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Neutralizing Chimeric Mouse-human Antibodies against *Burkholderia pseudomallei* Protease: Expression, Purification and Characterization

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A recombinant Fab monoclonal antibody (Fab) C37, previously obtained by phage display and biopanning of a random antibody fragment library against *Burkholderia pseudomallei* protease, was expressed in different strains of *Escherichia coli*. *E. coli* strain HB2151 was deemed a more suitable host for Fab expression than other *E. coli* strains when grown in media supplemented with 0.2% glycerol. The expressed Fab fragment was purified by affinity chromatography on a Protein G-Sepharose column, and the specificity of the recombinant Fab C37 towards *B. pseudomallei* protease was proven by Western blotting, enzyme-linked immunosorbent assay (ELISA) and by proteolytic activity neutralization. In addition, polyclonal antibodies against *B. pseudomallei* protease were produced in rabbits immunized with the protease. These were isolated from high titer serum by affinity chromatography on recombinant-Protein A-Sepharose. Purified polyclonal antibody specificity towards *B. pseudomallei* protease was proven by Western blotting and ELISA.

Keywords: *Burkholderia pseudomallei*, Phage display, Polyclonal antibodies, Protease, Recombinant monoclonal antibodies

Introduction

Melioidosis, an important health problem for humans and livestock, is a potentially fatal disease caused by a soil bacterium, *Burkholderia pseudomallei* (formerly *Pseudomonas pseudomallei*). The incidence of melioidosis correlates with rainfall, and it is endemic in Southeast Asia and Northern Australia, although melioidosis cases have been encountered in temperate areas such as France (Thibault *et al.*, 1996). Melioidosis is associated with underlying predisposing conditions, such as, diabetes and renal failure, and can range in presentation from a fulminant septicemic illness to an indolent local infection (Chaowagul *et al.*, 1993). *B. pseudomallei* virulence factors have been investigated since 1950, but as yet have been poorly characterized. Although *B. pseudomallei* isolates are capable of expressing an impressive array of both secreted and cell-associated antigens, the role(s) of these products in the pathogenesis of melioidosis are relatively ill defined (Leelarasamee, 2000). These products include exotoxin (Ismail *et al.*, 1987), protease (Sexton *et al.*, 1994), haemolysin, acid phosphatase (Heckley, 1964) and cytotoxic exolipid (Haussler *et al.*, 1998), and have been reported to have cytotoxic and proteolytic properties (Heckley, 1964; Tumwarsorn *et al.*, 1994), although the roles played by these various secreted exoproducts upon the virulence of *B. pseudomallei* are unclear. The involvement of *B. pseudomallei* metalloprotease in the pathogenesis of melioidosis has been fairly well studied, and it has also been postulated that the importance of the immunodominance of this protease may surpass that of exotoxin (Sexton *et al.*, 1994). The *B. pseudomallei* extracellular protease has previously been shown to be present in culture supernatants as an enzyme with a molecular weight of 36,000 Da that is optimally active at 60°C and pH 8.0 (Sexton *et al.*, 1994). The protease was also shown to be a metalloenzyme requiring zinc for maximal activity; moreover, this activity was inhibited in the presence of ethylenediaminetetraacetic acid (EDTA) and 1,10-phenantroline (Sexton *et al.*, 1994).

The use of antibodies as tools for diagnosis and/or as therapeutics for melioidosis has so far been limited to polyclonal and monoclonal antibodies towards the outer membrane or to uncharacterized bacterial proteins.

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Nevertheless, to date, there is no gold standard diagnostic strategy apart from bacterial culture, and treatment by combined antibiotic therapy is still practised in most endemic areas (Leelarasamee, 2000).

We previously reported on the synthesis and expression of recombinant single chain variable fragment (scFv) antibodies towards the exotoxin of *B. pseudomallei* (Nathan *et al*., 2002; Su *et al*., 2003). We have also utilized these antibodies to purify the antigenic determinant of the exotoxin molecule by antibody mediated affinity chromatography (Lim *et al*., submitted). In addition to this, we produced several recombinant Fragment Antibody Binding (Fab) monoclonal antibodies (Fabs) isolated from a combinatorial recombinant antibody (Fab) phage display library biopanned against the *B. pseudomallei* protease (Nathan *et al*., submitted). The heavy and light chain domains of antibodies from mice immunized with the protease were fused to human constant heavy and light chain regions and cloned into the pComb3H phage display vector to produce mouse-human chimeric recombinant antibodies. Based on an initial characterization of selected Fabs, Fab clone C37 found to be was able to neutralize proteolytic activity by up to 80%, and therefore, was selected for further expression and characterization in this study. The vast number of vectors available for expression, the manipulation of cloned genes, and complete information on the *Escherichia coli* genome, have encouraged the use of *E. coli* as a protein expression host (Karu *et al*., 1995). Both polyclonal and monoclonal antibodies allow for the analysis of the structure-function relationship of the protease in the pathogenesis of melioidosis. One way to achieve this would be by using antibodies to epitope map the antigen and to subsequently purify the epitope.

This study reports on the expression and purification of recombinant Fabs, and on the concomitant purification and characterization of polyclonal anti-protease antibodies. Both purified Fabs and polyclonal antibodies will, in the near future, be utilized as tools for the epitope mapping of the *B. pseudomallei* protease molecule, to determine antigenic determinants for the future development of vaccine and diagnostic candidates.

