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# Adjuvant and immunogenic properties of pneumolysin of *Streptococcus pneumoniae*

A thesis submitted to the University of Glasgow for the degree of Master of Sciences

By

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# Abbreviations

| | |
|---|---|
| α | Anti |
| °C | Degree Celsius |
| µg | micro gram |
| µl | micro litre |
| µm | micrometer |
| µM | micro molar |
| AEC | Anion Exchange Chromatography |
| AOM | Acute Otitis Media |
| APS | Ammonium persulphate |
| CDC | Cholesterol-dependent Cytolysin |
| DH5α *E.coli* | Library Efficiency® DH5α™ chemically competent *E.coli* |
| DNA | Deoxyribonucleic acid |
| dNTP | deoxyribonucletide triphasphate |
| E. coli | Escherichia coli |
| eGFP | Enhanced Green Fluorescent Protein |
| ELISA | Enzyme Linked Immunosorbent Assay |
| EU | Endotoxin Units |
| FPLCS | Fast protein liquid chromatography system |
| g | Gram |
| g | Centrifugal force |
| d $H_2O$ | distilling water |
| $H_2O$ | water |
| $H_2O_2$ | Hydrogen peroxide |

| | |
|---|---|
| His-Tag | Histidine Affinity Tag |
| HU | Haemolytic Units |
| IFN-γ | Interferon gamma |
| Ig | Immunoglobin |
| ILY | Intermedialysin |
| IPTG | Isoproyl-β-D-Thiogalactopyranoside |
| Kb | Kilobase |
| kDa | Kilodalton |
| L | Litre |
| LB | Luria Broth |
| LPS | Lipopolysaccharide |
| M | Molar |
| mg | Milligram(s) |
| ml | Millilitre |
| mM | Millimolar |
| MW | Molecular weight |
| OD | Optical Density |
| PBS | Phosphate Buffer Saline |
| PCR | Polymerase Chain Reaction |
| PFO | Perfringolysin O |
| PLY | Pneumolysin |
| PV | Polysaccharide Vaccine |
| Psa | Pneumococcal surface adhesion protein |
| Psp | Pneumococcal surface protein |

| | |
|---|---|
| rpm | Revolutions per minute |
| S.pneumoniae | Streptococcus pneumoniae |
| SDS-PAGE | Sodium Dodecyl Sulphate-Polyarylamide Gel Electrophoresis |
| V | Volts |
| WT | wild type |

# Abstract

The Gram-positive bacterium *S. pneumoniae* (the pneumococcus) is a major pathogen of man causing community -acquired diseases worldwide. The 23-valent polysaccharide (PS) vaccine can protect most of adults and children older than five, but it cannot protect children under age 2, immunodeficiency patients and the elderly. The PS conjugate vaccine which is complementary to the 23-valent PS vaccine is highly immunogenic and protects children under the age of 2 against invasive diseases, but its efficacy is threatened by strain replacement and serotype switching since it only protect against the limited number of serotypes contained within the vaccine. Therefore, it is important to develop new vaccines that can cope with those disadvantages.

Pneumococcal virulence proteins have been studied extensively for their possible application in production of an alternative vaccine. A protein-based vaccine is expected to be immunogenic in all age groups including infants and the elderly. Pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), pneumolsyin (PLY) are the current leading candidates, among them PLY is a very promising candidate for developing new vaccines.

A promising road to protection against *S. pneumoniae* would be production of fusion protein-based mucosal vaccine. A mucosal vaccine, which can break the transmission chain of infection by preventing nasopharyngeal colonization, should protect children under 2 and immunodeficiency patients. Prevention of

nasopharyngeal colonisation by pneumococci can prevent horizontal spread of the pathogen, thus improving herd immunity to protect non-vaccinated people.

Previous work in our group demonstrates that PLY can act as a good adjuvant in PLY fused antigens to induce both systemic and mucosal immunity. The results from this project show truncated PLY lost this adjuvant property in antigen fused with truncated PLY; however, the adjuvant property in antigen fused with truncated PLY was restored in the presence of free PLY. NMR Chemical shift perturbation experiment showed a tryptophan residue maybe involved in the interaction between PLY and water-soluble cholesterol.

# Chapter 1

# Introduction

## 1.1. Streptococcus pneumoniae

The Gram-positive bacterium *S. pneumoniae* (the pneumococcus) is a major pathogen causing community -acquired disease worldwide. Sternberg and Pasteur were the first to identify *S. pneumoniae* simultaneously in 1881 (Mitchell and Kerr 2002). *S. pneumoniae* was named as pneumococcus (Austrian 1999). The peumococcus asymptomatically colonises in the upper respiratory tract of human tract, and mainly infects patient at extreme age and those with immunodeficiency. The pneumoccocus can cause both non-invasive diseases, such as acute otitis media and sinusitis, and invasive diseases, such pneumonia, meningitis and sepsis. Pneumococcus infection causes high morbidity and mortality in both developing and developed countries. Every year in the USA, the pneumoccocus is estimated to cause 3000 cases of meningitis, 50,000 cases of bacteraemia, 500,000 cases of pneumonia and 7,000,000 cases of otitis media (Obaro 2000). Even with advanced antimicrobial treatments and medical facilities, 40,000 people still die of pneumoccocal infection in the USA per year (Obaro, S. and R. Adegbola 2002). In developing countries, 1 million children younger than 5 years old die from pneumoccocal infection per year ( Obaro, S. and R. Adegbola 2002). In recent years, antibiotic resistance has become a worldwide problem and further exacerbated the conditions since it limits the choices of antimicrobial agents; further more, the antimicrobial agents have to be used in the early stages of infection for high-risk groups such as children, elderly and immunodeficiency patients. Therefore, prevention of pneumococcal diseases by vaccination has become of great interest. Until now, many research groups have focused on the development of vaccines for particular high-risk groups.

## 1.2. Pneumococcal vaccine

Sir Almroth E. Wright using killed, whole organisms developed the first pioneering vaccine in 1911 (Watson et al., 1999), but the application failed because it only covered one or two serotypes. The first successful pneumococcal vaccine (reviewed (Bogaert, Hermans et al. 2004)) was developed using pneumococcal capsular polysaccharide (PS) after Felton and Bailey ( reviewed in Bogaert, Hermans et al. 2004) first isolated pneumococcal capsular PS. The vaccine was later abandoned because of the application of antibiotic therapy that is more effective than the vaccine to treat pneumococcal infections at that time(Austrian 1981). Emergence of the multidrug-resistant pneumococcus worldwide finally lead production of a 14-valent pneumococcal PS vaccine in 1977 (Bogaert, Hermans et al. 2004), which in turn was developed to a 23-valent PS vaccine in 1983. Merck Research Laboratories USA developed a licensed 23-valent PS vaccine named Pneumovax 23, which use 23-selected CPS from the 90 known serotypes. The 23-valent PS vaccines can only induce a B cell response that is T-cell independent. Though the 23-valent PS vaccine can protect most adults and children older than five against infection by serotype included in the vaccine, it has a very limited effect on the morbidity and mortality in some groups due to its poor immunogenicity in these groups, such as the elderly, children under age two and immunodeficiency patient(Butler, Shapiro et al. 1999). It does not cover all pneumococcus serotypes and is not effective against acute otitis media (Wadwa and Feigin 1999).

Due to these above disadvantages associated with PS vaccine, the selected PSs are conjugated with a protein carrier including tetanus toxoid or diphtheria toxoid to form the polyssacchride (PS) conjugate vaccine with improved efficacy(Dagan, Eskola et al.

1998). Although different number of PSs has been conjugated to carrier protein and evaluated, only one 7-valent PS conjugate vaccine has been licensed (Bogaert et al., 2004). Though the PSP conjugated vaccine is highly immunogenic and protective against invasive diseases in young children, and induces long-term protection compared to the PS vaccine, which only induce relatively short-term protection, it does not show any improved protection in the elderly (Shinefield and Black 2000). It is still serotype specific and does not include some serotypes highly prevalent in Africa, Asia and Oceania (Wuorimaa and kayhty, 2000). The number of PSs connected to proteins is limited, because antigen competition and carrier protein epitope suppression, which can impair the antibody response to PSs (Dagan et al., 1998), the PS conjugate vaccine only covers certain vaccine serotypes. Further more, strain placement and serotype switching can cause long-term vaccine failure. A study of the efficacy of 7-valent conjugate vaccine against recurrent otitis media showed a shift in serotype distribution (Veenhoven, Bogaert et al. 2003). Finally, the PS conjugated vaccines are too expensive for developing countries ((Veenhoven, Bogaert et al. 2003); Obaro SK et al., 1996). Therefore, it is important to develop new vaccines that can cope with these disadvantages.

The PS conjugate vaccines are able to reduce nasopharynx colonisation of vaccine serotypes of the pneumococcus (Dagan et al., 1997 and 2000; Mbelle et al., 1999). As colonisation on nasopharynx is the first step of pneumococcal infection and asymptomatic colonisation in the upper respiratory tract is high, especially in children, elicitation of local mucosal immunity to eliminate nasopharynx colonisation of the pneumococcus is potentially a good way to protect against pneumococcus (Bogaert et al., 2004; Zhang and Finn, 2004). Prevention of nasopharynx colonisation by the

pneumococcus can prevent horizontal the spread of the organism, thus improving herd immunity (Obaro, 2000; Zhang and Finn, 2004; Dagan and Fraser 2000), which protects unvaccinated people against pneumococcal infection. *Haemophilus influenzae* type b conjugate vaccines can elicit good mucosal immunity against *H. influenzae* infection, which results in a dramatic reduce in the incidence of *H. influenzae* type b related invasive diseases and nasopharyngeal carriage (Zhang and Finn 2004).

## 1.3. Pneumococcal proteins as candidates of vaccine development

Pneumococcal proteins have been studied extensively as potential vaccine candidates. A protein-based vaccine is expected to immunogenic in all age groups including infants and the elderly. Broad and serotype independent protection can be elicited if highly conserved protein or protein epitopes or a combination of several proteins are included in vaccine. It is relatively cheap and simple to produce protein vaccine by recombinant DNA technology. The potential candidates include pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA), pneumolysin (Ply), pneumococcal surface protein C (PspC or CbpA), autolysin, neuraminidase and putative proteinase maturation protein A (PpmA) (Bogaert et al., 2004 and 2004a; Zhang and Finn, 2004). Antibody against PsaA, PspA, PspC and Ply has been demonstrated in human serum and mucosal secretions (Rapola et al., 2000).

PsaA, a member of the family of metal binding lipoproteins, is part of an ABC transporter and important for virulence (Bogaert et al., 2004). Immunisation studies with PsaA have shown significant protection against colonization but less protection in against invasive disease (Briles, Ades et al. 2000); (Seo, Seong et al. 2002). It was

showed that oral vaccination of mice with PsaA encapsulated in microalginate microspheres induced significant protection against colonisation, pneumonia and septicaemia from an oral vaccination (Seo et al., 2002). Recently, Holmlund and co-workers showed the increase of level of IgG concentration to PsaA were detected in the 7 weeks old infant, and the IgG concentration to PsaA in pneumococcal carriage infants is significant higher than that in non-pneumococcal carriage groups (Holmlund et al., 2006). Mice intraperitoneally challenge with DNA vaccine plasmid expressing the PsaA induce humoral and cellular immune response against the pneumococcus (Miyaji, Ferreira et al. 2002).

PspA and PspC are the members of the choline-binding group of proteins, which connect to the cell surface by non-covalent interactions with cell wall phosphorylcholine (Mitchell and Kerr, 2002). Intranasal immunisation of mice with PspA is protective against nasopharyngeal colonisation, and prevents pneumococcal diseases (Wu et al., 1997). The immunisation studies with PspA showed significant protection against invasive disease but reduced efficacy against mucosal disease and nasopharyngeal carriage (Wu et al., 1997; Ogunniyi et al., 2000; (Arulanandam, Lynch et al. 2001). Mice intraperitoneally immunised with DNA vaccine plasmid expressing the truncated PspA can protect against intraperitoneal challenge with the virulent pneumococcus (Miyaji, Dias et al. 2003). Antibody selected from people vaccinated with PspA induces significant protection against pneumococci producing heterologous PspA (Briles, Hollingshead et al. 2000). Although PspA is structurally variable, the protection elicited by two or three PspA variants should be enough to induce protection against most clinical strains ((Briles, Hollingshead et al. 2000)).

However, development of new vaccine from PspA has slowed down because it has recently been found that PspA has the similarity to short sequences of human cardiac myosin throughout the molecule (Holmlund et al., 2006).

PspC is also named Choline binding protein A (CbpA) and is important for colonization and antibody induced by PspC can interfere with colonization (Balachandran, Brooks-Walter et al. 2002). PspC is variable among different strains and antibody elicited by PspC is cross-reactive with PspA (Brooks-Walter, Briles et al. 1999). Mice intraperitoneally immunized with PspC were significantly better protected than mice immunized with Ply toxoid alone against intraperitoneal challenge of virulent pneumococcus (Ogunniyi et al., 2001). PspA and PspC are paralogous, and have a very similar domain structure and have similarity in much of their sequences (Brooks-Walter, Briles et al. 1999).

There are three neuraminidases in penumococci, NanA, NanB and NanC. All pneumococcal isolates to date produce neuraminidase, but not all three (Jedrzejas 2001). Previous immunization study in the mice intranasal challenged by recombinant neuraminidase suggests that PLY is more valuable than the neuraminidase A as a vaccine candidate (Lock and Frasch, 1998). Recently Tong and colleagues recently showed immunization with NanA could protect chinchillas against nasopharyngeal colonisation (Tong et al., 2005). Pre-immunization of mice with autolysin was shown to be effective against a virulent wild type pneumococcus (Berry et al., 1989).

Pneumococcal alpha-enolase, immunoglobulin A1 protease, streptococcal lipoprotein rotamase A, and PpmA were showed to elicit antibody in children under age 2

(Adrian, Bogaert et al. 2004). These proteins have potential as candidates for protein-based vaccines, especially PpmA, it can elicit species-specific opsonophagocytic antibodies that were cross-reactive with various pneumococcal strains (Overweg, Kerr et al. 2000), and the serum IgG antibody titers against PpmA is high in children with nasopharyngeal carriage of pneumococci (Vainio, Fagerlund et al. 2005).

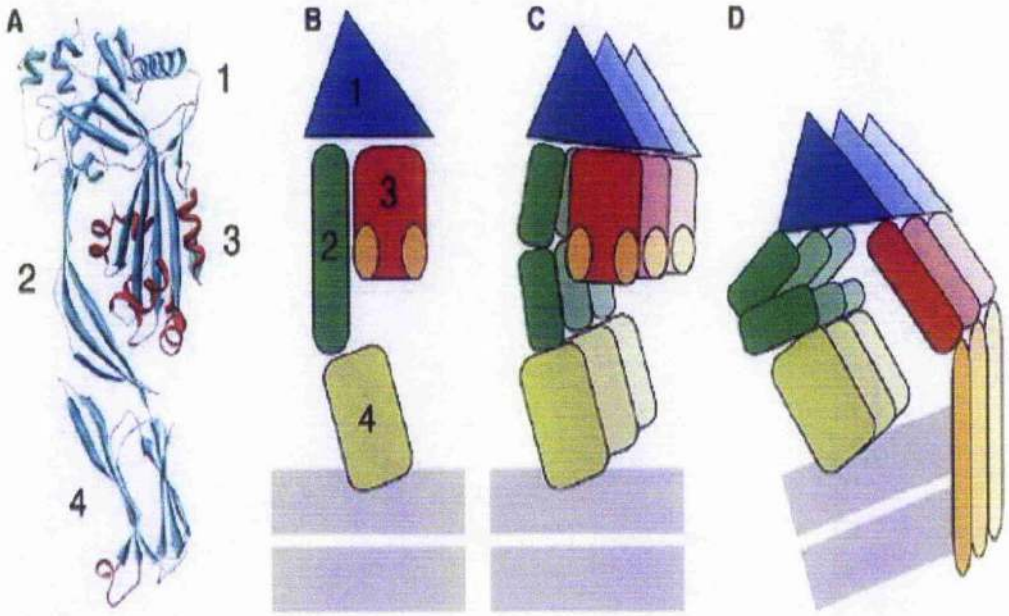
Immunization with combination of these proteins can induce enhanced protection than immunization with individual protein (Briles et al., 2000 and 2003; Ogunniyi et al., 2000 and 2001). The intranasal vaccination of mixture of PsaA and PspA can prevent nasopharyngeal carriage of pneumococci (Briles, Ades et al. 2000). Mice vaccinated with PLY and PspA can improve the protection against pneumococci compared to active vaccination with Ply alone (Briles, Hollingshead et al. 2003).

