

Purification of A *Burkholderia pseudomallei* Antigen Via Antibody Mediated Affinity Chromatography

Kue-Peng Lim, Rahmah Mohamed, Noor Embi and Sheila Nathan*

Centre for Gene Analysis and Technology, School of Biosciences and Biotechnology,
Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia

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Abstract. *Burkholderia pseudomallei* is the causative agent of melioidosis, a fulminating disease in South East Asia and northern Australia. *B. pseudomallei* is known to secrete various extracellular products related to its pathogenesis, such as lethal exotoxin, protease and hemolysin. We have attempted to purify the exotoxin as studies have shown it to exhibit necrotic and cytotoxic activities and inhibit cellular protein synthesis. Purification was performed by antibody mediated affinity chromatography using previously generated single chain variable fragments (scFv) towards partially purified *B. pseudomallei* exotoxin coupled to a diaminodipropylamine column. *B. pseudomallei* was grown in BHIB + 2% glycerol under static conditions at 37°C for 7 days and crude extract was subjected to the scFv-linked column to capture the exotoxin. SDS-PAGE analysis exhibited a purified protein migrating as a single band bearing a size of approximately 37kDa. N-terminal protein sequencing and further bioinformatics analysis showed that the peptide shared some similarity with a putative decapeptide of the *Pseudomonas aeruginosa* exotoxin A and is present in both chromosomes 1 and 2 of *B. pseudomallei*. This suggests that the protein obtained could be the 37 kDa subunit of the *B. pseudomallei* exotoxin. Selected assays demonstrated that the purified product had no hemolytic, proteolytic or tyrosine phosphatase activity. The purified protein can aid in understanding the role of this virulence factor and serve as a candidate for vaccine development to combat melioidosis.

Keywords. affinity chromatography, *Burkholderia pseudomallei*, exotoxin, scFv

INTRODUCTION

Burkholderia pseudomallei (previously *Pseudomonas pseudomallei*) is the causative agent of melioidosis, a fulminating disease in South East Asia and northern Australia. Melioidosis has a high mortality rate (Chaowagul *et al.*, 1993) and mortality rates for acute melioidosis stand at 98% (Ismail *et al.*, 1987). *B. pseudomallei* is known to secrete various virulence factors such as protease (Sexton *et al.*, 1994), tyrosine phosphatase (Kanai *et al.*, 1994), hemolysin (Denisov *et al.*, 1996) and exotoxin (Ismail *et al.*, 1987). Antibodies towards the exotoxin have been detected in sheep exposed to *B. pseudomallei* therefore implying that the exotoxin is produced during *B. pseudomallei* infection (Ismail *et al.*, 1991) and plays an important role in the pathogenesis of *B. pseudomallei*.

The *B. pseudomallei* exotoxin inhibits protein synthesis by catalyzing the ADP-ribosylation of elongation factor 2 (EF-2) (Mohamed *et al.*, 1989), thus categorizing the exotoxin into the family of enzymes termed mono (ADP-ribosyl) transferase and more specifically, a NAD⁺-diphthamide ADP-ribosyl transferase (EC 2.4.2.36). Other toxins in this family include exotoxin A from *Pseudomonas aeruginosa* (Amstrong

et al., 2002), diphtheria toxin from *Corynebacterium diphtheriae* (Middlebrook & Dorland, 1984) and pertussis toxin from *Bordetella pertussis* (Locht *et al.*, 1986). The catalyzed reaction schematic is:



To date, purification of this lethal exotoxin has been laborious and only partially successful. Pure exotoxin could serve multiple functions including as a vaccine candidate or a tool for diagnosis. Thus, we initiated to purify the exotoxin based on an affinity chromatography approach utilizing antibodies. The anti-*B. pseudomallei* exotoxin monoclonal antibody, 6E6A8F3B, was generated by hybridoma technology and exhibited high binding activity towards partially purified exotoxin (Nathan *et al.*, 2000). Nevertheless, continuous analysis of these antibodies had demonstrated that the

*Author for Correspondence.