Materials and Methods

Bacteria culture and protease purification All experiments pertaining to bacteria culture and harvesting were performed in a level 2 biosafety laboratory (Vidal *et al*., 1993). The extracellular protease was purified from a human *B. pseudomallei* strain, D286 (obtained from the Pathogen Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia). Protease was purified from brain-heart infusion (BHI, HispanLab, Cuba) broth cultures grown at 37°C for 7 days. Cultures were sterilized with formalin overnight and subjected to ammonium sulphate precipitation (70% saturation), DEAE-Cellulose Ion Exchange chromatography (pH 8) and CM-Cellulose Ion Exchange chromatography (pH 6). Chromatographic fractions demonstrating protease activity were pooled and dialyzed in phosphate-buffered saline (PBS). Aliquots of samples from each purification step were analyzed by 12% Sodium Dodecyl Sulphate-Polyacrilamide gel electrophoresis (SDS-PAGE).

Protein profile Protein profiles were determined during purification by measuring absorbance at 280 nm (A_{280}) by spectrophotometry (Biophotometer Eppendorf, Germany).

Exoprotease activity Exoprotease activity was determined using a modified azocasein test (Ling *et al*., 2001). 100 µl of purified protease, 50 µl of azocasein (0.2 g/l in water), and 20 µl of 250 mM Tris-HCl (pH 7.5) were incubated for 2 h at 37°C. Azocasein cleavage was then stopped by precipitating the proteins with 150 µl of 10% (w/v) trichloroacetic acid. After 5 min, samples were centrifuged for 5 min at $9,000 \times g$ and 120 µl aliquots of the supernatants were transferred to microtitre plates. Absorbance (A_{405}) was determined with a microplate reader after adding 150 µl of 1 N NaOH.

Immunization of rabbits Purified *B. pseudomallei* protease (200 µg) was electrophoresed by 12% SDS-PAGE and the section of the gel containing the *B. pseudomallei* protease with a molecular size of ~36,000 Da was excised. The gel fragment was then disintegrated through a syringe and denatured in 0.5 ml PBS (95°C, 10 min) (Harlow and Lane, 1988). Denatured samples were mixed with 0.5 ml of incomplete Freunds Adjuvant (IFA). Two New Zealand White rabbits were treated over a period of 2-3 months with 4 subcutaneous injections of 1 ml emulsion samples (200 µg purified protein in IFA). Immunizations were administered on days 0, 14, 28 and 42. Blood samples were taken on days 0 (pre-bleed), 21, 35 and 56 to monitor antibody titres.

Rabbit sera ELISA Microtitre plate wells (Nunc, Roskilde, Denmark) were coated overnight at 4°C with denatured (95°C, 10 min) purified *B. pseudomallei* protease (1 µg in 25 µl 1.0 M $NaHCO_3$, pH 8.6). The coated plates were blocked with 100 µl 5% skimmed milk for 1 h at 4°C, and washed thoroughly with distilled water. Serial dilutions of sera (1 : 2 to 1 : 512) in 5% skimmed milk were then added and incubated for 1 h at 37°C. The plates were washed prior to adding 50 µl of peroxidase-conjugated goat anti-rabbit immunoglobulins (diluted 1 : 30,000). The chromogenic substrate ABTS: peroxidase B (1 : 1; Kirkegaard & Perry Lab. Inc, Gaithersbug, USA) was added colour formation was measured at an optical density of 405 nm.

Purification of polyclonal antibodies Anti-*B. pseudomallei* protease immune serum was harvested and polyclonal (IgG) antibodies were purified using the MonoAb rec-Protein A Column Purification System (Zymed Laboratories, Inc, San Francisco, USA), according to the manufacturers protocol. The purified antibodies were analyzed by SDS-PAGE and by immunoblotting with peroxidase-conjugated anti-rabbit IgG.

Fab C37 expression Fab C37 is a recombinant Fab monoclonal antibody expressed by the antibody variable and constant region construct (V_L-C_L and V_H-C_H) cloned into a phagemid vector pComb3HSS (Fig. 1; Nathan *et al*., 2002). This fusion phage was

used to infect 50ml of various strains of *E. coli*, i.e, ER2537 {F *lacI*^{q} *Δ(lacZ)M15 proA*$^{+}$*B*$^{+}$ *zzf::Tn10(Tet*^{R}*)/fhuA2 supE thi Δ(lac-proAB) Δ(hsdMA-mcrB)5* (r_k^- m_k^- McrBC); New England Biolabs, Beverly, USA}, TG1{*supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM) 5* ($r_k^-m_k^-$) [F *traD36 proAB lacI*^{q}*ZΔM15*]; Stratagene, La Jolla, USA}, XL1-Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]^{c}; Stratagene}, and HB2151 {K12, Δ(*lac-pro) ara* NaI^{r} *thi* [F *pro*A^{+}B^{+} *lac*I^{q} *lac*ZΔM15]; Amersham Pharmacia, Piscataway, USA}. Infected cells were incubated for 6 h at 37°C with shaking at 250 rpm before 0.05 mM isopropyl-β-D-thiogalactopyransoside (IPTG) addition with or without carbon (0.2% glucose or glycerol) supplementation. The induction was allowed to continue overnight after which cells were separated (10,000 × *g* for 15 min at 4°C) from the culture medium. The supernatant (containing the extracellular soluble proteins) was then transferred to a clean container, filtered (0.45 μm filter) and stored at 4°C. And, the bacterial pellet was resuspended in ice-cold 1x tris-buffered saline (TBS, 2 ml) and subjected to 5 rounds of freeze-thawing (5 min in liquid nitrogen and 5 min at 37°C) to extract soluble proteins within the periplasm. Cell debris was pelleted by centrifugation at 10,000 × *g* for 30 min at 4°C. Supernatants which contained the soluble periplasmic proteins were analyzed by SDS-PAGE, immunoblotting, and indirect ELISA.

