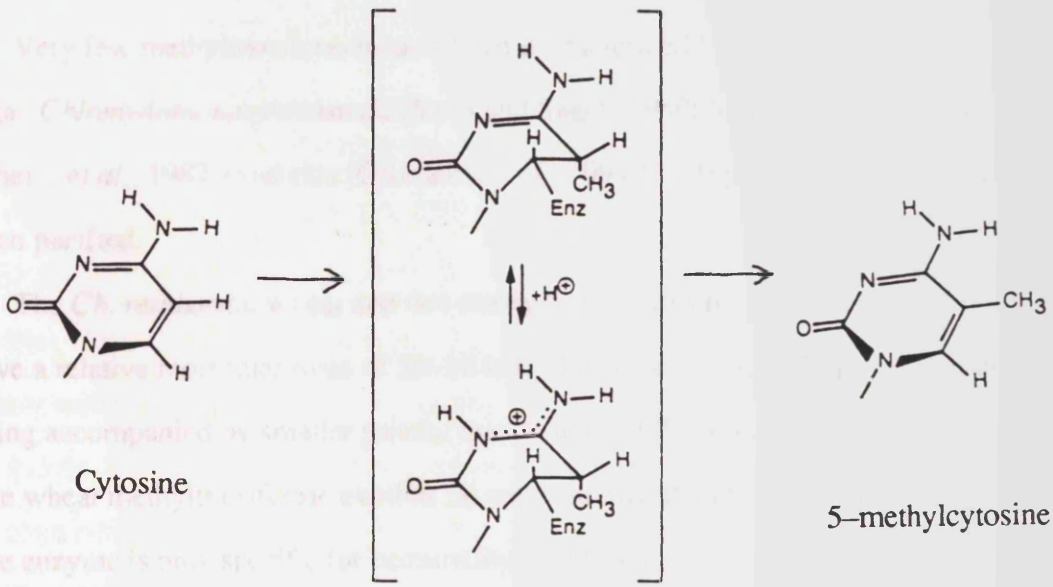
1.3 Mechanism of DNA methyltransferase action

It was proposed by Santi *et al.* (1983) that the mechanism of DNA methyltransferase catalysis involves the formation of a covalent enzyme–DNA complex. Such a complex has been detected in the prokaryotic H*ha* I methyltransferase (Osterman *et al.*, 1988) and captured in human methyltransferase using 5–fluorodeoxycytidine as a suicide substrate (Smith *et al.*, 1992).

The mechanism for human methyltransferase action described by Smith *et al.* (1992) is as follows: a sulphydryl group of a cysteine residue at the active site of the enzyme acts as the catalytic nucleophile (as proposed by Wu and Santi, 1987) and saturates the cytosine C5–C6 double bond. S–adenosyl methionine donates a methyl group to C5 of cytosine to form S–adenosylhomocysteine and the enzyme–DNA intermediate. Elimination of the hydrogen atom at C5 and the enzyme at C6 results in the regeneration of the C5–C6 double bond to produce 5–methylcytosine (Figure 1.2). However, with the suicide substrate, the covalent complex between the duplex DNA and methyltransferase is maintained as the 5–fluorine cannot be lost by elimination. The ability of the DNA methyltransferase to accurately preserve genetic information was found to be comparable to the fidelity observed by Loeb and Kunkel (1982) for mammalian polymerases in copying DNA sequence.

Figure 1.2 **Methylation of cytosine residues**

(adapted from Smith *et al.*, 1992)



1.4 DNA methyltransferases

Mammalian methyltransferases have been purified from various sources including rat liver (Simon *et al.*, 1978), human placenta (Pfeifer *et al.*, 1983), Hela cells (Roy and Weissbach, 1975) and mouse Krebs II ascites tumour cells (Turnbull and Adams, 1976). They are large, single unit enzymes of 100–200 kDa in size. This range in size may be due to proteolysis which has been shown to occur readily both *in vivo* and *in vitro* in mouse ascites tumour cells (Adams *et al.*, 1986).

Very few methyltransferases have been characterized from plants. The green alga *Chlamydomonas reinhardii* (Sano and Sager, 1980; Sano *et al.*, 1981), wheat (Theiss *et al.*, 1987) and rice (Giordano *et al.*, 1991) methyltransferases have all been purified.

The *Ch. reinhardii*, wheat and rice methyltransferases have been estimated to have a relative molecular mass of 50–58 kDa; the wheat and green alga enzymes being accompanied by smaller protein components of 35 and 22 kDa respectively. The wheat methyltransferase exhibits *de novo* and maintenance methylation while the rice enzyme is only specific for hemimethylated DNA.

A cytosine specific methyltransferase of 135 kDa has been purified from the mealybug (*Planococcus lilacinus*). It differs from all other eukaryotic methyltransferases studied in that it methylates cytosine residues in CpA as well as CpG sequences (Devajyothi and Brahmachari, 1992). Methylation of cytosine residues in CpA would not be inherited as CpA is not symmetrical with the opposing strand. Selker (1990) has proposed an alternative model for inheritance of methylated states in fungi where repeat point mutations result in the methylation of all cytosine residues.

Eukaryotic DNA methyltransferases preferentially methylate hemimethylated DNA (Gruenbaum *et al.*, 1982) but bacterial cytosine specific methyltransferases do not discriminate between unmethylated and hemimethylated DNA (Kelleher *et al.*, 1991). In certain bacteria and bacterial viruses, DNA methyltransferases occur as

components of restriction modification (R–M) systems. Such systems comprise pairs of opposing intracellular activities: a restriction endonuclease and a DNA methyltransferase which interact with specific recognition sequences of nucleotides in DNA. R–M systems are classified according to differences in structure, cofactor requirements and recognition sites of these enzymes (reviewed by Wilson and Murray, 1991). However, methyltransferases can also occur alone in bacteria. *E. coli* possesses two unaccompanied methyltransferases, DNA–adenine methyltransferase (Dam) and DNA–cytosine methyltransferase (Dcm) (Marinus, 1987).

Partial proteolysis of the methyltransferase from mouse Krebs II ascites tumour cells has been found to result in an increase in *de novo* activity (Adams *et al.*, 1983). This led to the proposal that mammalian DNA methyltransferases contain two domains, one containing the active site and the other an allosteric site which regulates methylation of unmethylated DNA (Adams *et al.*, 1986). There is now further supporting evidence for this model. Mammalian methyltransferases have been found to contain a 500 amino acid C–terminal domain that is closely related to bacterial 5–methylcytosine methyltransferases (Bestor *et al.*, 1988). This domain is linked to a large N–terminal domain which contains a Zn binding site. Proteolytic cleavage between the N– and C– terminal domains stimulates *de novo* methylation without changing the rate of maintenance methylation indicating that the N–terminal domain acts to inhibit *de novo* methylation (Bestor, 1992).

1.5 Functions of DNA methylation

1.5.1 DNA methylation and gene expression

One of the major functions of DNA methylation in eukaryotes is postulated to be regulation of gene expression. In general, for tissue specific genes there is a correlation between expression and undermethylation. The first indication of this

relationship came from studying methylation patterns using the isoschizomer restriction enzymes, *Msp* I and *Hpa* II in a technique devised by Waalwijk and Flavell (1978). These enzymes cleave the sequence CCGG but in the case of *Hpa* II, only if the internal cytosine is not methylated. Comparison of the fragments produced by digestion with these enzymes allows the proportion of cytosines methylated in the CpG dinucleotide to be obtained. However, the recognition sequence CCGG represents only about 6% of all the CpG dinucleotides present in the genome and therefore important sites may not be detected by this method. This limitation has been overcome by the development of an alternative method involving genomic sequencing used in combination with the polymerase chain reaction (Saluz and Jost, 1989).

The association between gene activity and reduced methylation has been found in several genes including the chicken ovalbumin gene (Mandel and Chambon, 1979) and the mouse metallothionein I gene (Compère and Palmiter, 1981). The globin genes have also been extensively studied. In the rabbit (Waalwijk and Flavell, 1978), chicken (McGhee and Ginder, 1979) and in humans globin genes have been clearly shown to be unmethylated in the tissue of expression but heavily modified in DNA from other tissues (van der Ploeg and Flavell, 1980). In plants there is also an association between hypomethylation and gene expression in a number of genes including storage protein genes in maize (Spena *et al.*, 1983; Bianchi and Viotta, 1988), photosynthetic genes in non green plant cells (Ngernprasirtsiri *et al.*, 1989) and rRNA genes in pea (Watson *et al.*, 1987). Riggs and Chrispeels (1990) have demonstrated using restriction digestion that in the cotyledons of *Phaseolus vulgaris* the expression of seed storage proteins encoded by genes lec 1 and lec 2 is correlated with hypomethylation of sites upstream of the lec 1 gene. DNA methylation has also been correlated to transposon activity in tobacco (Martin *et al.*, 1989) and maize (Chandler and Walbot, 1986; Schwartz and Dennis, 1986). However, there are significant exceptions to hypomethylation being associated with expression. The chicken α 2 (I) collagen gene (Mckeon *et al.*, 1982) and rat albumin gene (Kunnath

and Locker, 1983) have been shown to be actively transcribed in both heavily methylated and unmethylated states, while the α fetoprotein gene of rats is transcribed only in foetal liver, where it is heavily methylated, and not in adult liver where it is less methylated (Kunnath and Locker, 1983).

Whether gene inactivation in plants requires methylation of cytosines at both CpNpG and CpG sequences remains to be clarified. Preliminary results from Hershkovitz *et al.* (1990) suggest that only methylation at CpG sites is necessary for inactivation and that methylation of CpNpG sequences may not be involved in gene silencing.

Further evidence indicating that DNA methylation inhibits gene expression has come from two different experimental approaches. One approach is gene transfer which involves methylating DNA *in vitro* and introducing it into cells. Using this method Yisraeli *et al.* (1988) showed that endogenous α globin genes in human fibroblasts are methylated and not expressed. However, unmethylated copies of the same gene transfected into the same cell type are transcribed.

In plants *in vitro* hemimethylation has been shown to block transient gene expression after transfection into protoplasts (Weber and Graessmann, 1989; Hershkovitz *et al.*, 1989). Linn *et al.* (1990) introduced the maize A1 gene under the control of the CaMV 35S RNA promoter into the white flowering mutant *Petunia hybrida*. Different phenotypes of either white, variegated or red flowers were observed among individual transgenic plants. The CaMV 35S promoter has two CCGG methylation sites and using restriction digestion Linn *et al.* demonstrated that the A1 gene is expressed if CCGG site 1 on the promoter is modified but is turned off when both sites 1 and 2 are methylated. They also demonstrated that integration of multiple copies of the chimeric gene resulted in methylation of the promoter sequences. Similar results have been observed by Matzke *et al.*(1989; Matzke and Matzke, 1991) who demonstrated that inactivation of T–DNA–1 genes by methylation of the promoter sequences occurred only in plants containing an additional T–DNA gene.

The second approach involves the use of the potent demethylating agent, 5–azacytidine. This compound is a cytosine analogue and once incorporated into DNA appears to bind irreversibly to DNA methylase in a manner similar to 5–flurodeoxycytidine (section 1.3), thereby inhibiting DNA methylation. Following exposure of chicken AEV cells to 5–azacytidine, a previously inactive virus gene is found to be active and gene sequences to have undergone demethylation (Groudine *et al.*, 1981). In tobacco plant cells silent T–DNA genes were demethylated by 5–azacytidine treatment and found to be active (Klaas *et al.*, 1989). Recently Bochardt *et al.* (1992) have demonstrated that a β–glucuronidase (GUS) gene in cultured transgenic tobacco plant cells is silenced by DNA methylation. Treatment with 5–azacytidine resulted in demethylation of the genome and reactivation of the GUS gene. As in the findings of Weber *et al.* (1990) the pattern of methylation was maintained and inherited during meiosis and regeneration.

1.5.2 Mechanism of gene regulation by DNA methylation

It is generally assumed that DNA methylation affects gene transcription by altering protein–DNA interactions. This could occur directly if essential transcription factors were unable to bind to DNA if their binding sites contain a methylated CpG or indirectly due to changes in chromatin conformation inhibiting the access of transcription factors. A number of transcription factors that are sensitive to methylation have been discovered providing support for a direct effect (Kovesdi *et al.*, 1987; Watt and Molloy, 1988; Iguchi-Ariga and Schaffner, 1989). However, not all transcription factors have recognition sites containing the dinucleotide CpG or are sensitive to methylation at the recognition site. The SpI transcription factor will bind and activate even when the recognition site is modified (Holler *et al.*, 1988).

Razin and Cedar (1977) first indicated that chromatin structure could be related to the level of DNA methylation and that methylated DNA was organized into a

specific chromatin structure which is resistant to nucleases. Direct evidence that methylation could cause DNA to adopt a nuclease resistant structure was provided by Keshet *et al.*(1986).

Antequera *et al.* (1989) observed that in bulk chromatin cleavage of methylated sites with *Msp* I (an enzyme that cleaves CCGG irrespective of methylation status) is blocked, possibly by methyl binding proteins. They observed that digestion gave some short oligonucleosomes that arose from CpG islands, whereas most of the chromatin remained intact. Based on this observation, a method for isolating CpG island chromatin was developed by Tazi and Bird (1990), who from analysis of the protein content of DNA derived from CpG islands concluded that CpG islands differed from bulk chromatin in that they are deficient in histone H1 and histones H3 and H4 are highly acetylated. Conversely, 80% of methylated cytosines have been located in nucleosomes containing histone H1 (Ball *et al.*, 1983).

Two methyl binding proteins, MeCP1 and MeCP2 have been identified in Bird's laboratory (Meehan *et al.*, 1989; 1992). MeCP1 has been shown to require at least 12 methylated sites for efficient binding and to repress transcription especially when bound to promoters containing a high density of methylated CpG sites (Boyes and Bird, 1992). In comparison, MeCP2 is able to bind a single methylated CpG and has been found to be more abundant in the nucleus than MeCP1 (Lewis *et al.*, 1992; Meehan *et al.*, 1992). It has been suggested that MeCP2 may play a role in the genome-wide protection of methylated CpG sites against nucleases and in the formation of specific chromatin structures. However, a role in transcriptional repression cannot be ruled out.

Several DNA binding proteins that recognize the CRE (cAMP response element) have been identified in the nuclear extracts of pea seeds, wheat germ, cauliflower and soybean leaves as well as in a broad bean cDNA clone (Inamdar *et al.*, 1991; Ehrlich *et al.*, 1992). Binding of these proteins *in vitro* is inhibited by cytosine methylation of CRE. Whether these proteins respond to changes in DNA methylation in gene regulatory regions *in vivo* is still to be determined.

Kobayashi *et al.* (1990) have suggested that the transcriptional silencing of tomato plastid genes is due to methylation of DNA in promoter regions suppressing transcriptional initiation. However, there is evidence that transcriptional regulation plays a very limited role in plastid differentiation and that gene expression is mostly controlled by the post-transcriptional processing and/or stability of constitutively synthesized plastid transcripts (Gruissem, 1989).

1.5.3 DNA methylation and development

It has been proposed that DNA methylation is an important epigenetic mechanism involved in the developmental program (reviewed by Holliday, 1987; Cedar and Razin, 1990). Most of the research into the role of DNA methylation in development has concentrated on vertebrates, especially the mouse embryo, and as a result little is known about developmental control in plants.

Monk *et al.* (1987) used restriction digestion with *Msp* I and *Hpa* II to compare genome wide methylation in germ cells and in early mouse development. They observed that oocyte DNA is undermethylated compared to sperm DNA. DNA methylation levels in the eight cell embryo were found to be the average of the maternal and paternal genomes. Further embryo development is characterized at the blastocyst stage by overall demethylation followed by *de novo* methylation around the time of implantation and X–chromosome inactivation. This *de novo* methylation appears to occur independently in embryonic and extra-embryonic linages and not at all in the germ line (Monk, 1990).

As mentioned above, *de novo* methylation occurs around the time of X–chromosome inactivation. This occurs in female mammals and involves the transcriptional silencing of one X–chromosome. This inactive state is permanently maintained in somatic cells. It has been proposed that DNA methylation plays a role in this inactivation process (Riggs, 1975; Holliday and Pugh, 1975). Several

studies have confirmed that inactivation of housekeeping genes on the X–chromosome is correlated with hypermethylation. Examples of genes showing these correlations are those encoding hypoxanthine phosphoribosyltransferase (HPRT) and phosphoglycerate kinase (PGK) (reviewed by Monk, 1990).

Pfeifer *et al.* (1990) using PCR-aided genomic sequencing demonstrated that 120 CpG residues in the CpG island on the mouse *Pgk–1* gene were methylated on the inactive X–chromosome while they were totally unmodified on the active X–chromosome. However, DNA methylation does not appear to be the initiator of X–chromosome inactivation. Lock *et al.* (1987) have demonstrated that *de novo* methylation of the X–linked hprt gene occurred after the completion of the inactivation process. This has led to the suggestion that DNA methylation of the X–chromosome is a secondary event which acts to maintain inactivation. This is supported by evidence from studies using 5–azacytidine. Mohandas *et al.* (1981) demonstrated that genes on the inactive X–chromosome can be reactivated following treatment with 5–azacytidine.

During development, functional differences on parental chromosomes are conferred by genomic imprinting. This may prevent either the male or female genome becoming totipotent by itself so that both germ lines are required for normal development (reviewed by Surani *et al.*, 1990). It has been suggested that genomic imprinting is mediated by differential DNA methylation of the maternal and paternal genomes (Ridley *et al.*, 1988). Swain *et al.* (1987) have demonstrated in the off-spring of transgenic mice that a functional IgG/myc construct was active in heart muscle, but only when inherited from the father. The transgene derived from the mother was found to be fully methylated in all cell types. Similar results have been obtained from studies of other gene sequences in transgenic mice (Reik *et al.*, 1987; Sapienza *et al.*, 1987).

Although there is widespread *de novo* methylation during development, CpG islands appear to remain unmodified. Kolsto *et al.* (1986) demonstrated that the *Thy–1* gene contains a CpG island that is hypomethylated in all tissues during the

development of transgenic mice. Szyf *et al.* (1990) transfected embryonic stem (ES) cells (a totipotent cell line isolated from mouse blastocytes with known *de novo* methylation activity) with either a control construct or a construct containing a 214 base pair region from the *Thy–1* gene promoter sequence. The *Thy–1* CpG island and flanking sequences were found to be protected from *de novo* methylation whereas the control plasmid was methylated.

However, it appears that CpG islands undergo *de novo* methylation in cells in culture. A number of CpG island containing genes, such as thymidine kinase (Wise and Harris, 1988), metallothionein (Compere and Palmiter, 1981) and human α globin (Antequera *et al.*, 1989) appear to become inactive and methylated in culture.

It has been suggested that during the establishment of a cell line there is a progressive process of *de novo* methylation resulting in the emergence of an immortal line. This has been demonstrated by Jones *et al.* (1990) who, using restriction digests and ligation mediated PCR, examined the methylation state of the *Myo D1* CpG island in adult mouse tissues, mouse embryo cells in culture and the immortal fibroblastic cell line 10 T1/2. This gene is required for differentiation and growth control and has the potential to induce mitotic death. The CpG island was found to be unmethylated in all mouse tissues tested, unmethylated during the initial culture period but methylated in the immortal cell line. It has been proposed that this system selects for cells in which the CpG islands of non essential genes or of genes whose expression could result in cell death in culture have undergone fortuitous *de novo* methylation and have been inactivated. Further evidence for this type of process has come from studies by Antequera *et al.* (1990). They observed that CpG islands of many non-essential genes are highly methylated in tissue culture cells.

Szyf (1991) has proposed a model to explain the spreading of new methylation patterns by *de novo* methylation. It is speculated that nuclear factors bind to methylated DNA sites or centres of methylation (CM). These factors target the DNA methylase to methylate adjacent sites to the CM which in turn act as centres of methylation resulting in the spreading of *de novo* methylation.

It has been suggested that the process of cell differentiation in higher plants differs greatly from that known for mammals. Cell differentiation continues throughout the life of the plant and does lead to the appearance of highly specialized organs and tissues (reviewed by Goldberg *et al.*, 1989; Fedoroff, 1989; Sussex, 1989). An example of this would be the aleurone layers of cereal grains which are specialized in the synthesis of secretory proteins and are found in the endosperm (Tomaszewski *et al.*, 1991). These differences between plant and animal development could indicate differences in the mechanisms of developmental control.

Palmgren *et al.* (1991) have shown in *Daucus carota* that there is no correlation between the age or the differentiated stage of a tissue and its level of methylation. However, they suggested that the large differences in the level of methylation between tissues indicated that there is a considerable change in the methylation pattern during development.

However, there is evidence that developmental changes are not related to changes in DNA methylation. Tomaszewski *et al.* (1991) have demonstrated that a 5 kb segment is deleted from the DNA of terminally differentiated wheat endosperm cells compared to wheat embryo DNA. They have proposed that this deletion occurs during cell differentiation in the developing wheat grain and results in gene rearrangement. Recently however, Tomas *et al.* (1992) have demonstrated using restriction digestion that the decrease in protein synthesis observed during leaf growth and maturation in barley (Vera *et al.*, 1990) is not caused by rearrangement, loss or gain of plastid DNA or by changes in DNA methylation.

It has been proposed that DNA methylation may play a role in fruit ripening in higher plants (Kobayashi *et al.*, 1990). This process involves differentiation of chloroplasts into non-photosynthetic chromoplasts and is related to a decrease in expression of several plastid genes required for photosynthesis. Kobayashi *et al.* (1990), using restriction digestion, demonstrated that tomato chromoplast DNA contained 5–methylcytosine and this was correlated with transcriptional silencing of plastid genes. However, Marano and Carrillo (1991) have found that DNA

methylation is not involved in the process of ripening in tomato fruits. They suggest that this discrepancy may reflect genetic differences in the cultivars used.

1.5.3.1 Demethylation

The *de novo* methylation observed in the early mammalian embryo must be balanced by demethylation of DNA during early embryo development to produce the regions of undermethylation found in the genome.

Yisraeli *et al.* (1986) transfected *in vitro* methylated muscle specific actin gene constructs into fibroblasts and L8 myoblasts by DNA mediated gene transfer. L8 myoblasts are a cell line used as a model system for studying the activation and structure of muscle specific genes. Those genes inserted into this cell line underwent demethylation at unique CpG sites which was correlated with gene activation. No demethylation was observed in fibroblasts. Similar results have been obtained for the rat insulin gene in an insulinoma cell line and for immunoglobin κ chains in mouse lymphocytes (Frank *et al.*, 1990).

These studies suggest that some differentiated cell types retain the ability to recognise and demethylate genes, presumably in a manner similar to that which occurs during normal development *in vivo*.

Bianchi and Viotti (1988) have demonstrated in maize using restriction digestion that a set of storage protein genes coding for zeins and glutins are undermethylated in endosperm DNA. These sequences were found to be heavily methylated in mature pollen cells and in all sporophytic tissues investigated suggesting that the storage protein genes undergo demethylation in early endosperm development. Recently, Sørenson (1992) has demonstrated that expression of hordein genes in the developing barley endosperm is accompanied by hypomethylation.

There is evidence to suggest that a mechanism exists to demethylate CpG islands. Frank *et al.* (1991) introduced a methylated hamster aprt gene into mouse

oocytes and analysed the methylation status of the gene in the transgenic mice. The 5' CpG island region of the gene was found to be demethylated whereas the 3' end of the gene remained modified, the pattern of methylation being essentially identical to that of the endogenous hamster gene. They also demonstrated that this demethylation event was restricted to embryonic cells as the aprt gene remained methylated when transfected into fibroblasts. Demethylation of CpG islands may be involved in the reactivation of the X chromosome following *de novo* methylation in the germline.