Mailing address: Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia. Tel: 60-3-8921-3862; Fax: 60-3-8925-2698; E-mail: sheila@pkrisc.cc.ukm.my

hybridoma cell line was not very stable in culture and furthermore, these antibodies were also capable of binding to other extracellular products of *B. pseudomallei*. To circumvent these problems, the heavy and light chain variable domains of the monoclonal antibody were cloned into the phage display vector pComb3H to specifically select anti-exotoxin antibodies and improve binding affinities of the antibody towards the partially purified exotoxin (Nathan *et al.*, 2002). Subsequently, the scFv in pComb3H was subcloned into pComb3X, a vector that contains an amber codon, therefore requiring only a non-amber codon suppressing strain of *E. coli* to express the soluble protein. This vector also allows for the detection and purification of the expressed antibodies due to the presence of a hemagglutinin and histidine tag respectively. The expressed scFv protein was successfully purified via nickel mediated affinity chromatography (Lim *et al.*, 2004). In this study, we describe the purification and initial characterization of a putative antigenic exotoxin via the scFv mediated affinity chromatography.

MATERIALS & METHODS

Bacterial strains and culture conditions. *B. pseudomallei* (human strain D286) was obtained from the Faculty of Science & Technology, Universiti Kebangsaan Malaysia. The bacterial cells were grown on Brain Heart Infusion Agar (BHIA, Hispanlab, Spain) for 48 hours at 37°C. A single colony was inoculated into 10ml Brain Heart Infusion Broth (BHIB, Hispanlab, Spain) and grown at 37°C overnight. The overnight culture was subcultured into 500ml of BHIB containing 1% mucin and 2% glycerol and grown at 37°C under static conditions for 7 days. 5ml of 37% formalin was added to the culture and left overnight at 4°C to kill the bacteria. The culture was harvested by centrifugation at 13000g for 45 minutes at 4°C. The resulting supernatant is referred to as the *B. pseudomallei* crude extract throughout the text.

Affinity chromatography. The pComb3X bearing the C4 scFv clone (C4X) was transformed into *E. coli* Top10F' (Invitrogen, USA). The culture was grown at 30°C until log phase, induced with 1 mM IPTG and induction was continued for a further five hours at 30°C. Expressed scFv protein was purified via nickel mediated affinity chromatography (Lim *et al.*, 2004). Purified scFv protein was dialyzed against phosphate buffered saline overnight prior to freeze-drying. scFv protein (1mg) was coupled to the AminoLink® Plus Coupling Gel according to the manufacturer's instructions (Pierce, U.S.A.). The coupled column was equilibrated to room temperature and 1.5 ml *B. pseudomallei* crude extract in Sample buffer (BupH™ Phosphate Buffered Saline, Pierce, U.S.A.) was allowed to flow through the column. The protein of interest was eluted by applying Elution Buffer (0.2 M glycine-HCl, pH 2.5) and 1ml fractions were collected.

Alternatively, the scFv protein was immobilized to the diaminodipropylamine column using EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) according to the manufacturer's protocol (Pierce, USA). The column was stored upright at 4°C and 2 ml of *B. pseudomallei* crude extract was added to the column and purified based on protocols provided by the manufacturer (Pierce, USA). The fractions collected from both chromatography strategies were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and spectrophotometric absorbance at 280nm.

N-terminal protein sequencing and bioinformatics.

Affinity purified proteins were blotted onto PVDF membrane and N-terminal protein sequencing was performed by Midwest Analytical, St. Louis, USA. The 10-mer peptide sequences obtained were subjected to the ARTEMIS software to determine the predicted open reading frames (ORF) against the completed *B. pseudomallei* genome sequence at the Wellcome Trust Sanger Institute database (www.sanger.ac.uk). The predicted ORFs were subsequently subjected to a tBLASTp analysis against selected bacterial exotoxin sequences obtained using the PEDANT database followed by alignment of all sequences using ClustalX.