Fab purification Expressed Fab was purified by high performance liquid chromatography (HPLC) using a 5 ml HiTrap™ Protein G column and an AKTApurifier™ system, according to the manufacturer's recommendations (Amersham Pharmacia). Briefly, antibody samples were concentrated using a Vivaspin 2 fractionating column preconditioned with PBS (MW 30,000; Vivascience, UK) to a final volume of 500 μl. Unbound proteins were washed with PBS, and bound Fab was eluted with elution buffer (0.5 mM acetic acid). Fractions were checked for protein content by monitoring absorption at 280 nm.

Fab ELISA To determine the presence of expressed Fab, microtitre plates were coated overnight at 4°C with 50 μl of Fab C37 recombinant antibodies in 1.0 M $NaHCO_3$, pH 8.6. The coated plates were blocked with 100 μl 5% skimmed milk for 1 h at 4°C, wells were then washed thoroughly with distilled water, and HRP-conjugated goat IgG anti-human $F(ab)_2$ (1 : 1,000) was added. The wells were then washed again prior to adding 50 μl ABTS: peroxidase B (1 : 1; Kirkegaard & Perry Lab. Inc). Colour formation was measured at 405 nm.

To determine the specificity of the antibodies to *B. pseudomallei* protease, microtitre plates were coated overnight at 4°C with native or heat denatured samples of the protease (1 μg in 25 μl 1.0 M $NaHCO_3$, pH 8.6). The coated plates were blocked with 100 μl 5% skimmed milk for 1 h at 4°C, wells were washed thoroughly with distilled water, and blocked with 5% skimmed milk. Primary antibody (Fab C37 or polyclonal IgG) was added and incubated for 1 h at 37°C. The plates were washed and 50 μl HRP-conjugated goat anti-rabbit IgG (diluted 1 : 30,000) or HRP-conjugated goat IgG anti-human $F(ab)_2$ (1 : 1,000) was added. The wells were then washed and 50 μl ABTS: peroxidase B (1 : 1) substrate was added. Absorbance was measured at 405 nm.

SDS-PAGE and immunoblotting Protein samples were analysed using 12% polyacrylamide gels (Laemmli, 1970) stained with Coomassie brilliant blue R-250. Unstained gels were used for Western blotting using horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (diluted 1 : 30,000) and HRP-conjugated goat anti-human IgG [$F(ab)_2$] (diluted 1 : 1,000; Pierce Chem Co, Rockford, USA) to detect the polyclonal antibodies or the recombinant monoclonal antibodies, respectively, by adding SuperSignal West Pico Chemiluminescent Substrate (Pierce Chem Co). To determine the specificity of both antibodies towards *B. pseudomallei* protease, partially purified protease preparations transferred onto membranes were probed with both purified polyclonal antibodies (diluted 1 : 250) or Fab C37 (undiluted) for 2 h at room temperature.

Digestion of physiological proteins Selected physiological proteins (transferrin, myosin, IgA, and actin) were treated separately with protease, Fab C37, or a Fab C37-protease cocktail for 2 h at 37°C. Treated samples were electrophoresed by 12% SDS-PAGE and proteins were visualized by staining with Coomassie Brilliant Blue.

Results and Discussion

Theoretical and diagnostic applications of anti-*B. pseudomallei* recombinant antibodies for the study of the structure-function relationship of bacterial extracellular proteins require recombinant antibody fragments in large amounts, and several expression systems are now available to produce antibody fragments in various recombinant formats (Barbas *et al.*, 2001). In this study we utilized the pComb3H system and *E. coli* host strains to express and characterize anti-protease Fab fragment C37 antibodies. Polyclonal antibodies were also purified and characterized as positive controls for Fab analysis.

Characterization of the polyclonal antibodies specific to *B. pseudomallei* protease Initially, *B. pseudomallei* protease was purified, and this purification was monitored by measuring the specific activity of the protease using a modified azocasein test (Ling *et al.*, 2001) and by spectrophotometry at 280 nm. Ammonium sulphate precipitated material was applied to a DEAE-cellulose column and the protease activity was found to be associated with eluted fractions. The collected fractions were pooled, dialyzed and further purified using a CM-cellulose column. Protease activity was again found to be associated with the flow-through fractions. An SDS-PAGE profile of these pooled fractions exhibited the presence of impurities within the sample, which was indicated by the presence of more than one protein band when stained with Coomassie Blue. Therefore, pooled fractions were re-purified using a second CM-cellulose column where, once again, protease activity was associated with the flow-through. When samples were separated under denaturing, reducing conditions, the purified protease migrated as a band of 36,000 Da (data not shown), which agreed with the previously reported molecular weight by

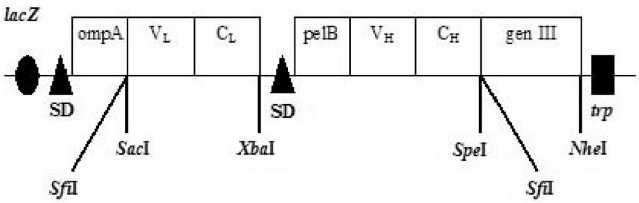

Fig. 1. Schematic of the FabC37 construct. The Fab (V_HCH_1-V_LC_L) construct was inserted upstream of the carboxyl terminal of the phage gpIII coat protein gene, and was displayed on the phage particles surface. *ompA* and *pelB* are bacterially derived leader sequences.