PLY, one of the most important virulent factors of *S. pneumoniae*, is a member of the thiol-activated toxins and produced by almost all clinical isolates of the pneumococcus; PLY is a soluble 53 kDa monomer. PLY also belongs to the cholesterol-dependent cytolysin (CDC) family. It binds to cholesterol-containing cell membrane, and lyses all cells containing cholesterol in their membrane, PLY forms oligomeric pores containing 30 to 50 PLY monomers, which are believed to lyse cells by osmotic force. PLY has different biological effects in different organs and tissues. PLY is able to interfere with human immunity and inflammatory response(Mitchell T J 2006). Sublytic concentrations of PLY are able to trigger inflammation and activate the complement pathway. PLY can slow ciliary beats in the bronchus and cause apoptosis in neutrophils, macrophages and neuronal cells (Mitchell T J 2006).

The structure of PLY is highly conserved among its variants, there is not much different with the primary structure (Mitchell T J 2006). How PLY binds cells and forms oligomeric pores are still not clear. There are two possible ways for pore formation by the CDC family. Studies from streptolysin O suggests that oligomerisation starts to form the pore after monomers bind and insert to the membrane (Palmer et al., 1998). In contrast, studies from perfringolysin O (PFO) suggest that before the entire oligomer inserts to form the final pore, a prepore forms after monomers bind to the membrane. (Hotze et al., 2001; Hotze et al.,2002). However, streptolysin O also could form a prepore (Heuck et al., 2003). More recently work with PLY suggests PLY forms a prepore before insertion of oligomer takes place (Tiley S J et al., 2005). PFO which is one of virulence factors of *Clostridium perfringens* has almost the same molecular mass as PLY. There are 48% sequence identity and 67% sequence similarity between PLY and PFO. The structure of PFO (Rossjohn et al., 1997) provides a model of the conformational transition into the pore form of model for PLY. Current understanding of PLY molecular mechanism is guided by a model built on the basis of the crystal structure of PFO (Rossjohn et al., 1997). PLY contains four domains (Figure 1.1.A); each of them plays a different role in the pore-forming process. Domain 1 plays a scaffold role that the other domains can reorganize during pore forming. Domain 4 is responsible for initially binding to cholesterol containing cell membrane by inserting a Trptophan rich loop into the upper layer of a lipid bilayer. As the more and more toxin molecules bind to the membrane, these monomers start to assemble a pre-pore due to interaction of domain 1 and 3 among the toxins. Domain 2 begins to bend and dissociate with domain 3, and two sets of three α-helices in domain 3 begin to refold into two long β hairpins. Finally, the β hairpins insert into the lipid bilayer and form pores. These proposals are

supported by the fact that the domain 4 of PLY is essential for PLY binding to sheep blood cell, and PLY without domain 4 (domains 1-3) is unable to bind to sheep erythrocyte membrane (Baba et al., 2001).

**Figure 1.1. Pore formation by Pneumolysin (Walz, 2005)**




(A) crystal structure of the soluble perfringolysin monomer (Rossjohn et al., 1997), the loop in 4 is tryptophan rich. (B-D) the structural rearrangements accompanying the pore formation (Tilley et al. 2005), (B) monomer, (C) prepore, (D) membrane inserted pore.

Purified human antibody to PLY can protect mice against virulent pneumococcal strains (Musher et al., 2001). Immunization of mice with PLY elicits protection against challenge with the pneumococcus (Ogunniyi et al., 2000 and 2001). PLY has been used as carrier in PS conjugate vaccines (Kuo et al., 1995; Lee et al., 2001), and immunization of mice with PS PLY toxoid conjugate vaccine showed rapid clearance

of the penumococcus in the blood and provided a cross-protection against challenge with heterologous serotypes of virulent pneumococci (Lee et al., 2001), which means PS conjugate vaccine using the pneumococcal protein as the carrier can provide a broader protective immunity than that using the non-pneumococcal proteins. However, PLY is too toxic to be used as vaccine directly, its mutant, PLY toxoid, is suitable candidate for generating new vaccine. PLY toxoid, ΔA146PLY lacking total hemolytic activity, is available in our group, and it remains as immunogenic as PLY in mice immunisation study (Kirkham et al., 2006). The PSP conjugate vaccines may induce mucosal immunity in addition to systemic immunity. Pneumococcal virulence proteins can act as carriers in the PSP conjugate vaccine. PS conjugate vaccine produced from pneumococcal proteins can provide a broader protective immunity than that produced from non-pneumococcal proteins (Simell et al., 2001). A new PS PLY toxoid conjugate vaccine has been produced and being evaluated in our group (unpublished data). Importantly, the Ply is not only a conjugate carrier but also induce an additional protection for broad protection against pneumococcal infection, it may elicit immunity complementary to PSs.

## 1.4. The advantage of PLY as a vaccine candidate

PLY is an ideal candidate for developing new penumococcal vaccine. Firstly, PLY is produced by almost all clinical isolates and its primary structure varies little; secondly, PLY elicits T cell dependent immune responses and induce circulatory IgG and secretary IgA antibodies (Simell et al., 2001); third, PLY toxoid is a very effective immunogenic carrier in PS conjugate vaccines; finally, even if the vaccine from PLY

toxoid can not prevent all serotypes, vaccine elicited immunity to PLY can possibly result the less severe disease.

Previous work in our group showed that intranasal challenging mice with very low amount of PLY fusion protein resulted fast production of a significant large amount of specific IgG antibody in mice serum to the carried protein, and high levels of specific IgA to the carried protein present in both the mucosal surface of nasopharynx and pulmonary tracts of mice. This suggested PLY could act as a good adjuvant in PLY fused antigens to induce both systematic and mucosal immunity (unpublished result). A protein-based mucosal vaccine becomes possible if the fusion protein that contain PLY and the other virulence proteins of *S. pneumoniae* remains highly and mucosally immunogenic.

A mucosal vaccine, which can break the transmission chain of infection by preventing nasopharyngeal colonization, should protect children under 2 and immunodeficiency patients. Prevention of nasopharynx colonisation of pneumococci can prevent horizontal spread of pathogen, thus improving herd immunity. The mucosal vaccine also has several advantages since it is administered intranasally and orally, it would avoid pain, and accidents associated with needles and transmission of infection, and save budget on medical equipment and skilled personnel (Zhang and Finn, 2004). A mucosal vaccine may also elicit systematic immunity, i.e. oral polio vaccine, which would further strengthen the immune system for protection against pathogen. Studies have suggested that oral or intranasal vaccine retain the same effectiveness as systemic application (Wu et al., 1997; Seo et al., 2002).

Native PLY is highly haemolytic, so we plan to fuse the carrier protein to specific domains of PLY. The aims of my project are (i) determine which regions of PLY are required for generating immune response, as we need to reduce toxicity while maintaining immunogenicity, (ii) determine whether we can use this technology to develop mucosal response is to other more relevant antigen, (iii) determine the structure of PLY in order to help understanding the mechanism of pore forming and above adjuvant property of PLY.

# Chapter 2

# Materials and Methods

## 2.1. Construction of pET33b-eGFPD4ePLY, pET33b-eGFPD4PLY and pET33b-eGFPD123PLY

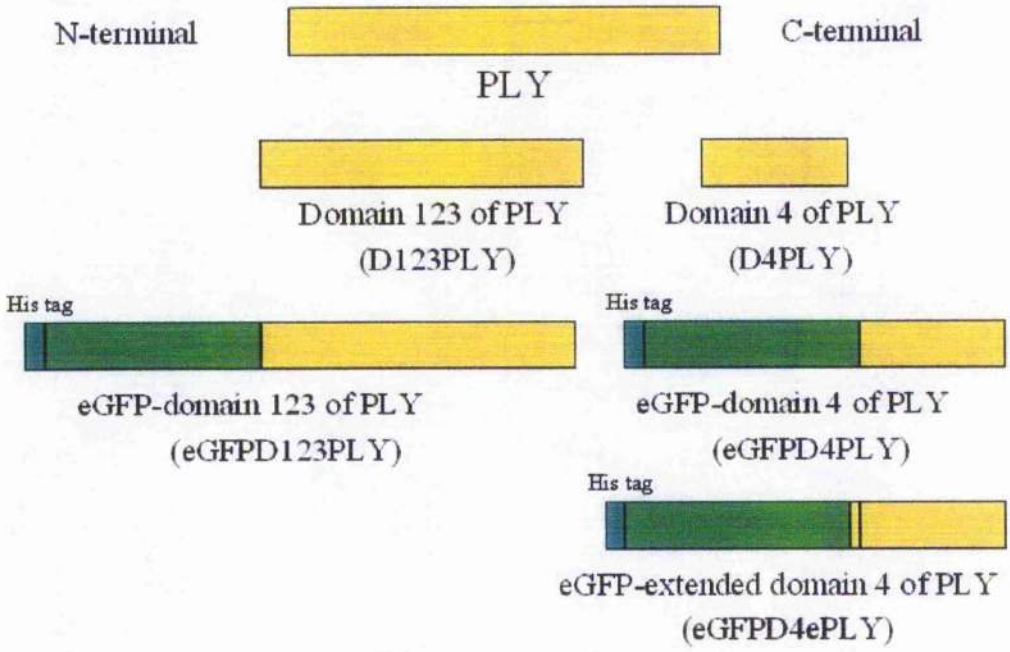
### 2.1.1. Plasmids

Plasmid pET33b (Merck Biosciences, Nottingham, UK), containing a kanamycin resistance gene, was used. pET33b-D4PLY, pET33b-D4ePLY, pET33b-123PLY and pET33b-eGFPPLY contains gene sequence of domain 4 of PLY (D4PLY), extended domain 4 of PLY (D4ePLY), domain 123 of PLY (D123PLY) and eGFPPLY respectively (Figure 2.1. gene sequence of extended domain 4 of PLY is 15 bp longer than that of domain 4PLY). pET33b-eGFPPLY and pET33b-PLY separately were constructed by Mr Graeme J. M. Cowan. 50µl of plasmids were prepared separately from Library Efficiency® DH5α™ chemically competent (DH5α) *E.coli* (Invitrogen, Paisley, UK) using QIAprep Spin Miniprep Kit (Qiagen, West Sussex, UK) following the manufacturer instructions, DNA quality and quantity was measured by agarose gel electrophoresis. The eGFP coding sequence was amplified from plasmid pET33b-eGFPPLY by Polymerase Chain Reaction (PCR, Table 2.1.1.) using Taq polymerase (Taq; Promega) and primers 20G and 20H (Table2.1.2. ).

**Table 2.1.1. The PCR for amplifying eGFP gene**

| Reagent | Concentration and Volume µl |
|---|---|
| Forwards primer (20G) | 10µM, 3µl |
| Reverse primer (20H) | 10µM, 3µl |
| Template (pET33b-eGFPPLY) | 2µl |
| DNTPs | 10mM, 3µl |
| DNase free d $H_2O$ | 78µl |
| 10 × Thermo poly buffer | 10µl |
| Taq | 1µl |

**Figure 2.1. A simple illustration of the differences between eGFPD4ePLY, eGFPD4PLY and eGFPD123PLY**




## 2.1.2. Enzyme digestion and ligation

The purified plasmids were then digested with 2µl each of *Nhe*I and *BamH*I (Figure 2.1.1) restriction enzymes (Promega, Sounthampton, UK) in 6µl of Muti-core™buffer at 37°C for 2 hours. The cut plasmids were treated with 1µl of calf intestinal alkaline phosphatase (promega) at 37°C for 30mins and cleaned up using gel purification kit (Qiagen). The PCR products from pET33b-eGFPPLY were cleaned using gel purification kit (Qiagen), digested using 2µl each of restriction enzyme *Nhe*I and *Bg*III (Promega) at 37°C for 2 hours and purified again using gel purification kit. 1µl of cleaned cut PCR products were ligated with 3µl of cut plasmids using 1µl of T4 DNA ligase in 1µl of ligation buffer plus 4µl of Dnase free $dH_2O$ at room temperature for 4 hours.

### 2.1.3. Transformation and DNA sequencing

Ligated plasmids were transformed into Library Efficiency® DH5α™ chemically competent *E. coli* following the instructions manufacturer provided (Invitrogen). Bacteria were grown overnight on the Luria-Bertani broth ( LB broth; Sigma-Adrich, Doreset, UK) agar plate containing 50μg/ml kanamycin at 37°C. PCR was performed in order to confirm the transformation using primers 7F and 7G (Table 2.1) flanking to the gene inserted. The PCR confirmed clones were grown in 10ml LB containing 50μg/ml kanamycin overnight at 37°C with shaking at 220rpm. Bacterial stock with 10% glycerol was made and 1ml aliquots were then stored at -80°C. Plasmids were purified from culture (Qiagen) and then sent for DNA sequence. 30μl of all three plasmids were sent to the Molecular Biology Support Unit, University of Glasgow and to DBSGENOMICS at Durham University for sequencing using primers 33Z, 34A and 7F (Table 2.1.1). Data for DNA sequence were analysed by Vector NTT™ Advance 10 software (Invitrogen). After the DNA sequences were proven to be correct, Plasmids including pET33b-eGFPD4PLY, pET33b-eGFPD4PLY, pET33b-eGFP123PLY, pET33b-123PLY and pET33b-D4cPLY were electro-transformed into BL21 *E.coli* (Stratagene) for expression by electroporation using BioRad Gene Pulser following the manufacturer instructions provided.

**Table 2.1.2. Primers used for construction expression vector**

| Primer Ref | Primer Name | Sequence (5' to 3') |
|---|---|---|
| 20G | GFP pET33b Fwd | GTC AGG CTA GCA TGA GTA AAG GAG AAG AAC |
| 20H | GFPpET33b Rev | CCA CGC AGA TCT TTG TAT AGT TCA TCC |
| 7F | T7 old Fwd | TAA TAC GAC TCA CTA TAG GG |
| 7G | T7 old Rev | GCT AGT TAT TGC TCA GCG GTG |
| 33Z | eGFPseq1 | TTGCACTACTGGAAAACTAC |
| 34A | eGFPseq2 | CATGGCAGACAAACAAAAAGAA |

## 2.2. Proteins expression and purification

### 2.2.1 Protein expression

The BL21 *E.coli* containing different plasmids separately were grown in 1 litre of terrific broth (TB) or M9 Minimal medium (M9, Appendix I) containing 50μg/ml kanamycin with shaking at 220rpm at 30°C. 1mM Isopropyl-β-D-thio-galactopyranoside (IPTG) was added to induce expression of the recombinant proteins when the $OD_{600}$ of cell cultures was reached 1in TB or the $OD_{600}$ of cell cultures was reached 0.8 in M9; The bacterial cells were harvested by centrifugation at 4°C after they were grown for a further 6 hours in TB or 10 hours in M9 at 30°C with shaking at 220rpm. Cell Pellets were resuspended in 4°C 50 ml 1 × phosphate buffered saline PBS (Oxoid, UK) with 5μg of the proteinase inhibitor Benzamidine and DNAnase I (Sigma-Aldrich, Dorset, UK) per litre of cell pellets, Bacterial pellet suspension were sonicated 4 times for 30 seconds at 100% by cell sonicator (Constant systems Ltd, Warwick, UK), one minute break between each time. The lysates were centrifuged at 18000g for 30 minutes at 4°C to remove cell debris. The supernatant were filtered by syringe using 0.22μm filter (Sartorius, Hannover, Germany).