Hemolytic activity assay. Hemolytic activity was determined using human erythrocytes as substrate as previously reported in the modified methods of Peters *et al.* (1982) and Nakazawa *et al.* (1987). 100ml of erythrocytes were washed 3 times with 1ml Calcium saline (10mM Tris-HCl, pH7.5, 5mM CaCl₂, 0.9% NaCl). Red blood cells were reconstituted to the original volume of blood (1ml) in calcium saline and diluted to a 1% suspension of erythrocytes. 100ml (10mg) sample solution and 100ml of diluted erythrocytes were co-incubated at 37°C for 1 hour and the reaction was stopped by incubation on ice for 2 minutes followed by centrifugation at 1500g for 2 minutes. The absorbance of the supernatant at 540nm was measured on by UV spectrophotometry (Shimadzu, Japan).

Proteolytic activity assay. Proteolytic activity was determined using azocasein as the substrate as previously described in the modified method of Percheron *et al.* (1995). 100µl (10µg) of sample solution was added to 50µl 0.5% azocasein and 20µl of 250mM Tris-HCl, pH8.0. The reaction mixture was incubated at 37°C for 30 minutes. The reaction was stopped by adding 150µl of 10% trichloroacetic acid and incubated at room temperature for 5 minutes followed by centrifugation at 9000g for 5 minutes. 120µl of the supernatant was transferred to a microtiter plate containing 150µl of 1N NaOH. Colour development was measured at 405nm using the MRX ELISA Reader.

Phosphatase assay. Phosphatase activity was determined using *p*-nitrophenyl phosphate (PNPP) as the substrate based on the modified method of Kanai *et al.* (1997). 100 μ l (10 μ g) sample solution was added to 100 μ l 0.1M Tris-HCl, pH7.5 and 50 μ l 0.2% PNPP. The reaction mixture was incubated at 40°C for 30 minutes and the reaction was stopped by adding 500 μ l of 0.5M NaOH. Colour development was measured at 420nm by UV spectrophotometry (Shimadzu, Japan).

RESULTS AND DISCUSSION

Affinity chromatography Purified exotoxin can contribute to the understanding of the mechanism of this enzyme and the infection mode of *B. pseudomallei*. Purification of exotoxin based on ammonium sulphate precipitation and Sephadex G-100 chromatography gave only a pool of protein with ADP-ribosylation activity but failed to produce a purified exotoxin (Ismail *et al.*, 1987). Thus, we believe that monoclonal antibodies specific towards *B. pseudomallei* or partially purified exotoxin are a suitable reagent for antigen purification by immunoaffinity, based on the specific and reversible interaction between substrate and ligand. In this study, we used both monoclonal antibody (6E6A8F3B) and scFv as a ligand to purify the exotoxin.

Both diaminopropylamine and AminoLink[®] columns were utilized during purification. The AminoLink[®] coupling gel was activated to form aldehyde functional groups and these groups react with primary amines present on proteins, antigens or antibodies. Reductive amination of the resulting Schiff's base forms a stable covalent linkage (Pierce, USA). Protein concentration determination indicated that 91 μ g of scFv protein had been covalently coupled to the AminoLink[®] support. *B. pseudomallei* crude extract was loaded onto the column and the eluted fractions were monitored by reading the absorbance at 280nm and by SDS-PAGE. Absorbance values (A_{280nm}) were almost zero for all the eluted fractions while no conclusive protein bands were visible by SDS-PAGE. This observation implied that all the protein was most likely eluted during the washing step with none bound to the matrix. As the AminoLink[®] matrix reacts with primary amines present on proteins, it is possible that amines present on the scFv had coupled to the gel matrix within the column. If the scFv protein binds to the exotoxin via amine groups, antigen-antibody bonds failed to form, thus proteins, including the exotoxin, were eluted during the washing step. To overcome this problem, the diaminopropylamine column was selected for the purification.