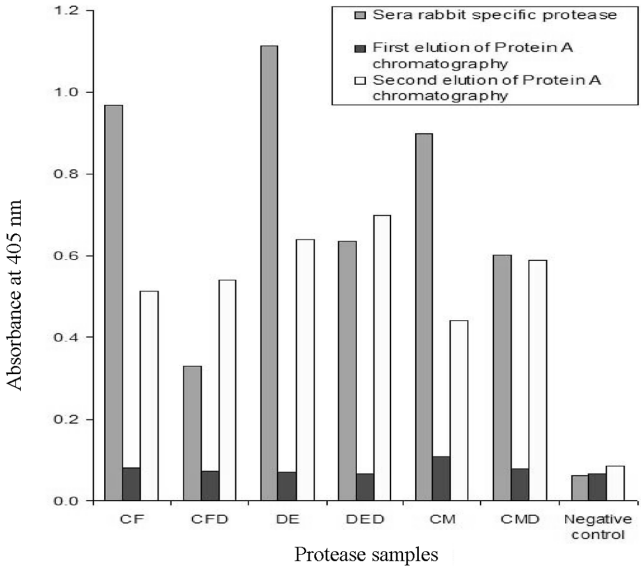

Fig. 2. Indirect ELISA on rabbit sera and elution products from Protein A chromatography for *B. pseudomallei* protease. Protease (1 μg) from all purification steps was coated into the microtitre plate wells. Crude rabbit sera, and first elution and second elution fractions obtained by Protein A chromatography were incubated as primary antibody and HRP-conjugated anti-rabbit IgG as secondary antibody. CF, post-ammonium sulphate precipitate (non-denatured); CFD, denatured CF; DE, non-denatured flow-through of DEAE-cellulose; DED, denatured DE; CM, non-denatured flow-through of CM-cellulose; CMD; denatured CM; negative control, 1% BSA/TBS.

Sexton *et al.* (1994). The procedure devised for the purification of *B. pseudomallei* protease resulted in a highly purified preparation with a significantly higher specific activity (130.0 U/mg) than the starting material (1.36 U/mg), and a 96-fold increase in purity. Yields of 1.5 mg protease per litre of culture were routinely obtained.

The purified protease was electrophoresed and the band corresponding to 36,000 Da on the SDS gel was excised, homogenized, and mixed with incomplete Freunds Adjuvant prior to being injected into the rabbits. Sera collected during the course of the immunization protocol were assayed for the presence of protease-specific antibodies by ELISA. Both

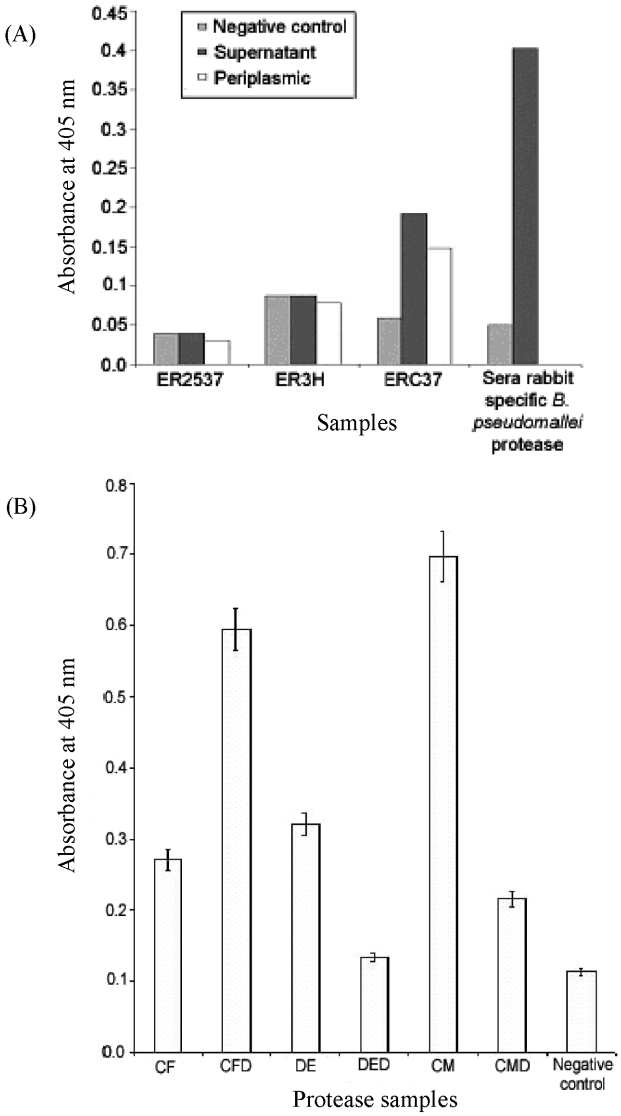

Fig. 3. (A) Indirect ELISA of Fab C37 antibody expressed in ER2537 (ERC37). ER2537 and ER2537 infected with vector pComb3HSS (ER3H) were used as negative controls in the study of antibody fragment expression. Serum from rabbits immunized with *B. pseudomallei* protease was used as a positive control. (B) ELISA of a FabC37 sample obtained from ERC37. Protease samples from a various purification steps were coated on microtiter plate wells. Goat IgG anti-human $F(ab)_2$ conjugated HRP was used as the secondary antibody. CF, post-ammonium sulphate precipitate (non-denatured); CFD, denatured CF; DE, non-denatured flow-through of DEAE-cellulose; DED, denatured DE; CM, non-denatured flow-through of CM-cellulose; CMD; denatured CM; negative control, 1% BSA/TBS.

immunized rabbits produced a reasonable protease-specific antibody response, with highest antibody titers (1 : 12,800) at bleed 4, as was expected (data not shown).