## 2.2.2 Protein purification

### 2.2.2.1. Nickel affinity chromatography (NAC)

Because expression vector pETT33b is designed to leave six histidines (His tag) at the N-terminus of the proteins, proteins can be purified by immobilised metal affinity chromatography since His tag can interact tightly with metal. The bacterial cell supernatants were passed through a Nickel-charged NTA ( Nitrilotriacetic acid resin) Superflow column (Qiagen) by fast protein liquid chromatography system (FPLCS). The His tagged proteins stayed on the column, and the other proteins and bacterial component were eluted. The His-tagged proteins then were eluted with a 0-250mM continuous gradient of imidazole in phosphate buffered saline (PBS)(Oxford). 25 fractions with increased concentration of imidazole were collected. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to select the right fraction which only contains one right size protein, the other fractions with contaminants were discarded.

### 2.2.2.2. Anion exchange chromatography (AEC)

Purified recombinant proteins were further purified by AEC using Poros ® HQ20 Micron media on a BioCad 700E workstation (Applied Biosystems Ltd, Warrington, UK) in order to exclude DNA and lipopolysacchrides (LPS). The recombinant proteins were eluted with 0.1M NaCl while DNA, LPS and the other contaminants still bound to the column.

### 2.2.2.3. Dialysis

Dialysis tubing with 8 KDa molecular weigh cut off (Medicell International Ltd, London, UK) was boiled in 10mM sodium bicarbonate solution with 1mM EDTA for 10 mins, washed with sterile PBS and stored at 4°C with 20% ethanol. The proteins were dialysed three times against a greater than 50-fold volume of 1 × PBS at 4°C. Amicon ultra centrifugal filters (Millipore, Watford, UK) were used to concentrate the proteins; Amicon ultra centrifugal filters were also used to cut off the small amount of contaminating proteins during purification of eGFPD123PLY. All proteins were filtered by syringe using 0.22μm filter (Sartorius) before aliquoted and stored at -80°C.

## 2.3 Analysis of purified proteins

### 2.3.1 Protein concentration

The amount of protein can be determined by measuring the absorbance by spectrophotometer at 280nm. Purified samples have a low A260 (DNA concentration) and a low A320 ( protein aggregates). The absorbance at 280nmm was divided by the extinction coefficient (E) of proteins. Protein concentration was also measured using a standard Bradford assay. A standard curve was prepared from 2mg/ml of Bovine serum albumin (BSA) to 0mg/ml. 10 μl of standard and samples in duplicate were transferred into a flat bottom 96 well plate, 200μl of Bradford's reagent was then added (Bio-rad, Hertfordshire, UK). Absorbance was measure at 570nm and the protein concentration was determined by the standard curve.

### 2.3.2. SDS-PAGE and Western Blotting

Proteins were analysed using 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting for presence of other contaminating proteins or degradation of the proteins. 2-10µl of samples were mixed with 5 × loading buffer and boiled for 5 min at 95°C before loading on gels. Precision Plus Protein standards Kaleidoscope™ marker (Bio-Rad) was used for indicating proteins' size. All gels were run at 200 Volts for 40 min, then either stained or transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) and blotted at 100 Volts for 60 min. The membranes were blocked in 3% skimmed milk (in Tris-NaCl PH 7.4) overnight at 4°C with shaking. Membranes were then incubated with first antibody (1:2000 dilution in 3% skimmed milk) for two hours at 37°C with shaking. Anti-PLY, anti-eGFP and anti-His-tag antibody were used in Western blotting as the first antibody. Membranes were then washed four times with Tris-Nacl (PH 7.4) buffer and incubated with HRP-Linked second antibody (1:2000 dilution in 3% skimmed milk; Amersham Biosciences ) for two hours at 37°C with shaking. Membranes were washed four times and finally developed with developer. For more details, please see Appendix 1.

### 2.3.3. Haemolytic assay

Round- and flat-bottomed 96-well plates (R-plate and F-plate; Nunc™, Danmark) were both used for haemolytic assay. 1ml of horse blood was centrifuged for 1 min at 13k rpm in an eppendorf tube. The supernatant was removed and the pellet was resuspended in same volume of PBS. Above process was repeated 5 times in order to move lysed cell and antibodies completely. 2% horse blood was prepared by adding 400µl washed horse blood to 20 ml PBS. 100µl of PBS was added into required wells

of a R-plate by multi-channel pipette.100µl of PBS (negative control) and samples were then added into the wells in row 1, and 100µl of solution was transferred into row 2 from row 1. The tips were changed and 100µl of solution was transferred into row 3 from row 2. Above step was repeated until 100µl was discarded from bottom row of plate. 100µl of 2% horse blood was added into each well. The plate was covered by a lid and incubated at 37°C for 30 minutes. The R-plate was centrifuged at 1000rpm for 1 minute and 100µl of supernatant from each well was transferred into a fresh F-plate by multi-channel pipette. The absorbance was measured by the spectrophotometer (96 well plate reader) at 540nM.

### 2.3.4. Cell binding assay by fluorescence microscopy

Horse blood was prepared following the method in haemolytic assay. Lysed horse erythrocytes were also prepared with sterile water. 1ml of horse blood was centrifuged for 1 min at 13k rpm in an eppendorf tube. The supernatant was removed and the pellet was resuspended in same volume of water. The cell suspension was centrifuged and the supernatant was removed again. The same process was repeated 3 times. 10 µl of horse blood and 1µl of proteins were added into 500 µl eppendorf tubes, and incubated at 37°C for 15 minutes. 1 µl of each sample was put on microscope slide and covered with a cover glass after the samples were washed 3 times using PBS in order to clean the excess proteins. The specimens were observed under microscope with ultraviolet (UV) light.

### 2.3.5. Cell binding assay by fluorescence-activated cell sorter (FACS)

Flow cytometry was performed using FACS. 4% horse blood was prepared by adding 800µl washed HBC into 20ml PBS. All proteins were diluted to 14nM/L, 140nM/L and 1400nM/L concentration. 500 µl of 4% horse blood and 500µl of protein sample were added into the eppendorf tubes. The mixture was transferred into FACS tubes and read by FACS after they were incubated at 37°C for 15 minutes.

### 2.3.6. Lipopolysacchride (LPS) levels in purified recombinant proteins

*E.coli* is a Gram-negative bacteria, LPS is the naturally of component of cell wall of Gram-negative bacteria. LPS is highly immunogenic, high level of LPS will apparently interfere mouse immunisation study. An in vitro kinetic, colorimetric, quantitative chromogenic, limulus amebocyte lysate (LAL) assay (Kinetic-QCL®) (Cambrex, Nottingham, UK) was used to determine the level of contaminating Gram-negative LPS in all protein samples. The assay was run following manufacturers instructions and with help of Dr. Gill R. Douce.

## 2.4 Structure study of PLY

D4ePLY and D123PLY purified from terrific broth were concentrated to 10 mg/ml in sterile PBS by amicon ultra centrifugal filters (Millipore); the concentration was determined by measuring OD value by spectrophotometer. 0.5 ml of 10mg/ml D4ePLY and D123PLY were mixed with different solvents in order to obtain crystal, which is used for X-ray crystallography analysis. Dr Allen Ribaldi Tunnicliffe helped with all experiments with X-ray crystallography. 0.5 ml of 10mg/ml D4ePLY was

analysed by the high field Nuclear Magnetic Resonance (NMR) spectroscopy. $N^{15}$ labelled D4cPLY and PLY purified from M9 minimal medium were concentrated to 5 mg/ml and 3mg/ml separately, and then were analysed by NMR spectroscopy. Water-soluble and insoluble cholesterol (Sigma) was used to map the binding site of PLY. Dr Brian Smith from University of Glasgow generated all data with NMR.

## 2.5. Mice immunisation study of purified recombinant proteins

In vivo experiment were performed in accordance with the UK Animals 1986 (Scientific Procedures Act). Food and water *ad libitum* were provided and mice were left at a constant room temperature of 20-22 °C with a 12 hours light/dark cycle. All animal was handling was done by Dr. Gill R. Douce. The sample processing, data acquisition and interpretation was carried out by the author, Jiang Tao Ma.

### 2.5.1. Intranasal challenge of mice

Six to eight week-old female mice (Harlan Olac,UK) were intranasally immunised 3 times with purified proteins on days 1, 27 and 54 in the groups shown in table 2.5.1. The experiment was based on giving 0.2μg of wild type (WT) PLY toxin and ten times equimolar concentration of D123PLY and D4PLY, and the final vaccine dose is 30μl. Group 1 was immunised with 0.2μg of PLY and 0.1μg of eGFP, which ensure the ratio of PLY and eGFP was identical to that present in an equivalent dose of the eGFPPLY protein in group 2; group 2 was immunised the equimolar concentration of eGFPPLY,; group 3 was immunised ten times equimolar concentration of D4PLY and eGFP; group 4 was immunised ten times equimolar concentration of eGFPD4PLY and eGFP; group 5 was immunised ten times equimolar concentration of D123PLY and eGFP; group 6 was immunised ten times equimolar concentration of

eGFPD123PLY and eGFP; group 7 was immunised ten times equimolar concentration of eGFPD4PLY and 0.2μg of PLY; group 8 was immunised ten times equimolar concentration of eGFPD123PLY and 0.2μg of PLY. Mouse sera from the day before vaccination were collected. Anti-eGFP and anti-PLY IgG response within individual serum samples were measured by enzyme linked immunoasorbent assay (ELISA).

**Table 2.5.1. Mice immunisation study of purified recombinant proteins**

| Group | Antigens used | Number of mice |
|---|---|---|
| 1 | eGFP, PLY | 6 |
| 2 | eGFPPLY | 5 |
| 3 | eGFP, D4ePLY | 5 |
| 4 | eGFPD4ePLY | 5 |
| 5 | eGFP, D123PLY | 5 |
| 6 | eGFPD123PLY | 5 |
| 7 | eGFPD4ePLY, PLY | 5 |
| 8 | eGFPD123PLY, PLY | 5 |

## 2.5.2. Measuring anti-eGFP by ELISA

Flat-bottomed 96-well plates (Nunc™) were used for ELISA. Plate wells were coated 100μl of 1μg/ml eGFP in PBS overnight at 4°C. The plates were washed three times in PBS-T (PBS plus 0.05% Tween 20) and dried thoroughly. The plates were blocked with 200μl/well of 1% BSA in PBS (Sigma-Aldrich) and incubated for 1 hour at 37°C. The plates were washed 3 times in PBS-T and dried thoroughly. The plates were then incubated at 37°C with 100μl/well of serial dilution of the serum in duplicate (started at 1:50) in PBS for 2 hours. The plates were washed 3 times in PBS-T and dried thoroughly. The plates were incubated for 1 hour at 37°C with 100μl/well of a 1:1000

dilution of anti-mouse anti-IgG HRP conjugate (Amersham, UK) in washing buffer. In the negative control, 100µl/well of fresh coating buffer was added; in the positive control, a mouse serum that has been tested active was added. The plates were washed 3 times in PBS-T and dried thoroughly. 50µl /well of developing solution (Appendix I) were added into each well. The plates were incubated for 5 min at 37°C and the reaction was stopped with 50µl/well of 3M Hydrochloric acid. The absorbance was read by spectrophotometer (96 well plate reader) at 490nm and titres were calculated as the reciprocal of the highest serum dilution, which gave an absorbance of 0.3.

### 2.5.3. Measuring anti-PLY IgG by ELISA

All the steps are the same as above except that plate wells were coated with100µl of 2.5µg/ml WT PLY in coating buffer (Appendix I) overnight at 4°C.

## 2.6 Statistical analysis

The GraphPad® Instat (GraphPad® Instat Software Inc., San Diego, CA) were used for analysing data. Antibody production experiment fit normal distribution, but only 5 samples in each group, so unpaired t-test was used to comparing antibody production between different groups.

# Chapter 3

# Results

## Construction and purification of pneumolysin derivatives

## 3.1. Construction of pET33b-eGFPD4ePLY, pET33b-eGFPD4PLY and pET33b-eGFPD123PLY

pET33b-eGFPD4ePLY, pET33b-eGFPD4PLY and pET33b-eGFPD123PLY were constructed in this work. pET33b-D4PLY, pET33b-D4ePLY, pET33b-123PLY and pET33b-eGFPPLY were constructed by Mr Graeme J.M.Cowan. The results from PCR (Figure 3.2.) confirmed the transformation of plasmids was successful. The size of PCR product for pET33b-eGFPD4PLY, pET33b-eGFPD4PLY and pET33b-eGFPD123PLY should be 1382 bps, 1397 bps and 2117 bps (Invitrogen Vector NTI Advance ™ 10). The result from PCR (Figure 3.2.) suggests eGFPD4ePLY, eGFPD4PLY and eGFPD123PLY gene has been constructed.