Carbodiimides were used to link carboxyl groups of the monoclonal antibody to the primary amine group contained in the diaminodipropylamine column, thus the amino groups of the monoclonal antibodies are free to couple to the exotoxin. Specific interaction between monoclonal antibodies and exotoxin enables isolation of the exotoxin from the crude extract of *B. pseudomallei* and the interaction is then

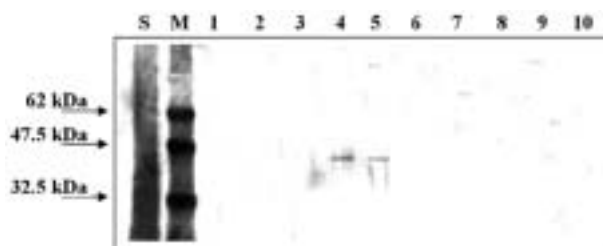


Figure 1. SDS-PAGE of the eluted fractions from the scFv immobilized affinity column. *B. pseudomallei* crude extract was loaded onto the column and bound protein was eluted with Pierce ImmunoPure[®] Elution Buffer. S: *B. pseudomallei* crude extract; M: Broad range molecular weight marker; 1-10: Sequential eluted fractions.

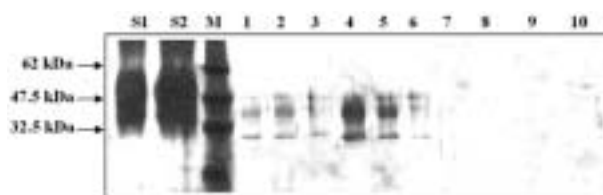


Figure 2. SDS-PAGE of the eluted fractions from the 6E6A8F3B monoclonal antibody immobilized affinity column. *B. pseudomallei* crude extract was loaded onto the column and bound protein was eluted with Pierce ImmunoPure[™] Elution Buffer. S1, S2: *B. pseudomallei* crude extract; M: Broad range molecular weight marker; 1-10 Sequential eluted fractions.

broken by elution with a low pH elution buffer. Both 6E6A8F3B and C4X scFv monoclonal antibodies were immobilized separately onto the diaminodipropylamine column to compare their ability to purify the exotoxin. Figure 1 shows the SDS-PAGE profile of the eluted fractions from the scFv-immobilized column. Fractions 4 and 5 exhibit a single protein band with a size of about 37 kDa. Figure 2 represents the SDS-PAGE profile of the eluted fractions from the 6E6A8F3B-immobilized column. Fractions 4 and 5 exhibited two proteins with sizes of approximately 37 kDa and 50 kDa that had been purified. Previously, Isa *et al.* (1983) had reported the purification of a 37 kDa exotoxin from *B. pseudomallei*. The variation shown between using 6E6A8F3B antibodies produced by hybridoma culture and scFv, by phage display, support our earlier findings that the 6E6A8F3B monoclonal antibodies are not stable in culture and are capable of binding to other extracellular products of *B. pseudomallei*. The phage display technology has allowed us to specifically select antibodies against the exotoxin and enabled us to improve the binding affinities of the antibody towards the exotoxin through the biopanning process (Nathan *et al.*, 2002).

The binding affinities of the purified protein towards the C4X scFv and 6E6A8F3B monoclonal antibodies were



Figure 3. Western blot of *B. pseudomallei* antigens with the scFv antibody. 1: *P. aeruginosa* exotoxin A (negative control); 2: Purified *B. pseudomallei* exotoxin from 6E6A8F3B immobilized column; 3: Purified *B. pseudomallei* exotoxin from scFv immobilized column; 4: Partially purified *B. pseudomallei* exotoxin from Sephadex G-100 chromatography; 5: Partially purified *B. pseudomallei* hemolysin from Sephadex G-100 chromatography; 6: *B. pseudomallei* crude extract; M: Broad range protein marker.