Antiserum directed against *B. pseudomallei* protease was purified by Protein A affinity column chromatography to obtain polyclonal (IgG) antibodies. Purified fractions were

tested for reactivity to protease by ELISA and antibodies specific for the protease were found in the second elution from the Protein A column (Fig. 2). We noticed that the purified antibodies (from the second elution) reacted significantly more with heat denatured protease samples than with non-denatured protease samples. These findings indicate that these polyclonal antibodies have a higher specificity towards protease under denaturing conditions, thus duplicating previous findings with anti-protease monoclonal and polyclonal antibodies (Nathan *et al*., in prep.). Harlow and Lane (1988) suggested that if a non-denatured immunogen is used for immunization, the immunogen would almost certainly undergo denaturation or partial denaturation *in vivo*.

Expression, purification, and characterization of Fab C37

To further enable the characterization of the recombinant Fab monoclonal antibody, C37, preliminary its experiments aimed at determining expression parameters were carried out in *E. coli* ER2537 cells infected with the fusion phage C37. The Fab gene was inserted downstream of the lac promoter, between the leader sequence OmpA and gene III (gpIII), to construct Clone C37 (Nathan *et al*., in prep.). The expressions of genes cloned downstream of the lac promoter can be conveniently induced by the addition of IPTG (de Bellis and Schwartz, 1990). All of the host cells used had the lac I^q genotype to limit the background expression of the lac promoter, to ensure that Fab expression only occurs upon induction by IPTG, as constitutive expression of Fab C37 is likely to be detrimental and debilitating to the host (Carrier *et al*., 1993). Fab C37 expression was optimal after incubating for 6 h at 37°C in the presence of a final concentration of 2 mM IPTG. Expressed Fab C37 was found both in the supernatant of the cell lysate and within the periplasmic space of the bacterial cells (Fig. 3a). This indicates that most of the Fab antibody fragments were secreted into the bacterial culture media, and this secretion was probably caused by bacterial cell death or partial lysis of the outer bacterial membrane (Barbas *et al*., 2001). The Fab C37 construct consisted of the Fab gene fused to the phage gpIII coat protein gene. The presence of pIII should allow the fusion protein to be visualized on the cell surface although minute amounts of Fab protein may be secreted into the surrounding culture. The supernatant of the ER2537 bearing C37 cell lysate was tested

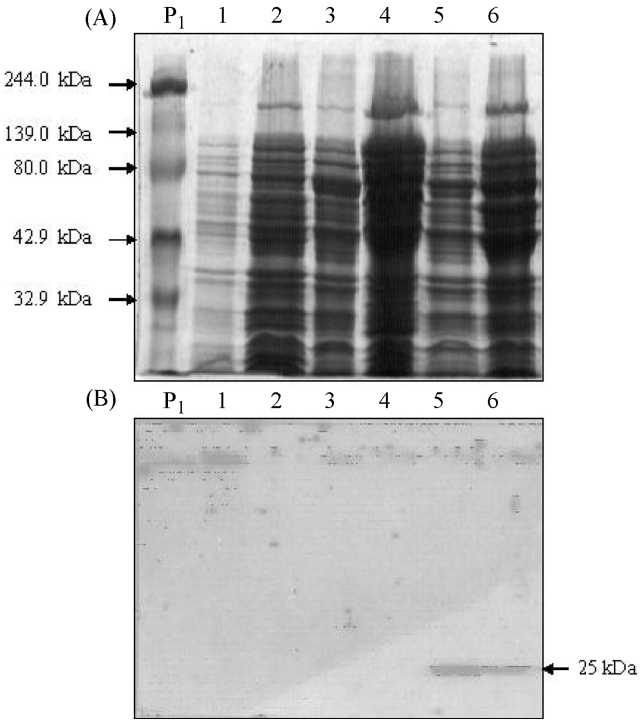

Fig. 4. (a) SDS-PAGE and (b) Western blot profile of expressed FabC37. Expressed FabC37 was detected by incubation with HRP-conjugated goat IgG anti-human $F(ab)_2$. P1, Kaleidoscope protein marker (BioRad); 1, ER2537 supernatant; 2, ER2537 periplasmic fraction; 3, ER2537 + pComb3H supernatant; 4, ER2537 + pComb3H periplasmic fraction; 5, ER2537 + C37 supernatant; 6, ER2537 + C37 periplasmic fraction.

Table 1. Yield of FabC37 produced by various *E. coli* strains under different conditions of growth and induction

Sample	Protein concentration (μg/μl)						
	0.2% glucose + 0.05 mM IPTG		0.2% glycerol + 0.05 mM IPTG		0.01 mM IPTG	0.1 mM IPTG	0.5 mM IPTG
	Supernatant	Periplasmic fraction	Supernatant	Periplasmic fraction	Periplasmic fraction	Periplasmic fraction	Periplasmic fraction
ER2537C37	1.60	5.05	1.40	5.95	-	-	-
HB2152C37	1.45	4.30	1.05	6.40	4.60	4.95	5.30
TG1C37	3.00	4.90	1.20	4.50	-	-	-
XL1-BlueC37	0.90	3.15	0.85	3.30	-	-	-
ER2537	1.40	2.90	1.05	1.90	-	-	-
HB2151	0.95	2.40	1.30	2.85	1.85	1.30	2.09
TG1	1.10	1.90	1.30	3.10	-	-	-
XL1-Blue	1.15	2.15	0.90	2.00	-	-	-
ER2537/pComb3H	1.10	1.9	1.15	2.10	1.80	2.59	2.10