1.5.3.2 Mechanism of demethylation

It has been assumed that demethylation *in vivo* takes place via a passive mechanism involving the failure to methylate newly replicated DNA. This is a slow process and for double stranded demethylation would require two cell divisions and occur in only 50% of cells (Razin and Riggs, 1980).

Taylor and Jones (1979) demonstrated that passive methylation takes place following treatment of cells with 5–azacytidine but there is increasing evidence that demethylation occurs via an active mechanism *in vivo* which is rapid and does not require DNA replication.

Razin *et al.* (1985) observed that mouse erythroleukemia cells undergo genome-wide demethylation in response to treatment by hexamethylene bisacetamide, a chemical responsible for inducing differentiation in these cells. This demethylation occurs at times in the cell cycle when DNA is not undergoing replication. Similar rapid demethylation has been observed in δ–crystallin (Sullivan and Grainger, 1986), vitellogenin genes (Saluz *et al.*, 1986) and in the Epstein-Barr virus in Burkitt's lymphoma cells (Szyf *et al.*, 1985).

Paroush *et al.* (1991), following transfection of the α actin gene into L8 myoblasts, observed demethylation to occur on integrated plasmid molecules derived

from bacteria. These carried a methylated adenine at all GATC sites and, by using restriction enzymes, demethylation was shown to occur without the molecules undergoing replication.

It has been postulated that the biochemical mechanism of demethylation occurs through an enzyme system which replaces methylcytosine with cytosine without breaking the phosphdiester backbone of the DNA (Razin *et al.*, 1986). However, there is evidence of demethylation occuring via a very short patch (vsp) excision repair like mechanism which replaces methylcytosine with cytosine (Adams, 1990; Hughes *et al.*, 1989).

1.5.4 DNA methylation and cancer

5–methylcytosine can potentially deaminate to thymidine resulting in T.G mismatchs causing C.G to T.A transition mutations. It has been proposed that this deamination is responsible for the observed 4–5 fold depletion of CpG sites in the genome while CpG islands are present in DNA because they are never methylated in the germline. A 5–methylcytosine deamination repair system has been detected in vertebrate cells (Brown and Jiricny, 1987). This system is thought to work through a 200 kDa protein which binds to T.G mismatches. The aberrant thymidine monophosphate is excised and the gap filled to generate a CpG pair (Wiebauer and Jiricny, 1989). However, this system is not error free and it has been suggested that in the human germline 30–40% of all point mutations can be accounted for by CpG methylation (Cooper and Youssoufian, 1988; Barker *et al.*, 1984). It has been demonstrated that the most common mutations found in the factor IX gene in haemophilia patients were transitions particularly at the CpG nucleotide (Koeberl *et al.*, 1990).

It has been proposed that these spontaneous mutations arise as methylation of target CpG sites in the germline increases the mutational frequency. Recently it has

been calculated that methylation of a CpG increases the possibility of a transition occurring at the cytosine by a factor of 12 (Sved and Bird, 1990). Since the paternal and maternal germlines are differentially methylated it is probable that a high percentage of spontaneous mutations arise from the more heavily methylated paternal germline with the maternal germline playing a relatively minor role (Driscoll and Migeon, 1990; Jones and Buckley,1990).

5–methylcytosine has also been found to induce mutations in the *p53* tumour suppressor gene which has been found to be altered in approximately half of all human tumours. In colon cancer there are two hot spots for mutation. These mutations represent C to G or G to A transitions at CpG sites which are shown to be methylated in somatic cells (Rideout *et al.*, 1990). Point mutations in the *p53* gene in human tumours occur in different patterns in different tumour types and may be the result of both 5–methylcytosine induced mutations and the direct action of environmental carcinogens (reviewed in Jones *et al.*, 1992).

1.5.5 Additional functions of DNA methylation

As well as its role in gene expression, DNA methylation has been speculated to function in a variety of other roles. These other roles have mainly been observed in prokaryotes rather in eukaryotes and some have not been studied in plant systems.

1.5.5.1 DNA replication

Although there is little evidence that DNA methylation plays a role in control of replication in eukaryotes, DNA methylation has been demonstrated to play a role in the initiation of replication in prokaryotes such as *E.coli* and for plasmid P1. The origin of replication in these systems contains multiple copies of the sequence GATC, the recognition site for DNA adenine methylase. It has been demonstrated that the absence of this enzyme disturbs the timing of replication (Bakker and Smith,

1989), suggesting that methylation is important for regulation of origin function (Campbell and Kleckner, 1990; Boye and Løbner-Olesen, 1990).

There have been several alternative suggestions as to how DNA methylation affects the initiation of replication in *E. Coli*. Barras and Marinus, (1989) proposed that either an initiation protein recognises fully methylated GATC sites or methylation reduces the stability of the duplex, thereby allowing unwinding to occur. However, Ogden *et al.* (1988) proposed that the hemimethylated GATC sites bind to a membrane protein involved in chromosome separation. Landoulsi *et al.* (1990) demonstrated that this membrane fraction inhibits the initiation of DNA synthesis at the origin of replication (*ori C*) on hemimethylated DNA templates *in vitro*. This is consistent with the findings of Russell and Zinder (1987) that replication cannot initiate from a hemimethylated *ori C*.

1.5.5.2 Mismatch repair

When a normal but incorrect base is introduced into DNA at replication it is usually removed by a proof reading mechanism before the next nucleotide is added. However, if this mechanism fails it results in a mismatch.

Mismatch repair systems are present in both prokaryotes and eukaryotes and use the lack of methylation on the newly synthesized (incorrect) daughter strand to determine which strand requires repair.

Mismatch mutations due to deamination of methylcytosine are frequent both in prokaryotes (Coulondre *et al.*, 1978) and eukaryotes (Barker *et al.*, 1984) and are believed to be one of the reasons why the vertebrate genome is deficient in the CpG dinucleotide (Bird *et al.*, 1979; Adams and Eason, 1984).

In plants, while there is a deficiency in CpG sites there is no such deficiency in the CpNpG trinucleotide (Hepburn *et al.*, 1987). This has led to the suggestion that either DNA methylation in plants and vertebrates is subject to different correction

constraints or that the CpG and CpNpG methylation systems in plants are themselves totally independent functions with different efficiencies of transition correction.

1.5.5.3 Recombination

It has been speculated that DNA methylation has a role to play in the evolution and stability of chromosome structure by preventing recombination events between highly repetitive sequences. These sequences are more highly methylated than other genomic fractions. As discussed in section 1.5.4, 5–methylcytosine is unstable and readily deaminates resulting in a GC to AT mutation.

Despite the presence of mismatch repair systems discussed in section 1.5.5.2, Kricker *et al.* (1992) have proposed that in mammals repeated sequences experience a high frequency of transition mutations at sites of cytosine methylation. These mutations are thought to be sufficient in reducing the degree of similarity between sequences by the few percent required to prevent recombination.

Recently, Hsieh and Lieber (1992) have proposed that CpG methylation specifies a chromatin structure that, upon DNA replication, is resistant to eukaryotic site-specific recombination.

1.5.5.4 Effect on foreign DNA in plants

Rogers and Rogers (1992) have proposed that DNA methylation may play a role in the protection of plant genomes from deletions induced by the insertion of foreign DNA. They introduced plasmid DNA carrying a reporter gene into barley cells. A high transformation efficiency and greater stability of foreign DNA was observed when an absence of N^6–methyladenine was coupled with methylation of cytosine residues in the dinucleotide CpG.

However, Brough *et al.* (1992) have demonstrated that *in vitro* methylation of the tomato golden mosaic virus (TGMV) introduced into tobacco (*Nicotiana tabacum*) protoplasts resulted in a reduction in the amount of viral DNA accumulated in transfected protoplasts. They proposed that cytosine methylation interferes with the first round of replication of TGMV DNA in plant cells.

1.6 Summary

Despite many years of research the role of DNA methylation is not fully understood. In general DNA methylation is correlated with gene silencing in both plants and animals but it still remains to be established whether methylation of DNA initiates the inactivation of genes or is a secondary event which acts to maintain inactivation as demonstrated by Lock *et al.* (1987).

Mammalian DNA methylases have been purified from a number of sources and shown to carry out both *de novo* and maintenance methylation (Bestor *et al.*, 1988). However, very few plant DNA methylases have been purified.

1.7 Project aims

It has been suggested that methylation of CpG sites alone is necessary for gene inactivation in plants (Hershkovitz *et al.*, 1990). Therefore methylation of CpNpG sites might serve a different function. If this is the case then it is possible that plants may contain more than one DNA methylase. DNA methylase has been previously partially purified from the nuclei of pea shoot tips (Yesufu *et al.*, 1991). Only one species of enzyme was detected. The aim of this project was to extend this work by refining the purification procedure so that separate enzyme species could be identified. Synthetic DNA substrates which contain CpI (representing CpG)

dinucleotides or CpNpG trinucleotides were used to assess the enzymic activity of purified fractions as well as to investigate the substrate specificity of the enzyme(s).

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Fine chemicals

Acrylamide, EDTA, glycerol, N N'-methylenebisacrylamide, phenol, perchloric acid, SDS and trichloroacetic acid (TCA) were supplied by FSA Laboratory Supplies (Fisons), Loughborough, England, U.K.

Bovine serum albumin, coomassie brilliant blue R, ethidium bromide, phenylmethylsulphonylfluoride (PMSF), Tween 20 (polyoxyethylene sorbitan monolaurate), salmon testis DNA, 3-[N-morpholino] propane-sulphonic acid (MOPS) and (poly dI-dC).(poly dI-dC) were supplied by Sigma Chemical Co., Poole, Dorset, England, U.K.

Ammonium persulphate, 4-aminosalicylic acid, bromophenol blue, 8-hydroxyquinoline, mercaptoethanol, N N N'-N'-tetramethylene diamine (TEMED) and the phosphoramidite derivatives for oligonucleotide synthesis with the exception of the methylated cytosine derivative were supplied by BDH Chemicals, Poole, Dorset, England, U.K.

Methylcytosine phosphoramidite derivative was obtained from Glen Research Corp., Herndon, V.A..

Triton-X 100 and *m*-cresol were supplied by Koch-Light Laboratories Ltd, Colnbrook, England, U.K.

Dithiothreitol (DTT) and T4 polynucleotide kinase were supplied by Boehringer Mannheim, East Sussex, U.K.

Heparin Sepharose and Q Sepharose fast flow were supplied by Pharmacia Ltd, Milton Keynes, England, U.K.

123 bp ladder DNA marker was obtained from Gibco BRL.

Peas (*Pisum sativum* var. Feltham First) were obtained from Booker Seeds, Sleaford, U.K.

Coir compost (ICI) was obtained from the local garden centre.

Detection liquids for Enhanced Chemiluminesence, hybond C+ extra and radiochemicals were obtained from Amersham International plc, Amersham, England, U.K.:

Radiochemicals:	Tritium TRK 236	15 Ci/mmole
	Tritium TRA 236	500 mCi/mmole

The above radiochemicals were mixed and diluted to give a specific activity of 4.6 mCi/μmole (1.1 μCi/10 μl).

2.1.2 Buffer M+

MOPS (pH 7.0)	50 mM
EDTA	1 mM
DTT	1 mM
NaN_3	0.01% (w/v)
glycerol	10% (v/v)
PMSF	0.06% (w/v)

2.1.3 SDS stop solution

SDS	1% (w/v)
butanol	5% (v/v)
EDTA	2 mM
p-amino salicylate	3% (w/v)
salmon sperm DNA	0.25 mg/ml
NaCl	0.125 M

2.1.4 SDS mix

SDS	2% (w/v)
EDTA	4 mM
butanol	10% (v/v)

2.1.5 Phenol mix

phenol	88% (v/v)
m-cresol	12% (v/v)
hydroxyquinoline	0.1% (w/v)

2.1.6 Bradford's reagent

Coomassie brilliant blue G	0.1 mg/ml
ethanol	4.75% (v/v)
phosphoric acid	8.5% (v/v)

2.1.7 SDS-PAGE buffers

Resolving gel buffer

Tris–HCl (pH 8.8)	1.5 M
SDS	0.4% (w/v)

Stacking gel buffer

Tris–HCl (pH 6.8)	0.5 M
SDS	0.4% (w/v)

2.1.8 SDS-PAGE electrode buffer (pH 8.3)

Tris	25 mM
glycine	191 mM
SDS	0.1% (w/v)

2.1.9 30% Acrylamide stock

acrylamide	30% (w/v)
NN'-methylenebisacrylamide	0.8% (w/v)

2.1.10 SDS-PAGE sample buffer

Tris–HCl (pH 6.8)	0.1875 M
glycerol	30% (v/v)
SDS	6% (w/v)
mercaptoethanol	15% (v/v)
bromophenol blue	0.1% (w/v)

2.1.11 Agarose gel sample buffer

SDS	1% (w/v)
glycerol	50% (v/v)
bromophenol blue	0.01% (w/v)

2.1.12 TBE buffer

Tris–HCl (pH 8.3)	0.9 M
boric acid	0.9 M
EDTA	0.025 M

2.1.13 Oligonucleotide annealing buffer

Tris (pH 7.6)	67 mM
$MgCl_2$	13 mM
DTT	6.7 mM
spermidine	1.3 mM
EDTA	1.3 mM

2.1.14 Phenol / chloroform

1:1 Phenol/choroform

Equal volumes of phenol and chloroform were mixed and 8–hydroxyquinoline was added to 0.1% (w/v). The mixture was equilibrated by extraction several times with TE buffer. The mixture was then stored under TE buffer in the dark.

TE buffer

Tris–HCL (pH 7.8)	10 mM
EDTA	1 mM

2.1.15 Phosphate buffered saline

Buffer A (pH 7.2)

NaCl	0.17 M
KCl	3.35 mM
Na_2HPO_4	10 mM
KH_2PO_4	1.84 mM

Buffer B

$CaCl_2.6H_2O$	6.8 mM

Buffer C

$MgCl.6H_2O$	4.9 mM

Buffers A, B and C were autoclaved separately and mixed in a ratio of 8:1:1 before use.

2.1.16 10X Imidazole buffer

Imidazole-HCl (pH 6.4)	100 mM
$MgCl_2$	180 mM
DTT	50 mM

2.1.17 10X T4 DNA ligase buffer

Tris–HCl (pH 6.4)	200 mM
$MgCl_2$	50 mM
DTT	50 mM
BSA	500 µg/ml

2.1.18 10X S1 nuclease buffer

Na Acetate (pH 4.5)	5 M
NaCl	2 M
$ZnSO_4$	10 mM
glycerol	5% (v/v)

2.1.19 Nitrocellulose transfer buffer

Tris (pH 7.2)	25 mM
glycine	0.19 M
SDS	0.02% (w/v)
methanol	20% (v/v)

2.1.20 Blotting buffer (TBS-T)

Tris–HCl (pH 7.6)	20 mM
NaCl	137 mM
Tween 20 (added just before use)	0.1% (v/v)

2.1.21 Stripping buffer

Tris–HCl (pH 6.7)	62.5 mM
mercaptoethanol	100 mM
SDS	2% (w/v)

2.1.22 2X SDS sample buffer

Tris–HCl (pH 6.8)	125 mM
mercaptoethanol	10% (v/v)
glycerol	20% (v/v)
SDS	4% (w/v)

2.2 Methods

2.2.1 Growing pea seedlings

Peas were washed, soaked for 3–4 hours and then planted in a closely packed monolayer into a garden tray (22 cm x 34 cm) containing an 1 inch deep layer of "coir compost". The pea seedlings were covered in a thin layer of coir and grown at 26°C under constant white light illumination. They were watered regularly with just sufficient water to keep them moist. The shoots were ready for harvesting after 5–6 days of growth. The 1.5–2.0 cm apical shoot tips were removed, weighed and stored at -20°C.

2.2.2 Preparation of pea nuclei

Pea nuclei were prepared as described by Yesufu *et al.*, 1991. All steps were carried out at 0-4°C. The pea apical shoot tips were homogenised in buffer M^+

pH 7.0 (2.1.2) by grinding in a mortar (2.5 ml buffer per gram material). The homogenate was filtered though 6 layers of muslin and centrifuged at 3000 rpm for 10 minutes, at 4°C in a Beckman bench top centrifuge, model GS-6R. The supernatant was removed. The pellet was resuspended in buffer M^+ using a 5 ml syringe, filtered though 1 layer of muslin and centrifuged as before. The resulting pellet was resuspended in buffer M^+ containing 1% Triton X–100 and left standing on ice for 20 minutes. This causes lysis of contaminating plastids in the nuclear suspension. The nuclei were centrifuged as before and continued to be washed in buffer M^+ containing Triton X–100 until no longer green. The isolated nuclei were stored at -20°C in an equal volume of buffer M^+ containing 50% glycerol.

2.2.3 Extraction of pea nuclei

2.2.3.1 Extract E1

The pea nuclei were stored in an equal volume of buffer M^+ (2.1.2) containing 50% glycerol. The NaCl and glycerol concentrations were adjusted to 0.2 M and approximately 10% respectively by the addition of 0.4 M and 0.2 M NaCl in buffer M^+ which contains no glycerol. The nuclei were mixed and left on ice for 30 minutes with occasional mixing. They were then centrifuged for 10 minutes, 3000 rpm at 4°C in a Beckman bench top centrifuge, model GS–6R. The resulting supernatant is referred to as E1. The salt extracted nuclei were resuspended in buffer M^+ containing 50% glycerol and were used for extract E2.

2.2.3.2 Extract E2

The salt extracted nuclei (2.2.3.1) were resuspended in buffer M^+ (2.1.2) (800 µl buffer per 200 µl nuclear pellet) and then incubated with micrococcal nuclease (400 Units/ml) and $CaCl_2$ (1 mM) for 10 minutes at 37°C. The reaction

was stopped with EGTA (2 mM). The partially digested nuclei were reextracted with 0.2 M NaCl (1 ml per 200µl of nuclear pellet). The reaction mixture was left on ice for 30 minutes and then centrifuged at 3000 rpm for 10 minutes at 4°C in a Beckman bench top centrifuge, model GS–6R. The resulting supernatant is referred to as E2.

Extracts E1 and E2 were dialysed overnight at -20°C against buffer M^+ containing 50% glycerol. After dialysis they were stored at -20°C with little loss of enzymic activity. If the extracts were to undergo purification procedures (2.2.13) they were not dialysed but used directly.

2.2.4 Assay for protein concentration

The protein concentrations in all samples were assayed by Bradford's method (Bradford, 1976). Bovine serum albumin was used as standard protein.
BSA (0–50 µg) was taken in 100µl of buffer M^+ and 1 ml Bradford's Reagent (2.1.6) was added, mixed and allowed to stand for 5 minutes. The absorbance at 595 nm was measured in a Cecil 2000 UV Spectrophotometer and used to plot a standard curve. 10 µl or 20 µl samples of pea DNA methylase fractions were diluted to 100 µl with buffer M^+ and 1 ml Bradford's Reagent was added, mixed and left to stand for 5 minutes and the absorbance measured at 595 nm. The protein concentration was estimated from the standard curve.

2.2.5 SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). A 8.75% resolving gel was prepared using 10 ml Resolving gel buffer (2.1.7), 11.7 ml 30% acrylamide stock (2.1.9), 18 ml dH_2O, 200 µl 10% ammonium persulphate and 50 µl TEMED which was added last. The

solution was mixed and poured into a pair of clamped gel plates. A thin layer of 1% SDS was layered onto the gel to allow it to set. A 6% stacking gel was prepared using 2.5 ml stacking gel buffer (2.1.7), 2.0 ml 30% acrylamide stock, 5.5 ml dH_2O, 150 µl 10% ammonium persulphate and 5 µl TEMED which again was added last. The layer of SDS was removed and the stacking gel mixed and poured on top of the resolving gel. The well–forming comb was inserted immediately and was removed as soon as the gel had polymerised. Samples were incubated with 0.5 volumes SDS–PAGE sample buffer (2.1.10) for 5 minutes in a boiling water bath and subjected to electrophoresis at 30–40 mA (<200 V). The electrode buffer was 25 mM Tris/glycine buffer (2.1.8) and protein markers (myosin 200 kDa, phosphorylase B 97.4 kDa, bovine serum albumin 69 kDa, ovalbumin 46 kDa, carbonic anhydrase 30 kDa, trypsin inhibitor 21.5 kDa and lysozyme 14.3 kDa) of known molecular weights were used. After electrophoresis the gel was either washed in 50% methanol overnight for silver staining (2.2.7) or was stained with Coomassie blue (2.2.8).

2.2.6 Agarose gel electrophoresis

A 1.5% agarose gel was prepared using 0.45 g agarose, 30 ml TBE buffer (2.1.12) and 1 µl ethidium bromide (10 mg/ml). The well–forming comb was inserted immediately and was removed as soon as the gel had set sufficiently. Samples were incubated with 0.2 volumes agarose gel sample buffer (2.1.11) for 5 minutes at 60°C and subjected to electrophoresis at 50 V. The electrode buffer was 1 x TBE and a 123 bp ladder was used as a molecular weight marker.

2.2.7 Silver staining of protein gels

The gel was fixed in 50% methanol overnight before treatment with solution C.

This solution consisted of solution A (0.8 g of $AgNO_3$ in 4 ml dH_2O) added dropwise whilst vigorously stirring to solution B (21 ml of 0.36% NaOH with 1.4 ml 14.8 M NH_4OH) until the saturation point was reached. Solution C was made up to 100 ml with distilled H_2O and used immediately to wash the gel for 10–15 minutes with gentle agitation at room temperature. The gel was then washed for 1–2 hours in distilled H_2O, again with gentle agitation. It was then washed with solution D (2.5 ml of 1% citric acid and 0.25 ml of 38% formaldehyde), made up to 500 ml with distilled H_2O and used immediately. The bands developed within 10 minutes and the gel was photographed immediately using a Polaroid C-U5 camera or imager. Development was stopped by washing in H_2O and then in 50% methanol.

2.2.8 Coomassie brilliant blue R staining of protein gels

The gel was immersed in Coomassie stain [0.1% (w/v) Coomassie brilliant blue R in 50% (v/v) methanol and 10% (v/v) acetic acid] for 1 hour at 37°C. The excess dye was poured off and the gel immersed in destain (10% glacial acetic acid and 10% methanol) overnight at room temperature and photographed with a polaroid camera or imager.

2.2.9 Extraction of native pea DNA

Pea nuclei were prepared as described in section 2.2.1. Nuclei from 100 g peas were suspended in 3 ml phosphate buffered saline prepared as in section 2.1.15 and 3 ml SDS mix (2.1.4). They were then incubated with proteinase K (50 µg/ml) for 1 hour at 37°C. An equal volume of phenol/chloroform (1:1) (2.1.14) was added to the nuclei. After gentle inversion, the mixture was centrifuged at 3000 rpm for 20 mins at room temperature in a Beckman bench top centrifuge, model TJ–6. The

supernatant was removed and the nuclei were again treated with phenol/choroform, centrifuged and the supernatant removed. The DNA was precipitated with 2 volumes of absolute ethanol precooled to -20°C. The DNA was washed twice in 70% ethanol and left to air dry before being dissolved in 1 ml 50 mM KCl. DNA concentration was estimated by reading the absorbance at 260 nm.