### Figure 3.1. Plasmid map of pET33b-eGFPD123PLY constructs.

The picture is made by Vector NTT™ Advance 10 software (Invitrogen)

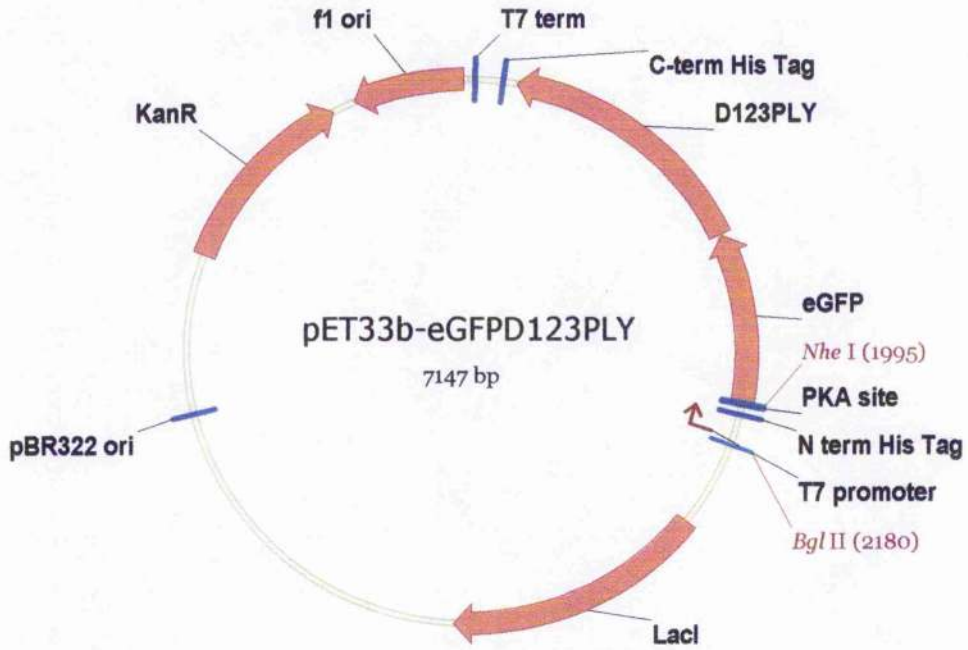
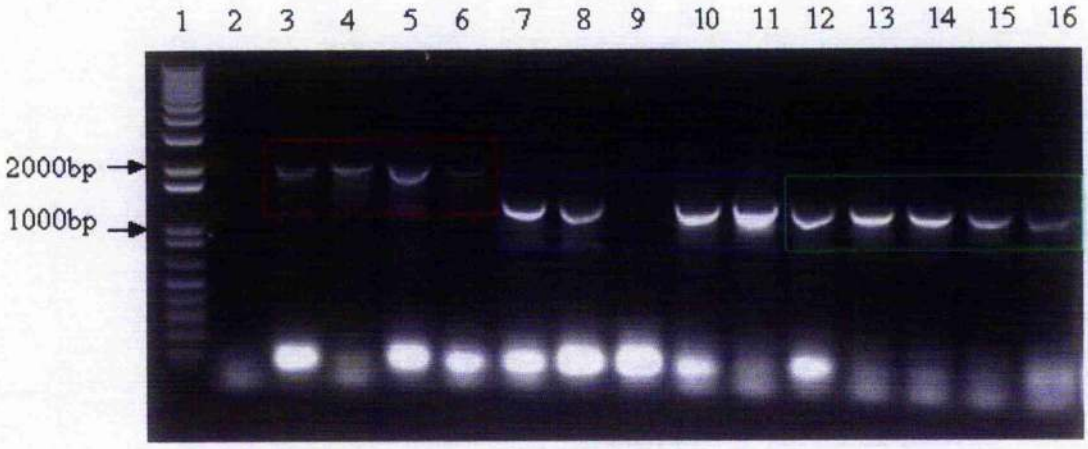


**Figure 3.2. Result of PCR**




Lane 1. 1Kb plus DNA ladder (Invitrogen); lane 2, control; lanes 3-6, eGFP-D123PLY is 2117 bp; lanes 7-11, eGFP-D4ePLY is 1397 bp; lanes 12-16, eGFP-D4PLY is 1382 bp.

Both data from the Molecular Biology Support Unit, University of Glasgow and the DBSGENOMICS at Durham University show no mutation in the sequence of eGFPD4PLY, eGFPD4PLY and eGFPD123PLY.

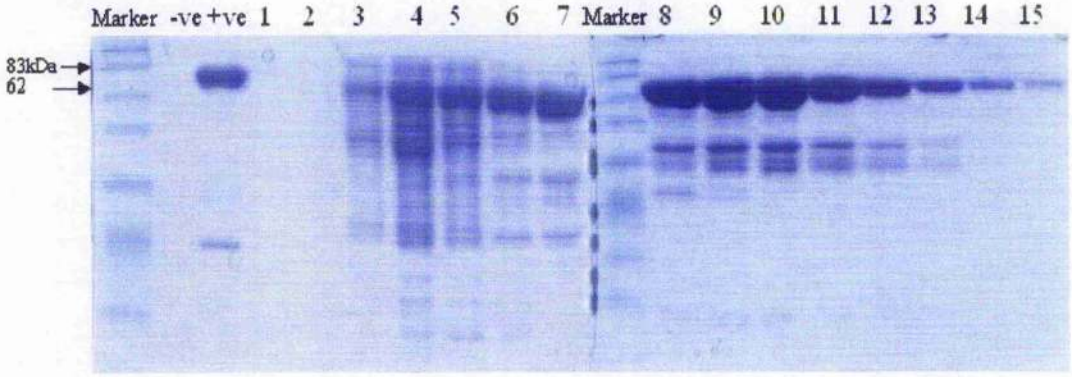
## 3.2. Protein purification

eGFPPLY, eGFPD123PLY, eGFPD4ePLY, PLY, D123PLY and D4ePLY, in total, six recombinant proteins were purified using a two-step method of Nickel affinity chromatography (NAC) and anion exchange chromatography (AEC). eGFPD4PLY was poorly expressed, on contrast, eGFPD4ePLY was expressed much better than eGFPD4PLY and no further experiment was done with eGFPD4PLY, which indicate the extension between eGFP and domain 4 of PLY can increase the solubility of recombinant protein.

### 3.2.1 Purification of eGFPD123PLY

Figure 3.3. Shows a representative NAC purification of eGFPD123PLY by SDS-PAGE. The different lane number represent different fractions, 8μl of samples were loaded in each lane. 20 fractions were collected during NAC purification of eGFPD123PLY. Marker is broad range marker (New England Biolabs), negative control is loading buffer, and positive control is previous purified recombinant eGFPPLY, whose molecular weight is 83 kDa. Fractions 3-8 contain a large amount of recombinant eGFPD123PLY, whose molecular weight is 70kDa, but contain a large amount of the other proteins, and were discarded. Fraction 9-15 were kept and used for next step AEC purification (Figure 3.2.1.2).

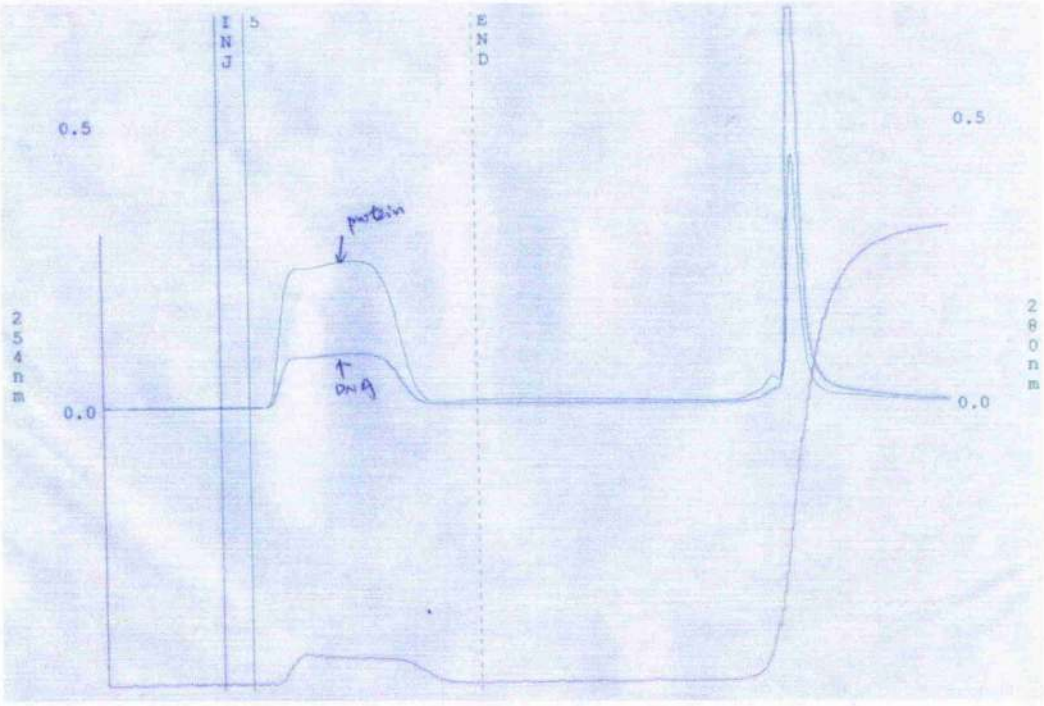
**Figure 3.3. SDS-PAGE gel of eGFPD123PLY after NAC purification.**




During AEC purification, eGFPD123PLY bound to the negatively charged column, and were washed completely, then was eluted with 0.1M NaCl. The column was finally cleaned with 3M NaCl. There are two peaks in the picture, the protein level is much higher than DNA in the first peak, the protein level is much lower than DNA level in the second peak, which mean there are much less DNA in the protein sample

and DNA has been efficiently moved by AEC because the first peak represents the fraction was collected and the second peak represent the waste.

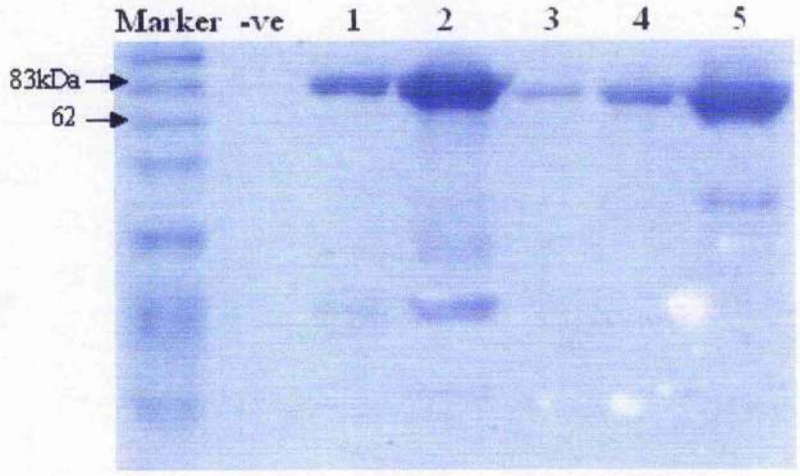
**Figure 3.4. AEC purification of eGFPD123PLY**




Anion exchange Chromatography of eGFPD123PLY. The green line is A280nm protein reading, which measures protein content. The blue line is A254nm DNA reading, which measures DNA content. The pink line represents NaCl concentration (M); the yellow line represents the pressure in the column.

The eGFPD123PLY peak from Figure 3.4. was dialysed three times at 4°C, 7 hours each time with 3L pre-chilled PBS buffer. 2ml of purified eGFPD123PLY was added into amicon centrifugation filters and were concentrated to almost 0.5ml. 0.5ml sterile PBS was added into filters and centrifuged again, this process was repeated 5 times. In Figure 3.5, SDS-PAGE gel shows the contaminant level of eGFPD123PLY was reduced significantly comparing to Figure 3.2.1.1. after using Amicon centrifuge tube.

**Figure 3.5. SDS-PAGE gel of eGFPD123PLY dialysis**




1µl of eGFPPLY protein was loaded in Lane 1, 8µl of eGFPPLY was loaded in Lane 2, 1µl of eGFPD123PLY protein was loaded in Lane 3, 3µl was loaded in Lane 4, and 8µl was loaded in Lane 5.

**Figure 3.6 SDS-PAGE gel of eGFPD4ePLY after NAC purification**

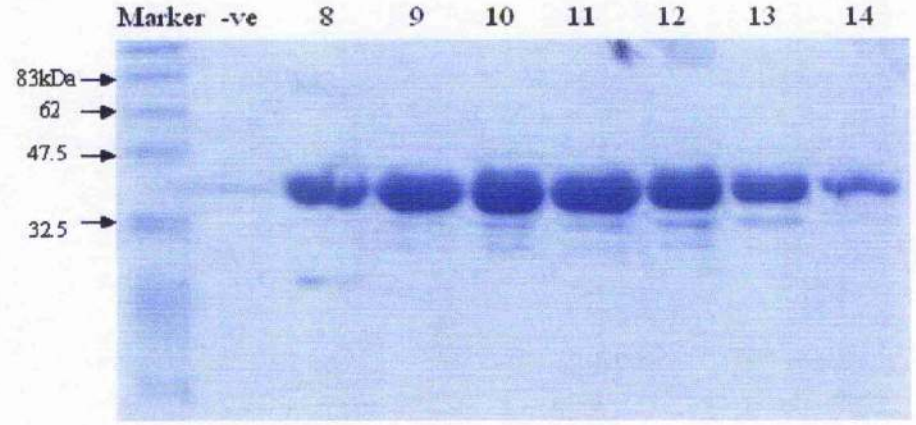
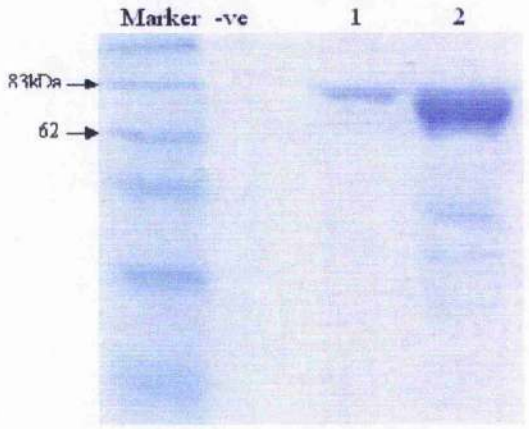


Figure 3.6. shows a representative NAC purification of eGFPD4ePLY by SDS-PAGE. The different lane number represent different fractions, 8µl of samples were loaded in each lane; MW of recombinant eGFPD4ePLY is 44kDa. 20 fractions were collected during NAC purification of eGFPD4ePLY. Fraction 9-16 were kept for further purification. The eGFPD4ePLY was further purified as eGFPD123PLY.

**Figure 3.7. SDS-PAGE gel of eGFPPLY after AEC purification**




In Figure 3.7. the samples were loaded in 10% SDS-PAGE gel, 1µl of protein was loaded in Lane 1, and 8µl was loaded in Lane 2. The gel was stained with coomassie brilliant blue solution. Negative control is loading buffer, MW of recombinant eGFPPLY is 83kDa. eGFPPLY was purified as the same as eGFPD123PLY.

**Figure 3.8 SDS-PAGE gel of PLY after NAC purification**

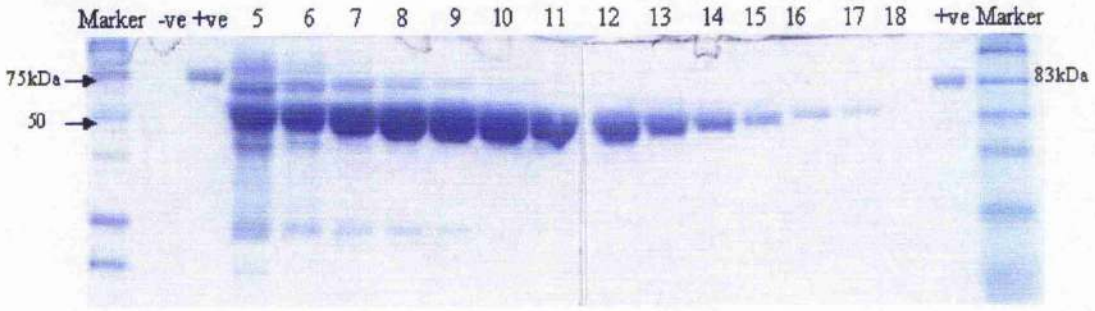



Figure 3.8. Shows a representative NAC purification of PLY by SDS-PAGE. The different lane number represent different fractions8µl of samples was loaded in each lane; MW of recombinant PLY is 57 kDa. 20 fractions were collected during NAC purification of PLY. Fractions 10-17 were kept for further purification. The PLY was further purified as eGFPD123PLY.