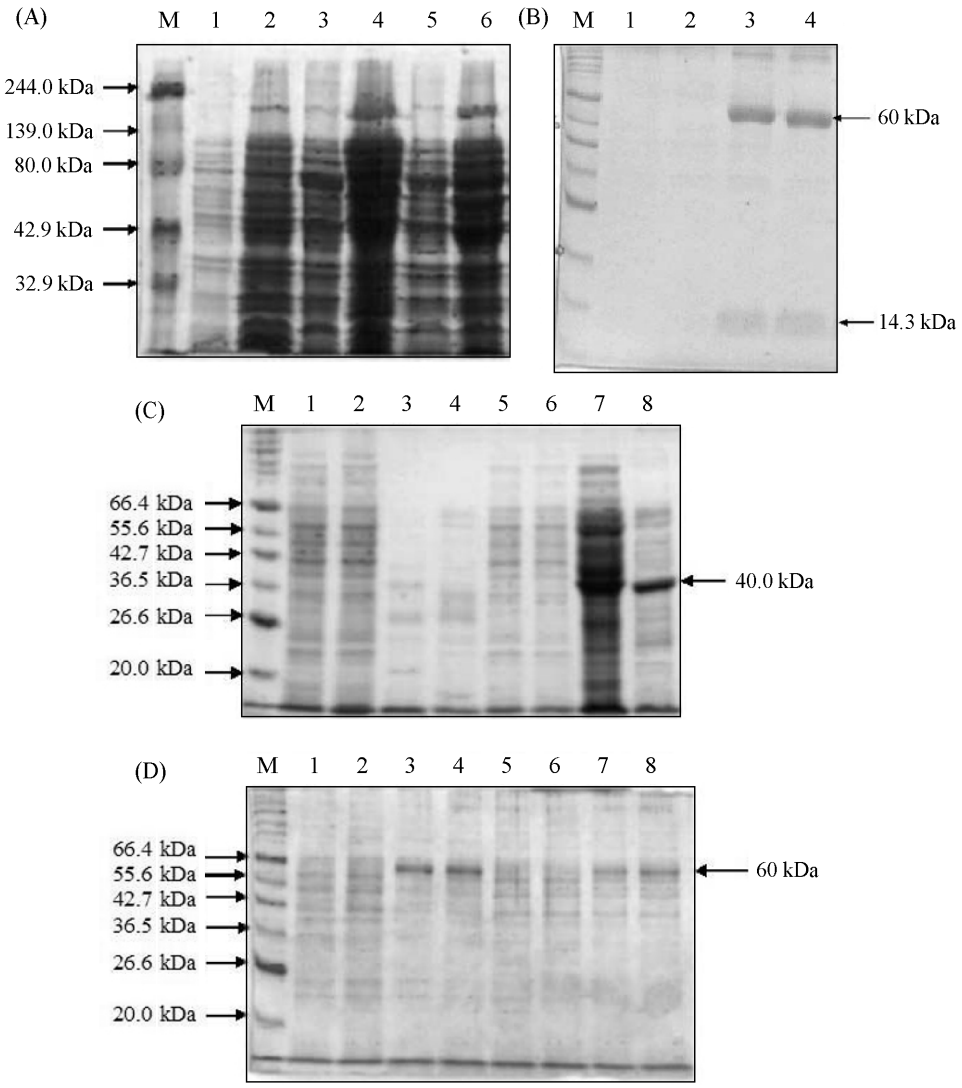

Fig. 5. SDS-PAGE (12%) of the expression products of Fab C37 antibody in different *E. coli* strains. Lane M, Marker. (A) ER2537 (+IPTG): Lane 1, host supernatant; Lane 2, host perisplasm; Lane 3, host + pcomb3HSS supernatant; Lane 4, host + pcomb3HSS perisplasm; Lane 5, host + C37 supernatant; Lane 6, host + C37 perisplasm, (B) HB2151: Lane 1, host supernatant (–IPTG); Lane 2, host supernatant (+IPTG); Lane 3, host + C37 (–IPTG); Lane 4, host + C37 (+IPTG), (C) XL-1 Blue: Lane 1, host supernatant (–IPTG), Lane 2, host supernatant (+IPTG), Lane 3, host + C37 supernatant (–IPTG), Lane 4, host + C37 supernatant (+ IPTG); Lane 5, host perisplasm (-IPTG); Lane 6, host perisplasm (+IPTG); Lane 7, host + C37 perisplasm (–IPTG); Lane 8, host + C37 perisplasm (+IPTG), (D) TG1: Lane 1, host supernatant (–IPTG); Lane 2, host supernatant (+IPTG); Lane 3, host + C37 supernatant (–IPTG), Lane 4, host + C37 supernatant (+IPTG); Lane 5, host perisplasm (–IPTG); Lane 6, host perisplasm (+IPTG); Lane 7, host + C37 perisplasm (–IPTG); Lane 8, host + C37 perisplasm (+IPTG)

for reactivity to protease in by ELISA (Fig. 3b). This supernatant of the cell lysate reacted with B. pseudomallei protease, and particularly with denatured (heat-treated) protease as compared to the non-denatured protein.