compared to that of partially purified hemolysin and exotoxin from Sephadex G-100 chromatography, crude extract of *B. pseudomallei* while the exotoxin A from *P. aeruginosa* was used as a negative control. The purified proteins and others extracellular products of *B. pseudomallei* were blotted with purified C4X scFv protein or 6E6A8F3B monoclonal antibodies with peroxidase conjugated anti-HA or goat anti-mouse IgG as the secondary antibody, respectively. The blots showed that only the purified protein from the scFv immobilized column and partially purified exotoxin obtained by Sephadex G-100 chromatography bound to the scFv antibody, where a 37kDa band was detected (Figure 3). The purified protein gave the highest intensity and this suggests that the purified protein is specific towards the scFv. The partially purified exotoxin from the second peak of Sephadex G-100 chromatography that has been previously reported to have ADP-ribosylation activity also showed binding capacity towards the scFv. These results have supported our earlier finding that the scFv protein is specific towards *B. pseudomallei* exotoxin (Nathan *et al.* 2002). The light and heavy chain variable domains of the C4X scFv were amplified from the 6E6A8F3B hybridoma cell line, thus we believe the protein purified by both scFv and 6E6A8F3B monoclonal antibodies share some similarities, but the 50kDa purified protein (from the 6E6A8F3B immobilized column) was not detectable by the scFv. This might be due to an error during the original dilution cloning of the initial hybridoma population and also the genetic variation of the hybridoma cell line after continuous subculture, where spontaneous mutation rates appear to be higher in vitro, associated with the high rate of cell proliferation and mutant cells are not eliminated unless their growth capacity is impaired (Freshney, 1990). Western blotting using crude extract of *B. pseudomallei*, which contains the exotoxin, exhibited no visible band(s) and this might be due to the fact that the *B. pseudomallei* crude extract consists of

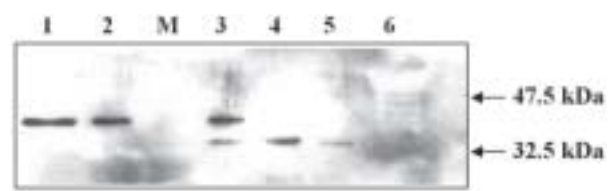


Figure 4. Western analysis of *B. pseudomallei* antigens blotted with the 6E6A8F3B monoclonal antibody. 1: Purified *B. pseudomallei* exotoxin from scFv-immobilized column; 2: Purified *B. pseudomallei* exotoxin from 6E6A8F3B immobilized column; 3: Partially purified *B. pseudomallei* exotoxin from Sephadex G-100 chromatography; 4: Partially purified *B. pseudomallei* hemolysin from Sephadex G-100 chromatography; 5: *B. pseudomallei* crude extract; 6: *P. aeruginosa* exotoxin A (negative control); M: Broad range protein marker.

various proteins and the concentration of a single protein is too low to be detected.

Western blotting using the 6E6A8F3B monoclonal antibodies as primary antibody demonstrated positive signals for the 37 kDa protein purified from both the scFv and 6E6A8F3B immobilized column, as well as partially purified exotoxin, hemolysin and the crude extract of *B. pseudomallei* (Figure 4). This further supports our earlier findings that these hybridoma derived antibodies are not specific towards exotoxin and can react with other extracellular products of *B. pseudomallei*. Nevertheless, the 6E6A8F3B monoclonal antibodies did demonstrate a higher affinity for the exotoxin, and judged by a higher intensity band when compared to the other extracellular products of *B. pseudomallei*.

Bioinformatics analysis of N-terminal derived peptide sequences The 37 kDa purified protein (E4A) and the 50 kDa-purified protein (E4B) from scFv-mediated affinity chromatography and 6E6A8F3B mediated chromatography respectively, were subjected to N-terminal protein sequencing. Analysis of the decapeptide of both E4A and E4B showed that both putative sequences are present in chromosomes 1 and 2 of the *B. pseudomallei* genome with homologies exceeding 80% (data not shown). The ARTEMIS software was utilized to determine the possible ORFs that carry these peptide sequences in the *B. pseudomallei* genome. A total of 6 potential ORFs were obtained and these were aligned against known exotoxin-like sequences from *E. coli*, *P. aeruginosa* and *H. influenzae* obtained from the PEDANT database of protein motifs and sequences. One potential *B. pseudomallei* ORF for E4A and two *B. pseudomallei* ORFs for E4B clustered well with *P. aeruginosa* exotoxin sequences (Figure 5a and 5b). The potential ORF of E4A (E4Aorf3) had a high degree of similarity with the *P. aeruginosa* Exoenzyme S

(a)