**Figure 3.9. SDS-PAGE gel of D123PLY after AEC purification**

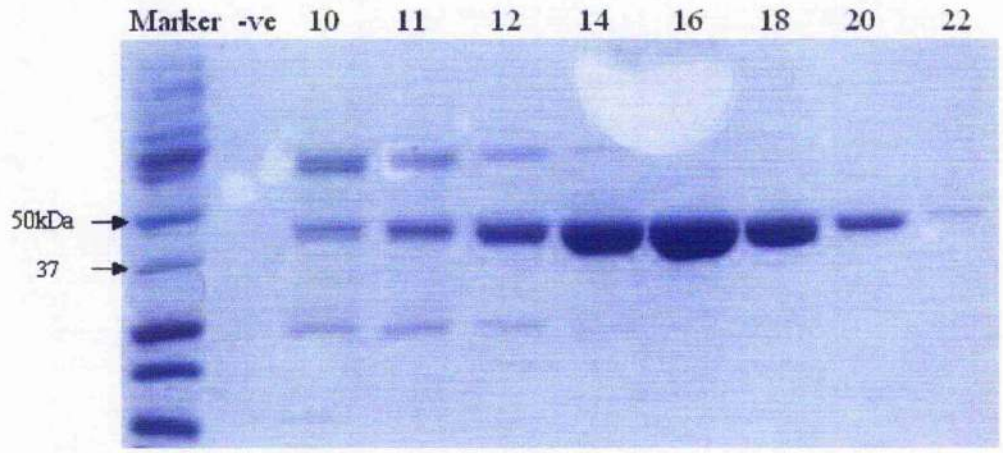



Figure 3.9. Shows a representative NAC purification of 123PLY by SDS-PAGE. The different lane number represent different fractions, 8µl of samples were loaded in each lane; MW of recombinant D123PLY is 44 kDa. 25 fractions were collected

during NAC purification of 123PLY. Fractions 16-22 were kept for further purification. The D123PLY was further purified as eGFPD123PLY

**Figure 3.10. SDS-PAGE gel of D4PLY after AEC purification**

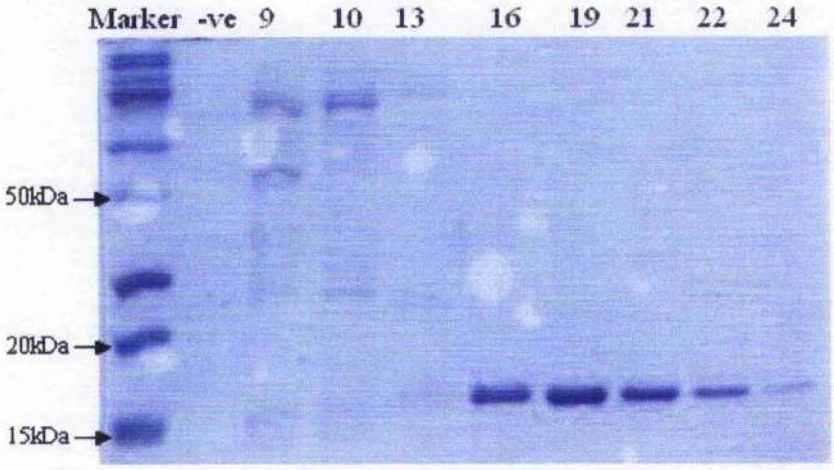



Figure 3.10. Shows a representative NAC purification of D4ePLY by SDS-PAGE. The different lane number represent different fractions, 8µl of samples were loaded in each lane; MW of recombinant D4ePLY is 18 kDa. 25 fractions were collected during NAC purification of D4ePLY. Fractions 16-25 were kept for further purification. The D4ePLY was further purified as eGFPD123PLY.

# Chapter 4

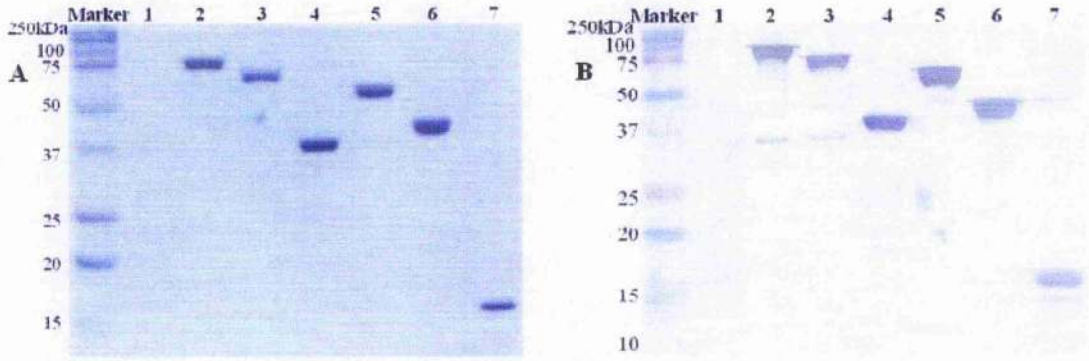
# Results

## In vitro properties and structural characterisation of pneumolysin and its derivative

## 4.1. SDS-PAGE and Western Blotting

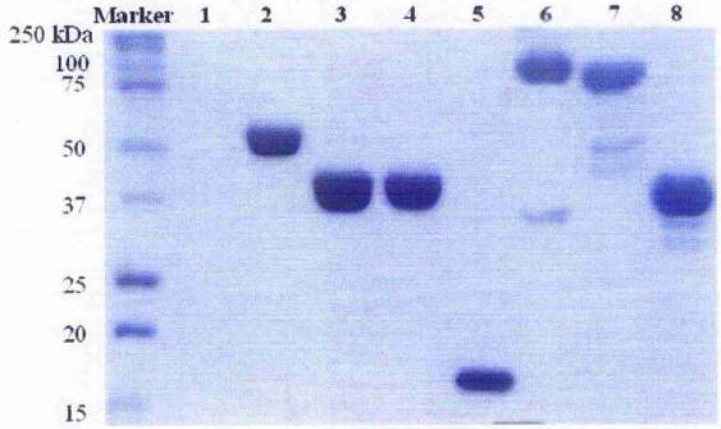
The purity of purified recombinant proteins were further analyzed by SDS-PAGE and Western Blotting. Figure 3.1. show highly pure recombinant proteins were produced. Figure 4.2. shows these purified protein are PLY or PLY derivatives and there are slight degradation in eGFP tagged proteins.

**Figure 4.1. SDS-PAGE (a) and Western Blotting (b) analysis of the purified recombinant proteins.**




1 μg of each sample was loaded on gel and the gel was stained with coomasie brilliant blue solution. Anti-Ply antibody was used for Western Blotting. Lane1, negative control; lane 2, eGFP-PLY is 83 kDa; lane 3, eGFP-D123PLY is 70 kDa; lane 4, eGFP-D4PLY is 42 kDa; lane 5, PLY is 57 kDa; lane 6, D123PLY is 44 kDa; lane 7, D4PLY is 18kDa.

**Figure 4.2. Overloaded SDS-PAGE analysis of the purified recombinant proteins**




In Figure 4.2., lane1, negative control; lane 2, PLY is 57 kDa; lane 3 & 4, D123PLY is 44 kDa; lane 5, D4ePLY is 18 kDa; lane 6, eGFPPLY is 83 kDa; lane 7, eGFPD123PLY is 70 kDa; lane 8, eGFPD4PLY is 42kDa.

## 4.2. Concentration of purified recombinant proteins

Protein concentration was measured using a standard Bradford assay (Figure 3). The concentration of eGFPPLY was 513 µg/ml, eGFP-D123PLY was 450 µg/ml, eGFP-D4ePLY was 434µg/ml, PLY was 347 µg/ml, D123PLY was 536µg/ml and D4PLY was 441µg/ml (Table 4.1.). eGFPPLY was expressed at 37°C, when expression temperature was changed to at 30°C, the production of eGFPPLY increases 20 fold. The other proteins except eGFPPLY and PLYcannot be expressed at 37°C.

**Table 4. 1. Concentration and yield of the purified recombinant proteins**

| Protein sample | Concentration ((μg/ml) | Yield (mg/L) |
|---|---|---|
| eGFPPLY | 513 | 80 |
| eGFPD123PLY | 450 | 100 |
| eGFPD4ePLY | 434 | 50 |
| PLY | 347 | 50 |
| D123PLY | 536 | 25 |
| D4ePLY | 441 | 20 |

## 4.3. Haemolytic assay

The purified proteins were analysed in a haemolytic assay to determine their specific activity. The purified recombinant PLY and eGFPPLY retains very strong haemolytic activity (Figure 4.3.C). 1nM of recombinant PLY and eGFPPLY was able to completely lyse 2% horse blood. The haemolytic activity of PLY and eGFPPLY are $3.9x10^4$ HU/mg and $2.75 x10^4$ HU/mg respectively. The same molar concentration of PLY and eGFPPLY has the same haemolytic activity, which indicates eGFP fusion does not interfere haemolytic activity of PLY. Truncated forms of PLY have no haemolytic activity at all even at 3000nM. Interestingly, the high concentration of D4ePLY and eGFPD4ePLY caused horse erythrocytes to form aggregate in PBS solution (Figure 4.3.A and B); the high concentration of D123PLY and eGFPD123PLY were able to clean the horse erythrocytes in the PBS, the supernatant in the high concentration of D123PLY and eGFPD123PLY is clearer than that its control (PBS) (Figure 4.3.A and B).

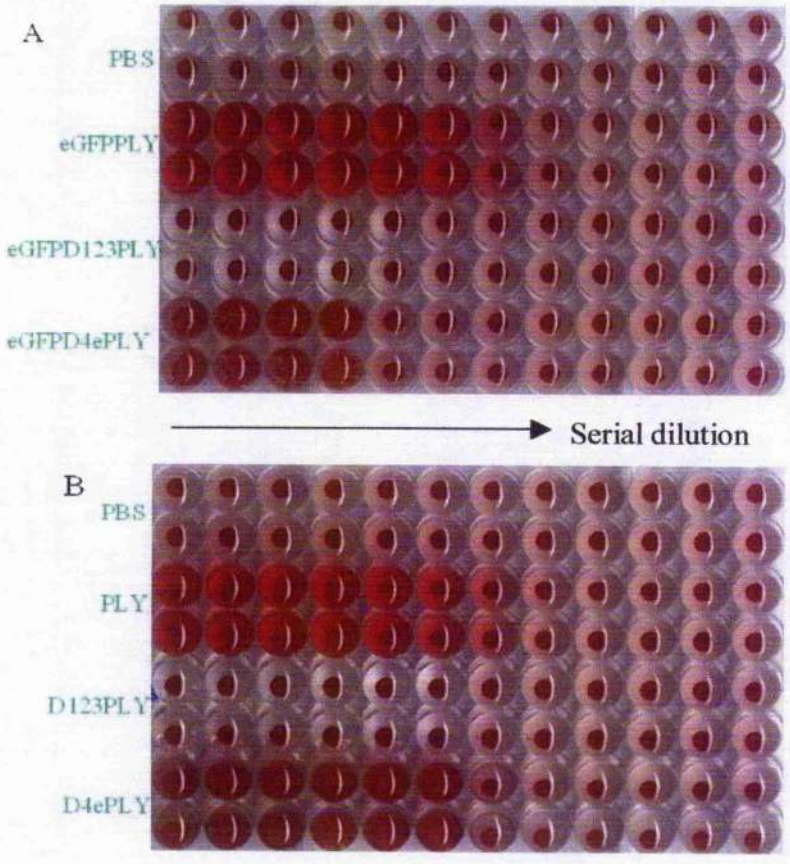
## 4.4. Cell binding assessed by Fluorescence Microscopy

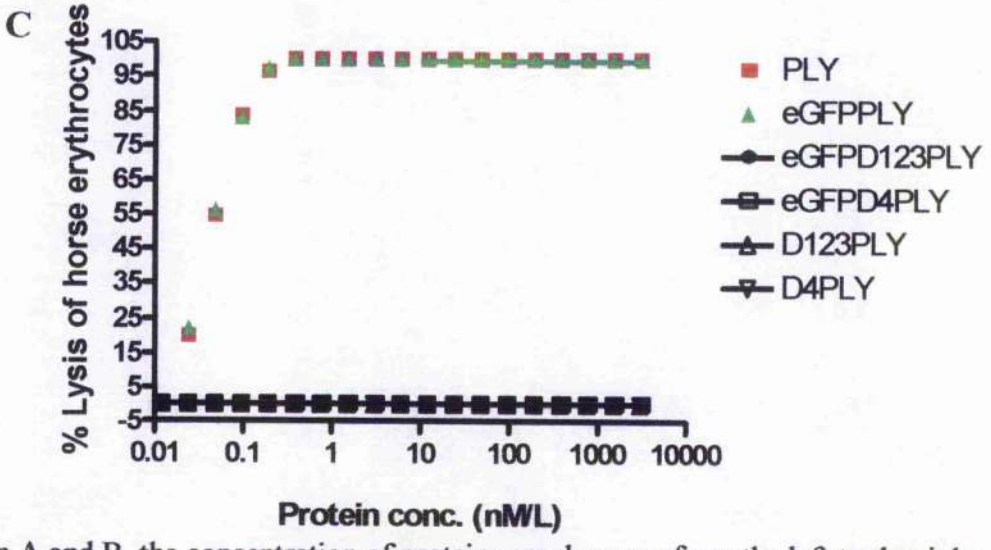
Florescence microscopy images (Figure 4.4.) showed eGFPPLY and eGFPD4PLY but not eGFPD123PLY bind to horse erythrocytes, and eGFPPLY lysed horse erythrocytes but eGFPD4ePLY did not. These results are consistent to the findings in the haemolytic assay. Furthermore, eGFPD4ePLY bound horse erythrocytes to form a large aggregate, which is also consistent to the findings in the haemolytic assay.

## 4.5. Cell binding assessed by FACS

In Figure 4.5., flow cytometry analysis further confirmed the finding with florescence microscope, eGFPD4PLY binds to horse erythrocytes, and eGFPD123PLY does not bind to horse erythrocytes. More fluorescent cells were detected with increasing of concentration of eGFPD4PLY, but not with eGFP and eGFPD123PLY; the horse erythrocytes were lysed at 7nM eGFPPLY since 1nM eGFPPLY is enough to lyse 2% horse erythrocyte.

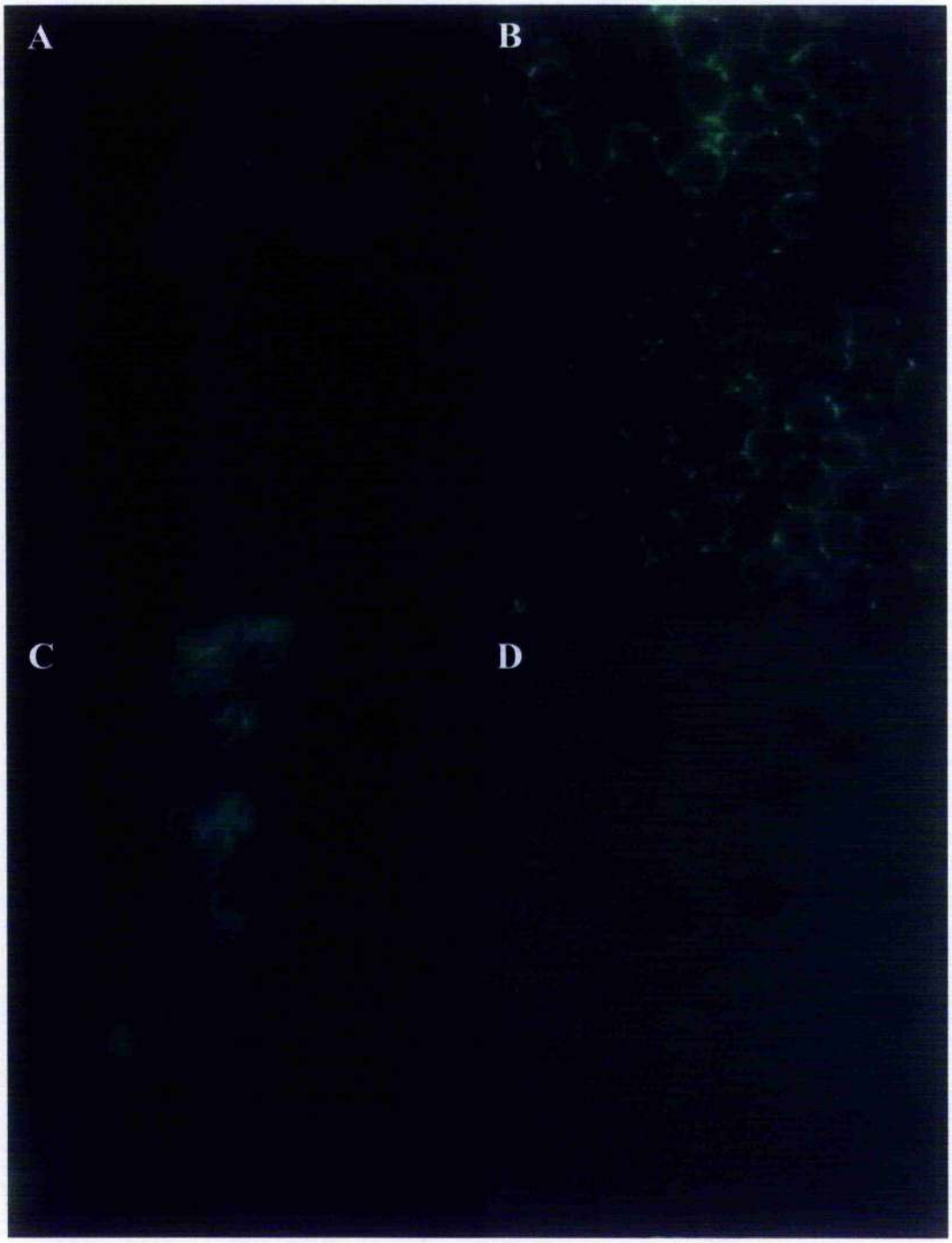
**.Figure 4.3. Haemolytic activity of purified recombinant proteins.**




In A and B, the concentration of proteins are decrease from the left to the right.

