The Journal of Microbiology, June 2004, p.126-132
Vol. 42, No. 2
Copyright © 2004, The Microbiological Society of Korea

Expression and Purification of a Recombinant scFv towards the Exotoxin of the Pathogen, *Burkholderia pseudomallei*

Kue-Peng Lim[1], HongBin Li[2] and Sheila Nathan[1,*]

[1]Centre for Gene Analysis and Technology, School of Biosciences and Biotechnology,
[1]Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia
[2]Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines, La Jolla, California 92037, USA

(Received November 25, 2003 / Accepted April 2, 2004)

A single chain variable fragment (scFv) specific towards *B. pseudomallei* exotoxin had previously been generated from an existing hybridoma cell line (6E6AF83B) and cloned into the phage display vector pComb3H. In this study, the scFv was subcloned into the pComb3X vector to facilitate the detection and purification of expressed antibodies. Detection was facilitated by the presence of a hemagglutinin (HA) tag, and purification was facilitated by the presence of a histidine tag. The culture was grown at 30°C until log phase was achieved and then induced with 1 mM IPTG in the absence of any additional carbon source. Induction was continued at 30°C for five h. The scFv was discerned by dual processes-direct enzyme-linked immunosorbent assays (ELISA), and Western blotting. When compared to *E. coli* strains ER2537 and HB2151, scFv expression was observed to be highest in the *E. coli* strain Top10F'. The expressed scFv protein was purified via nickel-mediated affinity chromatography and results indicated that two proteins a 52 kDa protein, and a 30 kDa protein were co-purified. These antibodies, when blotted against immobilized exotoxin, exhibited significant specificity towards the exotoxin, compared to other *B. pseudomallei* antigens. Thus, these antibodies should serve as suitable reagents for future affinity purification of the exotoxin.

Key words: scFv, phage display, monoclonal antibody, affinity chromatography, exotoxin

Burkholderia pseudomallei, previously known as *Pseudomonas pseudomallei,* is the causative agent of melioidosis, a fulminating disease found in the tropics, and also in subtropical regions. Melioidosis is associated with underlying predisposing conditions such as diabetes and renal failure, and presenting symptoms can range from merely a local infection, to septicemic illness. The pathogen is known to secrete various extracellular products that have been implicated in the melioidosis pathogenesis. These secreted products include protease (Lee and Liu, 2000), tyrosine phosphatase (Kondo *et al.,* 1991), and a lethal exotoxin (Isa *et al.,* 1983, Ismail *et al.,* 1987). Each of these purified products has the potential to serve as a vaccine candidate to combat the lethal effects of the pathogen.

We chose to isolate the lethal component of *B. pseudomallei,* the exotoxin which has been previously reported to inhibit cellular protein synthesis via ADP-ribosylation of elongation factor-2 (EF-2) (Domenighini and Rappuoli, 1996). The pure exotoxin would be a valuable tool, either in a diagnostic kit for active melioidosis, or as a potential vaccine. We propose to purify the exotoxin by antibody-mediated affinity chromatography using phage display-derived single-chain variable fragments (scFv clone C4), previously generated from an existing hybridoma cell line (Nathan *et al.,* 2000). Phage display technology enables the direct isolation of monovalent single-chain variable fragment (scFv) antibodies against any protein (Rader, 2001). The heavy and light-chain variable domains of the hybridoma line were amplified to construct a scFv, and cloned into the pComb3H phage display vector. Using this strategy, we successfully isolated an scFv towards the exotoxin. The scFv fragment is characterized by a unique CDR_H3 domain of ETTETS, which could represent a specific motif of the *B. pseudomallei* exotoxin-binding site (Nathan *et al.,* 2002).

Characterization of these scFv antibodies has been hampered by the low-level production of soluble fragments, and limitations in the availability of suitable secondary reagents for the detection of expressed antibodies. To circumvent these problems, we proposed to sub-clone the scFv into the pComb3X phage display vector. The pComb3X phage display vector contains an amber codon, thus requiring only a switch in the bacterial strain to express soluble proteins (Barbas *et al.,* 2001). Furthermore, this vector also allows for the detection and purification of expressed antibodies, due to the presence of

* To whom correspondence should be addressed.
(Tel) 60-3-8921-3862; (Fax) 60-3-8925-2698
(E-mail) sheila@pkrisc.cc.ukm.my

Hemagglutinin (HA) and Histidine tags, respectively. Here we report the successful expression of high levels of scFv upon induction by IPTG, with the purified scFv exhibiting good specificity to the exotoxin. Thus, this antibody could serve as a suitable reagent for antibody-mediated affinity purification of the exotoxin. Purified exotoxin will aid in understanding the structure-function relationship of the exotoxin and its role in pathogenesis. The pure exotoxin will also function as a diagnostic tool, and perhaps as a potential vaccine candidate.