2.2.10 DNA methylase assay

DNA methylase activity was assayed in a reaction mixture containing 10 µl S-adenosyl-L-[methyl-^{3}H] methionine (1.1 µCi at 4.64 Ci/mmole), 5 µl BSA, 15 µl buffer M^+ (2.1.2) and 10 µl appropriate DNA. The sample to be assayed was made up to 30 µl with buffer M^+ so that the final volume was 70 µl. After incubation at 30°C for 2 hours the reaction was stopped by the addition of 500 µl of SDS stop solution (2.1.3). The DNA was recovered by incubating the reaction mixture with 100 µg proteinase K for 10 minutes at 37°C followed by vortexing with 300 µl Phenol Mix (2.1.5) and then centrifugation at 14000 rpm for 5 minutes in a microfuge at room temperature. The aqueous layer was removed and thoroughly mixed with 2 volumes of ethanol. The DNA was pelleted by centrifugation at 14000 rpm for 5 minutes in a microfuge, the ethanol poured off and the DNA pellets left to dry. The DNA was redissolved in 50 µl 0.3 M NaOH for 1–2 hours at 37°C and then spotted onto filter paper squares (Whatman 3MM, 2.5 cm^2). The DNA was precipitated by washing 5 times with 5% TCA at 4°C for 10 minutes. The filters were then washed twice with ethanol and once with ether and dried under a stream of air before being transferred to scintillation vials. The DNA was solubilised in 500 µl of 0.5 M perchloric acid (PCA) at 60°C for 60 minutes. Ecoscint scintillation fluid (5 ml) was added to each vial and ^{3}H incorporation counted on a LKB 1209 Rackbeta liquid scintillation counter.

DNA methylase assay was performed according to the method of Turnbull and Adams (1976).

2.2.11 Oligonucleotide synthesis and annealing

Oligonucleotides were synthesized in the department of Biochemistry, University of Glasgow using an Applied Biosystems Machine. They were trinucleotide CpNpG oligonucleotides with the sequences $(CAG)_7(CTG)_7$ and $(CAG)_7(mCTG)_7$ where mC indicates a methylated cytosine residue. Both oligonucleotides were 21 mers. Following deprotection and precipitation with 3 volumes absolute ethanol and one tenth volume 3 M ammonium acetate, the oligonucleotides were redissolved in annealing buffer (2.1.13) and stored at -20°C for future use. When required the oligonucleotides were mixed in equivalent amounts for annealing. This was done by heating the mixture at 88°C for 2 min, 65°C for 5 min, 37°C for 5 min and at room temperature for 5 min. The amount of DNA present was estimated by recording the absorbance at 260 nm. The concentration was adjusted accordingly to 1 mg/ml and the annealed oligonucleotides stored at -20°C.

Hemimethylated molecules containing the dinucleotide target sequence (polydI-dC).(poly dI-dC/dmC) synthesized by annealing oligonucleotides (poly dI-dC).(poly dI-dC) and (poly dI-dC/dmC).(poly dI-dC/dmC) were found to be inactive as substrates for the pea DNA methylase (3.1.1) and so a substrate was made using *E. coli* DNA polymerase (Klenow fragment) by the method of Giordano (1991), except that DTT (1 mM) was present in the incubation. This hemimethylated (poly dI-dC).(poly dI-dC/dmC) was found to be an acceptable substrate (3.1.1).

2.2.12 Ligation of oligonucleotides

Synthetic oligonucleotides are synthesized without 5' phosphates. They were phosphorylated by end labelling using 'cold' ATP as follows: 14 µg of either hemi- or unmethylated CpNpG oligonucleotides were mixed with 80 µl 10 x imidazole

buffer (2.1.16), 200 µl PEG, 75 µl ATP (3 mM) and 1.2 units T4 polynucleotide kinase in a final volume of 380 µl. The mixture was incubated at 37°C for 30 min, warmed to 45°C for 5 min and then put on ice to chill.

Once phosphorylated, the DNA was ligated as follows: 70 µl 10 x T4 ligase buffer (2.1.17), 100 µl ATP (3mM) and 7 units T4 ligase were added to the above mixture and incubated for 3 hours at 16°C. The mixture was then split into two equal portions. One portion was treated with S1 nuclease as follows: 31 µl 10 x S1 nuclease buffer (2.1.18) and 400 units S1 nuclease were added and the mixture incubated at 37°C for 30 min.

Both ligated and ligated/S1 nuclease treated DNAs were recovered after phenol/choroform extraction (2.1.14). Extractions with phenol/chloroform were performed as follows: an approximately equal volume of phenol/chloroform was added to each of the DNA solutions to be extracted which were mixed and centrifuged for 5 min at 14000 rpm in a microfuge. The upper aqueous layer was transferred to a fresh tube and the DNA ethanol precipitated as follows: 3 M ammonium acetate (1/10 th volume of DNA solution) and 2 volumes cold absolute ethanol were added to the incubation mix and the DNA precipitated at -70°C for 2 hours. The DNA was pelleted by centrifugation at 14000 rpm for 10 min at 4°C in a microfuge and then washed twice in cold absolute ethanol, centrifuged as before and then left to dry at room temperature. The DNA pellet was redissolved in an appropriate volume of 50 mM KCl to give a DNA concentration of 0.1 mg/ml.

2.2.13 Purification of nuclear extracts, E1 and E2

2.2.13.1 Elution of methylase from Q Sepharose

Q Sepharose was washed twice with 0.6 M NaCl in buffer M^+ (2.1.2) and then twice with buffer M^+ containing no NaCl. The Q Sepharose was then resuspended in buffer M^+ producing a 25% suspension. This suspension was mixed with

various salt solutions of known concentration and an equal volume of E1 and E2 was added to each. These samples were mixed on ice for 20 min and centrifuged at 4°C, 13000 rpm for 5 min in a microfuge. The supernatants were dialysed and assayed for methylase activity as described in section 2.2.10.

2.2.13.2 Heparin Sepharose and Q Sepharose

Heparin Sepharose was washed in buffer M^+ (2.1 2) containing 0.2 M NaCl and mixed with nuclear extract (1.5 ml heparin Sepharose/mg protein) for 30 minutes on ice. The mixture was then centrifuged for 10 minutes at 3000 rpm at 4°C in a Beckman bench top centrifuge, model GS–6R. The supernatant was removed and saved for dialysis and assay. The heparin Sepharose pellet was resuspended in an appropriate volume of 0.2 M NaCl in buffer M^+ and the column poured by gravity settling at 4°C. A 10 ml NaCl gradient (0.2–0.7 M) was applied to the column at 10 ml/h and 1 ml fractions were collected. The optical density of these fractions at 280 nm was recorded and fractions 3–6 pooled after previously establishing that they had the highest level of methylase activity. The NaCl concentration of this pool was estimated to be 0.45 M. This was adjusted to 0.15 M with buffer M^+ as this had been found to be the required salt concentration for binding of the enzyme to Q Sepharose (4.3). At this stage a 1 ml sample of the diluted pool was set aside for dialysis and assay. The remainder of the pool was mixed with Q Sepharose (2 ml/ mg protein) and stirred on ice for 30 minutes. It was then centrifuged for 10 minutes at 3000 rpm at 4°C as before and the supernatant collected and saved for dialysis and assay. The Q Sepharose pellet was resuspended in a small volume of 0.15 M NaCl in buffer M^+ and the column poured by gravity settling at 4°C. A 10 ml NaCl gradient (0.15–0.4 M) was applied to the column at 10 ml/h and 1 ml fractions collected. The optical densities of these fractions at

280 nm were recorded. All samples to be assayed were dialysed against buffer M^+ containing 50% glycerol. They were assayed as described in section 2.2.10.

2.2.13.3 Modification of method 2.2.13.2.

Heparin Sepharose was mixed with E1 for 30 min on ice and then centrifuged at 4000 rpm, 4°C for 10 min in a Beckman bench top centrifuge, model GS–6R. The supernatant was removed and saved for assay while the pellet was washed twice in 0.25 M NaCl in buffer M^+ (2x vol E1) and was centrifuged as before. The supernatants were pooled and saved. The resulting pellet was washed twice in 0.7 M NaCl in buffer M^+ (0.5x vol E1), centrifuged and the supernatant diluted so that the salt concentration was 0.15 M. This supernatant was then mixed with 1 ml Q Sepharose and the purification procedure continued as in 2.2.13.2.

2.2.13.4 Gel filtration and Q Sepharose

A Superose 6 gel filtration column was cooled with iced water and equilibrated with buffer M^+ containing 0.15 M NaCl for 2 hours. Extract E1 was prepared as described in section 2.2.3.1 and was concentrated using centricon tubes by centrifugation at 2000 rpm for 10 min and then for 30 min at 4°C. The removed liquid was saved for dialysis and assay. The concentrated E1 sample (1–2 ml) was applied to the column which was eluted with buffer M^+ containing 0.15 M NaCl. 2 ml fractions were collected on ice and their optical density at 280 nm recorded. A peak of optical density was observed. A 1 ml sample of each fraction forming this peak was saved for dialysis and assay. The remainder of these fractions were pooled and mixed with 1.5 ml Q Sepharose for 30 min on ice. The purification procedure was then continued as described in section 2.2.13.2 except that a NaCl gradient of 0.15–0.5 M was applied to the Q Sepharose column.

2.2.14 Transfer of proteins to nitrocellulose: Western Blotting

A sheet of nitrocellulose impregnated nylon membrane (Hybond C^+ extra), cut to the same size as the gel, was soaked in transfer buffer (2.1.19) and placed on top of the gel which had been placed on a sheet of Whatman 3MM paper presoaked in transfer buffer. Care was taken to ensure there were no air bubbles between the gel and membrane. A second sheet of filter paper, again presoaked in transfer buffer was placed on top of the membrane and everything was sandwiched between two sponge pads soaked in transfer buffer and placed in a perspex cassette. The cassette was placed in a BioRad transblot tank filled with transfer buffer so that the nitrocellulose sheet was facing the cathode. The electrophoretic blotting was carried out at 40-60 mA overnight.

2.2.15 Enhanced Chemiluminesence (ECL)

Enhanced Chemiluminesence was carried out according to the manufacturers protocol. The electrophoretic blot was washed in blotting buffer containing 5% dried milk (2.1.20) for 1 hour with continuous shaking at room temperature to block non-specific binding to the membrane. The membrane was rinsed twice, then once for 15 minutes and then twice for 5 minutes with blotting buffer. The membrane was incubated with 5% normal goat serum and the primary antibody at a dilution of 1 in 2000 for 1 hour at room temperature and then washed with blotting buffer as before. The membrane was then incubated for 1 hour with 0.1% of the secondary antibody linked to horseradish peroxidase at room temperature and again washed with blotting buffer as above. The chemiluminesence was detected after the addition of an equal volume (final volume 0.125 ml/cm^2) of detection liquids 1 and 2 supplied by Amersham. The membrane was washed for 1 minute in the detection liquids in the dark room, wrapped in clingfilm, exposed to hyperfilm–ECL for 10 seconds and the film developed. The exposure time can be varied as required.

CHAPTER 3

Characterisation of Pea DNA Methylase

3.1 Sequence specificity

Plant DNA methylation differs from that found in mammals in that cytosines in the trinucleotide CpNpG are methylated as well as those in the dinucleotide CpG. This raises the possibility of there being more than one methylase enzyme.

In the following experiments only extract E1 (2.2.3.1) was used to study sequence specificity using synthetic oligonucleotides, as extract E2 (2.2.3.2) contains some endogenous native pea DNA (4.2.3). Extract E1 is a crude nuclear extract and should therefore contain a full complement of nuclear enzymes.

Enzyme affinity for CpNpG target sites was studied using the 21 mers $(CAG)_7(CTG)_7$ and $(CAG)_7(mCTG)_7$. A single stranded oligonucleotide $(CAG)_7$ was also studied. CpG target sites were represented by repeating (poly dI-dC).(poly dI-dC) and (poly dI-dC).(poly dI-dC/mC) sequences. In these oligonucleotides dG was replaced by dI as the bonding between a chain of (poly dG-dC).(poly dG-dC) is so strong as to prevent action of the methylase enzyme (Pfeifer and Drahovsky, 1986; Wells *et al.*, 1970). In all the oligonucleotides referred to, mC indicates a methylated cytosine and therefore the $(CAG)_7(mCTG)_7$ duplex is hemimethylated. In this way both maintenance and *de novo* methylation can be investigated using the above oligonucleotides.

Initially (poly dI-dC).(poly dI-dC/mC) was made by annealing oligonucleotides to (poly dI-dC).(poly dI-dC) (section 2.2.11) but this resulted in low levels of methylation when used as a substrate. The accepting strands of the annealed oligonucleotides contain alternating CI residues while the 'template' strands contain either alternating mCG or mCI residues. This results in a high concentration of alternating methylcytosines and is thought to render the duplex resistant to the partial denaturation which appears to be necessary for enzyme interaction (Adams *et al.*, 1979). Alternatively, the presence of a methyl group in an alternating sequence may cause the DNA to assume a left handed helical conformation (Van Lier *et al.*, 1983), in which form it is less able to accept methyl groups (Smith *et al.*, 1991).

Therefore, (poly dI-dC).(poly dI-dC/mC) was prepared by polymerase action (2.2.11). When made by this method it was found that (poly dI-dC).(poly dI-dC/dmC) was a good substrate for the enzyme and it was therefore used to investigate substrate specificity of the methylase enzyme.

3.1.1 Comparison of methylase activity with different DNA substrates

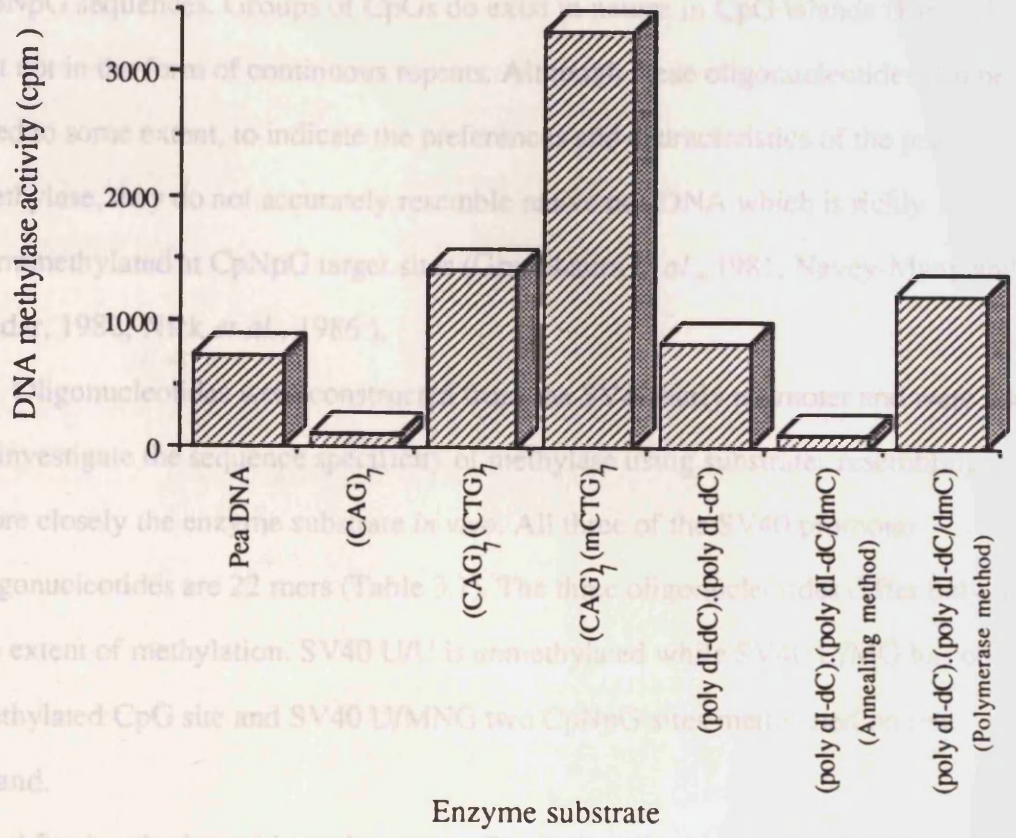
Extract E1 was incubated with various substrate DNAs and assayed for methylase activity as described in section 2.2.10. High levels of methylase activity were observed with all synthetic oligonucleotide substrates when compared to pea DNA, with the exception of (poly dI-dC).(poly dI-dC), single stranded $(CAG)_7$ and (poly dI-dC).(poly dI-dC/dmC) (annealing method) (Figure 3.1).

Single stranded $(CAG)_7$ DNA has been found to interfere with the methylation of duplex DNA. When both are present, only 27% of the activity obtained with double stranded DNA alone is recorded. It is possible that either the enzyme has a strong affinity for single stranded DNA, although it does not methylate it, or the single stranded DNA interacts with the hemimethylated duplex to form a structure that is not recognized by the enzyme.

In the case of CpNpG target DNA, with the hemimethylated substrate at least a two-fold increase in enzyme activity was observed, compared to that recorded with an unmethylated substrate. The highest level of enzymic activity overall was observed with the hemimethylated trinucleotide substrate. This and the unmethylated dinucleotide (poly dI-dC).(poly dI-dC) were used to assay methylase activity so that maintenance and *de novo* methylation could be investigated as well as enzyme specificity for CpG and CpNpG target sites.

Figure 3.1

Comparison of methylase activity with different DNA substrates



Extract E1 containing a total of 5 µg protein was incubated with 1 µg of the indicated substrate DNAs for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor. Each point represents the mean of two experiments.

3.1.2 Methylation of oligonucleotides derived from the SV40 early promoter

The oligonucleotides previously mentioned have all consisted of repeating units of either (poly dI-dC).(poly dI-dC) representing CpG target sequences or repeating CpNpG sequences. Groups of CpGs do exist in nature in CpG islands (Bird, 1986) but not in the form of continuous repeats. Although these oligonucleotides can be used to some extent, to indicate the preferences and characteristics of the pea methylase, they do not accurately resemble native pea DNA which is richly hemimethylated at CpNpG target sites (Gruenbaum *et al.*, 1981; Navey-Many and Cedar, 1982; Nick *et al.*, 1986).

Oligonucleotides were constructed from the SV40 early promoter and were used to investigate the sequence specificity of methylase using substrates resembling more closely the enzyme substrate *in vivo*. All three of the SV40 promoter oligonucleotides are 22 mers (Table 3.1). The three oligonucleotides differ only in the extent of methylation. SV40 U/U is unmethylated while SV40 U/MG has one methylated CpG site and SV40 U/MNG two CpNpG sites methylated on one strand.

After incubation with crude extract, E1, as described in section 2.2.10, a two fold difference in the level of methylation was observed between the unmethylated and hemimethylated SV40 oligonucleotide substrates (Figure 3.2). The highest level of methylation was obtained with the hemimethylated oligonucleotides and no significant difference in the level of methylation was observed between the SV40 U/MG and SV40 U/MNG oligonucleotides. This is consistent with previous results that indicate that pea DNA methylase has a preference for hemimethylated over unmethylated DNA and uses both CpNpG and CpG target sites.

Table 3.1

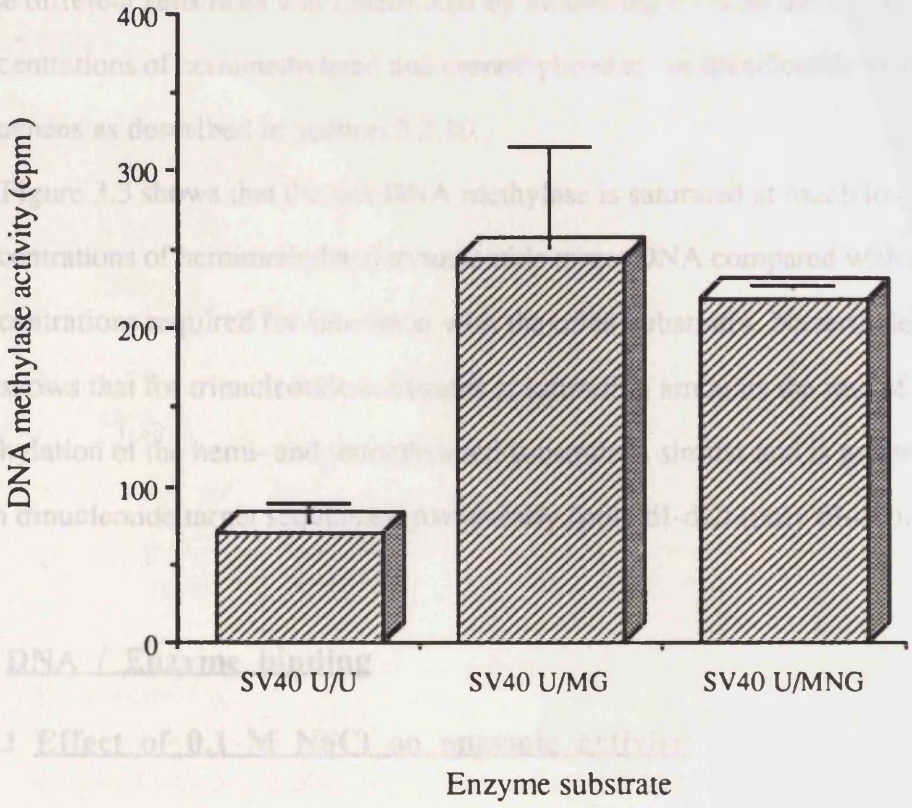
SV40 oligonucleotide sequences

SV40 oligonucleotide	Sequence
SV40 U/U	ATT CTC CGC CCC ATG GCT CAG T
SV40 U/MNG	ATT CT**C** CGC CCC ATG G**C**T CAG T
SV40 U/MG	ATT CTC **C**GC CCC ATG GCT CAG T

The sequence of the C–rich strand is given. The G–rich strand is complementary but unmethylated. **C** indicates a methylated cytosine residue.

Figure 3.2

Methylase activity with SV40 derived oligonucleotides



Extract E1 containing a total of 12 µg protein was incubated with 1 µg of the indicated SV40 oligonucleotide DNAs for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor. A blank value of 31.5 cpm was subtracted from the values obtained. The error bar represents one standard deviation.

3.2 Affinity of enzyme for target sites

It has been shown that the methylase enzyme methylates hemimethylated DNA to a greater extent than unmethylated DNA (3.1.1). The affinity of the enzyme for these different substrates was determined by incubating E1 with increasing concentrations of hemimethylated and unmethylated tri- or dinucleotide target DNA sequences as described in section 2.2.10.

Figure 3.3 shows that the pea DNA methylase is saturated at much lower concentrations of hemimethylated trinucleotide target DNA compared with the concentrations required for saturation with the other substrates. Nevertheless, Table 3.2 shows that for trinucleotide substrates at saturating amounts the rate of methylation of the hemi- and unmethylated substrate is similar and is greater than with dinucleotide target sequences, particularly (poly dI-dC).(poly dI-dC).