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pa4943      -----MFFERPGGGEQAVLVHLEGQNPEAREDPQEFQELVRSAGAETVAFVS
E4Aorf3    -----PEHAQLLPPE-----RLTAIERRARLQSR-----
exos       MHIQSLQQSPSFAVELHQAASGRLGQIEARQVATPSEAQQLAQRQDAPKGEGLLARLGA

pa4943      VSRHQPSAKYLIQS--GKVEELHDLVKAENVELVIFNHTLTSPQERNLERAFECRVLDR
E4Aorf3    LERNLQGFRRIG--GNVIDRHNLRDDD-----AAACPVP---
exos       LVRPFVAIMDWLKGKLLGSHARTGPPQPSQDAQPAVMSSAVVFKQMVLLQALPMTLKGDKA

pa4943      GLILDIFAQRARTHEGKLQVELAQLEHMSTRLVRGWT-----HLERQKGGIG
E4Aorf3    -----LFVQRVFR-----EILRDVLRQVGRELVPQAP-----LLERVV-DVD
exos       SELATLTPEGLAREHSRLASGDGALRSLSTALAGIRAGSQVEESRIQAGRLLERSIGGIA

pa4943      LRGPGET-----QLETDRLLRVRIRQIKQRLEKVRSQREQARRGRKRADIPAVS
E4Aorf3    -----DALPDRAQHAVILAPPDGHMEVPRAP-----
exos       LQQWGTGGAASQLVLDASPELREITDQLHQVMSEVALLRQAVESEVSRVSADKALADG

pa4943      LVGYTNAGKSTLFNSLTASEVYAANQLFATLDPTLRLQLDDLGPVVLADTVGFIHRLPH
E4Aorf3    -----LLALRCGDILEG-----R
exos       LVKRFGADAEEKYLGRQPGGIHSDAEVMALGLYTGIIHYADLNRALRQQEELDAG-----QK

pa4943      KLVEAFRATLEESSNADLLLHVIDAYEP-----ERDAQ
E4Aorf3    QLRETPAQRVEITLQAGFVLHVLAVRQR-----DHSIR
exos       LIDQMSAAFEKSGQAEQVVKTFRGRTRGDFAFNAVEEGKVGHDDGYLSTSLNPGVARVSGF

pa4943      VEQVLAVLGEIGANELPMLLEVYNKVDLLPSVEPHIQRDDSGKPVVWLSAQTGEGDLLR
E4Aorf3    SNRLTP-----
exos       QGTISTVFGRSGIDVSGISNYKNEKEILYNKETDMRVLLSASDEQGVTRRVLEEAALGEO
    
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(b)

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E4Borf4    MPASMRIRCCTDDTLARDAQRRTMRRSAVFARAVRVSPATGAAHAPGLPSLFRPPLHVAV
ETA        -----
E4Borf1    -----MERTMNFMSLSRIVPRALAAG---CLFAAAGASQAAGVYAPYVDVTLTYPT

E4Borf4    ALAMRPARPVFPFASFVSPRARGSFPLRSHAHGRGASPSVARPLARRKAPAAACGD
ETA        -----AQDQEPDARGR
E4Borf1    PLVDQIGVQQGIQQFMLAFVSGGNQCTPSWGGVQPIGNGAGDLLDKIATSVTAYRAKGG

E4Borf4    SNAPRLTLLYVHIKNHIPPAIDQPGHINGESMSQREAITDYLRLVHLTLLMERSVTRA
ETA        IRNGALLRVYVPRS-----SLPGFYR-----
E4Borf1    DVAVSFGGAAGQPLMQACSSVAALKGAYQTVIDTYSLTHVDFDIEG--ASQQDSAAVARN

E4Borf4    AVKLNQSQPAISAALRRLRDLITGDPLLVRGKSGMVPTEYGLRLLLEPVQNALREIERIKFQ
ETA        -TGLTLAAPEAAGEVERL---IGHPLPLRLDAITGPEE-----
E4Borf1    FQAVAQQLADYAAKGKPLHVTTLTPAMP TGLVQDGLNVLNAALANNVTLDAVNIMTMDYG

E4Borf4    QHNFDPATSI RCYRIGCPDYLNVLFVPTVVERFRQAAPNATLEFHSLSGP-----
ETA        -----
E4Borf1    PSGIDMGAASIAAQGLYSQLD TAYKSAGKPQTDAQLKQLVGVTPMIGVNDVAGEIFTLA