Materials and Methods

Bacteria host strains

Various *Escherichia coli* strains were transformed with either the vector pComb3X (Barbas *et al.*, 2001), or with scFv clone C4. The strains used were ER2537 (New England Biolabs, USA) [λ-F' *lac* I^qΔ(*lacZ*)*M15 proA*$^+$*B*$^+$*/fhuA2 supED*(*lac-proAB*) *thiD*(*hsdMS-merB*)*5*($r_k^-m_k^-$McrBC-) K12], HB2151 (Stratagene, USA) [*araD* (*lac, proAB*), *lac*I^q Δ(*lacZ*) *M15*, *thi*/ F' *proA*$^+$*B*$^+$*/fhua2*Δ(*thi*Δ(*hsdMS merB*) (r_k m_k^- *McrBC*$^-$) K12] and Top10*F'* [*F*{*lac*I^q,*Tn10*(Tet^R)}*mcrA*Δ *(mcr-hsdRMS-mcr-BC)*φ*80lacZ*Δ*M15*Δ *lacX74deoR recA1 araD139*Δ *(ara-leu) 7697galU galK rpsL* (*Str*^{R})*endA1 nupG*] (Invitrogen, USA).

Burkholderia pseudomallei *exotoxin*

Burkholderia pseudomallei exotoxin was prepared as previously described (Isa *et al.*, 1983). 7-day stationary cultures of *B. pseudomallei* strain D286 (Faculty of Science and Technology, Universiti Kebangsaan Malaysia) were prepared and ammonium sulfate-precipitated material was purified by Sephadex G-100 chromatography.

Subcloning

Both vector pComb3X (4.8 kb; Barbas *et al.*, 2001) and clone C4 (3.95 kb), a recombinant antibody clone consisting of the anti-*B. pseudomallei* exotoxin scFv (750 bp) cloned into pComb3H (Nathan *et al.*, 2002), were digested utilizing the restriction enzyme *Sfi*I. The restriction-digested scFv fragment and pComb3X vector were purified with a QIAGEN gel extraction kit (Qiagen, USA). Ligations between the scFv fragment and pComb3X were prepared in a ligation mixture of 5X buffer (Gibco BRL, USA) and 5U T4 Ligase (Gibco BRL, USA). Vector: scFv (w/w) ratios of 1:1, 2:1 and 3:1 were set up in the ligation mixture and incubation was done overnight at room temperature. 1 µl of the ligation mixture was electroporated into *E. coli* ER2537 cells (2.5 kV, 25 µF and 200 Ω). The electroporated cells were then grown and plated onto Luria-Bertani (LB) agar with carbenicillin. Colonies bearing the pComb3X-scFv construct were confirmed by restriction enzyme digestion and DNA sequencing.

Expression

Random subclones were transformed into different *E. coli* host cells, *i.e*, HB2151 and Top*10*F'. A single colony was selected and grown in 5 ml SuperBroth (SB, 3.5% trypton, 2% yeast extract, 0.5% NaCl), from which 100 µl of the overnight culture was added to 10 ml of fresh LB broth. 1 mM IPTG was added after 5 h, or at $O.D_{600nm}$ 0.6-1.0, and the cells were allowed to grow for a further 5 h. Optimization of scFv expression was performed utilizing different host cells, differing concentrations of IPTG ranging from 1 mM to 5 mM, or addition of carbon sources to the culture--concentrations of glucose ranged from 0% to 1.0%. The cells were subsequently harvested by centrifugation at 4000×g for 15 min at 4°C, and the resulting pellets were freeze-thawed 5 times and centrifuged. The resulting pellets (periplasm fraction) and supernatants were subjected to enzyme-linked immunosorbent assays (ELISA) and Western blotting, to determine expression levels and specificity with peroxidase-conjugated mouse anti-HA (1:500), which was utilized as the secondary antibody, and HRP Supersignal (Pierce, U.S.A.), which functioned as the substrate.