3.3 DNA / Enzyme binding

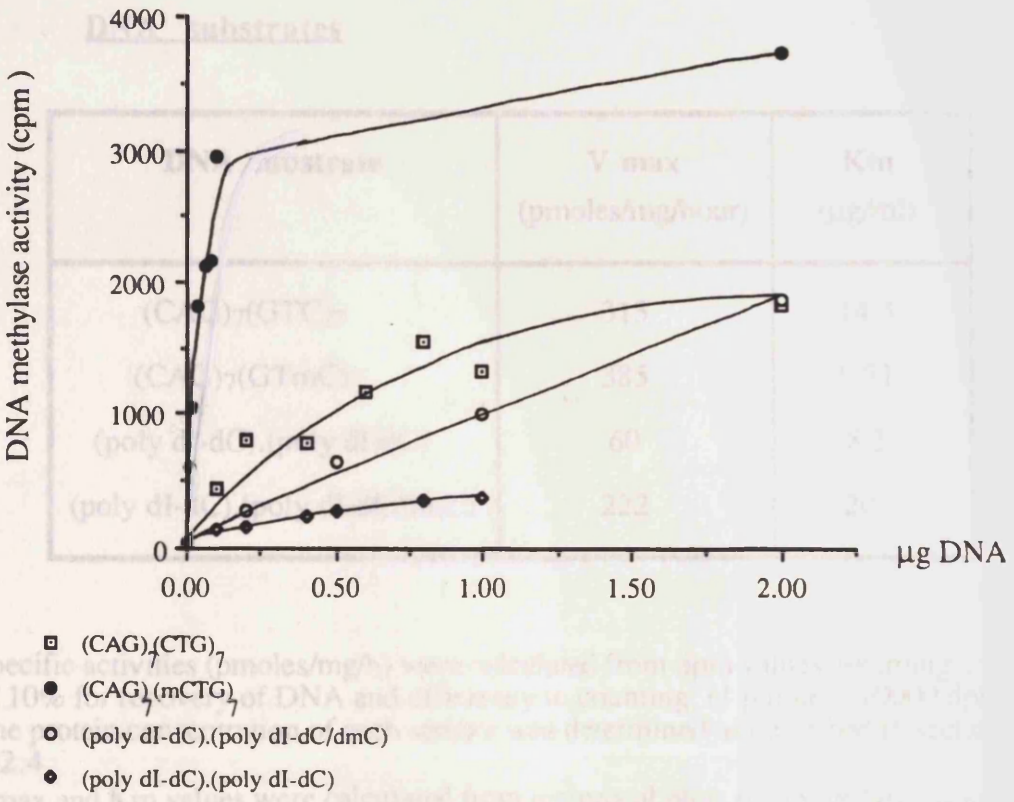
3.3.1 Effect of 0.1 M NaCl on enzymic activity

The addition of 0.1 M NaCl before a DNA–enzyme complex has formed has been found to inhibit the methylation reaction with native pea DNA (Yesufu *et al.*, 1991).

Figure 3.4 shows the effect of adding 0.1 M NaCl 10 min after the start of the incubation (2.2.10). Methylation of unmethylated $(CAG)_7(CTG)_7$ target DNA is found to be inhibited almost immediately after the addition of NaCl while there is no inhibition of methylation of hemimethylated $(CAG)_7(mCTG)_7$ target DNA over the full incubation period of 120 min. Methylation of unmethylated (poly dI-dC).(poly dI-dC) is inhibited after 50 min following addition of NaCl while methylation of hemimethylated (poly dI-dC/dmC).(poly dI-dC/dmC) DNA only ceases after 90 min of incubation.

Figure 3.3

DNA concentration curves



Extract E1 containing a total of 5 µg protein was incubated for 2 hours at 30°C with the indicated amounts of hemi- and unmethylated di- and trinucleotide DNAs, in the presence of [^{3}H] SAM as methyl donor.

Table 3.2

V max and Km values of methylase enzyme with different DNA substrates

DNA substrate	V max (pmoles/mg/hour)	Km (μg/ml)
$(CAG)_7(GTC)_7$	313	14.3
$(CAG)_7(GTmC)_7$	385	0.71
(poly dI-dC).(poly dI-dC)	60	8.3
(poly dI-dC).(poly dI-dC/dmC)	222	20

Specific activities (pmoles/mg/h) were calculated from dpm values assuming a value of 10% for recovery of DNA and efficiency in counting. (1 pmole = 10000 dpm). The protein concentration of each sample was determined as described in section 2.2.4.

Vmax and Km values were calculated from reciprocal plots of 1/V vs 1/[s] using the data obtained from figure 3.3 assuming Michaelis Menten kinetics.

Figure 3.4

Effect of 0.1 M NaCl on methylase activity

Key

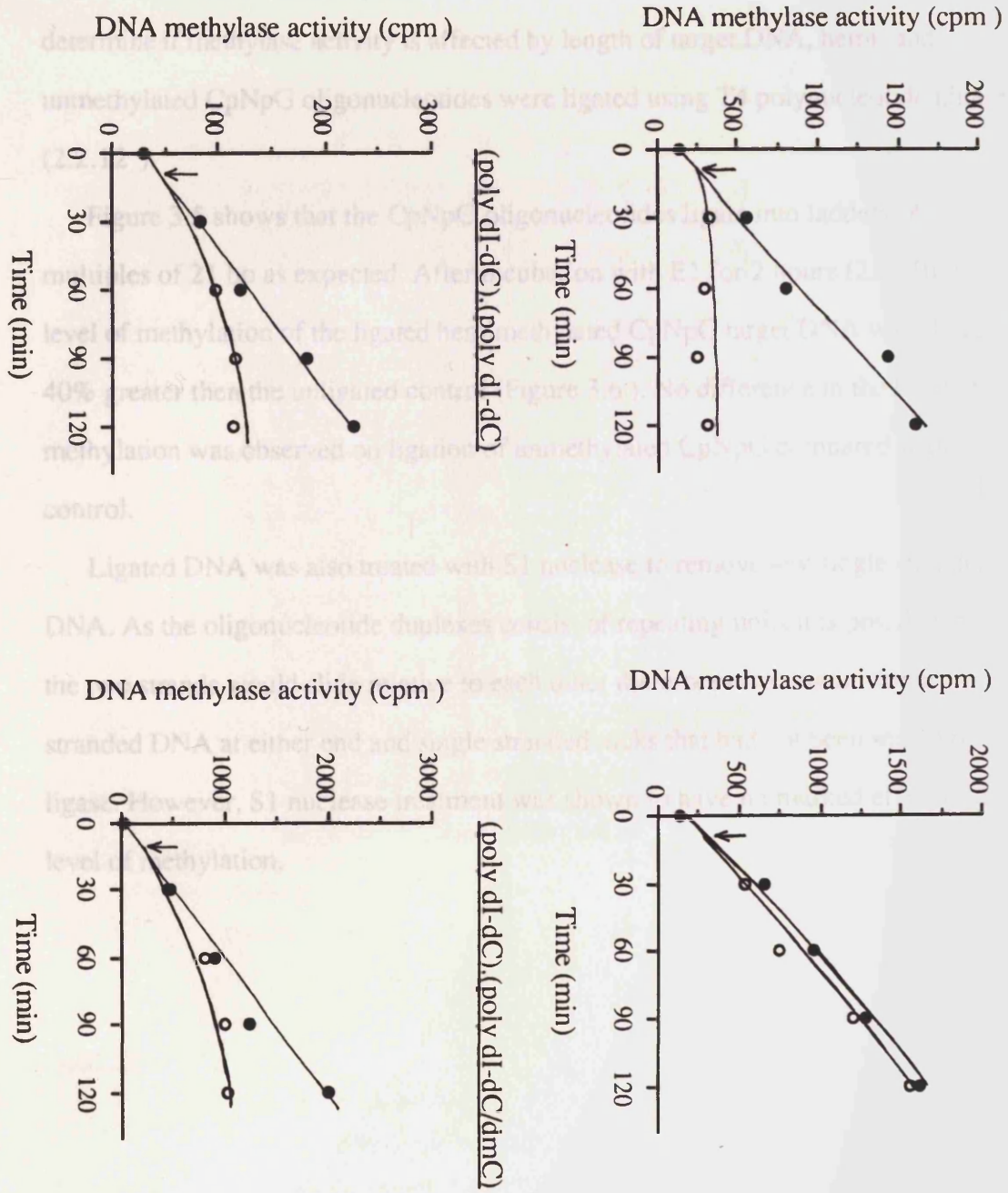
● – 0.1M NaCl

o + 0.1M NaCl added at 10 min as indicated by the arrow.

Extract E1 containing a total of 5 µg protein was incubated with the substrates indicated for 2 hours at 30°C. 10 min into the incubation, 0.1 M NaCl was added to the assay where indicated.

Figure 3.4

Effect of 0.1 M NaCl on methylase activity



3.3.2 Effect of ligation of oligonucleotide substrates on enzyme activity

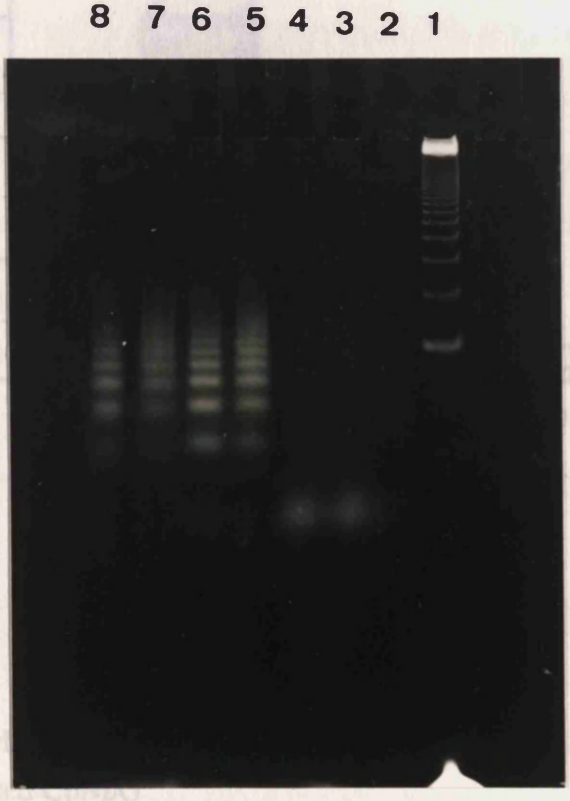
The oligonucleotides used to represent hemi- and unmethylated CpNpG target DNA are 21 mers. Those representing CpG residues are longer in length. To determine if methylase activity is affected by length of target DNA, hemi- and unmethylated CpNpG oligonucleotides were ligated using T4 polynucleotide kinase (2.2.12).

Figure 3.5 shows that the CpNpG oligonucleotides ligate into ladders of multiples of 21 bp as expected. After incubation with E1 for 2 hours (2.2.10) the level of methylation of the ligated hemimethylated CpNpG target DNA was about 40% greater then the unligated control (Figure 3.6). No difference in the level of methylation was observed on ligation of unmethylated CpNpG compared with the control.

Ligated DNA was also treated with S1 nuclease to remove any single stranded DNA. As the oligonucleotide duplexes consist of repeating units it is possible that the two strands would slide relative to each other therefore resulting in single stranded DNA at either end and single stranded nicks that had not been sealed by ligase. However, S1 nuclease treatment was shown to have no marked effect on the level of methylation.

Figure 3.5

Effect of ligation on CpNpG oligonucleotide DNA

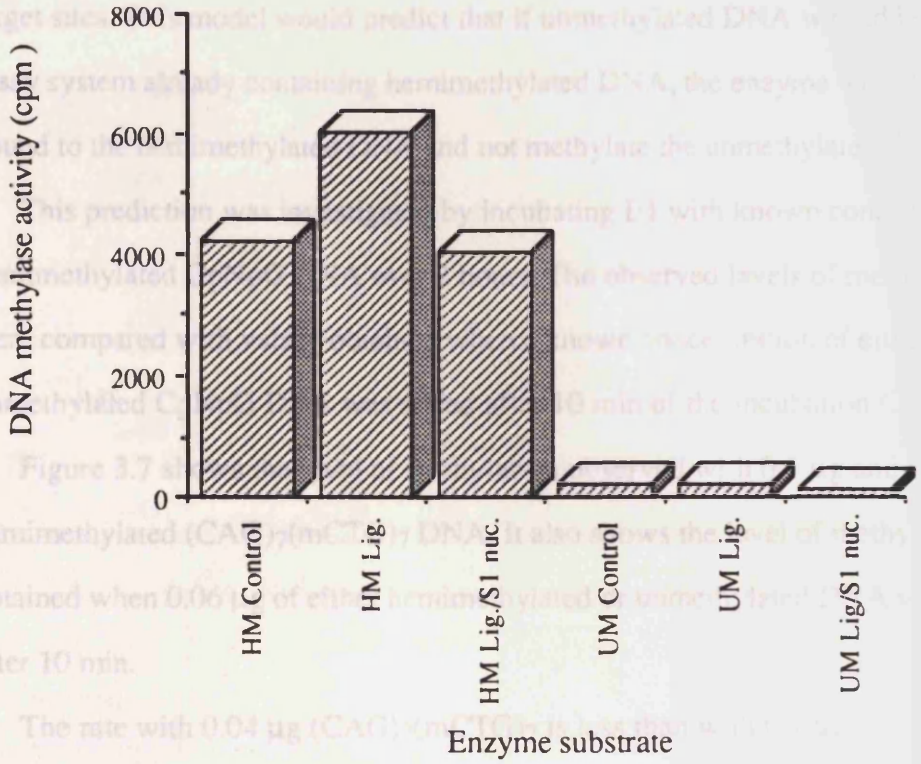


Lane number:

1	123 bp DNA marker
3	Unligated hemimethylated DNA
4	Unligated unmethylated DNA
5	Ligated hemimethylated DNA
6	Ligated unmethylated DNA
7	Ligated and S1 nuclease treated hemimethylated DNA
8	Ligated and S1 nuclease treated unmethylated DNA

Figure 3.6

Comparison of methylase activity on ligation of substrate DNA



Key:

HM: Hemimethylated CpNpG

UM: Unmethylated CpNpG

Lig.: Ligated

S1 nuc.:S1 nuclease treated

Extract E1 containing a total of 30 µg protein was incubated for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor with 0.1 µg of the substrates indicated

3.3.3 Effect of competition with hemi- or unmethylated DNA on levels of methylation

Results indicate that the methylase enzyme shows greater affinity for hemi-methylated than unmethylated DNA and remains bound to it while searching for target sites. This model would predict that if unmethylated DNA was added to an assay system already containing hemimethylated DNA, the enzyme would remain bound to the hemimethylated DNA and not methylate the unmethylated DNA.

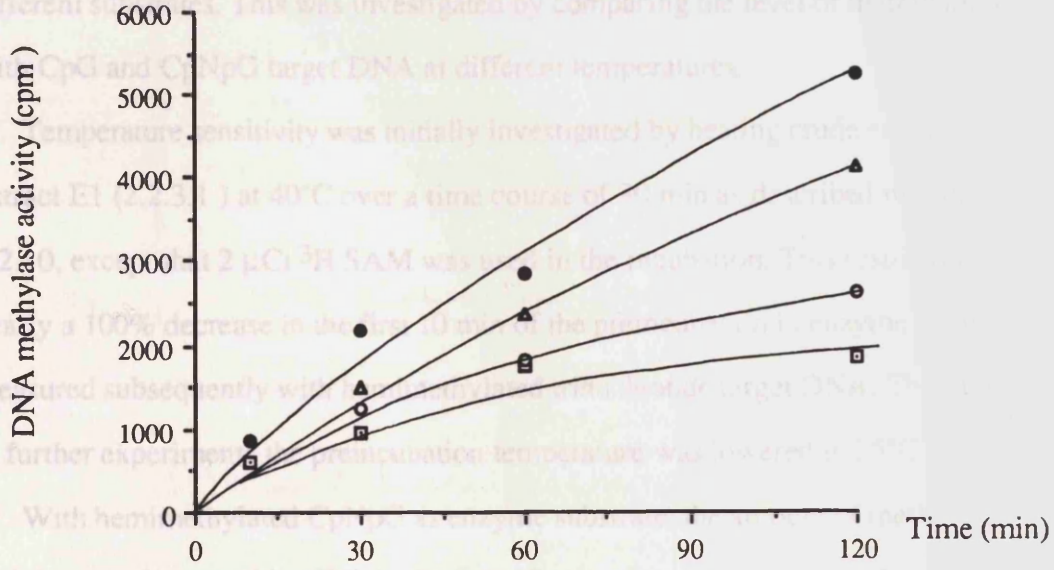
This prediction was investigated by incubating E1 with known concentrations of hemimethylated CpNpG DNA over 2 hours. The observed levels of methylation were compared with values obtained when a known concentration of either hemi- or unmethylated CpNpG DNA was added after 10 min of the incubation (2.2.10).

Figure 3.7 shows the level of methylation observed with 0.1 μg and 0.04 μg hemimethylated $(CAG)_7(mCTG)_7$ DNA. It also shows the level of methylation obtained when 0.06 μg of either hemimethylated or unmethylated DNA was added after 10 min.

The rate with 0.04 μg $(CAG)_7(mCTG)_7$ is less than with 0.1 μg $(CAG)_7(mCTG)_7$ confirming the results shown in Figure 3.3, that the lower amount of DNA is rate limiting. Increasing the amount of hemimethylated substrate from 0.04 μg after 10 min increases the rate to that seen with 0.1 μg indicating that there is free enzyme available to interact with the added DNA. As we have shown, this results in a tight and stable complex. No difference is observed on addition of unmethylated DNA. If the methylase enzyme had dissociated from the initial hemimethylated substrate and interacted to form a tight complex with the unmethylated DNA a decrease in the level of methylation would have been observed as unmethylated $(CAG)_7(CTG)_7$ has been shown to be a poor substrate compared to hemimethylated $(CAG)_7(mCTG)_7$ DNA (Figure 3.1). Therefore, this result is consistent with the idea that once bound to hemimethylated CpNpG DNA the enzyme remains bound.

Figure 3.7

Effect of competition with hemi- or unmethylated DNA on levels of methylation



- ▫ 0.04 µg HM
- ● 0.1 µg HM
- ▲ 0.04 µg HM + 0.06 µg HM
- ○ 0.04 µg HM + 0.06 µg UM

Key:

HM: Hemimethylated CpNpG
UM: Unmethylated CpNpG

Extract E1 containing a total of 5 µg protein was incubated with 0.1 and 0.04 µg hemimethylated CpNpG DNA for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor. After 10 min incubation, 0.06 µg hemi- or unmethylated CpNpG DNA was added to an assay already containing 0.04 µg hemimethylated CpNpG as indicated on the graph. A blank value of 156 cpm was subtracted from the values obtained.

3.4 Effect of temperature on enzyme activity

The pea DNA methylase enzyme has been shown to behave differently depending on whether hemimethylated or unmethylated CpG or CpNpG target sites are presented to it (3.3). This suggests a possibility of there being either more than one methylase enzyme, or one enzyme that exhibits differential activities with different substrates. This was investigated by comparing the level of methylation with CpG and CpNpG target DNA at different temperatures.

Temperature sensitivity was initially investigated by heating crude enzyme extract E1 (2.2.3.1) at 40°C over a time course of 30 min as described in section 2.2.10, except that 2 μCi ^{3}H SAM was used in the incubation. This resulted in nearly a 100% decrease in the first 10 min of the preincubation in enzyme activity measured subsequently with hemimethylated trinucleotide target DNA. Therefore, in further experiments the preincubation temperature was lowered to 35°C.

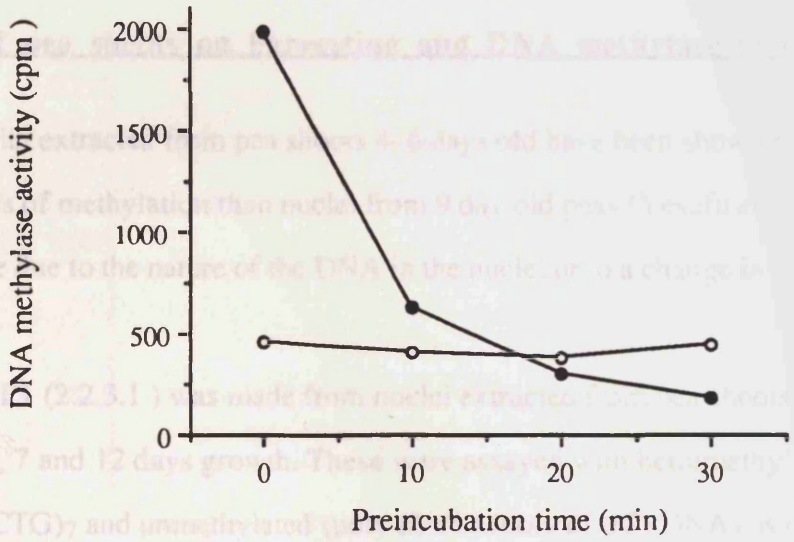
With hemimethylated CpNpG as enzyme substrate, the amount of methylase activity was decreased by 68% in the first 10 min of preincubation and by about 90% after 30 min of preincubation (Figure 3.8). However, no significant decrease was observed in enzyme activity with unmethylated (poly dI-dC).(poly dI-dC) target DNA. It is possible that these observed results were not due to temperature sensitivity of methylase, but to changes in some other constituents of E1 e.g.nucleases. Therefore, the above experiment was repeated with partially purified fractions of the enzyme prepared by ion exchange chromatography as described in section 2.2.13.3.

As with E1, there was a significant difference found when activity was subsequently assayed with the two different substrates. With hemimethylated CpNpG there was a decrease of 80% in enzyme activity in the first 10 min of the preincubation and after 30 min of preincubation a fall of 89%. No change in activity was seen with (poly dI-dC).(poly dI-dC). In these experiments the enzyme was preincubated in the absence of substrate (either DNA or SAM),

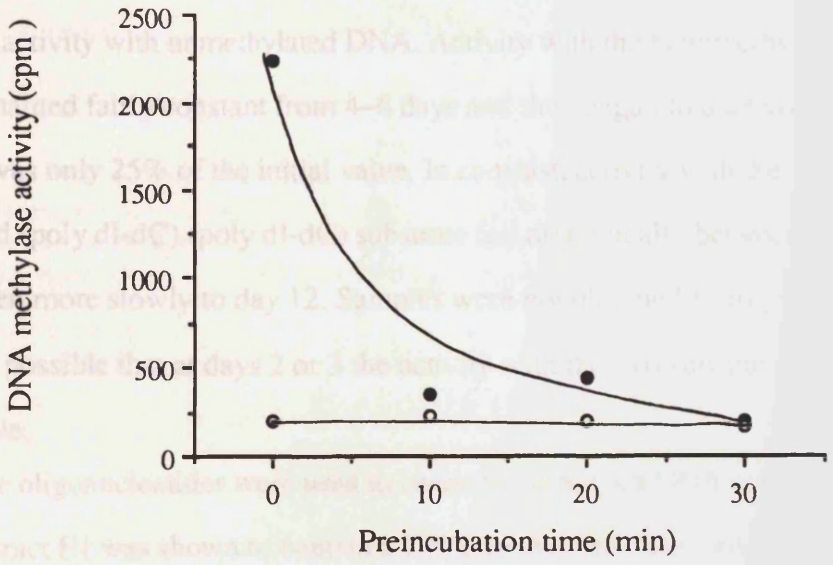
Figure 3.8

Effect of temperature on enzyme activity

a) With Extract E1



b) With partially purified enzyme fraction



- ● Hemimethylated $(CAG)_7(mCTG)_7$
- o Unmethylated (poly dI-dC).(poly dI-dC)

Extract E1 containing a total of 6 µg protein or partially purified enzyme containing 8 µg protein were incubated at 35°C for 0–30 min. They were then incubated with 1 µg $(CAG)_7(mCTG)_7$ or 2 µg (poly dI-dC).(poly dI-dC) for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor

whereas on incubation in the assay (in the presence of substrates) there is no loss of activity after 2 hours at 30°C (Figure 3.4).