E4Borf4    AFDYELALEDGKLDIVGNWPEPEQLHLSNLFVDEIVCLMSNSHPFAKRGGLTLDQYLN
ETA        -----EGGRLETILG-WP-----LAERTVVIPS-----
E4Borf1    NAQSVQTMANNNYGFVGIWSITRDKACDGSSQYASPICSGVAQQPYAFSSVFKQLGGHW

E4Borf4    APHLAFTP-YSVGQRGAIDVHLARERLKRHVVTLPYFNLA PYVLVKS DLI FT TTRLFAD
ETA        --AIP TDP-RNVG--GDLDPSIPD--KEQAISALPD-----
E4Borf1    GAGVTQDPNYGGGSDGGKPPGAPWSATQVYTAGATV TYQG--TTYQAQWWTQGDIPGQ
    
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Figure 5. (a) Alignment of a predicted ORF for E4A with *P. aeruginosa* section 468 of 529 of the complete genome (pa4943, GenBank Acc. No: AE004907) and Exoenzyme S (exos, Gen Bank Acc. No. X99471). (b) Alignment of two predicted ORFs for E4B with Exotoxin A ADP-ribosyltransferase domain (ETA, Gen Bank Acc. No. AF227424).

(exos), an ADP-ribosyltransferase (Frithz-Lindsten *et al.*, 1997) whilst the potential ORFs for E4B demonstrated high similarity to the ADP-ribosyltransferase domain (domain III) of Exotoxin A (Gallant *et al.*, 2000). These results suggest that the purified protein was coded for by sequences that are similar to known ADP-ribosyltransferase domains of exotoxin genes and could possibly be the corresponding domain for the exotoxin of *B. pseudomallei*. The presence of the exotoxin sequences in both chromosomes of *B. pseudomallei* indicates the importance of exotoxin in the survival and pathogenesis of *B. pseudomallei*. This also shows that the exotoxin might be coded by more than one gene or has high copy numbers in the *B. pseudomallei* genome, implying that the exotoxin might not only serve as a virulence factor of *B. pseudomallei* but the expression of this protein might also be important in the active growth of this pathogen.

Activity assay Purified protein (37 kDa) from the scFv mediated affinity chromatography was pooled and dialysed with phosphate buffered saline. Hemolysin (Denisov *et al.*, 1996), protease (Lee & Liu, 2000) and tyrosine phosphatase (Kanai *et al.*, 1997) are known extracellular products of *B. pseudomallei*. Due to the technical difficulties in performing an ADP-ribosylation assay to prove the identity of the purified protein, we chose to assay the protein for haemolytic, proteolytic and phosphatase activities to eliminate the possibility that the purified 37kDa protein could be either of these proteins.

The hemolysin activity assay showed that the 37 kDa purified protein from the scFv mediated column had low hemolytic activity, about 2 fold higher than the negative control, while hemolytic activity for partially purified hemolysin, partially purified exotoxin, protease and crude extract were 5 fold, 4 fold, 4 fold and 3 fold, respectively (Figure 6a), more than the negative control. The Phosphatase assay showed that the purified protein had very low phosphatase activity compared to hemolysin, crude extract, partially purified exotoxin and protease (Figure 6b). In the protease assay, the reaction buffer, Tris-HCl pH 8, was used as the negative control with commercial trypsin as the positive control. No proteolytic activity was detected from the purified protein, while trypsin, protease, partially purified hemolysin, partially purified protease and crude extract of *B. pseudomallei* all exhibited relatively high proteolytic activity when compared to the negative control (Figure 6c). Thus, the purified protein from the scFv mediated affinity column exhibited no protease activity and negligible hemolytic and phosphatase activities.