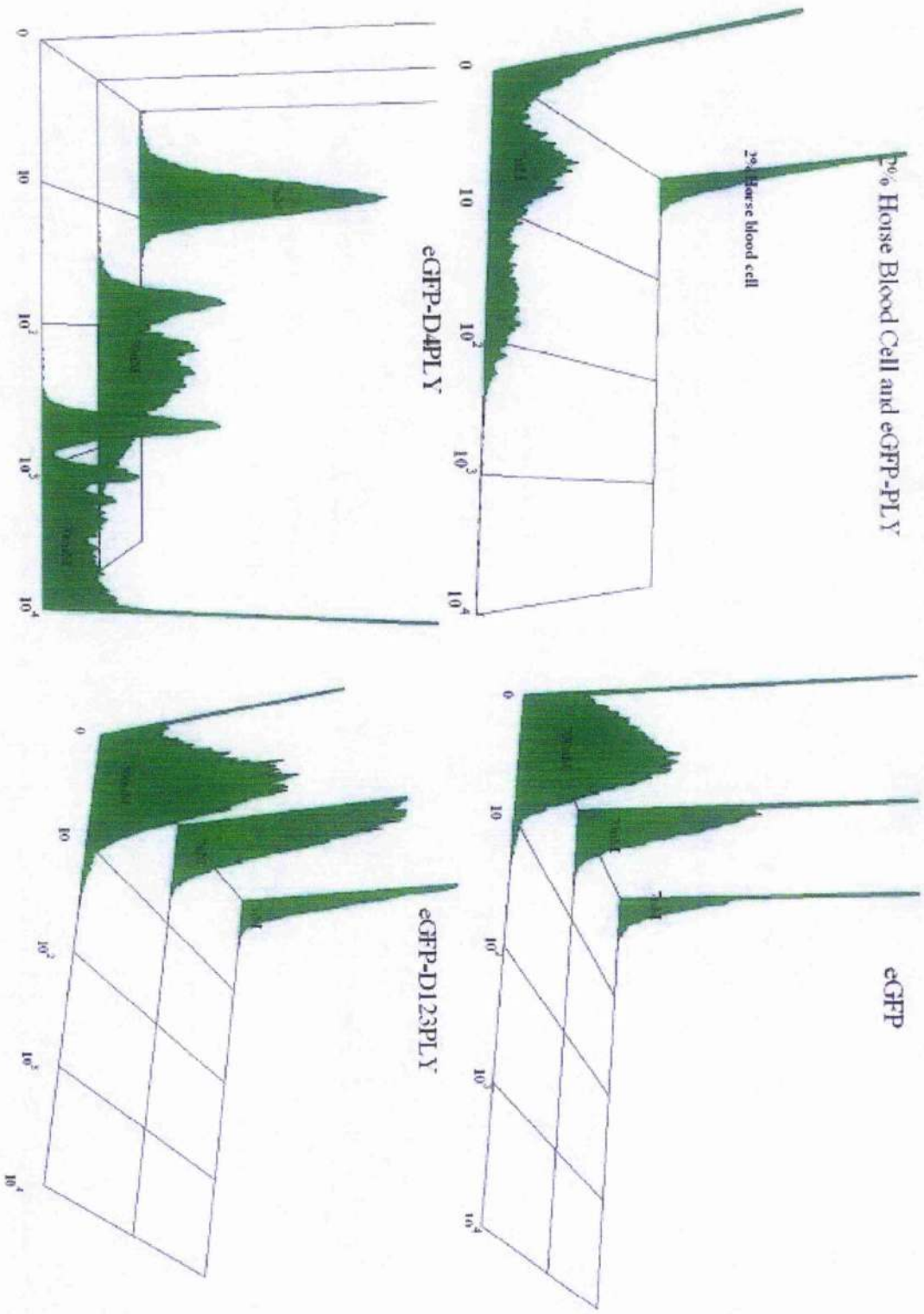
**Figure 4.4. Fluorescence microscopy images**




**X 100**

(A)&(C eGFPPLY binds to horse erythrocytes; (B) eGFPD4PLY binds to horse erythrocytes without lysis; (D) eGFPD123PLY does not bind to horse erythrocytes.

**Figure 4.5. Flow cytometry analysis**




Flow cytometry data was plotted using WinMDI (http://facs.scripps.edu)

## 4.6. Quantification of LPS contamination in purified recombinant proteins

**Table 3.2. LPS Levels in purified recombinant proteins**

| Protein samples | LPS Concentration (IU/μg) |
|---|---|
| eGFPPLY | 0.35-0.37 |
| eGFPD123PLY | 0.334-0.373 |
| eGFPD4ePLY | 0.067 |
| PLY | 0.103 |
| D123PLY | 0.047 |
| D4ePLY | 0.065-0.068 |
| eGFP | 1.88 |

Purified recombinant eGFPD4ePLY, PLY, D123PLY and D4ePLY have very low LPS level, which less than or equal to 0.1. LPS level in eGFPPLY, eGFPD123PLY and eGFP are much higher than the others, but still acceptable for immunization studies.

## 4.7 Structural characterisation of PLY

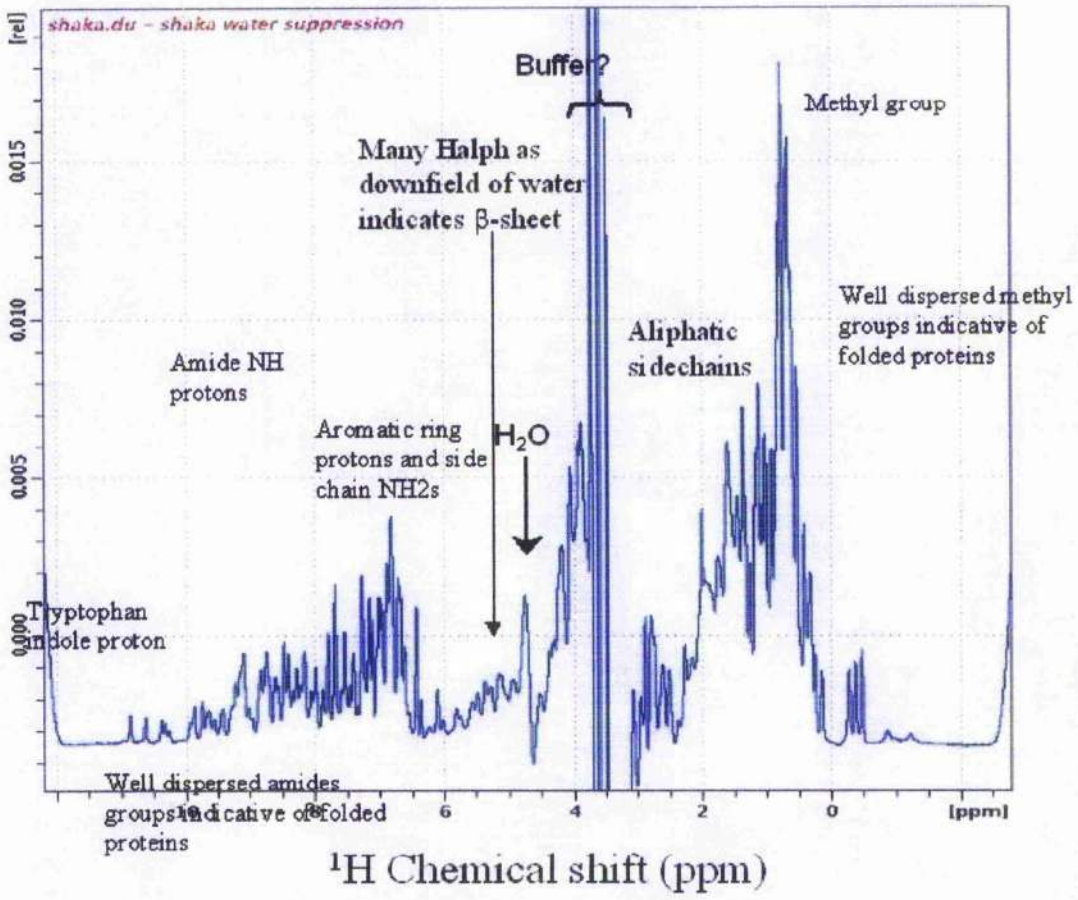
In protein precipitation experiment for X-ray crystallography, D123PLY precipitated heavily and quickly, and no crystal could form, which mean the concentration of D123PLY is too high. About 50% of samples with D4ePLY started to precipitate, but no crystal was formed.

In the parallel to X-ray crystallography, NMR spectroscopy was used to solve the structure of PLY. Figure 4.7.1. shows D4ePLY was well folded since the protein signals were well visible, well dispersed and sharp. Chemical shift (frequency) gives you information about which line comes from which chemical group, but proteins are

made up of many thousands of atoms, which have similar chemical environments, so spectra are overlapped. $^{15}N$ labelled D4ePLY and PLY were purified and analysed by high field NMR spectroscopy. 2-D $^{15}N$ heteronuclear single quantum correlation (HSQC) of spectrum of D4cPLY (Figure 4.7.2.) and PLY (Data not show) were generated, which can be used for mapping the binding site of PLY. Recombinant PLY and eGFPPLY maintain properties of native PLY, and NMR data show purified recombinant proteins are well folded, so immunogenic and structural properties of PLY can be studied extensively with these proteins.

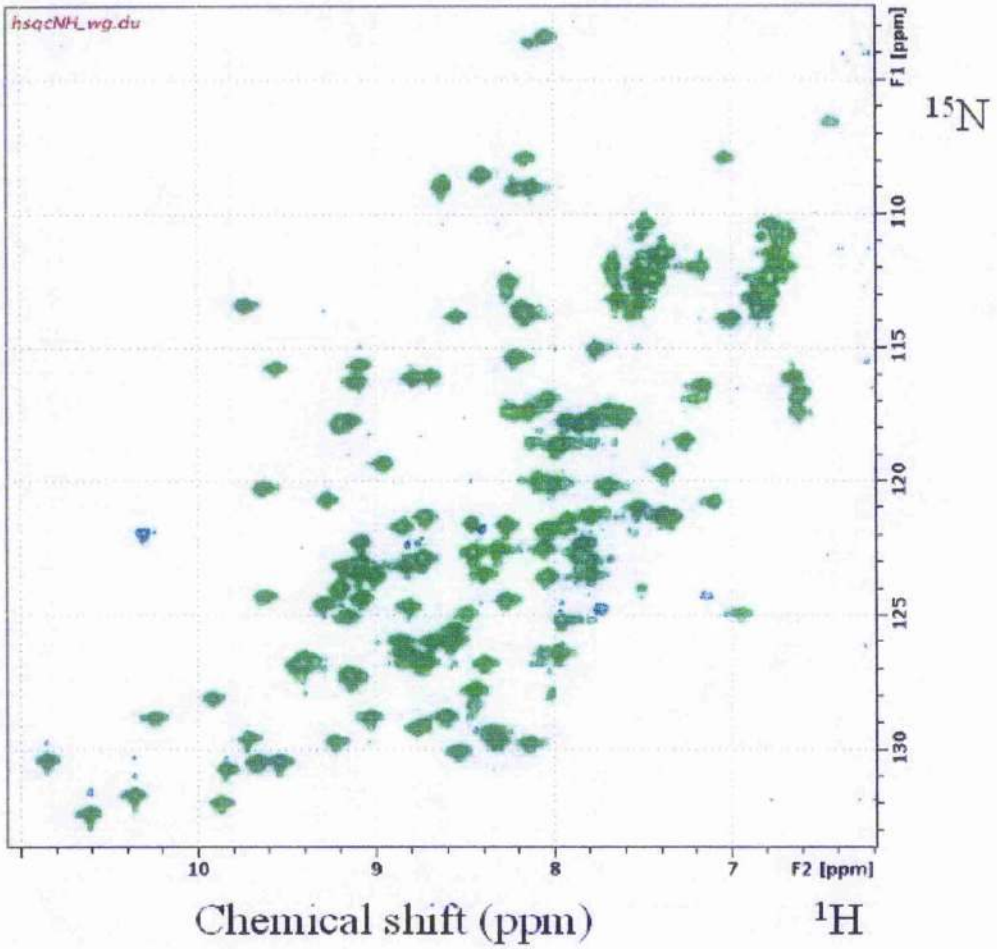
Cholesterol in the cell membrane has been considered to be the binding site for PLY. So next we sought to map the binding site of the interacting amino acids in D4ePLY to cholesterol by chemical shift perturbation. The difference of HSQC spectrum between samples is called chemical shift perturbation spectroscopy. Figure 4.7.3. shows overlayed 2-D $^{15}N$ HSQC experiments from the free D4ePLY and the mixture of D4ePLY with water insoluble cholesterol. Each spot is derived from an amide nitrogen and its attached proton so there is a spot for each residue in the protein. Shifts in the position of the spots when the peptide binds indicate changes in environment. There is no significant shifts on adding cholesterol, which implies no binding, but it could be solubility problem of cholesterol we used. Figure 4.7.4. shows overlayed 2-D $^{15}N$ HSQC experiments from the free D4ePLY and the mixture of D4PLY with water-soluble cholesterol. There are small, but significant shifts on adding water soluble cholesterol, which could imply interaction between water soluble cholesterol and individual amino acid. A tryptophan is the most perturbed residue (see the arrow). However, the experiments need to be repeated and controlled for pH.