3.5 Age of pea shoots on harvesting and DNA methylase activity

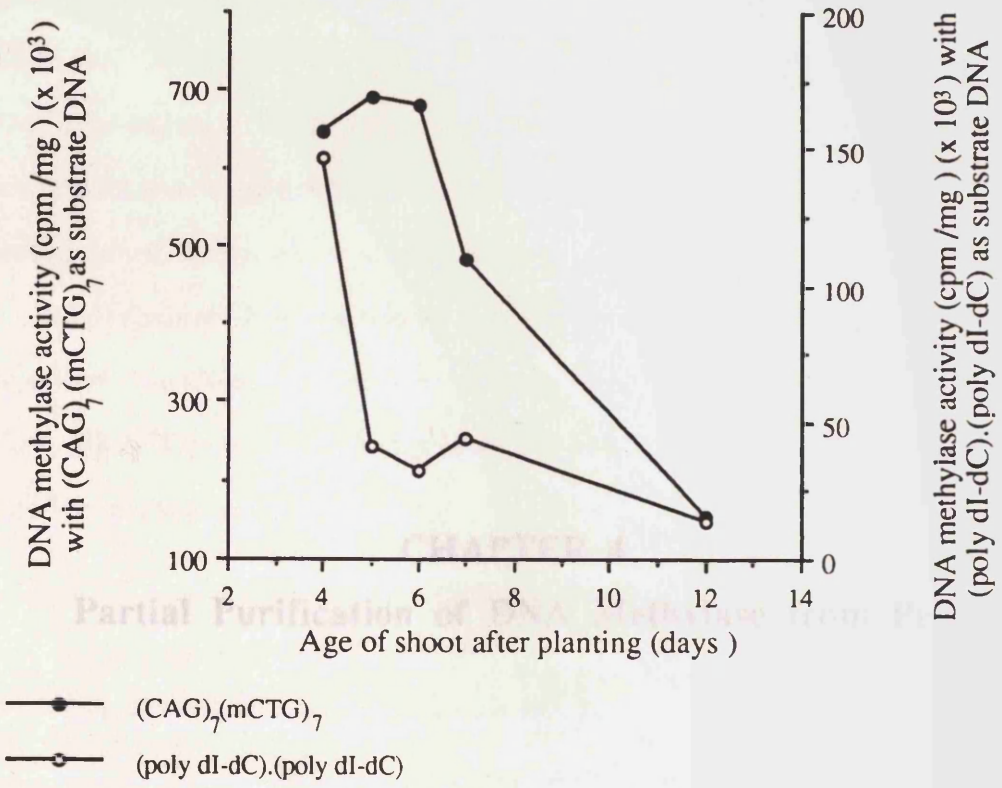
Pea nuclei extracted from pea shoots 4–6 days old have been shown to exhibit higher levels of methylation than nuclei from 9 day old peas (Yesufu *et al.*, 1991). This may be due to the nature of the DNA in the nuclei or to a change in the enzyme.

Extract E1 (2.2.3.1) was made from nuclei extracted from pea shoots harvested after 4, 5, 6, 7 and 12 days growth. These were assayed with hemimethylated $(CAG)_7(mCTG)_7$ and unmethylated (poly dI-dC).(poly dI-dC) DNAs as described in section 2.2.10. Figure 3.9 shows how methylase activity decreased with age of the pea shoots. However, activity with hemimethylated DNA decreased at a slower rate than did activity with unmethylated DNA. Activity with the hemimethylated substrate remained fairly constant from 4–6 days and then began to decrease so that at day 12 it was only 25% of the initial value. In contrast, activity with the unmethylated (poly dI-dC).(poly dI-dC) substrate fell dramatically between days 4 and 5 and then more slowly to day 12. Samples were not obtained from younger peas but it is possible that at days 2 or 3 the activity with the two substrates might be comparable.

Synthetic oligonucleotides were used to characterise the pea DNA methylase enzyme. Extract E1 was shown to contain a DNA methylase(s) that methylates both CpG and CpNpG sites with a preference for hemimethylated DNA. It would appear that the enzyme(s) has a higher affinity for DNA with a hemimethylated trinucleotide target sequence and forms a more stable complex with this substrate than with the other DNAs investigated. The different behaviour of the enzyme with DNAs with a hemimethylated CpNpG or unmethylated CpG target sequence on heat treatment suggests the possibility of there being two different enzymes. This will be discussed further in Chapter 4.

Figure 3.9

Age of pea shoots and DNA methylase activity



Extract E1 was prepared from nuclei extracted from peas harvested on different days as indicated. E1 (30 µl) was then incubated with 1 µg hemimethylated CpNpG and 2 µg unmethylated (poly dI-dC).(poly dI-dC) DNA for 2 hours at 30°C with [^{3}H] SAM present as methyl donor.

CHAPTER 4

Partial Purification of DNA Methylase from Pea

4.1 Introduction

Previous attempts to purify DNA methylase from pea have been carried out (Yesufu *et al.*, 1991). One peak of enzymic activity was obtained by gel filtration on Superose 6 and by sequential elution from DEAE–Sephacel and Mono Q with 0.2 M NaCl and 0.35 M NaCl respectively. This peak fraction was run on a SDS–PAGE gel and on silver staining a major band of 160 kDa was observed. However, this protein was found to be unstable and to be degraded when subjected to a second gel electrophoresis within 48 hours.

In this chapter further procedures to purify the methylase enzyme are described. The eluted fractions were assayed with (poly dI-dC).(poly dI-dC) and/or $(CAG)_7(mCTG)_7$ so that CpG, CpNpG, maintenance and *de novo* methylation could be investigated.

4.2 DNA methylase activity of extract E2

4.2.1 Effect of micrococcal nuclease treatment on E2 activity

E2 extract was prepared as in section 2.2.3.2 except that the salt extracted nuclei sample was split and one half treated with 3.72 mM EGTA at the same time as 400 U/ml micrococcal nuclease, while EGTA was added after the nuclease digestion to the other half. Micrococcal nuclease requires Ca^{2+} ions in order to be active. These ions are chelated by EGTA thereby rendering the micrococcal nuclease inactive. After 0.2 M NaCl treatment and dialysis the E2 extracts were assayed for methylase activity with or without hemimethylated $(CAG)_7(mCTG)_7$ substrate DNA as described in section 2.2.10.

The E2 extract prepared from nuclei treated with inactive micrococcal nuclease exhibited low levels of enzymic activity regardless of whether substrate DNA was added to the assay system (Table 4.1). This shows that there is no

Table 4.1

Effect of micrococcal nuclease treatment on E2 DNA methylase activity

	DNA methylase activity (cpm) when micrococcal nuclease is:			
+/– DNA	Inactive		Active	
– DNA	55	79	822	750
+ DNA	35	41	1252	1379

Extract E2 was prepared from salt extracted nuclei that had been treated with either active or inactive micrococcal nuclease. The E2 samples were assayed for methylase activity using 1 μg $(CAG)_7(mCTG)_7$ as substrate DNA for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor. Duplicate results are given.

extractable methylase enzyme available. However, E2 prepared from nuclease digested nuclei was found to have methylase activity even when there was no synthetic substrate DNA present. This suggests that the methylase enzyme binds tightly to the nuclei and this nuclear DNA has to be partly digested in order to release the enzyme. It is clear however, that the methylase activity is solubilized along with DNA by this treatment.

4.2.2 Extraction of pea nuclei with 0.2 M NaCl and micrococcal nuclease

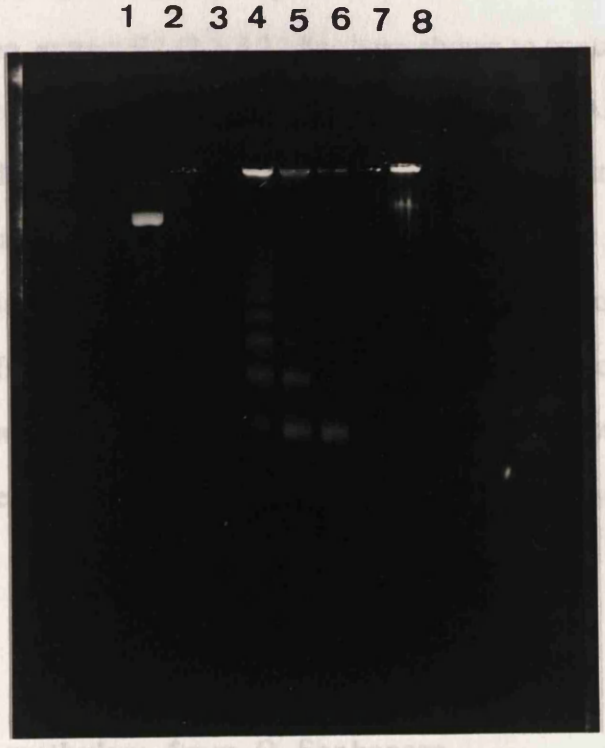
The optimum concentration of micrococcal nuclease required for digestion of nuclear DNA was determined by incubating salt extracted nuclei (2.2.3.1) with increasing concentrations of micrococcal nuclease and then preparing E2 as described in section 2.2.3.2.

Figure 4.1 shows that the native pea DNA was progressively digested with higher concentrations of micrococcal nuclease until at 400 U/ml no nucleosomal DNA was detected. The absence of DNA in the 0 U/ml sample was considered to be due to all the DNA pelleting during centrifugation. This did not occur in other samples because the DNA has been digested into smaller pieces.

All subsequent E2 preparations were carried out using 400 U/ml micrococcal nuclease. However, the possibility of the extract containing native pea DNA was investigated further.

Figure 4.1

Digestion of nuclei with an increasing concentration of micrococcal nuclease



Lane number

1 Marker
2 E1
3 0 U/ml micrococcal nuclease
4 100 U/ml micrococcal nuclease
5 200 U/ml micrococcal nuclease
6 300 U/ml micrococcal nuclease
7 400 U/ml micrococcal nuclease
8 Salt extracted nuclei

10 µl of each nuclei sample treated with the indicated concentrations of micrococcal nuclease and then extracted to give E2, was mixed with 2 µl DNA sample buffer and incubated for 5 min at 60°C. The samples were then loaded onto a 1.5% agarose gel and run at 50V.

4.2.3 Contamination of E2 extract with native pea DNA

E1 (2.2.3.1) has been shown by agarose gel electrophoresis to contain no native pea DNA. When assayed with no added substrate DNA no measurable enzymic activity was observed. However, on addition of substrate DNA there was a large increase in methylase activity (Figure 4.2).

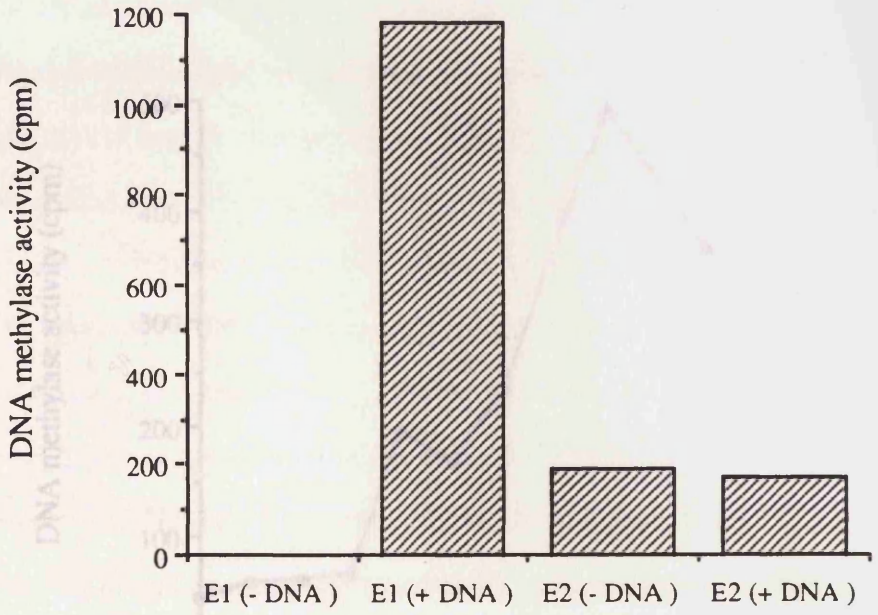
In comparison, extract E2 (2.2.3.2) has been shown to contain native pea DNA (Figure 4.2). When assayed with no added substrate DNA, enzymic activity was observed. Addition of substrate DNA to the E2 assay had little effect on the level of methyl transfer. It was considered that the enzyme binds tightly to the native pea DNA which is protected from nuclease action. Several methods to remove native pea DNA from E2 were attempted. These included treatment with DNase I, DEAE, ammonium sulphate, changes in pH and centrifugation on a sucrose cushion. All these methods were unsuccessful in recovering enzyme activity (results not shown).

4.3 Elution of methylase from Q Sepharose

Extracts E1 and E2 were prepared as described in section 2.2.3 and pooled. They were then mixed with Q Sepharose as described in section 2.2.13.1. DNA methylase was found to bind to Q Sepharose at salt concentrations below 0.15 M. At higher salt concentrations enzyme activity was found in the supernatants after mixing enzyme and Q Sepharose (Figure 4.3). Therefore, in all purification procedures where Q Sepharose was used, the enzyme was bound to the column material at 0.15 M NaCl and eluted with a NaCl gradient of 0.15-0.4 M. A gradient of 0.15–0.5 M was found to be too steep for sufficient separation of proteins (4.4.1).

Figure 4.2

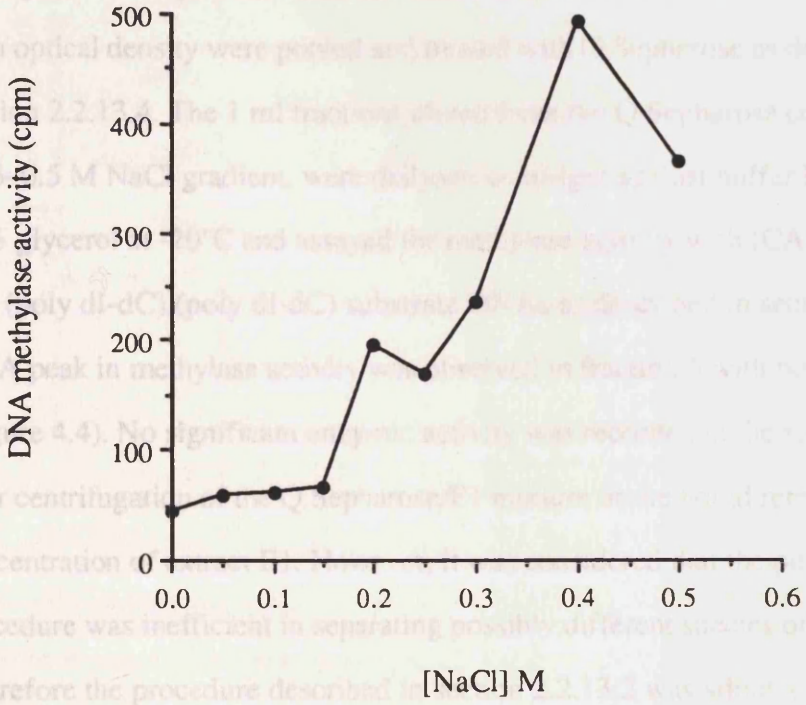
Methylase activity of extracts E1 and E2 +/- substrate DNA



30 µl of each extract was incubated with or without 1.4 µg pea DNA for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor.

Figure 4.3

Elution of DNA methylase from Q Sepharose with increasing NaCl concentrations



250 µl Q Sepharose in a 25% suspension was mixed with an equal volume of E1/E2 at various salt concentrations as indicated. After centrifugation the supernatants from each sample were dialysed and assayed for methylase activity with pea DNA as enzyme substrate for 2 hours at 30°C. [^{3}H] SAM was present as methyl donor.

4.4 Purification of extract E1

4.4.1 Gel filtration followed by treatment with Q Sepharose

A Superose 6 gel filtration column and extract E1 were prepared as described in sections 2.2.13.4 and 2.2.3 respectively. Extract E1 was concentrated using Centricon tubes as described in section 2.2.13.4 and then loaded onto the column. The optical density of the fractions was recorded at 280 nm and those fractions with high optical density were pooled and treated with Q Sepharose as described in section 2.2.13.4. The 1 ml fractions eluted from the Q Sepharose column with a 0.15–0.5 M NaCl gradient, were dialysed overnight against buffer M^+ containing 50% glycerol at -20°C and assayed for methylase activity with $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) substrate DNAs as described in section 2.2.10.

A peak in methylase activity was observed in fraction 5 with both substrates (Figure 4.4). No significant enzymic activity was recorded in the supernatant saved after centrifugation of the Q Sepharose/E1 mixture or the liquid removed during concentration of extract E1. However, it was considered that the purification procedure was inefficient in separating possibly different species of the enzyme. Therefore the procedure described in section 2.2.13.2 was adopted.

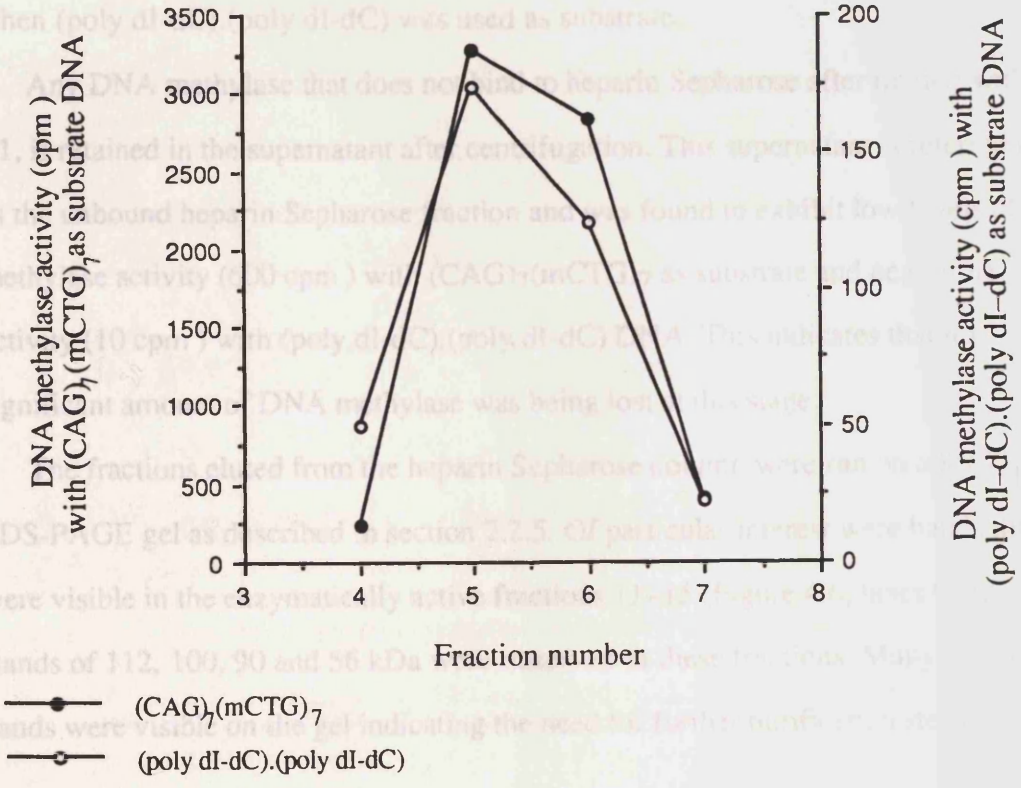
4.4.2 Elution of DNA methylase from a heparin Sepharose column

Heparin Sepharose was used to purify the DNA methylase enzyme as it is negatively charged and mimics DNA in that it binds to DNA binding proteins.

Extract E1 was prepared as described in section 2.2.3.1 and was mixed for 30 min on ice with 10 ml heparin Sepharose in buffer M^+ containing 0.2 M NaCl. A column was prepared and a 40 ml 0.2–0.7 M NaCl gradient was applied as described in section 2.2.13.2. The 2 ml fractions collected were dialysed overnight against buffer M^+ containing 50% glycerol at -20°C before assay with

Figure 4.4

Elution from a Q Sepharose column following gel filtration on a superose 6 column



30µl of each sample was incubated with 0.1 µg $(CAG)_7(mCTG)_7$ or 1 µg (poly dI-dC).(poly dI-dC) substrate DNAs for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor. Blank values of 48 and 30 cpm were subtracted from the values obtained with $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) respectively.

$(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) as substrate DNAs as described in section 2.2.10.

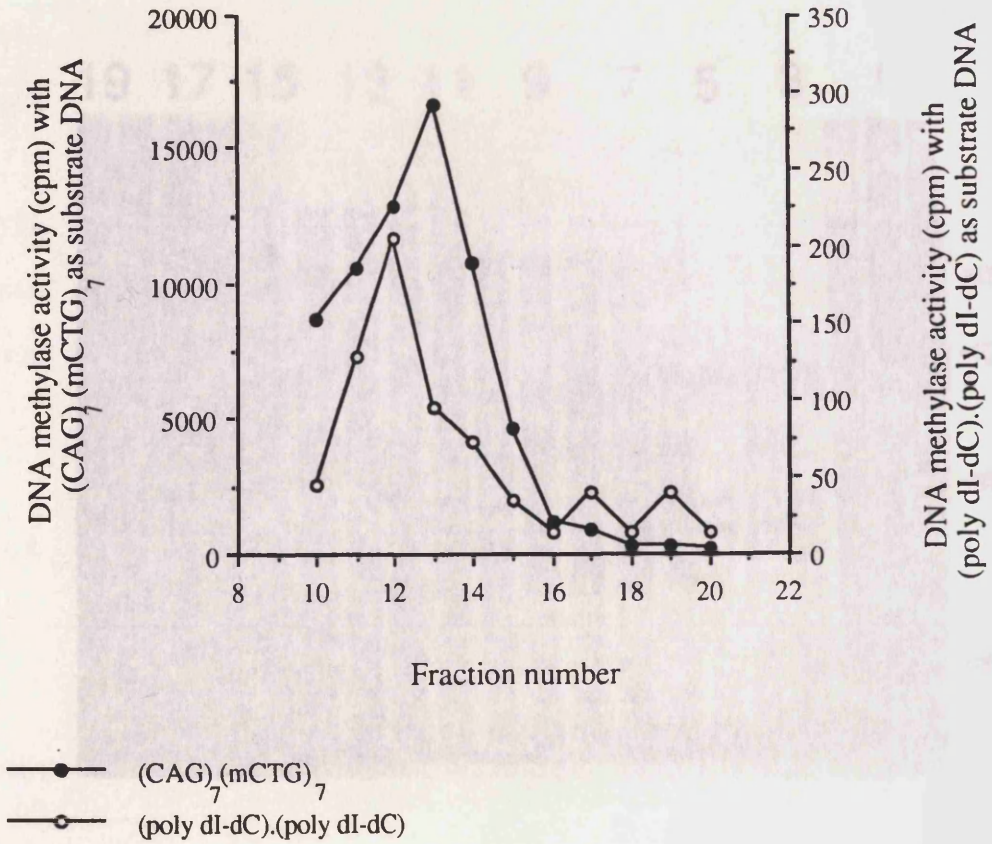
The greatest level of enzyme activity with either substrate DNAs was observed to occur in different fractions (Figure 4.5). With $(CAG)_7(mCTG)_7$ substrate DNA the highest level of methylation was recorded in fraction 13 compared to fraction 12 when (poly dI-dC).(poly dI-dC) was used as substrate.