ELISA and Western blotting

In the ELISA, 1 µg of expressed scFv protein samples was coated overnight at 4°C followed by blocking with 50 µl of 5% skim milk, at 37°C, for 1 h. The wells were then washed 5 times with 0.1% Phosphate Buffered Saline (PBS)-Tween 20 (PBST). After washing, peroxidase-conjugated mouse anti-HA (1:500, Roche) was utilized as the secondary antibody and incubated for 1 h at 37°C. After 5X washing with PBST, a mixture of peroxidase and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (1:1) was used as the reaction substrate, and colour development was measured at 405 nm.

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed according to the Laemmli method (1974). 10 µg protein samples in a 3X sample buffer (0.5 M Tris-Cl, pH6.8, 0.2 M EDTA, 10% SDS, β-mercaptoethanol, glycerol) were heated at 95°C for 10 min, and electrophoresed on a 12% SDS-PAG at 150 V for 1 h, utilizing the EC 120 Mini Vertical Gel System (EC Apparatus Corporation, USA). Western blotting was performed according to the methods of Towbin *et al.* (1979). Proteins in the SDS-PAGE gel were transferred to a PVDF membrane using the EC 140 Mini Blot Module (EC Apparatus Corporation, USA) at 100 mA for 1 h and 45 min. The membrane was then blocked overnight with 5% skim milk in PBS. After 5X extensive washing with PBST, the membrane was incubated with peroxidase-conjugated mouse anti-HA (1:500) for 1 h at 37°C. Finally, the membrane was again washed 5X with PBST, incubated with 500 µl peroxidase Supersignal substrate (Pierce, USA), and exposed to autoradiography film.

Nickel-affinity chromatography

Expressed scFv was purified via high-performance liquid chromatography (HPLC) using the HiTrap™ HP column

on the AKTAprime^{TM} system, according to the manufacturer's recommendations (Amersham Pharmacia, USA). The column was briefly preconditioned with binding buffer (20 mM Tris, 0.5 M NaCl, pH 7.0), and protein samples were loaded onto the column. Nickel-bound scFv was eluted with an elution buffer (20 mM Tris, 0.5 M NaCl, 0.5 M imidazole, pH 7.0), and all fractions were monitored for protein content by absorption at 280 nm.

Results and Discussion

Subcloning

A 750 bp purified scFv fragment and 3.2 kb purified digested pComb3X vector were successfully ligated with a DNA ratio of 1:1 (w/w). Positive transformants bearing a size of 3.95 kbp were digested with *Sfi*1, and the presence of the scFv (750 bp) was confirmed (data not shown). The scFv fragment consists of the variable heavy-chain (V_H) and variable light-chain (V_L) domains, which were amplified from the monoclonal antibody, 6E6A8F3B. The scFv fragment was specific towards *B. pseudomallei* exotoxin (Nathan *et al.*, 2002). The V_H and V_L were joined by a 15-amino acid $(Gly_4Ser)_3$ linker, via an overlap extension polymerase chain reaction. This linker permits inter-chain, but not intra-chain, pairing of the variable domains (Dall'Acqua *et al.*, 1998). The linker ameliorates the low stability of the F_V fragment, thus extending its usefulness in therapeutic and diagnostic applications. Fig. 1 shows a schematic of Clone C4X. The histidine (HIS) peptide tag, at the carboxyl terminus of the displayed protein, facilitates purification of proteins with immobilized metal (nickel) affinity. The influenza hemagglutinin (HA) epitope tag, YPYDVPDYAS, facilitates detection of the protein with commercially available anti-HA antibody-enzyme conjugates (Barbas *et al.*, 2001). An amber stop codon, TAG, is located at the junction of the regions encoding the peptide tags and pIII. Propagation of phage in suppressor strains, such as XL1-Blue and ER2537 (*supE* or *supF* are also acceptable), allows the production of *gene III* fusion proteins for phage display. However, if a male non-suppressor strain, such as HB2151, is infected with the phagemid phage, the stop codon will be read, and soluble proteins will be produced (without fusion of the phage coat protein, pIII) (Barbas *et al.*, 2001).

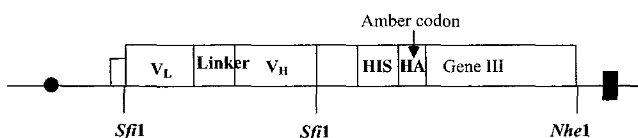

Fig. 1. Schematic diagram of pComb3X-scFv (clone C4X). The variable immunoglobulin domains of the light (V_L) and heavy chain (V_H) were fused by a peptide linker (L) to form the scFv. The scFv construct was fused to the carboxy-terminal domain of the minor coat protein (pIII) and was displayed on the phage particle surface. The pComb3X vector carries an amber codon inserted between the 3 *Sfi* I restriction site and the 5 end of gene III. The 6X histidine (HIS) tag was used for scFv purification whilst the hemagglutinin (HA) decapeptide tag at the 3 end of the HIS tag was for detection of expressed scFv using an anti-HA antibody.