**Figure 4.7.1. Chemical shift experiments for unlabelled D4ePLY**



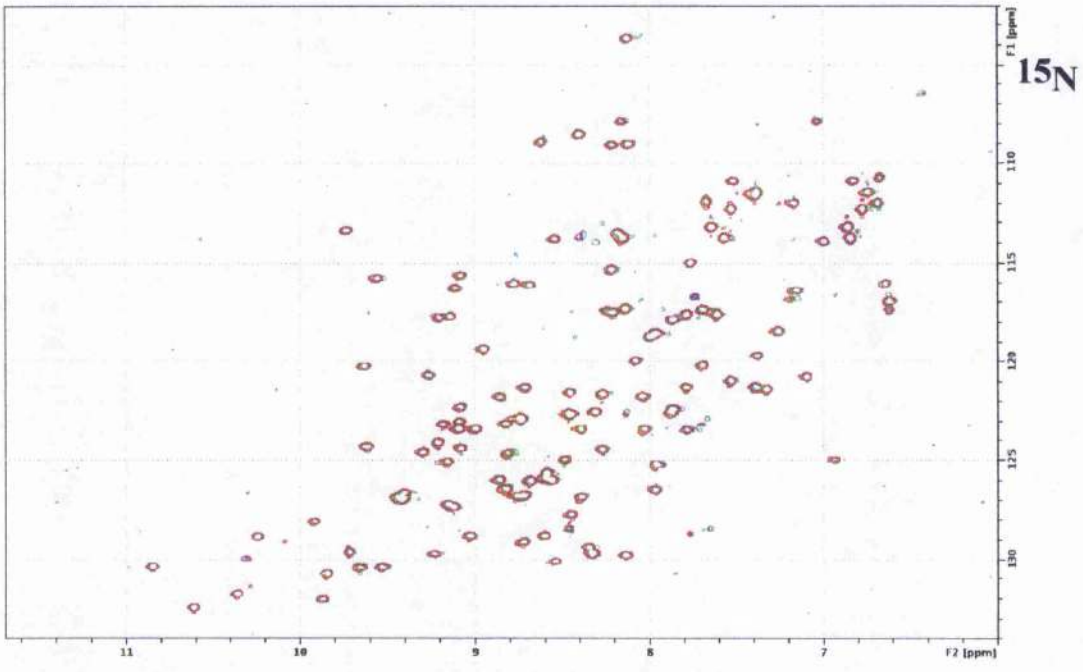

1-D proton NMR spectrum of D4ePLY recorded at 600 MHz and 298K in PBS on a Bruker AVANCE spectrometer equipped with cryoprobe.

**Figure 4.7.2. Chemical shift experiments for $^{15}N$ labelled D4ePLY**



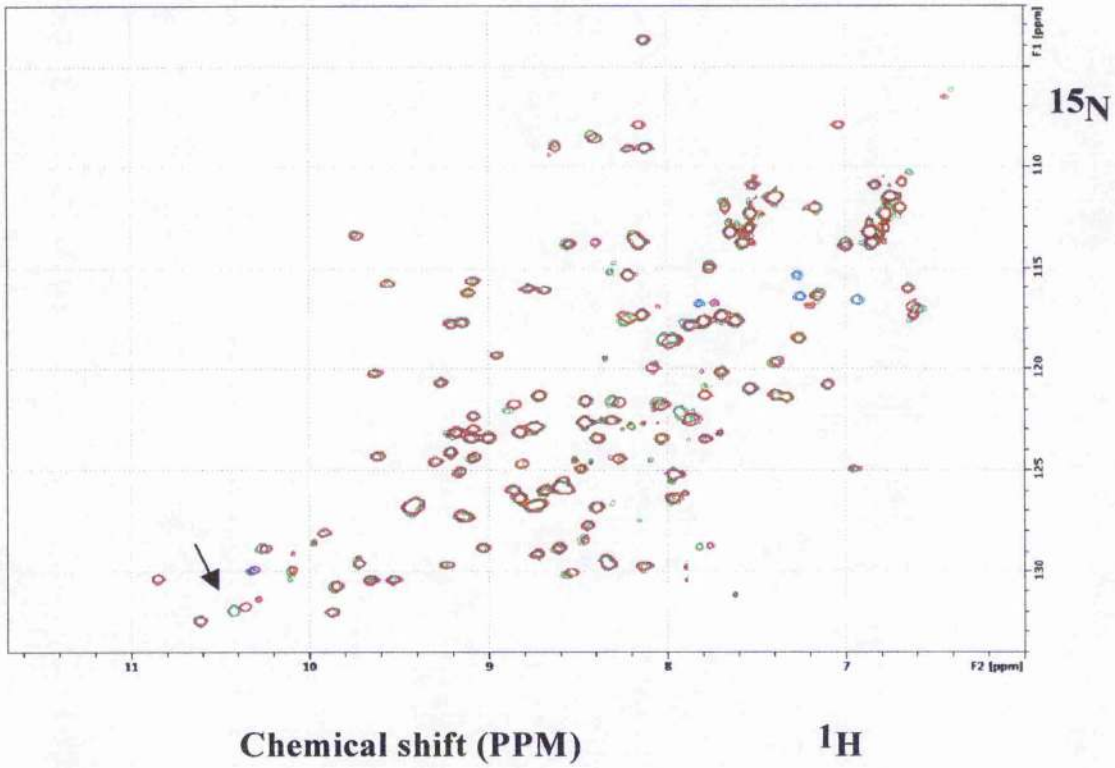

2-D $^{15}N$ heteronuclear single quantum correlation (HSQC) of spectrum of D4ePLY recorded at 600MHz ($^{1}H$) and 298K in PBS on a Bruker AVANCE spectrometer equipped with cryoprobe. Each spot is derived from an amide nitrogen and its attached proton so there is a spot for each residue in the protein.

**Figure 4.7.3.** Chemical shift perturbation experiments for water insoluble cholesterol binding of $^{15}N$ labelled D4ePLY




2-D $^{15}N$ HSQC of spectrum of D4ePLY recorded at 600MHz and 298K in PBS on a Bruker AVANCE spectrometer equipped with cryoprobe. Each spot is derived from an amide nitrogen and its attached proton so there is a spot for each residue in the protein.

**Figure 4.7.4. Chemical shift perturbation experiments for water-soluble cholesterol binding of $^{15}N$ labelled D4ePLY**




2-D $^{15}N$ HSQC of spectrum of D4ePLY recorded at 600MHz and 298K in PBS on a Bruker AVANCE spectrometer equipped with cryoprobe. Each spot is derived from an amide nitrogen and its attached proton so there is a spot for each residue in the protein.

# Chapter 5

# Results

## Mouse immunisation study of purified PLY and its derivative proteins

## 5.1. Immunising mice with purified proteins

Group 1 to 6 (Harlan Olac, UK) were intranasally immunised 3 times with purified proteins on days 1, 27 and 54. Group 7 (table 5.1.) and 8 (table 5.1.) were intranasally immunised 2 times on days 1 and 54. Bleeds were taken one day before each immunisation; the last bleed was taken two weeks after the third boost (day 54). However, group 7 and 8 were bled 4 times the same as the others (day 0, 26, 53 and 67), but immunised twice (day 1, day 54). One mouse died in-group 2 and three mice died in group 7 (Table 5.1.) the day after first immunisation. Anti-eGFP and anti-PLY IgG antibody were measured in all samples of every bleed (Figure 5.1.D and Figure5.2.E).

### Table 5.1. Groups of mice used for immunisations

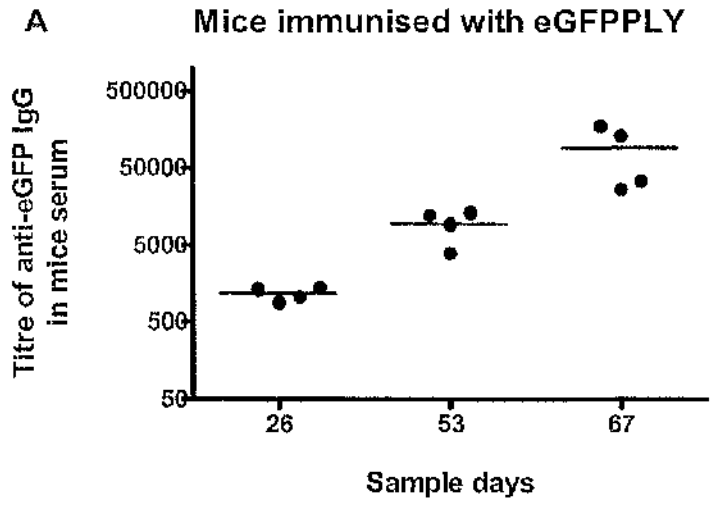
| Group | Antigens used | Number of mice |
|---|---|---|
| 1 | eGFP, PLY | 6 |
| 2 | eGFPPLY | 4 |
| 3 | eGFP, D4ePLY | 5 |
| 4 | eGFPD4ePLY | 5 |
| 5 | eGFP, D123PLY | 5 |
| 6 | eGFPD123PLY | 5 |
| 7 | eGFPD4ePLY, PLY | 2 |
| 8 | eGFPD123PLY, PLY | 5 |

## 5.2. Immune response in mice immunised with purified proteins.

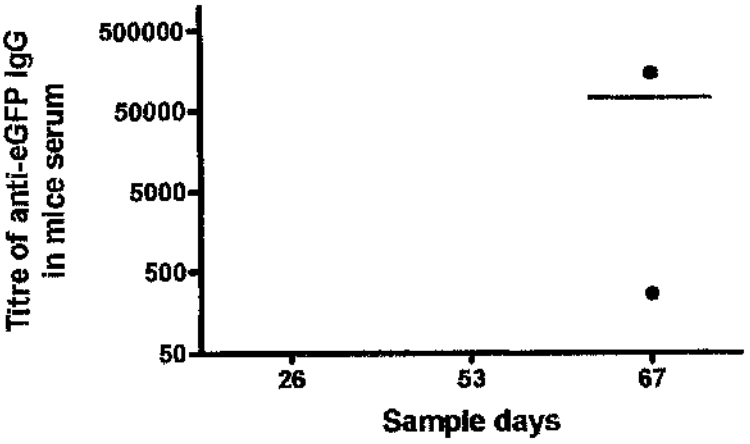
No anti-eGFP IgG antibody was detected in the first bleed of all the groups (Figure 5.1.D), and no anti-eGFP IgG were detected in the group 3 and 5 (Figure 5.1.D). ). Mice intranasally challenged with very low level of eGFPPLY (0.2$\mu$g) rapidly produced of high levels of IgG antibody to eGFP, but intranasal administration of mixture of eGFP and PLY were unable to induce the above response (Figure 5.1.D).

There were detectable levels of anti-eGFP IgG antibody in-group immunised with eGFPPLY after the first immunisation (day 26), and the level of ani-eGFP IgG increased significantly after each boost (Figure 5.1.A and D), but no anti-eGFP IgG was detected in group immunised with eGFP and PLY (Figure 5.1.D). Interestingly, no anti-eGFP IgG antibody was produced in group 4 (eGFPD4ePLY) and 6 (eGFPD123PLY), but high level of anti-eGFP IgG and detectable level of anti-eGFPP IgG were found after one boost in group 7 and 8(Figure 5.1.B-D). The results for group 4, 6, 7 and 8 showed intranasal administration of eGFP tagged truncated form of PLY are unable to induce anti-eGFP IgG response in mice unless the intact PLY was administrated simultaneously (Figure 5.1B-D).

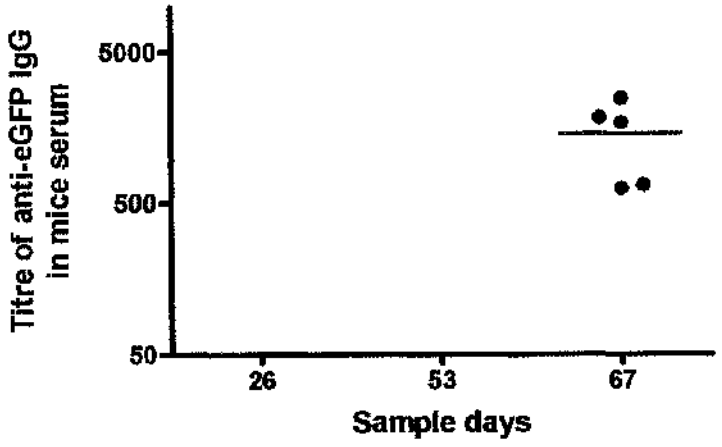
**Figure 5.1. Anti-eGFP IgG response over time in mice immunised with different of antigens**



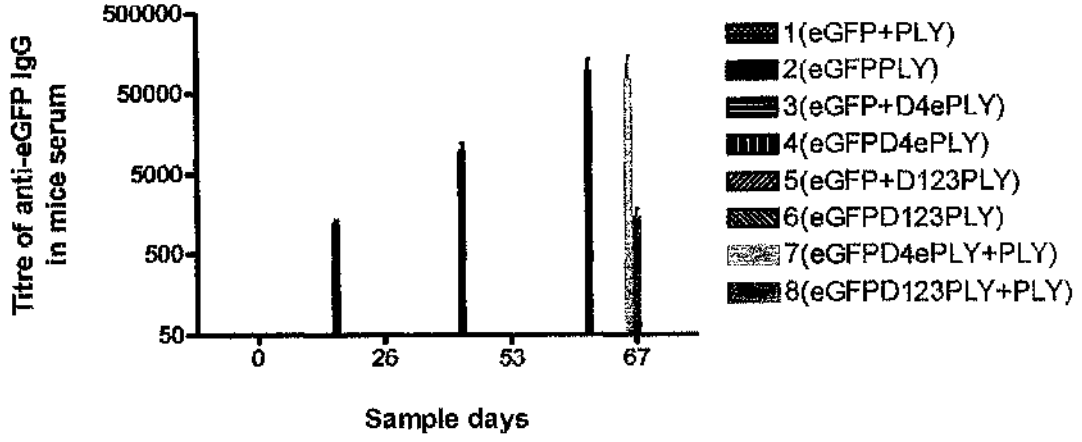
B Mice immunised with mixture of eGFPD4ePLY and PLY




C Mice immunised with mixture of eGFPD123PLY and PLY

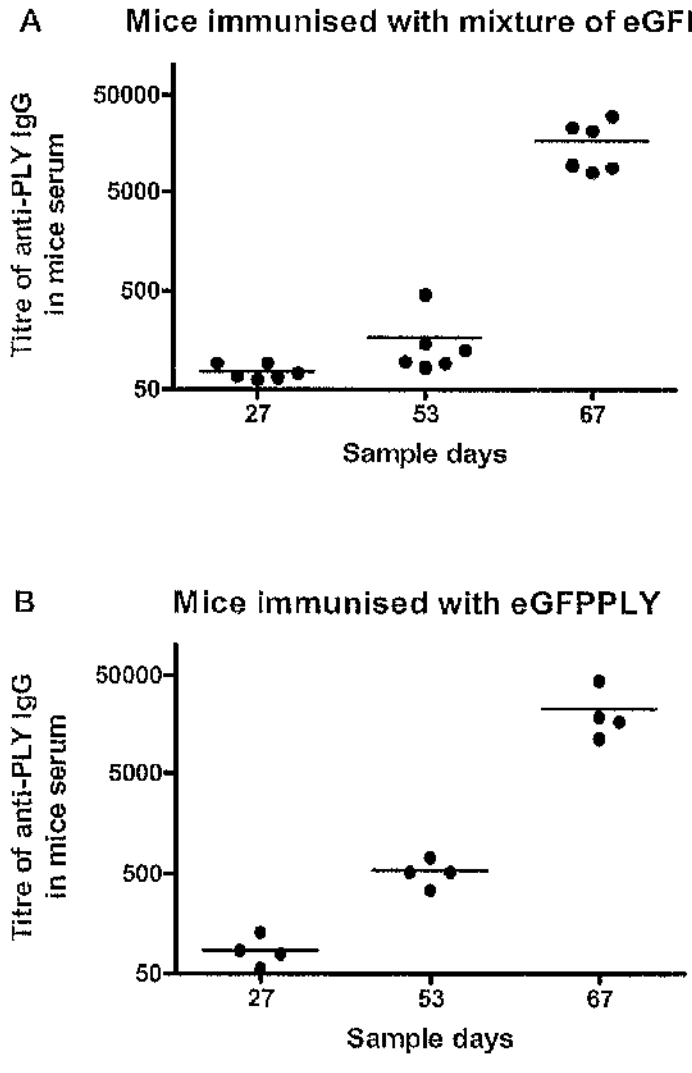



D Summary

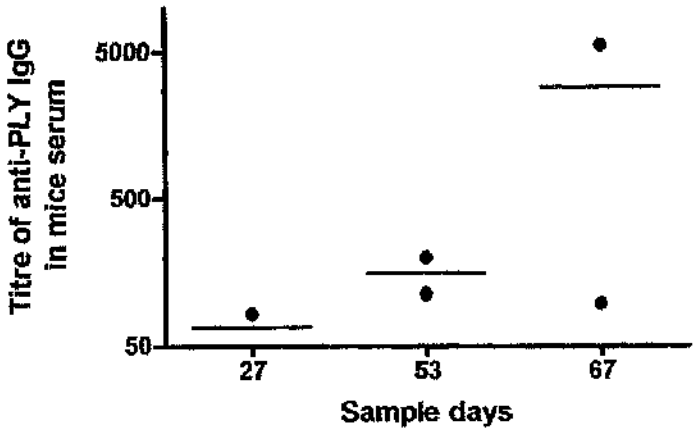


In A, B and C, Each dot represents an individual mouse and each line presents the mean response of total mice. D shows mean anti-eGFP IgG titre at OD490nm0.3 (±SEM) in mice before each challenge. Each bar represents the geometric mean titre of total mice and each error bar the standard deviation from the mean. The detection limit of anti-PLY IgG titre is 50.