Any DNA methylase that does not bind to heparin Sepharose after mixing with E1, is retained in the supernatant after centrifugation. This supernatant is referred to as the unbound heparin Sepharose fraction and was found to exhibit low levels of methylase activity (600 cpm) with $(CAG)_7(mCTG)_7$ as substrate and negligible activity (10 cpm) with (poly dI-dC).(poly dI-dC) DNA. This indicates that no significant amount of DNA methylase was being lost at this stage.

The fractions eluted from the heparin Sepharose column were run on a 8.75% SDS-PAGE gel as described in section 2.2.5. Of particular interest were bands that were visible in the enzymatically active fractions 11–15 (Figure 4.6, lanes 9–13). Bands of 112, 100, 90 and 56 kDa were observed in these fractions. Many resolved bands were visible on the gel indicating the need for further purification steps.

Figure 4.5

Elution of DNA methylase from a heparin Sepharose column with a 0.2–0.7 M NaCl gradient

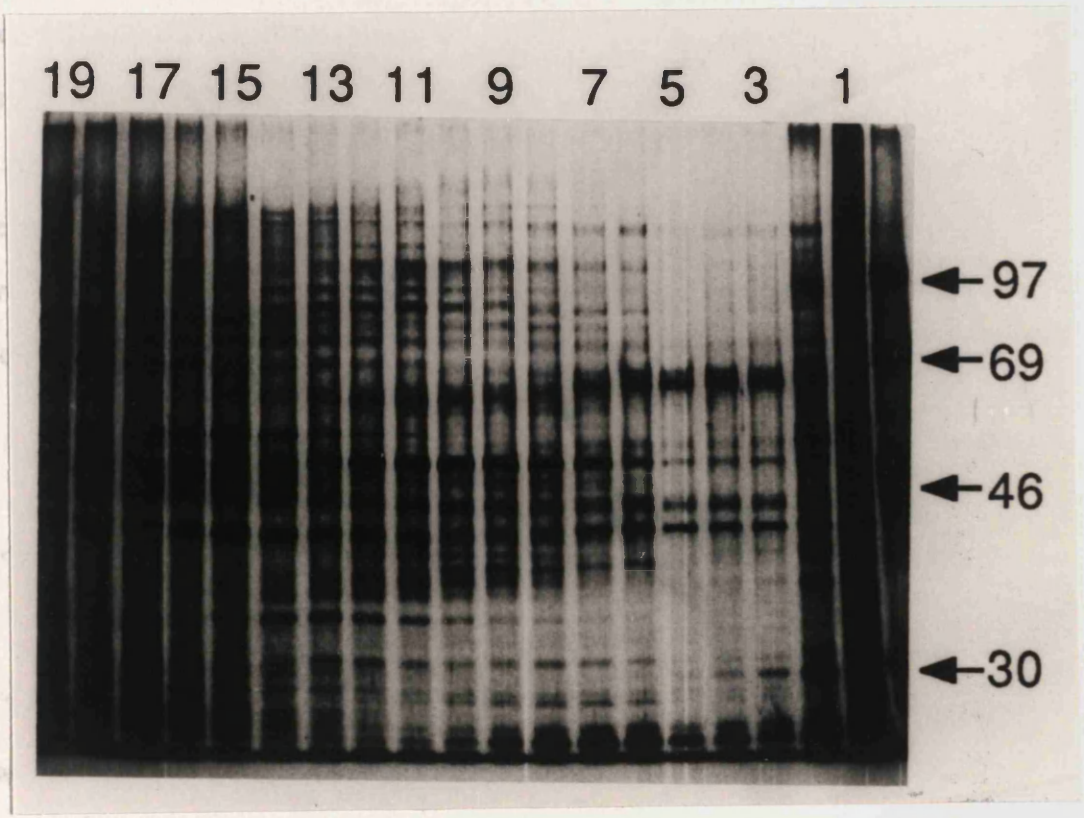


20 µl of each fraction eluted from a heparin sepharose column was incubated with 1 µg $(CAG)_7(mCTG)_7$ substrate DNA. 2 µg (poly dI-dC).(poly dI-dC) DNA was incubated with 35 µl of each fraction. Incubation was for 2 hours at 30°C with [^{3}H] SAM as methyl donor. Blank values of 54 and 61 cpm were subtracted from the values obtained with $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) respectively.

Assays were carried out in duplicate with negligible error.

Figure 4.6

SDS–PAGE gel of fractions eluted from a heparin Sepharose column



30 µl of each fraction sample was mixed with 15 µl protein sample buffer and boiled for 5 min. They were then loaded onto a 1.5 mm thick 8.75% SDS–PAGE gel. The gel was run at 40 mA, 200 V and then silver stained.

4.4.3 Standardization of heparin Sepharose column

Heparin Sepharose has been shown to separate DNA methylase from many other proteins in extract E1 (4.4.2). The procedure was standardized using 1 ml of heparin Sepharose and a 10 ml 0.2–0.7 M NaCl gradient and carried out using extract E1 (2.2.3.1) as described in section 2.2.13.2. The ten 1 ml fractions collected were dialysed overnight at -20°C against buffer M^+ containing 50% glycerol and assayed for methylase activity using hemimethylated trinucleotide DNA as substrate as described in section 2.2.10.

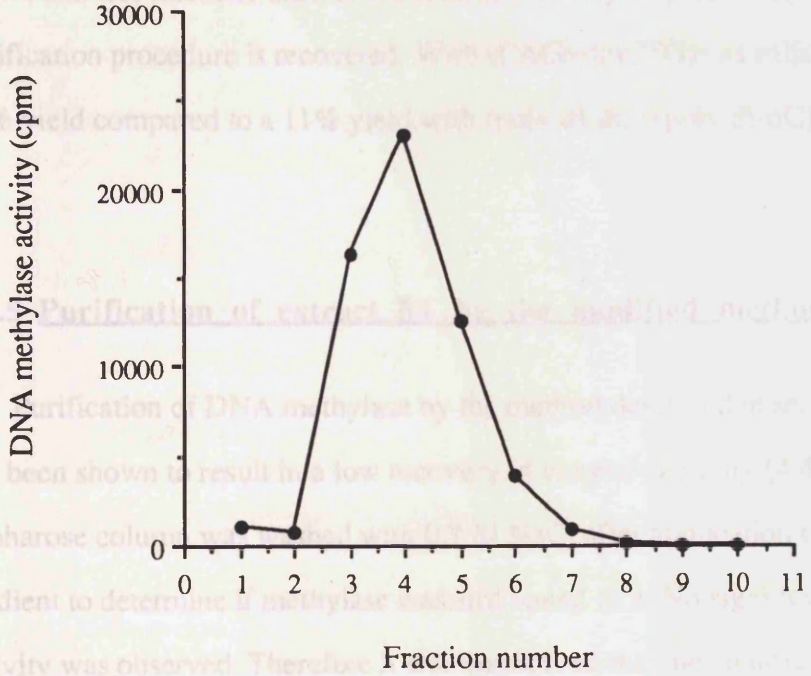
A sharp peak of methylase activity was observed in fractions 3–5 (Figure 4.7). Therefore, in all subsequent purification procedures using this method fractions 3–6 were pooled in order to retain as much methylase enzyme as possible before proceeding to the next purification step.

4.4.4 Elution from a Q Sepharose column following treatment with heparin Sepharose

The optimum salt concentration for DNA methylase to bind to Q Sepharose fast flow has been previously found to be 0.15 M (section 4.3). The combined NaCl concentration of fractions 3–6 pooled from a heparin Sepharose column (4.4.3) was estimated to be 0.45 M. Therefore this pool was diluted to 0.15 M with buffer M^+ (2.1.2) before mixing with Q Sepharose for 30 min on ice as described in section 2.2.13.2. A column was prepared and a NaCl gradient of 0.15–0.4 M applied. Previously a gradient of 0.15–0.5 M had been found to be too steep for sufficient separation (4.4.1). The 1 ml fractions eluted from the Q Sepharose column were dialysed overnight at -20°C against buffer M^+ containing 50% glycerol and then assayed for methylase activity using $(CAG)_7(mCTG)_7$ or (poly dI-dC).(poly dI-dC) as substrate DNAs (2.2.10).

Figure 4.7

Standardization of the procedure for elution of DNA methylase from a heparin Sepharose column



30 µl of each fraction eluted from a heparin Sepharose column was incubated with 1 µg $(CAG)_7(mCTG)_7$ for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor. A blank value of 43 cpm was subtracted from the values obtained.

Enzyme activity was observed to peak in different fractions depending on the substrate DNA (Figure 4.8). With hemimethylated trinucleotide DNA the highest level of methylation occurred in fraction 6 and remained relatively high in fraction number 7. In comparison, enzyme activity with unmethylated dinucleotide substrate DNA increased in fraction 4 to a peak in fraction 5.

The percentage yield of DNA methylase activity recorded with both substrates shows that less than one third of the total active enzyme present at the start of the purification procedure is recovered. With $(CAG)_7(mCTG)_7$ as substrate there is a 31% yield compared to a 11% yield with (poly dI-dC).(poly dI-dC) DNA.

4.4.5 Purification of extract E1 by the modified method 2.2.13.3

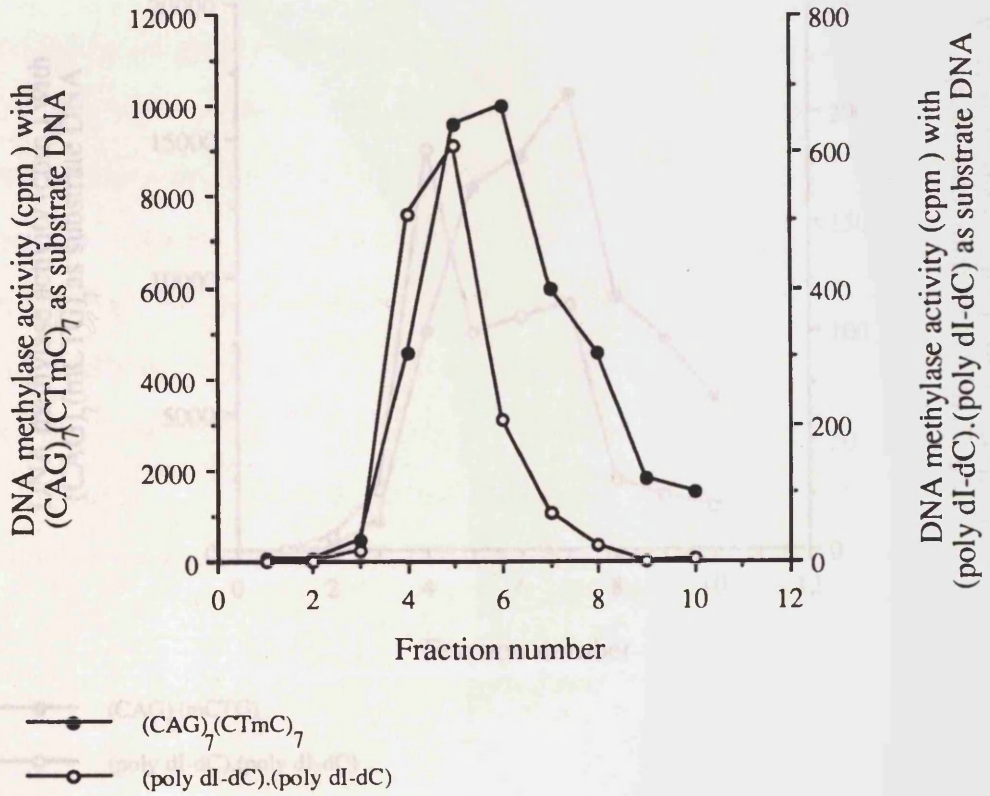
Purification of DNA methylase by the method described in section 2.2.13.2 has been shown to result in a low recovery of enzymic activity (4.4.4). The Q Sepharose column was washed with 0.7 M NaCl after application of the NaCl gradient to determine if methylase was still bound to it. No significant enzymic activity was observed. Therefore it was considered that the significant loss of DNA methylase occurred during the treatment with heparin Sepharose and so the procedure with heparin Sepharose was modified as described in section 2.2.13.3.

Extract E1 (2.2.3.1) was treated with heparin Sepharose and Q Sepharose as described in section 2.2.13.3. The 1 ml fractions eluted from the Q Sepharose column were dialysed overnight at -20°C against buffer M^+ containing 50% glycerol and then assayed for methylase activity using $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) as substrate DNAs as described in section 2.2.10.

Methylase activity was observed over several tubes with the two substrates (Figure 4.9). This could be interpreted as 3 peaks of activity. With both $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) DNAs there was a broad peak of

Figure 4.8

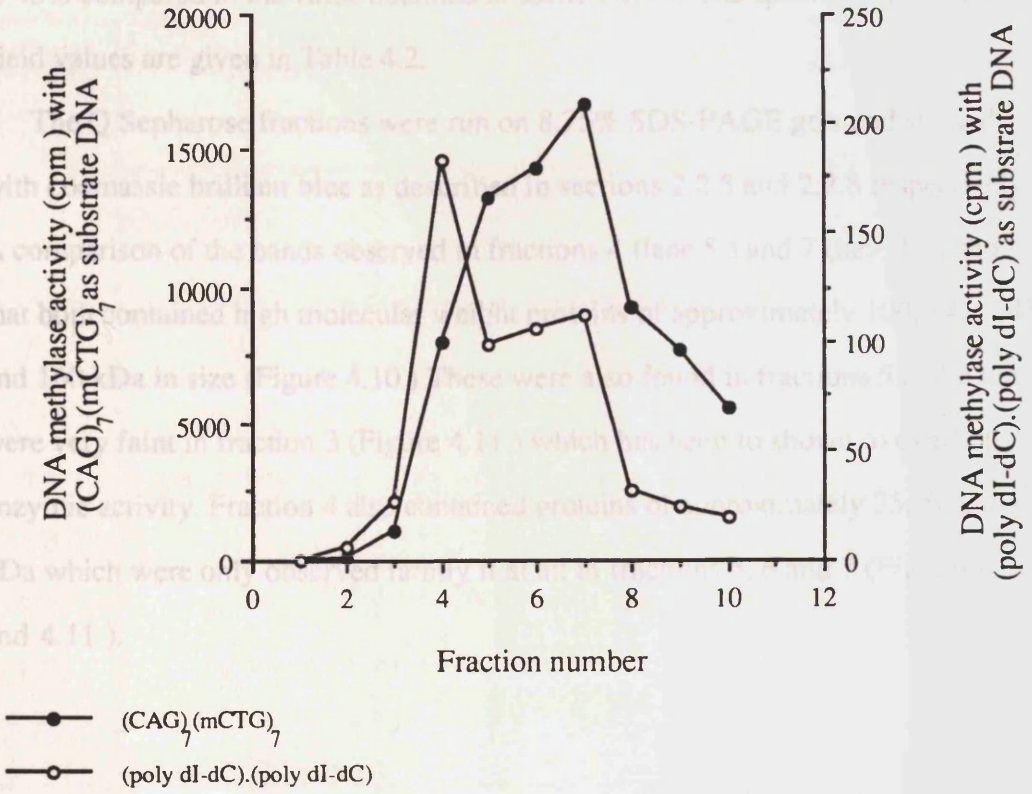
Elution of DNA methylase from a Q Sepharose column following treatment with heparin Sepharose



30 µl of each fraction eluted from the Q Sepharose column was incubated with 1 µg $(CAG)_7(mCTG)_7$ or 2 µg (poly dI-dC).(poly dI-dC) DNAs for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor. Blank values of 55 and 43 cpm were subtracted from the values obtained with $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) respectively.

Figure 4.9

Elution of DNA methylase from a Q Sepharose column prepared by modified method 2.2.13.3



20 µl of each fraction eluted from a Q Sepharose column were incubated with 1 µg $(CAG)_7(mCTG)_7$ or 2 µg (poly dI-dC).(poly dI-dC) substrate DNAs for 2 hours at 30°C with [^{3}H] SAM as methyl donor. Blank values of 69 and 50 cpm were subtracted from the values obtained with $(CAG)_7$ $(mCTG)_7$ and (poly dI-dC).(poly dI-dC) respectively.

methylase activity observed over fractions 5, 6 and 7. However, with the unmethylated dinucleotide the highest level of methylation was observed in fraction 4.

Only 52% of the enzyme activity was recovered with $(CAG)_7(mCTG)_7$ as substrate DNA, while recovery with (poly dI-dC).(poly dI-dC) increased four fold to 43% compared to the value obtained in section 4.4.4. The specific activity and yield values are given in Table 4.2.

The Q Sepharose fractions were run on 8.75% SDS-PAGE gels and stained with coomassie brilliant blue as described in sections 2.2.5 and 2.2.8 respectively. A comparison of the bands observed in fractions 4 (lane 8) and 7 (lane 11) showed that both contained high molecular weight proteins of approximately 100, 141, 143 and 160 kDa in size (Figure 4.10) These were also found in fractions 5 and 6 but were very faint in fraction 3 (Figure 4.11) which has been to shown to exhibit low enzymic activity. Fraction 4 also contained proteins of approximately 35, 50 and 90 kDa which were only observed faintly if at all in fractions 5, 6 and 7 (Figures 4.10 and 4.11).

Table 4.2

Specific Activity and % yield of samples that have undergone the procedure described in section 4.4.5

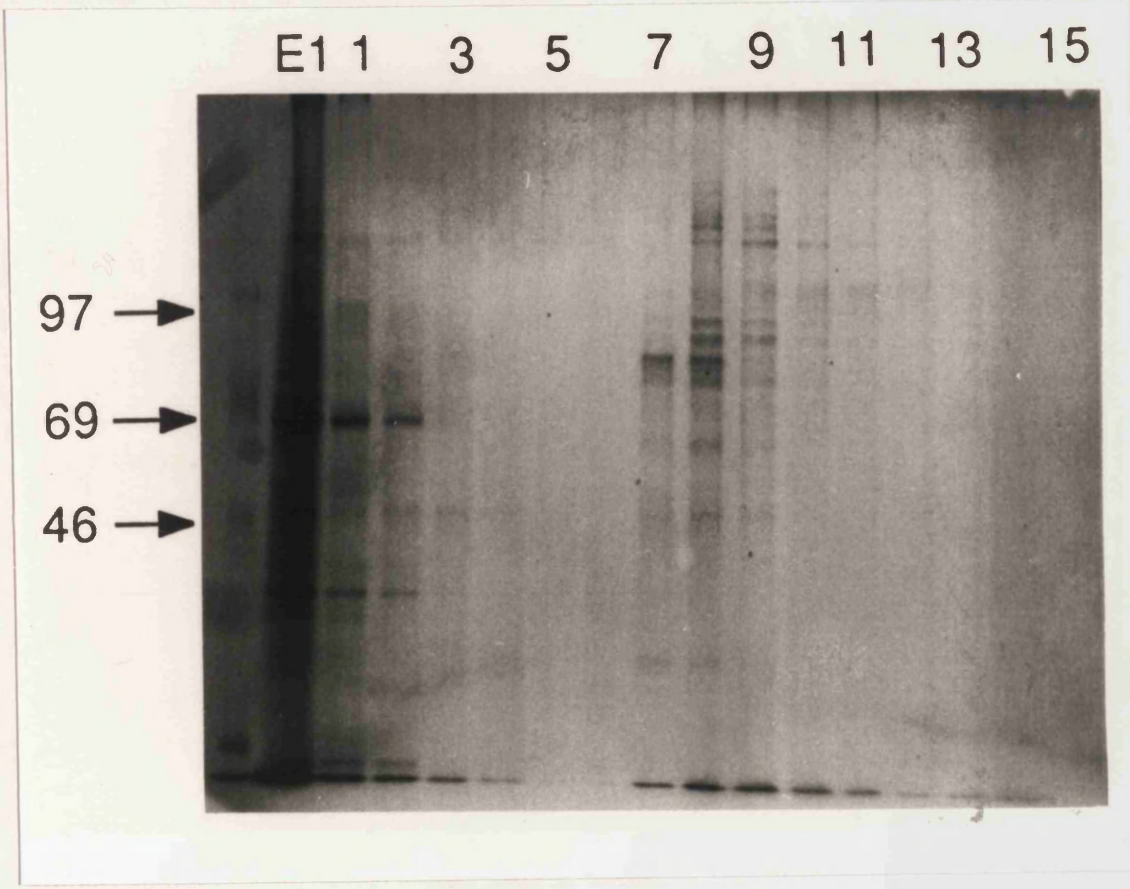
Sample	Specific Activity (U/mg)		% Yield	
	$(CAG)_7(mCTG)_7$	(poly dI-dC). (poly dI-dC)	$(CAG)_7(mCTG)_7$	(poly dI-dC). (poly dI-dC)
E1	382	5	100	100
HSU	34	1	9.4	27
EP	349	5	86	102
QSU	5	3.5	0.9	47
F 4	3333	78		
F 7	16667	112		
QS Pool	4630	37	54	33

Key

E1	Extract E1
HSU	Unbound heparin Sepharose fraction
EP	Enzyme pool from heparin Sepharose
QSU	Unbound Q Sepharose fraction
F4	Fraction number 4
F7	Fraction number 7
QS Pool	Specific activity or total yield of the Q Sepharose pool

Figure 4.10

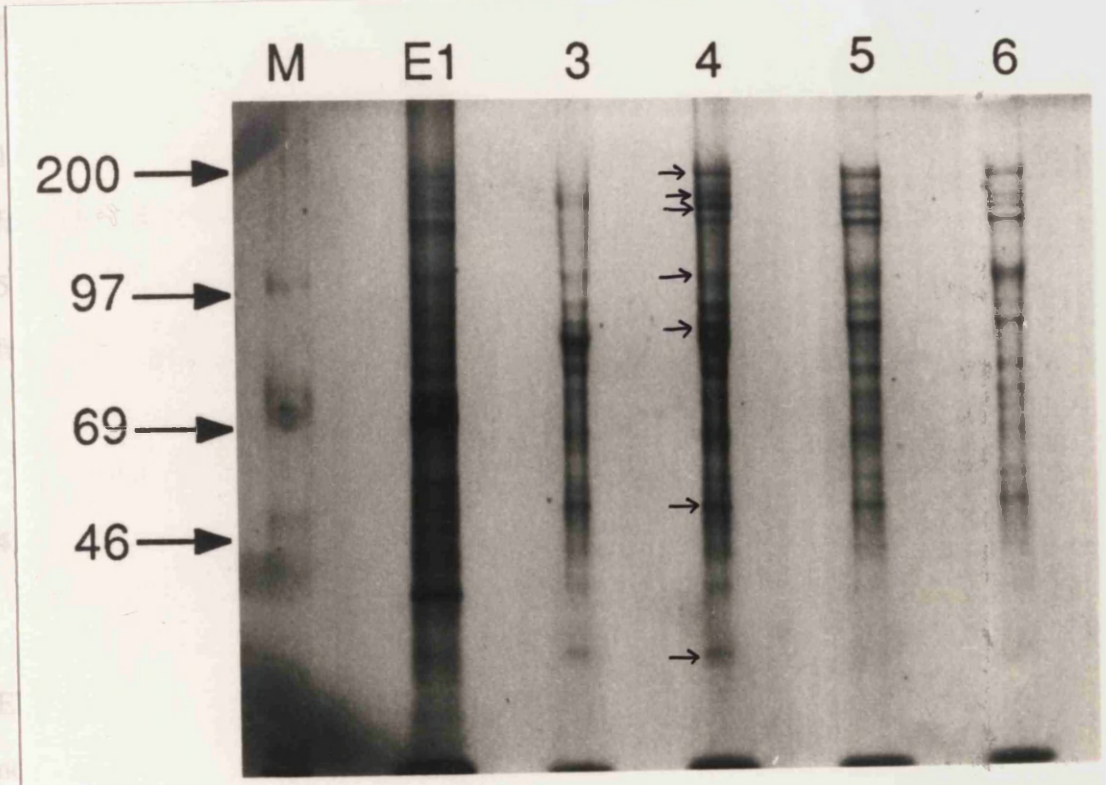
SDS–PAGE gel (1.5 mm thick) of fractions eluted from a Q Sepharose column prepared by modified method 2.2.13.3.