Figure 4 is a representative profile of Fab C37 expression under different culture conditions. The Fab protein band is not clear due to the presence of host proteins. However, the detection of the Fab C37 fragment by Western blot indicated a molecular weight of 25,000 Da, showing that the recombinant monoclonal antibodies were successfully expressed in *E. coli.* The secondary antibody utilized HRP-conjugated goat IgG anti-human $F(ab')_2$ recognizes the human heavy chain constant region within the mouse-human chimeric construct of C37. The 25,000 Da band was produced under reducing conditions due to the cleavage of the disulfide bonds between the light and heavy chains of the antibody.

The presence of a large amounts of host cell proteins that were also secreted under these expression conditions resulted in limited success in purifying Fab C37 (not shown). Thus, we chose three other *E. coli* strains to optimize expression i.e. TG1, XL1-Blue, and HB2151. Supernatants from cell lysates and the periplasmic fractions of all four strains carrying the

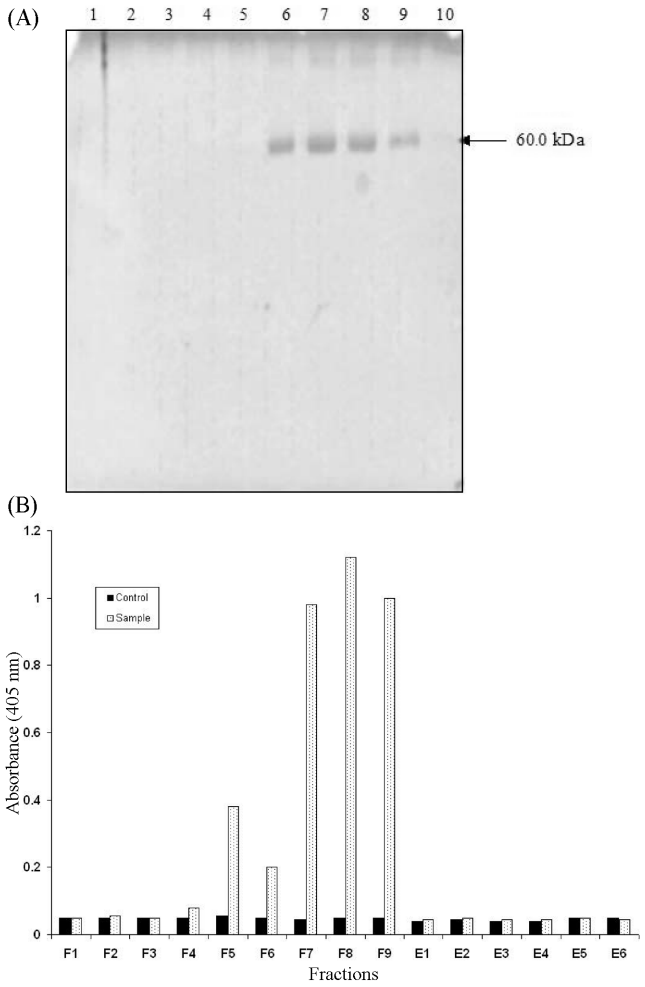

Fig. 6. (A) SDS-PAGE (12%) of Fab C37 products purified by Protein G chromatography. Lanes 1-10, fractions 1 to 10. Fractions 6 to 9 were pooled into one tube. (B) Corresponding ELISA of flow through (F1-F9) and eluted (E1-E6) fractions against *B. pseudomallei* protease.

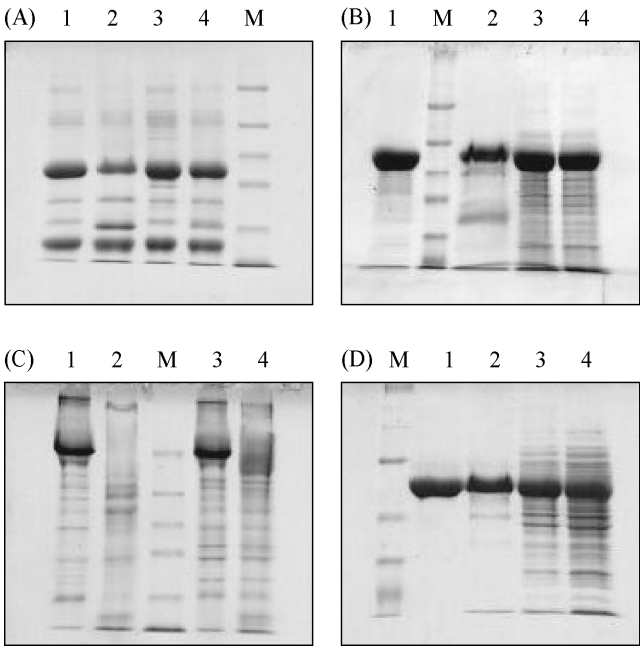

Fig. 7. Digestion of physiological proteins with *B. pseudomallei* protease only or with a protease - purified Fab C37 cocktail. (A) transferrin (B) actin (C) myosin (D) IgA. 1: protein; 2: protein + protease; 3: protein + Fab C37; 4: protein + protease + Fab C37; M: Broad Range molecular weight marker.