Selected clones were sequenced, in order to confirm the reading frame of the scFv sequence. The *ompseq* primer (Barbas *et al.*, 2001) was used to sequence the whole scFv, where the primer binds to the leader sequence *ompA* (between 2620-2640 bp in pComb3X), while the *HRLM-*F primer (which binds to the internal linker sequence), was used to sequence the V_H domain. Both full-length scFv and V_H gene sequences showed 99% identity with the previously reported C4 scFv sequence (Nathan *et al.*, 2002; GenBank Accession No: AY054296-7) (data not shown).

Expression

As mentioned in the above section, selected phage display vectors such as pComb3X are typified by the possession of an amber codon, located between the scFv antibody gene, and gene *III* of the phage. Growth in an amber non-suppressor strain, such as HB2151, elicits the soluble expression of scFv proteins. However, in an amber suppressor strain, such as ER2537 or TG1, the suppression of the amber codon is incomplete, and both complete pIII-antibody fusion protein and soluble antibody protein are synthesized in the same cell (Su *et al.*, 2003). Soluble proteins are normally transported to the periplasmic space and, from there, leaked into the growth media (Barbas *et al.*, 2001). Hence, soluble antibodies can normally be harvested either from the growth culture or the periplasmic space. In this report, initial scFv expression in a non-suppressor host showed that the production of soluble proteins in the culture medium was generally insufficient. The quantity of protein from the periplasm (after 5 rounds of freeze-thawing), however, was approximately 100 times the quantity of protein which was extracted from culture media (data not shown). Hence, reading of the amber codon by the non-suppressor host strain was most likely incomplete, and some of the scFv was retained in the periplasmic space as fusion proteins.

scFv protein expression levels were monitored in Top10F', HB2151 and ER2537 by ELISA, where the presence of the scFv was detected by the anti-HA antibody. This antibody is also known to bind to a 9-amino acid sequence of a 60 kDa *E. coli* protein, and this binding most probably contributed to the (albeit low) absorption readings observed in the samples of host cells alone and host cell bearing vectors (Table 1). Considering these background values, it is evident that scFv proteins were present in the C4X samples and results demonstrate that Top10F' cells expressed the highest amount of scFv protein (Table 1). Expression levels in Top10F' were 11-fold higher than in the negative control (host cell alone), while expression levels for HB2151 were 5 times higher, and expression levels for ER2537 were twice as high as those of the negative control. This suggests that the non-sup-

Table 1. Optimization of scFv expression. The culture was grown at 30°C until log phase, induced with 1 mM IPTG and induction was continued for another five hours at 30°C in Top10*F'* unless stated otherwise. scFv expression levels were determined by ELISA, and peroxidase conjugated anti-HA was used as the secondary antibody. 1% BSA was used as control for the ELISA. C4X-1 and C4X-2 are 2 clones bearing the scFv insert.

		Absorbance values (O.D. 405 nm)			
		C4X-1	C4X-2	pComb3X alone	Host cell alone
Temperature and time	37°C/ overnight	0.094 ± 0.006	0.152 ± 0.040	0.086 ± 0.009	0.064 ± 0.002
	30°C/ 5 h	0.241 ± 0.039	0.203 ± 0.026	0.113 ± 0.035	0.065 ± 0.002
Host cell	Top10F'	0.816 ± 0.062	0.596 ± 0.095	0.107 ± 0.145	0.058 ± 0.004
	ER2537	0.136 ± 0.049	0.160 ± 0.089	0.093 ± 0.009	0.067 ± 0.008
	HB2151	0.386 ± 0.256	0.245 ± 0.106	0.108 ± 0.040	0.063 ± 0.008
Glucose concentration	0%	0.304 ± 0.056	0.274 ±0.0.27	0.117 ± 0.013	0.060 ± 0.006
	0.5%	0.197 ± 0.008	0.178 ± 0.020	0.073 ± 0.016	0.059 ± 0.008
	1.0%	0.152 ± 0.014	0.131 ± 0.007	0.057 ± 0.003	0.059 ± 0.002
	1.5%	0.147 ± 0.021	0.120 ± 0.017	0.058 ± 0.003	0.058 ± 0.006
	2.0%	0.132 ± 0.006	0.129 ± 0.014	0.060 ± 0.008	0.060 ± 0.002
IPTG concentration	1 mM	0.399 ± 0.077	0.256 ± 0.070	0.108 ± 0.020	0.121 ± 0.070
	2 mM	0.189 ± 0.015	0.186 ± 0.026	0.101 ± 0.020	0.084 ± 0.003
	3 mM	0.114 ± 0.044	0.106 ± 0.025	0.086 ± 0.001	0.089 ± 0.001
	4 mM	0.086 ± 0.004	0.089 ± 0.001	0.085 ± 0.005	0.079 ± 0.001
	5 mM	0.090 ± 0.007	0.070 ± 0.013	0.070 ± 0.002	0.086 ± 0.000