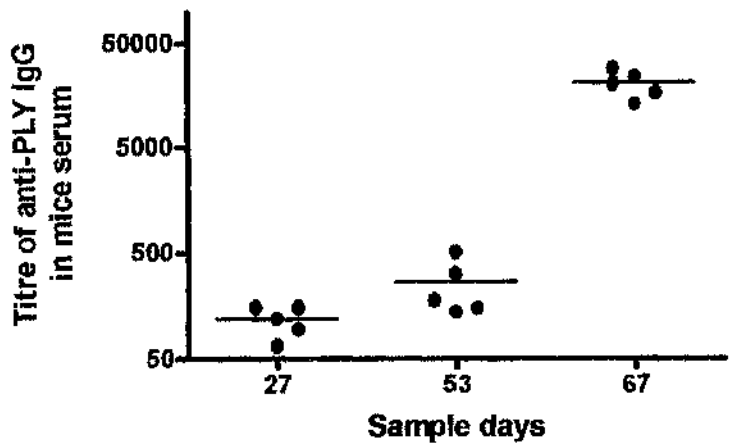
**Figure 5.2. Anti-PLY IgG response over time in mice immunised with different combination of antigens**



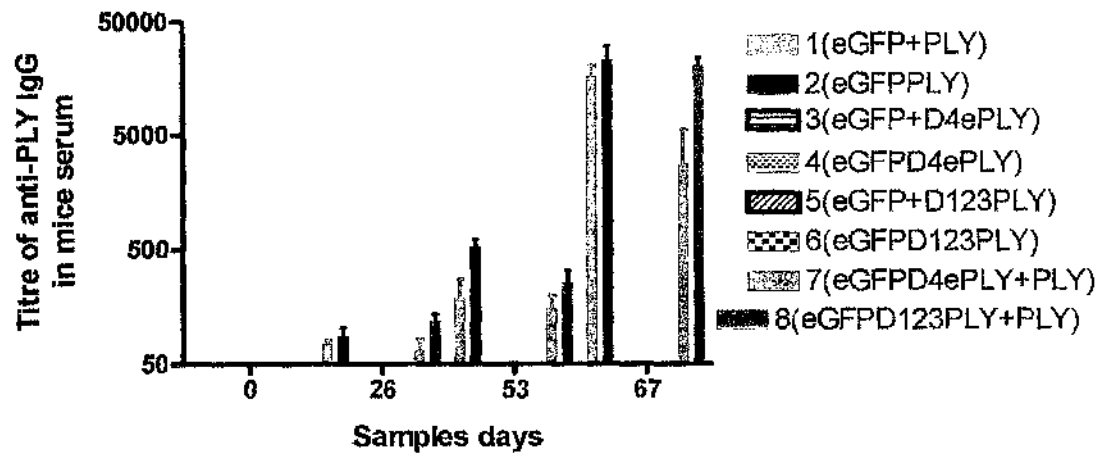
C Mice immunised with mixture of eGFPD4ePLY and PLY




D Mice immunised with mixture of eGFPD123PLY and PLY




E



In A, B, C and D, each dot represents an individual mouse and each line presents the mean response of total mice. E shows mean anti-PLY IgG titre at OD490nm0.3 (±SEM) in mice before each challenge. Each bar represents the geometric mean titre of total mice per group and each error bar the standard deviation from the mean. The detection limit of anti-PLY IgG titre is 50.

No PLY specific IgG was detected in the first bleed of all mice, and no PLY specific IgG was detected in the serum of group 3, 4, 5 and 6 (Figure 5.2.E). Low level of anti-PLY IgG was detected in the second bleed of group 1, 2, 7 and 8, but high level anti-PLY IgG was detected after boost (Figure 5.2.A-E). These results means intranasal administration of PLY but no truncated form of PLY is able to elicit immune response in mice.

# Chapter 6

# Discussion

Vectors pET33b-eGFPD4PLY, pET33b-eGFPD4PLY and pET33b-eGFPD123PLY were successfully constructed. A large amount, highly pure recombinant of eGFPPLY, eGFPD123PLY, eGFPD4PLY, PLY, D123PLY and D4PLY with low levels of contaminating LPS were obtained using NAC and AEC two step purification. All protein can be expressed at 37°C and 30°C, but can not be purified due to formation of inclusion body at 37°C except eGFPPLY and PLY, and the yield of eGFPPLY and PLY protein at 30°C increased 20 and 5 fold respectively comparing to that at 37°C (data not show), which suggests the solubility of protein increases with decrease of temperature. The results from fluorescence microscopy and flow cytometry showed that D4ePLY is capable of binding to horse erythrocyte membrane, but D123PLY is not, and both D4ePLY and D123PLY are not able to lyse horse erythrocytes, which is consistent to results from Baba *et al*. However, Baba and his colleagues (Baba, Kawamura et al. 2001) used immunoblotting for detecting cell binding property of PLY and D4cPLY, and the maximum concentration in their haemolytic assay is about 100nM,the maximum concentration in our assay is over 3mM.

In our haemolytic assay, the phenomenon that D4ePLY caused horse erythrocytes to form aggregates in PBS solution has also been found in PLY and PLY mutant (unpublished data); fluorescence microscopy images (Figure 4.4.) further showed this phenomenon with D4PLY and PLY. The reasons why D4PLY cause horse erythrocytes to aggregate still remain unclear, further investigation will be conducted.

An alternative membrane binding site, rather than the D4ePLY cholesterol binding site has been suggested. High concentrations of D123PLY were shown to induce γ-interferon and NO in mouse spleen cells(Baba, Kawamura et al. 2002). The results

suggested that was independent of cholesterol binding and pore forming ability of PLY. The clearance property of D123PLY in haemolytic assay (Figure 4.3.A and B) has been found in wild type non-haemolytic PLY (unpublished data). Thought the mechanism of causing clearance is not clear, it suggests physical contact showed occurred between horse erythrocyte and D123PLY, which means the second cell membrane binding site may exist in domain 123 of PLY except the cholesterol binding site in domain 4 of PLY. This will be further investigated by rat mast cell degranulation assay and cell binding assays.

Results of Chemical shift experiments for both $^{15}N$ labelled and unlabelled D4ePLYfurther show D4ePLY is well folded, and Chemical shift experiment for $^{15}N$ labelled PLY show PLY is well folded. A tryptophan was suggested to be involved interaction of PLY with water-soluble cholesterol (Figure 4.7.4.) However, $^{13}C$ and$^{15}N$ double labelled PLY and D4ePLY needs to be purified in order to obtain NMR structure of PLY for further mapping mechanism of binding of PLY to cholesterol.

Mice intranasally challenged with very low level of eGFPPLY (0.2$\mu$g) rapidly produced of high levels of IgG antibody to eGFP, but intranasal administration of mixture of eGFP and PLY were unable to induce the above response (Figure 5.1.), which confirmed the previous finding that PLY can act as a mucosal adjuvant to eGFP when eGFP and PLY are genetically linked but not unlinked. Intranasal administration of eGFP tagged truncated form of PLY are unable to induce anti-eGFP IgG response in mice unless the intact PLY was administrated simultaneously (Figure 5.1.B-D). These results mean that free PLY could act on an adjuvant when eGFP

linked to truncated form of PLY, and part of PLY maintain adjuvant properties of PLY. High level of anti-PLY IgG was detected in the groups immunised with PLY and eGFPPLY, but not found in groups immunised with truncated form of PLY (Figure 5.2.), which means PLY remain highly immunogenic after genetically linked to another protein.

The pervious work in our group also suggests ΔA146PLY, a mutant which has lost haemolytic property totally but as immunogenic as native PLY without its associated effects (Kirkham, L. A. et al. 2006), can still act as a adjuvant in eGFPΔA146PLY fusion during immunisation study. Since the adjuvant property of native PLY is better PLY mutant, so the adjuvant property: PLY> ΔA146PLY > truncated PLY( D123PLY and D4ePLY). Because the PLY mutant retains the adjuvant property of native PLY, it would be interesting to see if anti-eGFP antibody can be produced in mice immunised with mixture of ΔA146PLY eGFP tagged truncated PLY.

For the next step, the eGFP protein needs to be replaced with a pneumococcal protein. One or more fusion proteins are expected to elicit serotype independent protection, which would provide a broader protection against *S. pneumoniae*. The adjuvant property of PLY may exist in the other member of cholesterol dependent cytolysin family. In addition, the fusion protein can also be used in polysaccharide conjugate vaccine. PsaA may be a good candidate to form a fusion protein with PLY. PsaA has shown significant protection against colonization but less protective in against invasive disease (Briles et al., 2000 and 1997; Seo et al., 2002). Fusing PsaA to PLY would be expected to eliminate nasopharyngeal colonization of pneumococcus and

provide a broad protection against the pnenumococcal diseases. The eGFP protein can also be replaced with proteins from the other pathogen for developing new vaccines.

A potential problem with mucosal vaccine is that cross-reactivity of mucosal vaccine can reduce, even eradicate of some species of nature microflora on the surface of gut and respiratory tract, these species can protect humans from diseases infected by the pathogens other than pneumococcus.

The availability of highly purified recombinant eGFPPLY, eGFPΔA146PLY, eGFPD4PLY and eGFPD123PLY in our group will accelerate investigation into the mechanism of pore forming with PLY, and toxin cell trafficking can also be studied. The truncated form of PLY will be used in structure studies of PLY.

# Appendix I

# Buffers and Recipes

# Appendix I (buffers and recipes)

## A. Media recipes

**LB broth**
10g Tryptone
10g NaCl
5g Yeast Extract
In 1L of dH2O, autoclave.

**LB Agar**
1 Litre of LB Broth as above plus 10g Bacteriological Agar
Autoclave, store at RT.
Melt in microwave, cool immediately or keep at 60-65°C in water bath, pour plate

**Terrific broth (TB)**
12g bacto-tryptone
24g bacto-yeast extract
4ml glycerol
Make up to 0.9L with $dH_2O$ and autoclave

In a separate flask dissolve in 100ml $dH_2O$:
2.31g $KH_2PO_4$ monobasic
12.54g $K_2HPO_4$ dibasic (for trihydrate 16.45 g)

**M9 minimal medium**
5 × M9 stock solution
64 $Na_2HPO_4.7H_2O$
15$KH_2PO_4$
2.5g NaCl
Make up to 1L with $dH_2O$ and autoclave

1L M9 minimal medium
200ml 5 × M9 (autoclaved)
2ml 50mM $CaCl_2$ (filter-sterilised)
2ml 1M $MgSO_4$ (filter-sterilised)
0.8ml 50mg/ml Thiamine(filter-sterilised)
800ml sterile $dH_2O$ (autolaved)

## B. Nickel affinity chromatography (NAC) buffer

**10 × PBS**
80g NaCl
2g KCl
14.4 $Na_2HPO_4$
2.4 $KI_2PO_4$
In 1 litre $dH_2O$

**250mM EDTA PH 7.5-8**
93.6g EDTA in 1 litre $dH_2O$

**250mM imidazole**
17.02g imidazole in 1L PBS

**100mM $NiSO_4$**
26.28g $NiSO_4$ in 1L $dH_2O$

**3M guanidine HCl**
286.59g guanidine HCl in 1L $dH_2O$

## C. BioCAD buffer

**1 × PBS**
8g NaCl
0.2g KCl
1.44 $Na_2HPO_4$
0.24 $KH_2PO_4$
In 1 litre $dH_2O$ and autoclave

**3M NaCl**
175.32g NaCl in 1L $dH_2O$

**20% Ethanol**
200ml Ethanol A. R.
Make up to 1L with $dH_2O$

## D. SDS PAGE gel

| **RUNNING GEL(two gels)** | 5% | 12% | 10% | 7.5% |
|---|---|---|---|---|
| $dH_2O$ | 2.35ml | 3.35 | 4.05 | 4.85 |
| 1.5M Tris pH8.8 | 2.5ml | 2.5 | 2.5 | 2.5 |
| 10% SDS | 0.1ml | 0.1 | 0.1 | 0.1 |
| 30% polyacrylamide | 5.0ml | 4.0 | 3.3 | 2.5 |
| 10% ammonium persulphate | 0.05ml | 0.05 | 0.05 | 0.05 |
| TEMED | 0.005ml | 0.005l | 0.005 | 0.005 |

| **STACKING GEL (two gels)** | |
|---|---|
| $dH_2O$ | 3.21ml |
| 0.5M Tris pH6.8 | 1.25ml |
| 10% SDS | 0.05ml |
| 30% polyacrylamide | 0.488ml |
| 10% ammonium persulphate | 0.025ml |
| TEMED | 0.005ml |

**5 × sample buffer**
0.6ml 0.5M Tris pH6.8
5.0ml 50% glycerol
2.0ml 10% SDS
1.0ml 1% bromophenol blue
0.9ml $dH_2O$

**10 × Running Buffer**
30g Tris
144g Glycine
10g SDS
Make up to 1L with $dH_2O$

## E.Western Blotting

**Transfer Buffer**
25mM Tris Base (3.03g)
192mM Glycine (14.4g)
20% Methanol (200ml)
Make up to 1L with $dH_2O$
(chilled to 4°C)

**Tris NaCl pH7.4**
Tris base 1.2g
NaCl 8.7g
make up to 1L with $dH_2O$
conc. HCl 800µl

**Developer (make just before use)**
Dissolve 30mg 4-chloro-1-napthol in 10ml Methanol
Add to 40ml Tris NaCl pH7.4
Add 30µl $H_2O_2$ (30% w/v)

## E.ELISA

**Developing solution (make just before use)**

| | |
|---|---|
| 0.1M citric acid | 12.25ml |
| 0.25M $Na_2HPO_4$ | 12.75ml |
| $H_2O_2$ | 20µl |
| Fast™ o-phenylenediamine dihydrochroide tablet | one |

Unless otherwise stated, all reagents and chemicals are from Sigma-Adlrich, Dorset, UK

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