Previous studies have reported the presence of secreted *B. pseudomallei* virulence factors of similar size i.e. a 33kDa metalloprotease (Chenthamarakshan, unpublished), a 36kDa metalloprotease (Sexton and Jones, 1994) and superoxide dismutase (36 kDa (Chenthamarakshan, unpublished). Nevertheless, as shown above, the purified protein lacks any

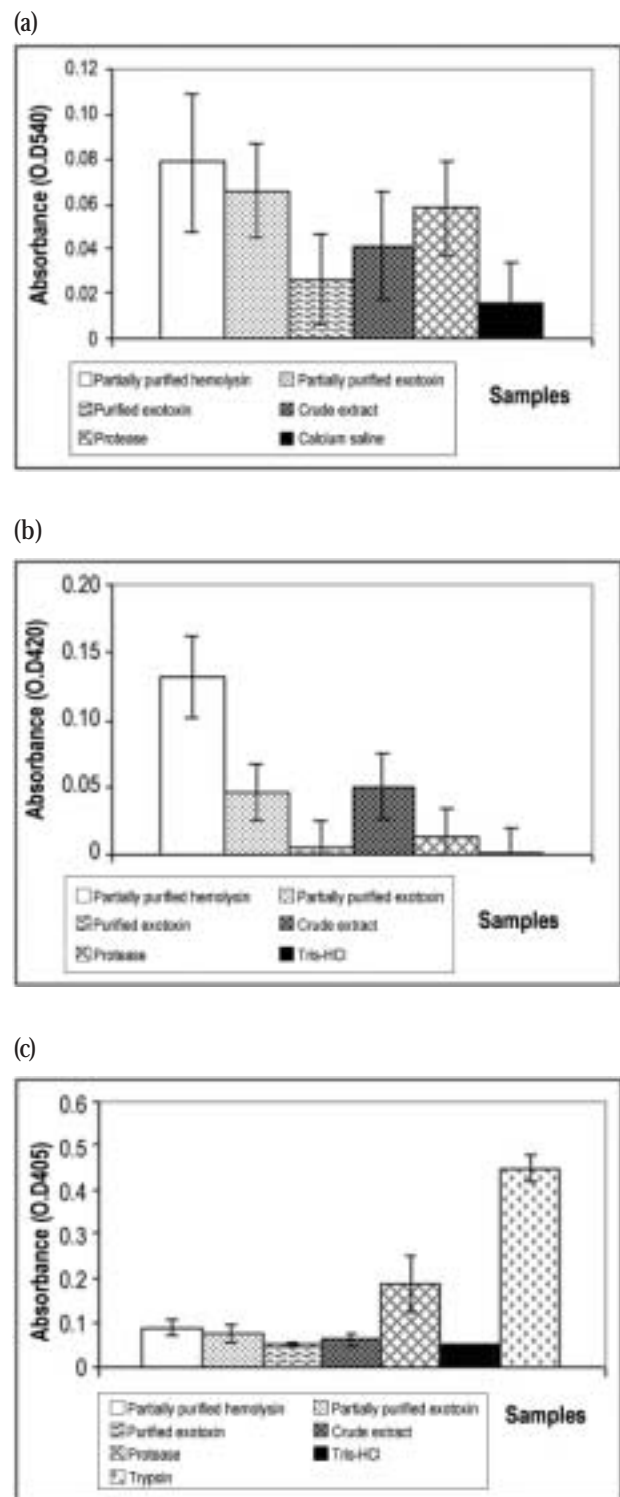


Figure 6. Protein enzymatic assays performed on the purified protein (a) Hemolytic assay (b) Phosphatase assay (c) Proteolytic assay.

proteolytic activity whilst the information on the presence of superoxide dismutase in the *B. pseudomallei* crude filtrate has yet to be verified. These reports and the results obtained in this study aid in substantiating our suggestion that the purified protein could be the putative exotoxin of *B. pseudomallei*.

CONCLUSION

scFv mediated affinity chromatography successfully purified a 37 kDa protein from crude *B. pseudomallei* extract. The decapeptide of this protein is present in both chromosomes of *B. pseudomallei* suggesting that this protein plays an important role in the survival and pathogenesis of the pathogen and is most probably coded by more than one gene or has high copy numbers. Analysis of the sequence showed that this decapeptide has good similarity to *P. aeruginosa* exotoxin A. As the purified protein has exhibited no proteolytic, hemolytic or phosphatase activities, the results from study strongly suggest that the 37kDa purified protein could be the putative exotoxin of *B. pseudomallei*.

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