30 µl of each fraction sample was mixed with 15 µl protein sample buffer and boiled for 5 min. The samples were loaded onto a 1.5 mm thick 8.75% SDS–PAGE gel which was run at 40 mA, 200 V. The gel was then stained with coomassie brilliant blue.

Figure 4.11

SDS–PAGE gel (2 mm thick) of fractions eluted from a Q Sepharose column prepared by modified method 2.2.13.3.



60 µl of each fraction sample was mixed with 20 µl protein sample buffer and boiled for 5 min. The samples were loaded onto a 2 mm thick 8.75% SDS–PAGE gel which was run at 40 mA, 200 V. The gel was then stained with coomassie brilliant blue. The fraction sample number is indicated. M=Marker.

4.4.5.1 Enhanced Chemiluminesence (ECL)

A duplicate gel to that shown in Figure 4.11 was Western blotted as described in section 2.2.14. The blot was probed with an antibody obtained from Dr. Follmann, to a wheat methylase protein 55 kDa in size and bands detected using enhanced chemiluminesence as described in section 2.2.15.

The antibody was observed to associate with several proteins (Figure 4.12). The strongest association was with a protein approximately 70 kDa in size in all four samples.

The antibody also reacted with high molecular weight bands in the range of 160–180 kDa and a doublet of 80–100 kDa. All of these bands were seen in fractions 3, 4, 5 and 6 but were of lower intensity in fraction 3. Only in fractions 4, 5 and 6 was a band of 112 kDa observed. This band increased in intensity in fractions 5 and 6.

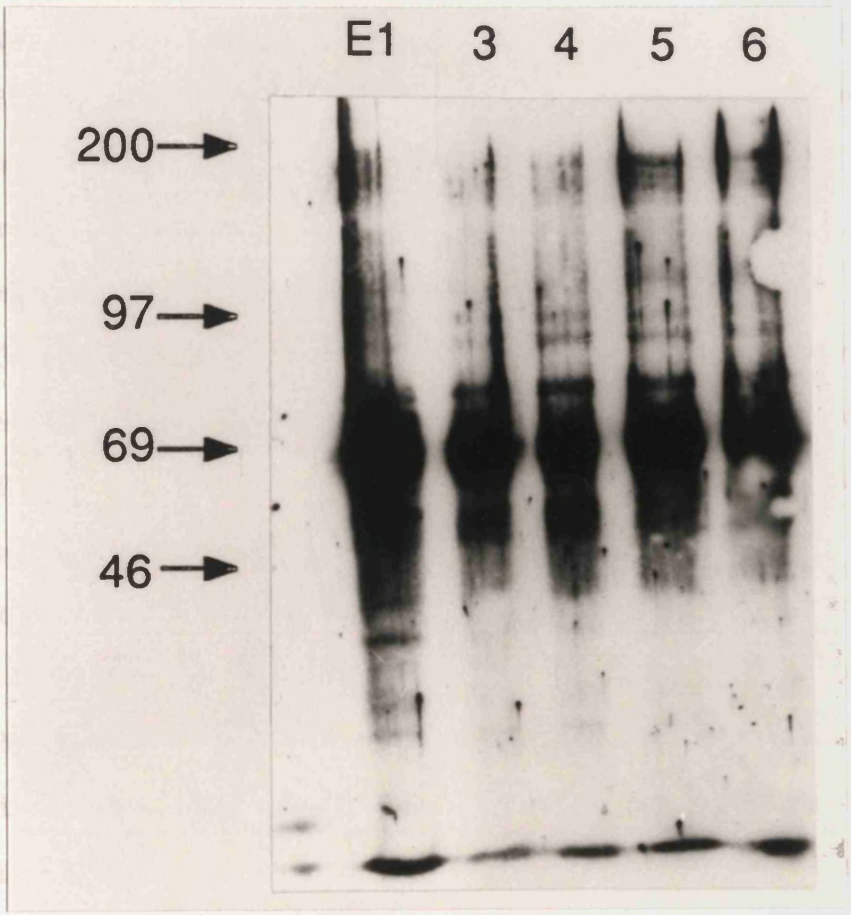
Wheat methylase antibody was obtained from Dr Follmann, Gesamthochschule Kassel, Germany.

4.5 Purification of Extract E1 in the presence of benzamidine

In all previous sections, extract E1 was purified with PMSF (0.6 mg/ml) and EDTA (1 mM) as the protease inhibitors. It was considered that these alone might not be sufficient to prevent degradation of the methylase enzyme and so benzamidine (1 mM) was used in conjunction with PMSF. It was introduced at different stages in the purification procedure.

Figure 4.12

Autoradiograph of samples probed with an antibody to a 55 kDa wheat methylase protein



60 µl of each fraction sample was mixed with 20 µl protein sample buffer and boiled for 5 min. The samples were loaded onto a 2 mm thick 8.75% SDS–PAGE gel which was run at 40 mA, 200 V. The gel was then Western blotted and probed with an antibody to a 55 kDa wheat methylase protein. The sample fraction number is indicated.

4.5.1 Introduction of 1mM benzamidine before Q Sepharose column

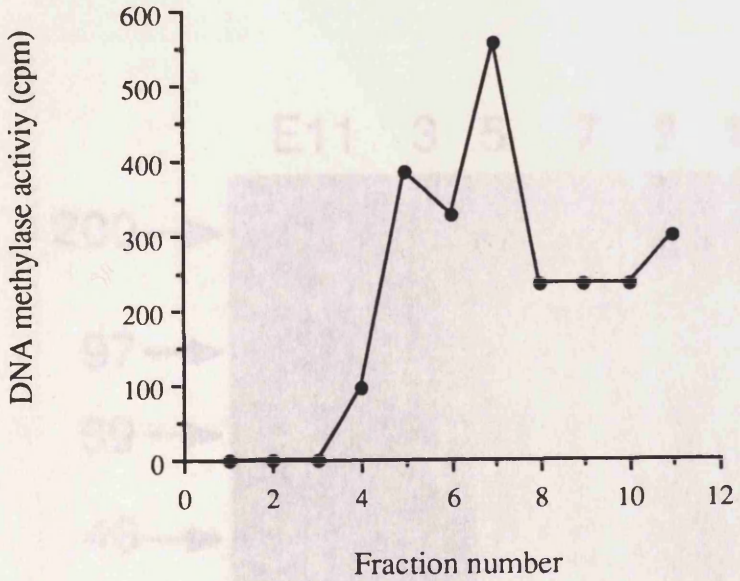
Extract E1 was prepared as described in section 2.2.3.1. It was mixed with heparin Sepharose as described in section 2.2.13.3 except that a 0.5 M wash instead of a 0.7 M wash was carried out. The appropriate fraction was diluted from 0.5 M to 0.15 M with buffer M^+ containing 1mM benzamidine. The diluted fraction was then mixed with Q Sepharose as described in section 2.2.13.2. The 1 ml fractions eluted from the Q Sepharose column were dialysed at -20°C overnight against buffer M^+ containing 50% glycerol and then assayed for methylase activity in the presence of benzamidine using $(CAG)_7(mCTG)_7$ as substrate DNA as described in section 2.2.10.

Two peaks of methylase activity were observed in fractions 5 and 7 (Figure 4.13). The highest level of methylation occurred in fraction 7 but there was a 20 fold decrease in the cpm value obtained compared to that for the same fraction in the purification of E1 in the absence of benzamidine (4.4.5). This suggests that benzamidine might inhibit DNA methylase activity in some way.

The fraction samples from the Q Sepharose column were run on two 8.75% acrylamide SDS-PAGE gels as described in section 2.2.5. One gel was stained with coomassie brilliant blue (2.2.8) and the other silver stained (2.2.7) as indicated. A strong band of approximately 180 kDa was observed in fraction 4 (Figure 4.14, lane 9) This band was seen to a lesser extent in fraction 5 (lane 10) along with two other bands of approximately 55 and 38 kDa. Fractions 7 (lane 12) was observed to contain 55 and 38 kDa bands. Figure 4.15 shows that 90, 63 and 50 kDa bands were also present in fractions 4, 5 and 6 and very faintly in fraction 7.

Figure 4.13

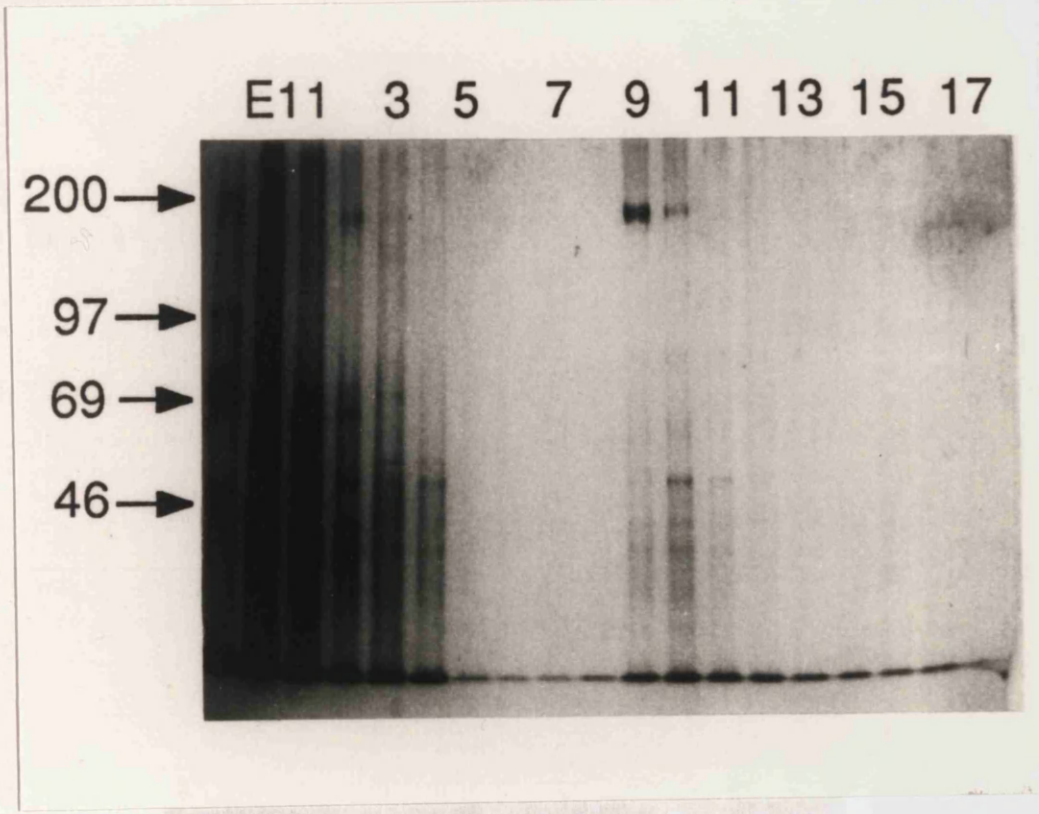
Elution of DNA methylase from Q Sepharose in the presence of 1 mM benzamidine



30 µl of each fraction eluted from a Q Sepharose column was incubated with 1 µg $(CAG)_7(mCTG)_7$ substrate DNA for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor. A blank value of 51 cpm was subtracted from the values obtained.

Figure 4.14

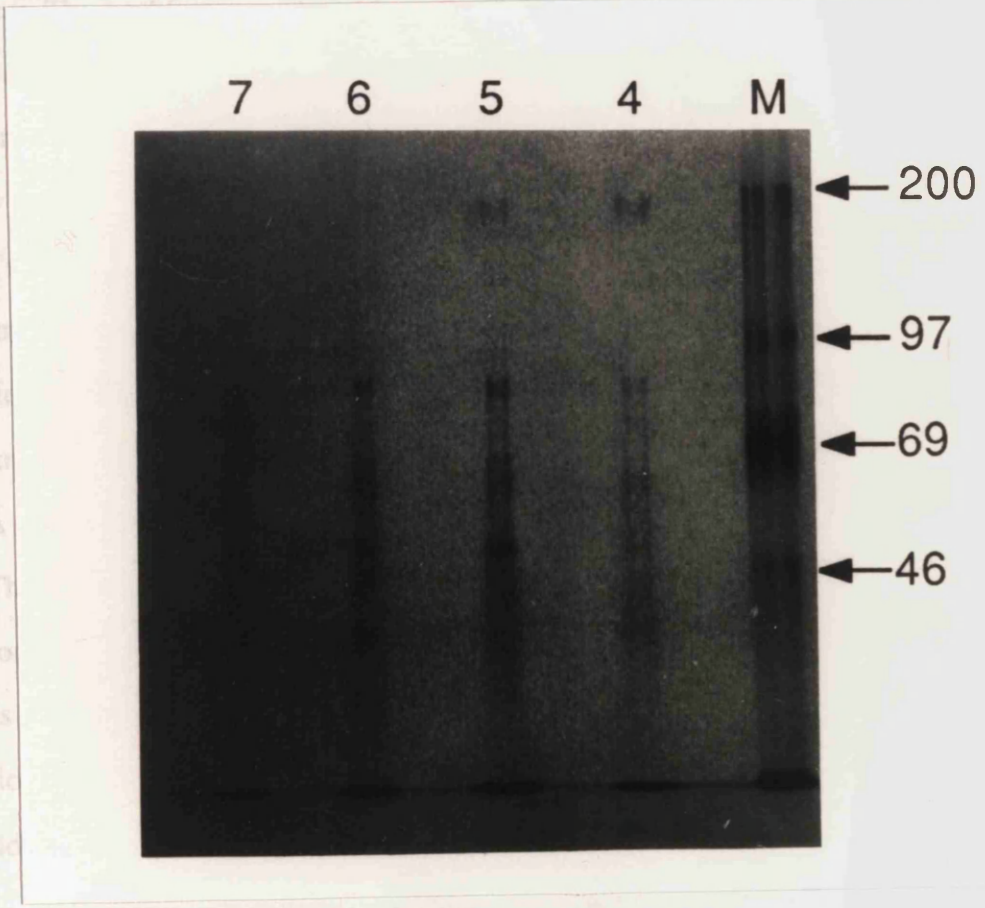
SDS–PAGE gel of fractions eluted from a Q Sepharose column in the presence of 1 mM benzamidine



30 µl of each fraction sample was mixed with 15 µl protein sample buffer and boiled for 5 min. The samples were then loaded onto a 1.5 mm thick 8.75% SDS–PAGE gel and run at 40 mA, 200 V. The gel was then silver stained.

Figure 4.15

SDS–PAGE gel of fractions eluted from a Q Sepharose column in the presence of 1 mM benzamidine



60 µl of each fraction sample was mixed with 20 µl protein sample buffer and boiled for 5 min. The samples were then loaded onto a 2 mm thick 8.75% SDS–PAGE gel and run at 40 mA, 200 V. The gel was then stained with coomassie brilliant blue. The sample fraction number is indicated. M=Marker.

4.5.2 Introduction of 1 mM benzamidine at the stage of E1 preparation

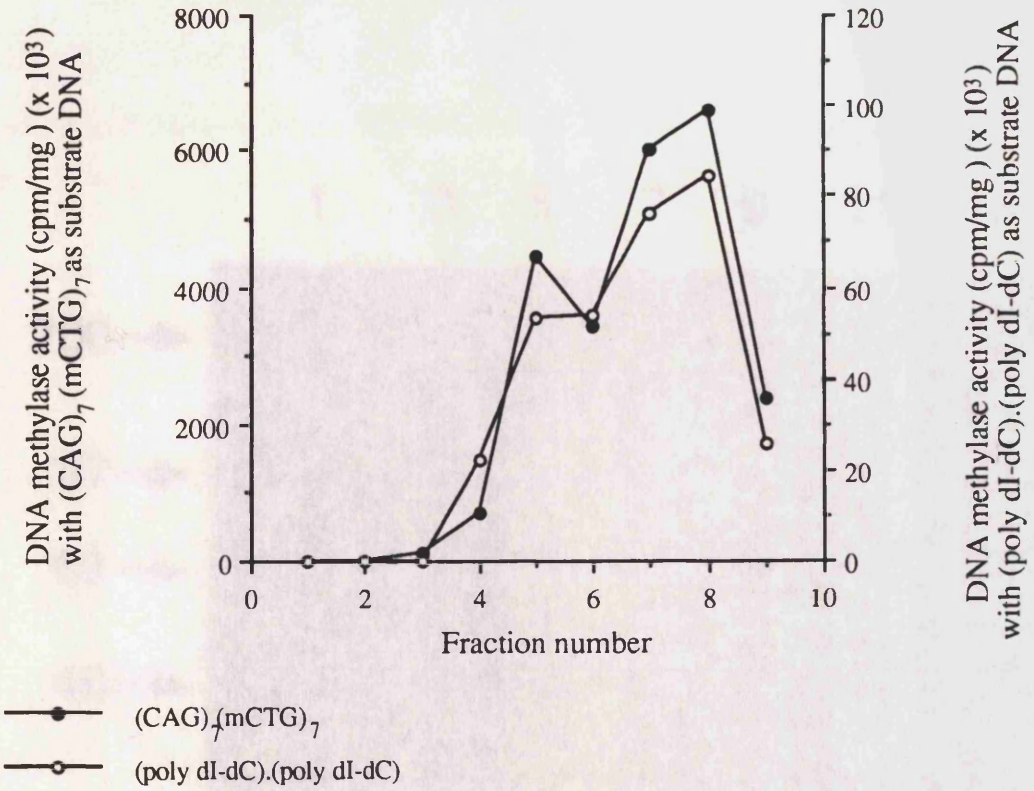
Pea nuclei were prepared as described in section 2.2.2 except that 1 mM benzamidine was present in all buffers. Extract E1 was prepared from these nuclei as described in section 2.2.3 and then treated with heparin Sepharose and Q Sepharose as described in section 2.2.13.3. At all stages 1 mM benzamidine was present. The 1 ml fractions eluted from the Q Sepharose column were dialysed overnight at -20°C against buffer M^+ containing 50% glycerol and then assayed, in the presence of benzamidine, for methylase activity using $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) as substrate DNAs as described in section 2.2.10.

Two peaks of methylase activity in fractions 5 and 8 were observed with both substrates (Figure 4.16). The highest level of methylation activity occurred in fraction 8 with both substrates. The percentage yield of active enzyme with each substrate was low. Only 20% of activity was recovered with $(CAG)_7(mCTG)_7$ DNA and as little as 11% with (poly dI-dC).(poly dI-dC) as substrate.

The samples were run on a 8.75% acrylamide SDS-PAGE gel as described in section 2.2.5. The gel was then silver stained as described in section 2.2.7. Many bands were visible on the gel (Figure 4.17). Comparison of active and less active fractions enabled certain bands of 50, 63, 70, 90, 100 and 160 kDa to be considered as possibly methylase or degraded fragments of the methylase enzyme.

Figure 4.16

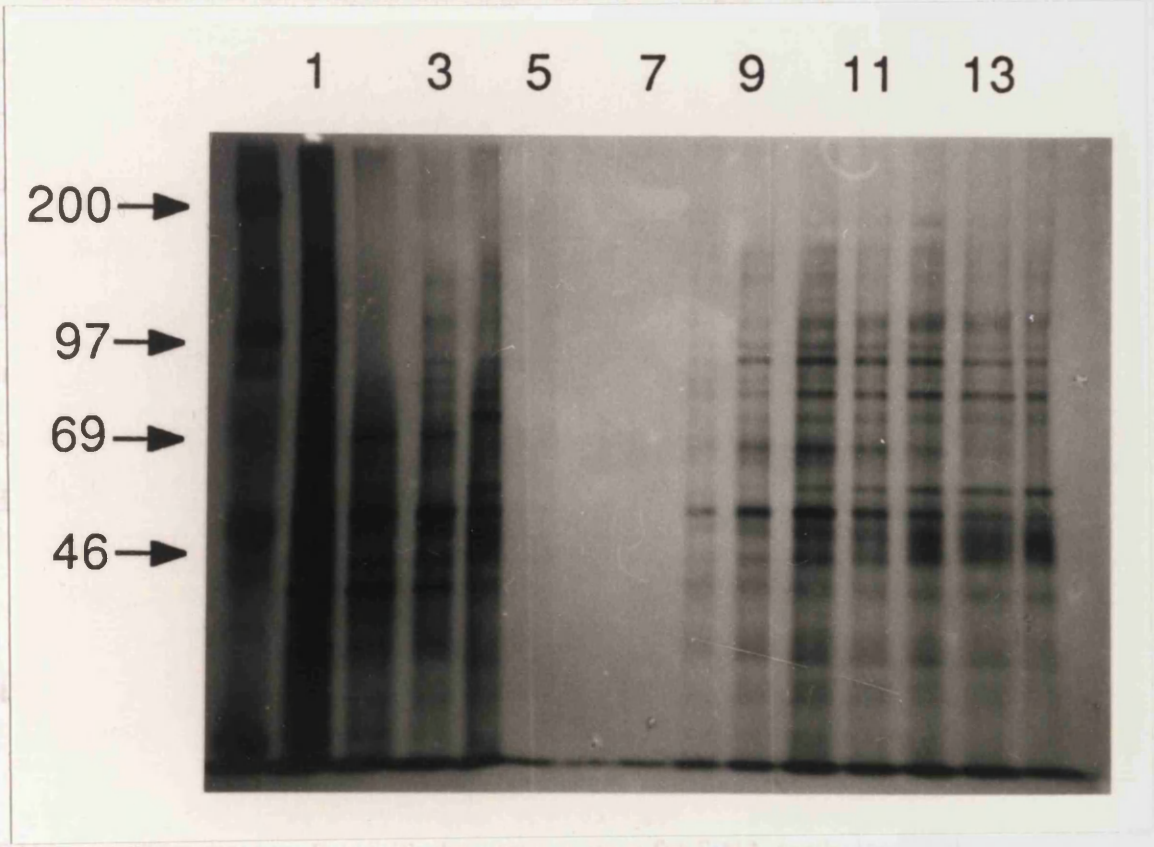
Elution from Q Sepharose of Extract E1 prepared in the presence of 1 mM benzamidine



30 µl of each sample was incubated with 1 µg $(CAG)_7(mCTG)_7$ or 2 µg (poly dI-dC).(poly dI-dC) substrate DNAs for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor. Blank values of 71 and 69 cpm were subtracted from the recorded values with $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) respectively.

Figure 4.17

SDS–PAGE gel of fractions eluted from Q Sepharose in the presence of 1 mM benzamidine following preparation of Extract E1 in the presence of 1 mM benzamidine



30 µl of each fraction sample was mixed with 10 µl protein sample buffer and boiled for 5 min. The samples were then loaded onto a 1.5mm thick 8.75% SDS–PAGE gel and run at 40 mA, 200V. The gel was then silver stained. Lanes 9 to 14 represent fractions 4 to 9 respectively

4.5.3 Possible inhibition of DNA methylase by benzamidine

The low levels of methylation recorded in the presence of benzamidine suggest that the DNA methylase is being inhibited by benzamidine. This was determined by incubating at 30°C, 0–10 mM benzamidine with 30 µl of an active sample fraction which had undergone purification in the absence of benzamidine and then assaying for methylase activity as described in section 2.2.10.