pressor host cell, Top10*F*, is most suitable for the expression of scFv, as it seems to exhibit relatively low interference from host cell proteins.

The yield of recombinant proteins can vary, depending on plasmid copy number, improper protein folding, and intracellular degradation or toxicity to the host cell (Stollar 1997). Fusion proteins, such as scFv which is fused to a histidine tag and hemagglutinin tag, may be present as inclusion bodies. Therefore, the growth rate of the subclone requires optimization to prevent the formation of these inclusion bodies, and for optimum expression. At 30°C, expression of scFv was higher than expression carried out at 37°C (Table 1). As it is generally recognized that inclusion bodies tend to be more readily formed at 37°C (Raffai *et al.*, 1999; Hunke and Betton, 2003), all further expression was performed at 30°C to prevent toxicity to the cells. An up-shift in temperature enhances the synthesis of plasmid-encoded proteins by increasing the plasmid copy number, and accelerates protein synthesis (Hoffman and Rinas, 2001). Production of recombinant proteins often interferes with the physiology of the host organism by causing stress responses (Raffai *et al.*, 1999). Expressed pro-protein is usually in a reduced form with improper folding, and has to be transported into the oxidized periplasm for disulfide bond formation and proper folding. Higher incubation temperatures tend to increase protein synthesis, but the rate of transportation of the pro-protein to the oxidized periplasm may be slower than the rate of protein synthesis. This, in turn, might cause the pro-protein to aggregate in the cytoplasm, and form inclusion bodies which are toxic to the host cells, and reduce the amount of active protein (Raffai *et al.*, 1999). The protein's ability to be transported to the periplasm depends on the leader sequence of 15-25 amino acids. After translocation, this leader sequence will be cleaved by specific peptidases, to form a mature protein (Barbas *et al.*, 2001). The pComb3X vector consists of a leader sequence, *ompA,* which transports the recombinant antibody into the periplasm for modification into an active scFv protein.

The expression of genes cloned downstream of the *lac* promoter can be conveniently induced by the addition of β-isopropylthiogalactoside (IPTG) (De Bellis and Schwartz, 1990). IPTG binds to the *lac* repressor molecule, inhibiting the binding of this molecule to the *lac* operator and allowing transcription to occur. In this study, inducing expression of the scFv protein with 1 mM IPTG was deemed to be the most suitable (Table 1) concentration for this purpose. The use of 1 mM IPTG has been also reported, by Stollar (1997), to induce the expression of anti-Z-DNA scFv. In the study reported here, scFv expression was inhibited when IPTG concentration exceeded 2 mM. In the presence of excess IPTG, cells have been reported to respond to the induction of protein synthesis by increasing rRNA synthesis. This upshift in RNA synthesis, in turn, redirects energy resources, thus contributing to an increased metabolite load in the host cell (Donovan *et al.*, 2000). Metabolite load occurs when host cell ATP or GTP and certain amino acids are used to stabilize and express the recombinant protein (Glick 1995).

Glucose was added to the bacterial culture as an alternative carbon source to optimize the scFv expression. The presence of glucose in the growth medium represses *lac* promoter expression, thus preventing basal expression from

this promoter, prior to the addition of IPTG (De Bellis and Schwartz, 1990). The highest scFv expression occurred in the absence of an additional carbon source (Table 1). Figure 2a shows that the scFv protein of around 30 kDa is detected when a concentration of 0-0.10% glucose was used, and not detected at glucose concentrations of 0.15-0.35% and 0.4-1.0% (data not shown). The scFv protein fused with pIII (52 kDa) is not detectable by SDS-PAGE. The 52 kDa scFv is a result of the co-expression of the phage coat protein pIII (20.3 kDa), His tag (0.66 kDa), HA tag (0.99 kDa), and the scFv (27.5 kDa), while the 30 kDa soluble scFv consists of the scFv-His-HA fusion, without the pIII protein.