C37 phage were tested for the expression of Fab C37 by quantifying the amount of protein secreted in the supernatant and periplasmic fractions (Table 1). Many factors can affect expression efficiency e.g. the choice of nutrients and environmental parameters such as temperature, dissolved oxygen tension etc. The expression of foreign proteins in a recombinant host cell often utilizes significant host cell resource, and places a metabolic burden on the host, which may decrease growth and reduce protein expression. Thus, to enhance protein expression in this study, 0.2% (w/v) glucose or 0.2% (v/v) glycerol was added to the growth medium. The presence of glucose in the growth medium represses expression from the *lac* promoter, thus blocking the basal expression of this promoter prior to the addition of IPTG (de Bellis and Schwartz, 1990). Growth was maintained at 37°C to enhance the production of correctly folded functional Fab antibodies (Glick, 1995). From Table 1, we noted that the protein concentration was higher in the periplasmic fraction than in the culture supernatant. This is in accord with the pComb3H system, which retains recombinant protein fused to the coat protein pIII. Fab molecules are initially produced as pro-proteins and their ability to be transported to the periplasm depends on the 15-25 amino acid leader sequence (*ompA*). After the translocation, this leader sequence is cleaved by specific peptidases to form a mature Fab-pIII fusion protein of approximately 50,000-60,000 Da (Karu *et al.*, 1995). In the pComb3X system, the presence of an amber codon upstream of the pIII gene and the use of non-amber suppressor *E. coli* can be exploited to encourage the production of soluble antibody (Barbas *et al.*, 2001). The presence of the an additional carbon source (0.2% glucose or 0.2% glycerol) in the growth media, resulted in the expression of similar quantities of Fab protein within a host, but overall, this expression was higher in the HB2151C37 clone than in the 3 other *E. coli* strains used. HB2151 was found to be the most suitable host cell for the expression system adapted in this study, as was demonstrated by its SDS-PAGE profile, which showed less host cell protein expression in HB2151 (Fig. 5). Under non-denaturing conditions, a band of ~50,000 to 60,000 Da was evident, which is representative of the theoretical molecular weight of a Fab antibody fragment (50,000 Da) (Ward *et al*., 1989; Barbas *et al.*, 2001). Indirect-ELISA of the HB2151C37 antibodies against *B. pseudomallei* protease showed higher binding between Fab and protease than the control HB2151 cell fraction alone (data not shown).

The optimization of IPTG concentrations for HB2151

carrying C37 induced with 0.5 M IPTG resulted in higher protein production (Table 1). Nevertheless, the overproduction of foreign proteins encoded by the DNA introduced into the host cell triggers the metabolic burden phenomenon (Carrier *et al.*, 1993). Metabolite overload occurs when host cell ATP or GTP and certain amino acids are used to stabilize and express recombinant protein. Therefore, although highest expression was observed with 0.5 mM 1PTG, 0.05 mM 1PTG was used in the present study as previously reported by Donovan *et al.* (2000). Currently, no rationale has been established for the different expression profiles observed in different *E. coli* strains. Raffai *et al.* (1999) previously reported that TG1 cells are more suitable for expressing 2E8 Fab than XL1-Blue, and suggested that this difference is due to different bacterial growth rates. Raffai *et al.* (1999) also observed a higher yield of Fab within the periplasm and suggested that the relative distribution of antibody fragments between the periplasmic space and the medium can be modulated by culture conditions, as was also proposed by Kipriyanov *et al.* (1997). Moreover, the accumulation of Fab within the oxidized periplasmic space allows for better functional Fab folding.

Purification of the Fab antibody fragment Fab C37 was carried out on a Protein G chromatography column. Protein G is a bacterial cell wall protein and was isolated from group G streptococci (Sjobring *et al.*, 1988) and Fab fragments have been shown to bind to recombinant protein G (Derrick and Wigley, 1994; Kwack, 2000). In the present study, crude IPTG filtrate induced Fab was loaded onto a Protein G column and most of the protein of interest was eluted in fractions 6 to 9 (Fig. 6). Those fractions contained over 98 % of the pure Fab antibody fragment, and were pooled, dialyzed against PBS, and lyophilized. Indirect ELISA was performed using the purified FabC37, polyclonal antibodies, and *B. pseudomallei* protease. The purified Fab C37 exhibited good specificity towards the denatured *B. pseudomallei* protease (data not shown). In addition, we also found that the purified Fab was able to partially neutralize the proteolytic cleavage activity of protease. *B. pseudomallei* protease has been reported to proteolytically digest physiological proteins such as transferrin, myosin and immunoglobulins (Sexton *et al.*, 1994; Ling *et al.*, 2001). This is demonstrated in Fig. 7, which shows that myosin was cleaved by the protease and that transferrin was only partially cleaved. However, Fab C37 was able to prevent the degradation of myosin when incubated with the protease, thus supporting our previous findings that Fab C37 is able to neutralize *B. pseudomallei* protease activity by up to 80% (Nathan *et al.*, 2002).

These experiments demonstrate that Fab C37 has high specificity for the protease. If both proteins can be produced in large amounts and purified to homogeneity, the co-crystallization of the antibody-protease complex should allow us to further study the structure-function relationship of the protease in our quest to understand the pathogenesis of *B. pseudomallei*.

Conclusion

Polyclonal antibodies and recombinant Fab monoclonal antibodies against *B. pseudomallei* protease were successfully obtained by immunization and bacterial expression, respectively. The purity and biological activity of the antibodies obtained were demonstrated by ELISA and Western blotting. The protocols adopted for the production and purification were sufficient to prepare large quantities of biologically active antibodies. However, the purification protocol utilized for the *B. pseudomallei* protease failed to purify the protein to homogeneity; Fab antibodies could be utilized for the large-scale affinity purification of the protease. The availability of pure *B. pseudomallei* protease will enable the further elucidation of the structure-function relationship of *B. pseudomallei* protease by X-ray crystallography.

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