No inhibition of the enzyme was observed. However, the sample used would have contained methylase that was already to some extent degraded. It was considered that benzamidine may inhibit not only the degradation of DNA methylase but also the activity of the non degraded enzyme while not affecting the activity of the degraded enzyme. This was investigated by treatment with trypsin in order to mimic the presumed degradation. Success would have been manifest as an increase in enzymic activity, however, a decrease in enzyme activity with $(CAG)_7(mCTG)_7$ substrate DNA was observed while there was no change with (poly dI-dC).(poly dI-dC) DNA (results not shown).

4.6 Purification of E2

Extract E2 (2.2.3.2) has been shown to contain native pea DNA (4.2.3). This DNA was shown to interfere with the assay system for DNA methylase with specific substrates. Attempts to remove it successfully and to retain enzymic activity failed (4.2.3). It was considered that the enzyme present in extract E2 might be more stable if mixed with extract E1 and therefore a mixture of extracts E1 and E2 or extract E2 alone were subjected to purification by affinity chromatography as described in section 2.2.13.2. This would determine if the presence of pea DNA affected the purification and if the DNA could be removed from extract E2 by this method.

4.6.1 Purification of a mixture of extracts E1 and E2

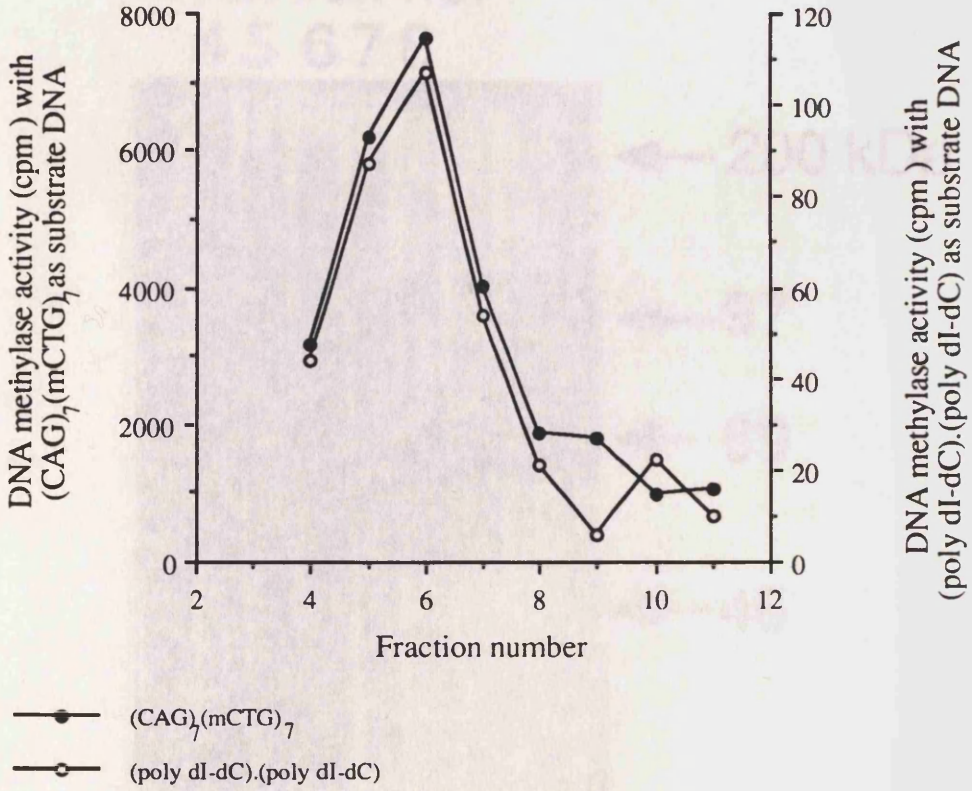
Extracts E1 and E2 were prepared as described in section 2.2.3. Equal volumes of E1 and E2 were treated with heparin Sepharose and Q Sepharose as described in section 2.2.13.2. The 1 ml fractions eluted from the Q Sepharose column were dialysed overnight at -20°C against buffer M^+ containing 50% glycerol and then assayed for methylase activity using $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) as substrate DNAs and with no DNA as described in section 2.2.10.

The highest level of methylation was obtained in fraction 6 for both substrates (Figure 4.18). However, the values recorded were lower than those obtained with equivalent concentrations of extract E1 especially with (poly dI-dC).(poly dI-dC) as substrate DNA. No enzyme activity was obtained when the Q Sepharose fractions were assayed in the absence of DNA, indicating that they contained no native pea DNA. The unbound Q Sepharose fraction was also found to contain no native pea DNA or active enzyme. However, significant levels of enzymic activity were observed in extract E2, the unbound heparin Sepharose fraction and the pool of fractions from the heparin Sepharose column when they were assayed with no added DNA indicating the presence of native pea DNA in these fractions. The Q Sepharose fractions were run on a 8.75% SDS–PAGE gel as described in section 2.2.5. This gel was silver stained (2.2.7) and revealed the presence of many bands (Figure 4.19). Comparison of active (fraction 6) and less active fractions indicated bands of 180–160, 100, 90 and 56 kDa as possibly being methylase or degraded fragments of the methylase enzyme.

With $(CAG)_7(mCTG)_7$ DNA as substrate less than one third of the available methylase was recovered. This value fell to less than 3% with (poly dI-dC).(poly dI-dC) DNA. The enzyme specific activity values (U/mg) and % yields for the relevant samples are given in Table 4.3 .

Figure 4.18

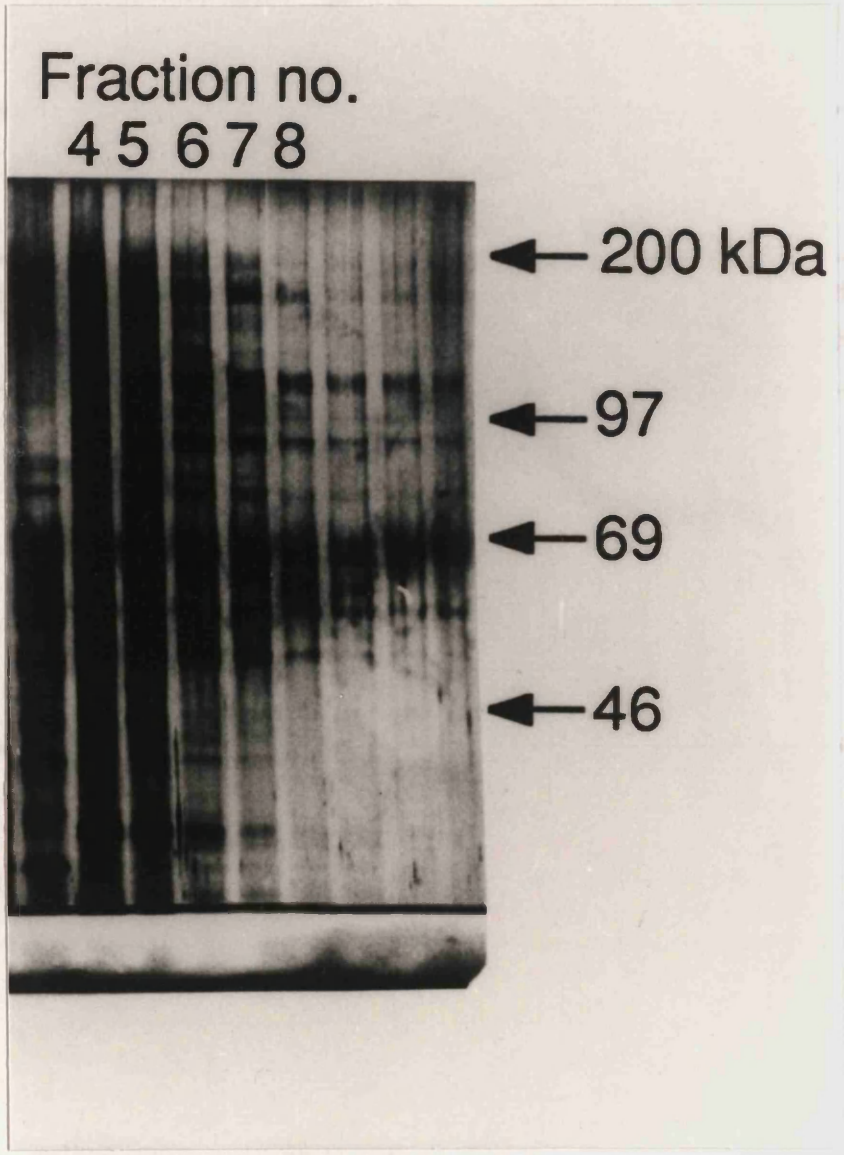
Elution of a mixture of extracts E1 and E2 from a Q Sepharose column



30 µl of fractions 4–11 was incubated with 1 µg $(CAG)_7(mCTG)_7$ or 2 µg (poly dI-dC).(poly dI-dC) substrate DNAs for 2 hours at 30°C in the presence of $[^3H]$ SAM as methyl donor. Blank values of 73 and 48 cpm were subtracted from the values obtained with $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) respectively.

Figure 4.19

SDS–PAGE gel of fractions eluted from a Q Sepharose column to which a mixture of extracts E1 and E2 were applied



30 µl of each fraction sample was mixed with 15 µl protein sample buffer and boiled for 5 min. The samples were then loaded onto a 1.5 mm 8.75% SDS–PAGE gel and run at 40 mA, 200 V. The gel was then silver stained. The sample fraction number is indicated.

Table 4.3

Specific Activity and % yield of samples that have undergone the procedure described in section 4.6.1.

Sample	Specific Activity (U/mg)		% Yields	
	$(CAG)_7(mCTG)_7$	(poly dI-dC). (poly dI-dC)	$(CAG)_7(mCTG)_7$	(poly dI-dC). (poly dI-dC)
E1	354	64	100	100
HSU	29	20	9.2	34
EP	91	12	40	30
F 6	850	9.7	12	0.7
QS Pool	370	4.9	31.3	2.3

Key

E1	Extract E1
HSU	Unbound heparin Sepharose fraction
EP	Enzyme pool from heparin Sepharose
F6	Fraction number 6
QS Pool	Specific activity or total yield of the Q Sepharose pool

4.6.2 Purification of extract E2 alone

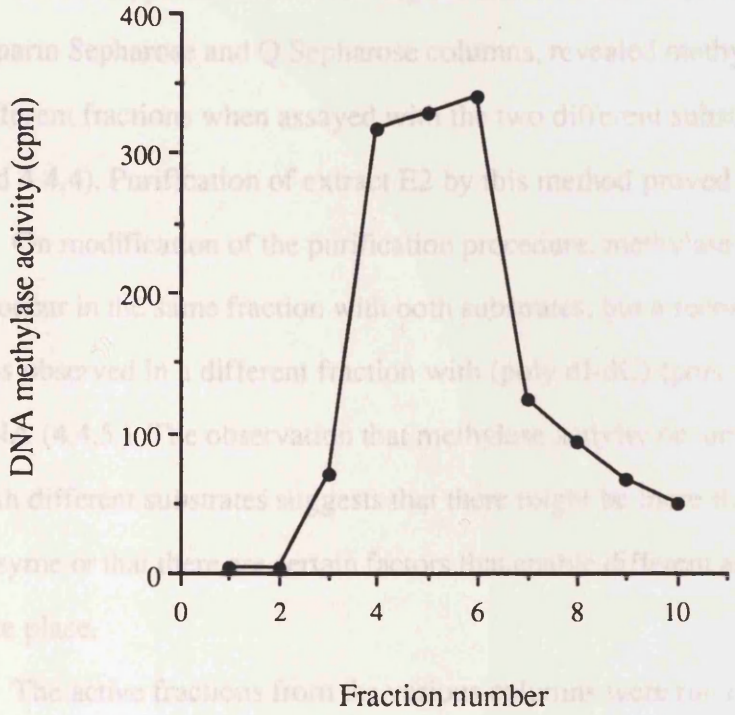
Purification of a mixture of extracts E1 and E2 (section 4.6.1) showed that active enzyme not contaminated with native pea DNA can be recovered. It was not clear however, whether any E2 enzyme had been recovered as it was possible that the E2 bound to DNA was retained on the column. To determine what proportion of the activity recovered was from extract E1 or if any methylase enzyme was recovered from E2, the same purification procedure was carried out as described in section 2.2.13.2.

Extract E2 was prepared as described in section 2.2.3.2 and then treated with heparin Sepharose and Q Sepharose as described in section 2.2.13.2. The 1 ml fractions eluted from the Q Sepharose column were dialysed overnight at -20°C against buffer M^+ containing 50% glycerol and then assayed for methylase activity using $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) as substrate DNAs and with no DNA as described in section 2.2.10.

No methylase activity was observed with either (poly dI-dC).(poly dI-dC) DNA or in the absence of added DNA. Methylation was observed with $(CAG)_7(mCTG)_7$ substrate DNA (Figure 4.20) but the values obtained were at least 10 fold lower than those recorded in section 4.6.1 where a mixture of E1 and E2 was exposed to the same treatment. This suggests that the methylase activity observed when extracts E1 and E2 were purified together was recovered predominantly from extract E1.

Figure 4.20

Elution of Extract E2 from a Q Sepharose column



30 µl of each fraction were incubated with 1 µg $(CAG)_7(mCTG)_7$ substrate DNA for 2 hours at 30°C in the presence of [^{3}H] SAM as methyl donor. A blank value of 50 cpm was deducted from each value.

4.7 Summary and Conclusions

In concordance with previous findings (Yesufu *et al.*, 1991), one broad peak of methylase activity was obtained by gel filtration on Superose 6 with both $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) as substrate DNAs (4.4.1).

However, purification involving the elution with NaCl of extract E1 bound to heparin Sepharose and Q Sepharose columns, revealed methylase activity in different fractions when assayed with the two different substrates (sections 4.4 2 and 4.4.4). Purification of extract E2 by this method proved unsuccessful.

On modification of the purification procedure, methylase activity was observed to occur in the same fraction with both substrates, but a secondary peak of activity was observed in a different fraction with (poly dI-dC).(poly dI-dC) as substrate DNA (4.4.5). The observation that methylase activity occurs in different fractions with different substrates suggests that there might be more than one methylase enzyme or that there are certain factors that enable different aspects of methylation to take place.

The active fractions from the various columns were run on SDS–PAGE gels and on staining several bands were observed. Although no definite methylase band or bands could be identified, bands of 180–160, 100–80, 112 and 70 kDa were found to occur in the active fractions investigated. They were also found to react with an antibody to a component of the wheat methylase enzyme (4.4.5.1). From these results, the 70 kDa band cannot be considered to be an active component of DNA methylase as it is found in an inactive fraction (Figure 4.12, fraction 3) but it might be a structural component of the protein. The large number of bands identified suggest degradation of the enzyme. However, addition of 1 mM benzamidine while preventing degradation was also found to result in a decrease in enzymic activity.

CHAPTER 5

Discussion

In mice a single DNA methylase gene has been detected. This could indicate that methylation patterns at CpG sites are established and maintained by a single species of DNA methylase. However, three closely related forms of the enzyme have been detected in murine erythroleukemia (MEL) cells. It is possible that they are a result of specific alternative splicing of mRNA precursors but is thought they are due to post–translational proteolytic cleavage modification of the enzyme as no difference in the size of mRNA transcripts was observed (Bestor *et al.*, 1988). In plants the existence of CpNpG methylation and the suggestion that CpG and CpNpG methylation may serve different functions raises the possibility of there being more than one DNA methylase involved (Hershkovitz *et al.*, 1990).

DNA methylase is a nuclear protein which on salt treatment can be partially extracted resulting in a crude nuclear extract, E1. The remaining enzyme is tightly bound within the nuclei. Treatment with micrococcal nuclease was found to be required to partly digest the DNA to release the enzyme. However, the enzyme was still found to be associated with the pea DNA (Table 4.1). Attempts to remove this DNA by affinity chromatography proved unsuccessful as the DNA bound enzyme was retained on the Q Sepharose column (sections 4.6.1 and 4.6.2). Therefore, crude nuclear extract E1 was used as a source of the enzyme for purification and to investigate sequence specificity so as not to lose any species of the enzyme.

Extract E1 has been shown to contain a DNA methylase(s) that methylates both CpG and CpNpG sites. The highest level of methylation was observed with hemimethylated di– and trinucleotide DNAs compared to the corresponding unmethylated substrates (Figure 3.1). This preference for hemimethylated DNA was also demonstrated when extract E1 was incubated with oligonucleotides derived from the SV40 early promoter. A two fold difference in the level of methylation was observed between hemi– and unmethylated SV40 oligonucleotide substrates (Figure 3.2). Preferential methylation of hemimethylated DNA has been observed for mammalian DNA methyltransferases (Gruenbaum *et al.*, 1982). In bacteria, type I

methyltransferases prefer hemimethylated DNA but type II show no discrimination between hemi– and unmethylated DNA (Kelleher *et al.*, 1991).

It would appear that the pea DNA methylase enzyme interacts with a higher affinity with hemimethylated trinucleotide DNA than with the other DNA substrates (3.2). Low concentrations of hemimethylated DNA containing the trinucleotide target sequence are required to saturate the enzyme. However, at saturating DNA concentrations, the rate of methylation is similar with hemi– or unmethylated target sequences but greater with trinucleotide target sequences than with dinucleotide target DNA.

It has been proposed that DNA methyltransferases form a covalent complex with their target sequences (Santi *et al.*, 1983). These DNA–enzyme complexes form only transiently and would be broken by reversal of the formation reaction due to hydrolysis or on the transfer of a methyl group from S–adenosyl methionine. Addition of 0.1 M NaCl 10 min after the start of the incubation has been found to inhibit the methylation reaction with native pea DNA (Yesufu *et al.*, 1991) and with di– or trinucleotide synthetic DNAs. The exception is hemimethylated trinucleotide substrate DNA where the methylation reaction continues linearly for 120 min irrespective of the addition of NaCl (3.3.1). This may be because the DNA–enzyme complex formed with CpNpG DNA is more stable than the complexes formed with the other DNA substrates. It would appear that inhibition of the other DNA substrates following addition of NaCl depends on the length of the target DNA. Unmethylated CpNpG is a 21 mer and the methylation reaction is inhibited almost immediately after the addition of NaCl. The dinucleotide DNA substrates are longer, the unmethylated substrate being 100–1000 bp and the hemimethylated substrate being approximately 5000 bp in length. The methylation reaction with these substrates is inhibited after 50 and 90 min respectively following the addition of NaCl. Therefore the longer the DNA the longer it takes for the NaCl to have an effect.

This has led to the suggestion that on interaction of the enzyme with unmethylated trinucleotide and hemi– and unmethylated dinucleotide DNAs there is a rapid equilibrium between DNA and unbound, loosely bound and covalently bound enzyme. This results in the reaction being rapidly inhibited by salt which traps the reactants in an unbound form. However, with hemimethylated trinucleotide target DNA, the initial DNA–enzyme complex is more stable and hence more likely to be converted into the covalent complex than to dissociate. Consistent with this idea that once bound to hemimethylated CpNpG DNA the enzyme remains bound are the experiments on competition (3.3.3) and ligation of CpNpG DNA (3.3.2). The latter shows that on ligation of hemimethylated CpNpG DNA the level of methylation increases by 40% compared to the unligated control. No difference in the level of methylation was observed on ligation of unmethylated CpNpG DNA. The readiness of the enzyme to form a complex with hemimethylated CpNpG DNA compared to other DNA will have the effect *in vivo* of effectively maintaining the pattern of CpNpG methylation. CpNpG sites are known to be heavily methylated in plants (Gruenbaum *et al.*, 1981; Navey-Many and Cedar, 1982; Nick *et al.*, 1986).

The different behaviour of the enzyme depending on whether hemi– or unmethylated CpG or GpNpG target sites are presented to it suggests a possibility of there being more than one methylase enzyme. In concordance with previous findings (Yesufu *et al.*, 1991), attempts to purify the pea DNA methylase by gel filtration resulted in a broad peak of enzyme activity with both $(CAG)_7(mCTG)_7$ and (poly dI-dC).(poly dI-dC) as substrate DNAs (Figure 4.4).

Affinity chromatography involving elution from heparin Sepharose and Q Sepharose revealed the peak of enzymic activity in different fractions when assayed with the two substrates (Figures 4.5; 4.8; 4.9). SDS–PAGE of such fractions eluted from a Q Sepharose column prepared by modified method 2.2.13.2 revealed many bands (Figures 4.10; 4.11). Comparison of bands observed in the active fractions showed that they all contained high molecular weight proteins ranging from 100–160 kDa. These bands were only faintly observed in fractions exhibiting low enzymic

activity. Additional low molecular weight proteins of 35, 50 and 90 kDa were observed in the fraction exhibiting the highest level of methylase activity with unmethylated dinucleotide DNA (Figure 4.11). However, it is not thought that they constitute an enzyme that is only specific for unmethylated DNA as enzymic activity with the unmethylated dinucleotide substrate was observed in all the active fractions.

Although no definite methylase band or bands could be identified, bands of 180–160, 100–80, 112 and 70 kDa were found to react with an antibody to a component of the wheat methylase enzyme (Figure 4.12). However, the 70 kDa band was also observed in an inactive fraction (figure 4.12, fraction 3) and therefore cannot be considered to be an active component of DNA methylase. However, it might be a structural component of the protein.

The large number of bands identified suggest degradation of the enzyme through proteolysis which has been observed in mammalian DNA methylases (Adams *et al.*, 1986). Attempts to reduce proteolysis by including further protease inhibitors such as benzamidine in the purification led to loss of activity that could not be recovered by limited treatment with trypsin suggesting that the benzamidine may also be reacting with the methyltransferase.

No distinct bands are visible on SDS–PAGE gels between fractions exhibiting different levels of methylation with different substrates. This is consistent with there being only one pea DNA methylase enzyme. The relative activity with different substrates during purification might be due to changes in conformation of the enzyme. It is possible that such changes in conformation could result in the observed decrease in the level of methylation of hemimethylated DNA following heat treatment of extract E1 and purified fractions. No change in the level of methylation of unmethylated DNA was observed (figure 3.8). Conformational changes of the methylase enzyme could also explain the observed decrease in the level of methylation in growing pea shoots. The decrease is slower with hemimethylated trinucleotide DNA than with unmethylated dinucleotide DNA over a period of 4–12 days. The relatively high level of methylation of unmethylated DNA at day 4 might indicate *de novo*

activity of the enzyme at early stages of development. This has been observed to occur in early mammalian embryo development (Monk *et al.*, 1987).

Very few DNA methylases have been purified from plant sources. The green alga *Ch. reinhardii*, wheat and rice methylases have all been purified and have been estimated to have a relative molecular mass of 50–58 kDa (Sano and Sager, 1980; Sano *et al.*, 1981; Theiss *et al.*, 1987; Giordano *et al.*, 1991). Therefore, these proteins are significantly smaller than any of those identified as possible candidates for pea DNA methylase. However, the wheat and green alga methylases are accompanied by smaller protein components of 35 and 22 kDa respectively suggesting degradation of a larger protein. Consistent with this idea is the fact that the rice enzyme is stimulated 50 fold by hemimethylated substrate DNA while methylation of unmethylated DNA is negligible (Giordano *et al.*, 1991). This suggests that either a component of the enzyme specific for methylation of unmethylated DNA has been removed or that a cofactor(s) required for *de novo* methylation has been removed during the purification procedure.

CHAPTER 6
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