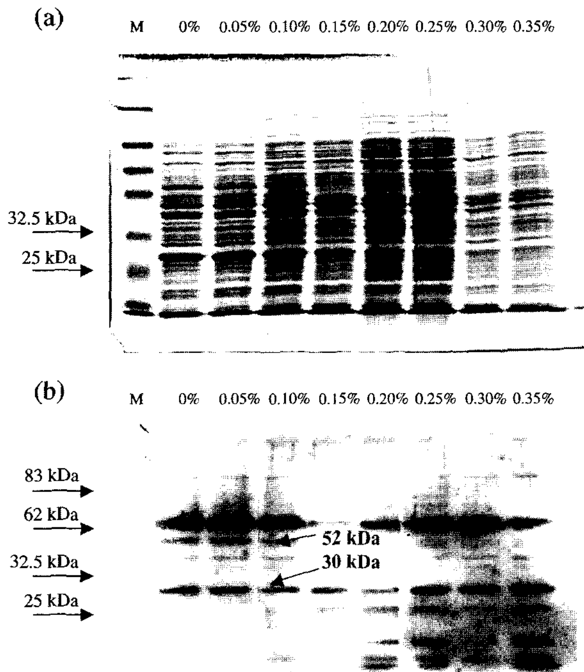

Fig. 2. SDS-PAGE (a) and western blot (b) of the proteins expressed in the presence of different concentrations of glucose (0-0.35%). Peroxidase conjugated anti-HA was used as the secondary antibody in the Western blot.

An increased concentration of glucose also results in non-specific expression as shown by detection of extra bands (besides 30 kDa and 52 kDa). Detected extra bands are most likely host cell proteins (Fig 2b) that, coincidently, can be recognized by anti-HA. In addition, the smaller fragments observed on the Western blot could be a result of proteolytic cleavage of scFv or host proteins, as no protease inhibitors were added during the expression procedure. The inhibition of scFv expression by glucose is most probably due to glucose inhibiting the uptake of IPTG via *lac* permease (Voet *et al.,* 1999). Therefore, when glucose is present in the culture medium, induction by IPTG is insufficient and expression of scFv is at a minimum level. Raffai *et al.* (1999) have previously reported that the addition of 0.4 M sucrose to the induced cultures also dramatically reduced the rFab yield. Conversely, when a rich medium was utilized, expression of host cell proteins was enhanced.

The scFv protein yield requires improvement, to avoid having to concentrate the protein for exotoxin purification purposes. Improving the expression apparently involves an increase in the proportion of soluble protein, as well as improved viability of host cells during induction. Expression of recombinant protein can be improved by humanization strategies, as in the report by Carter *et al.* (1992), in which it was found that the recruitment of the Framework Region (FR) residues from a well-expressed humanized fragment into a poorly-expressed murine fragment, resulted in up to 10-fold enhancement in the titer of the

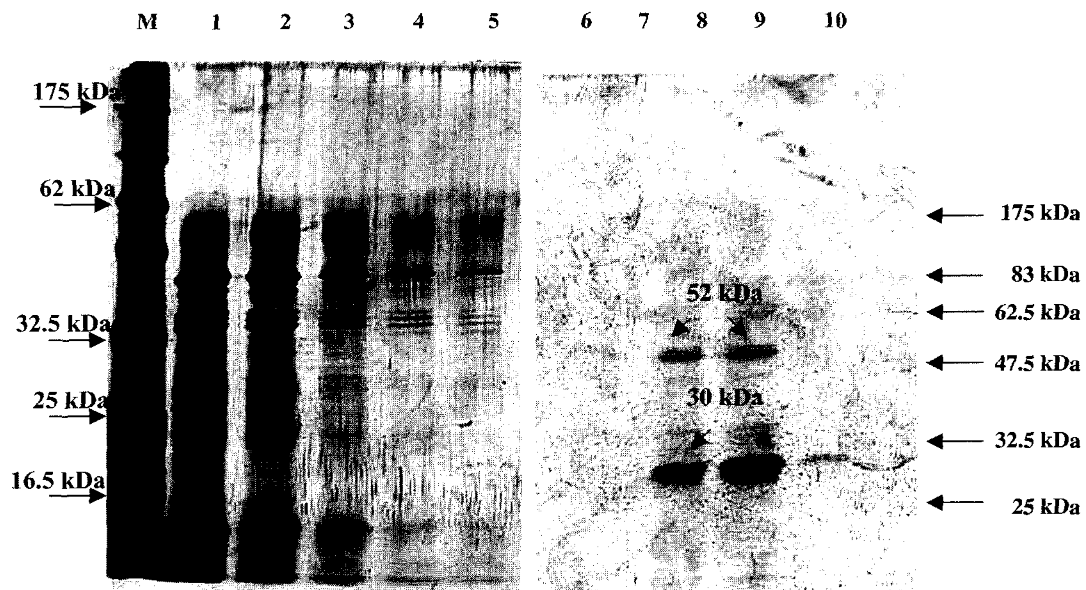

Fig. 3. SDS-PAGE of Ni-affinity chromatography purified scFv antibody M: Broad range protein marker, 1-5: Wash fractions, 6-10: Eluted fractions.

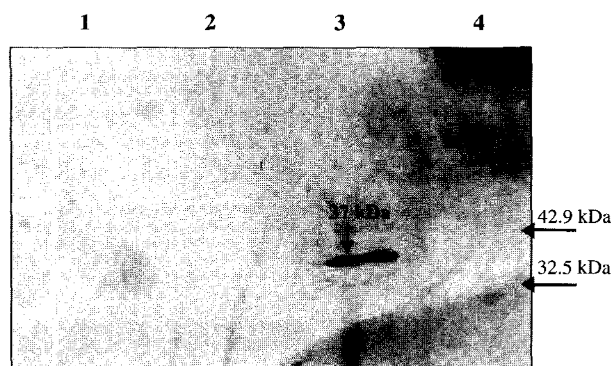

Fig. 4. Western blot of *B. pseudomallei* antigens with the purified scFv antibody. Peroxidase conjugated anti-HA was used as the secondary antibody. The purified antibody binds specifically to the 37 kDa exotoxin. (1: Crude extract, 2: Hemolysin, 3: Exotoxin, 4: Protease).

functional fragment (Dall'Acqua *et al.,* 1998). Sequence diversification of antibody fragments (for example, by using mutator cells to create sequence diversity, CDR mutagenesis, or randomization of the linker connecting the V_L and V_H domain in the scFv (Dall'Acqua *et al.,* 1998)), combined with phage display screening, has been shown to result in a 10-fold increase in scFv protein production. Recombinant scFv expression can also be improved by *in-vitro* transcription and translation of the scFv and the use of high-density fermentation (Dall'Acqua *et al.,* 1998).

The expressed scFv protein was purified via nickel-affinity chromatography with the HiTrap™ HP column. The SDS-PAGE profile of the elution fractions indicated that a 52 kDa and 30 kDa scFv had been co-purified (Fig 3). Specificity of scFv was determined by Western blotting with *B. pseudomallei* antigens. Results showed that the scFv protein was specific towards *B. pseudomallei* exotoxin, as a 37 kDa band (the reported size of the *B. pseudomallei* exotoxin (Isa *et al.,* 1983)) was detected in the presence of the exotoxin, and no band was detected when protease, hemolysin, and crude *B. pseudomallei* extract were used (Fig 4). The scFv specific to the *B. pseudomallei* exotoxin can serve, then, as a tool for the purification of lethal exotoxin from crude cultures of *B. pseudomallei* via antibody-mediated affinity chromatography. With the purified exotoxin, the pathogenic qualities of the toxin can be further researched. Eventually, this may also lead to the production of a vaccine to combat melioidosis.

Conclusion

An scFv towards *B. pseudomallei* exotoxin was successfully subcloned into the phage display vector, pComb3X. Expression of this protein was optimal when host cells were grown at 30°C for 5 h, or until an $O.D_{600nm}$ of 0.6-1.0, and induced with 1 mM IPTG, at 30°C, for a further five h. Higher concentrations of IPTG (>1 mM) inhibited scFv expression. Top10F cells were found to produce a higher scFv protein yield than did HB2151 and ER2537 cells. The use of an alternative carbon source (glucose) did not induce any significant increase in scFv expression; conversely, protein expression was inhibited in the presence of glucose. The scFv was successfully co-purified, as both a 30 kDa soluble and 52 kDa periplasmic protein, via nickel-mediated affinity chromatography. The purified scFv demonstrated binding with the *B. pseudomallei* exotoxin, and not to the other purified bacterial extracellular products, evidencing specificity for the exotoxin. Large-scale preparation of scFv under these conditions should be feasible to produce sufficient quantities of antibody as a tool for antibody-mediated affinity chromatography for exotoxin purification.

Acknowledgements

This project received funding from the Malaysian Government through an IRPA Top Down Grant (09-02-02-0123). Lim Kue Peng is a recipient of the National Science Fellowship Award.

References

Barbas III, C.F., D.R. Burton, J.K. Scott and G.J. Silverman. 2001. Phage Display: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Carter, P., R.F. Kelley, M.L. Rodrigues, B. Snedecor, M. Covarrubias, M.D. Velligan, W.L. Wong, A.M. Rowland, C.E. Kotts, and M.E. Carver. 1992. High-level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Bio/Technology* 10, 163-167.

Dall'Acqua, W. and P. Carter. 1998. Antibody engineering. *Curr. Opin. Struc. Biol.* 8, 443-450.

De Bellis, D. and I. Schwartz. 1990. Regulated expression of foreign genes fused to *lac* control by glucose levels in growth medium. *Nucleic Acids Res.* 18, 1311.

Domenighini, M. and R. Rappuoli. 1996. Three conserved consensus sequences identify the NAD-binding site of ADP-ribosylating enzymes, expressed by eukaryotes, bacteria and T-seven bacteriophages. *Mol. Microbiol.* 4, 667-674.

Donavan, R.S., C.W. Robinson and B.R. Glick. 2000. Optimizing the expression of a monoclonal antibody fragment under the transcriptional control of the *Escherichia coli lac* promoter. *Can. J. Microbiol.* 46, 532-541.

Glick, B.R. 1995. Metabolic load and heterologous gene expression. *Biotech. Adv.* 13, 247-261.

Hoffmann, F. and U. Rinas. 2001. Plasmid amplification in *Escherichia coli* after temperature upshift is impaired by induction of recombinant protein synthesis. *Biotech. Lett.* 23, 1819-1825.

Hunke, S. and J.M. Betton. 2003. Temperature effect on inclusion body formation and stress response in the periplasm of *Escherichia coli. Mol. Microbiol.* 50, 1579-1589.

Isa, W., M. Noor Embi, M. Rahmah, O. Othman, R. Nyonya, and I. Ghazally. 1983. The purification of *Pseudomonas pseudomallei* exotoxin. *Proc. Mal. Biochem. Soc. Conf.* 13, 241-247.

Ismail, G., M. Noor Embi, O. Omar, J.C. Allen, and C.J. Smith. 1987. A competitive immunosorbent assay for detection of *Pseudomonas pseudomonas* exotoxin. *J. Med. Microbiol.* 23, 353-357.

Kondo, E., V. Petkanchanapong, P. Naigowit, K. Kurata, and K. Kanai. 1991. Demonstration of acid phosphatase activity in antigenic glycoprotein fractions obtained from culture filtrate of *Pseudomonas pseudomallei. Jpn. J. Med. Sci. Biol.* 44, 213-224.

Laemmli, U.K. 1970. Cleavage of a structural protein during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lee, M.A. and Y. Liu. 2000. Sequencing and characterization of a novel serine metalloprotease from *Burkholderia pseudomallei. FEMS. Microbiol. Lett.* 1, 67-72.

Nathan, S., R. Mohamed, R. Pillai, K.Y. Lai, and N. Embi. 2000. Production and characterisation of anti-*Burkholderia pseudomallei* exotoxin monoclonal antibodies. *J. Biochem. Mol. Biol. Biophys.* 4, 17-25.

Nathan, S., H. Li, R. Mohamed, and N. Embi. 2002. Phage display of recombinant antibodies towards *Burkholderia pseudomallei* exotoxin. *J. Biochem. Mol. Biol. Biophys.* 6, 45-53.

Rader, C. 2001. Antibody libraries in drug and target discovery. *DDT.* 6, 36-43.

Raffai, R., J. Vukmirica, K.H. Weisgraber, and E. Rassart. 1999. Bacterial expression and purification of the Fab Fragment of a monoclonal antibody specific for the low-density Apolipoprotein E. *Protein. Expr. Purif.* 16, 54-90.

Stollar, B.D. 1997. Bacterial expression of anti-DNA antibody domain. In Methods: *A Companion to Methods. Enzymol.* 11, 12-19.

Su, Y.C., K.P. Lim, and S. Nathan. 2003. Bacterial expression of the scFv fragment of a recombinant antibody specific for *Burkholderia pseudomallei* exotoxin. *J. Biochem. Mol. Biol.* 36, 493-498.

Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.* USA 76, 4350-4354.

Voet D., J.G. Voet, and C.W. Pratt. 1999. Fundamentals of Biochemistry. John Wiley & Sons Inc., New York.