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Bacterial Expression of the scFv Fragment of a Recombinant Antibody Specific for *Burkholderia pseudomallei* Exotoxin

Yu-Ching Su, Kue-Peng Lim and Sheila Nathan*
Centre for Gene Analysis and Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia,
43600 UKM Bangi, Selangor D.E., Malaysia

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The scFv antibody towards the *Burkholderia pseudomallei* exotoxin was previously constructed by phage display and exhibited good specificity towards the exotoxin. We report here the optimization of the scFv expression in an *E. coli* expression system. Four different *E. coli* strains (ER2537, TG1, HB2151, and XL1-Blue) were examined for optimal expression of the scFv protein. Two types of carbon source (i.e. 0.2% glucose and 0.2% glycerol) were also tested for their ability to induce the scFv expression. Cells that carried the scFv construct were grown at 30ºC and induced with 0.05 mM IPTG. The expression was then monitored by SDS-PAGE, Western blotting, and indirect ELISA. The Western blot profile showed different levels of the scFv expression among the host strains; XL1-Blue exhibited the highest level of the scFv protein expression. Glycerol at a concentration of 0.2% (v/v) significantly increased the scFv protein expression level when compared to 0.2% (w/v) glucose. Further optimization demonstrated that the scFv protein expression in XL1-Blue was the most optimal with a glycerol concentration as low as 0.05%. However, by indirect ELISA, only the scFv protein that was expressed in 0.2% (v/v) glycerol exhibited high specificity towards the *Burkholderia pseudomallei* exotoxin.

Keywords: Antibody, *E. coli*, Exotoxin, Protein expression, ScFv

Introduction

Melioidosis, an important health problem for humans and livestock, is a potentially fatal disease that is caused by a soil bacterium, *Burkholderia pseudomallei* (formerly *Pseudomonas pseudomallei*). Although the disease is endemic in Southeast Asia and Nothern Australia with incidences that correlate with rainfall, 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 they are still poorly characterized. The bacterium is known to secrete various extracellular products which have been implicated in the pathogenesis of this disease. These products include exotoxin (a heat labile lethal toxin) (Ismail *et al*., 1987b), hemolysin (Ashdown and Koehler, 1990), phosphatase acid (Kanai and Kondo, 1994) and protease (Ismail *et al*., 1987a; Vorachit *et al*., 1995; Ling *et al*., 2001). Recently, the purification and characterization of cytotoxic exolipid has been described (Hausesler *et al*., 1998). It is believed that the exotoxin is responsible for the rapid onset of the disease and dramatic course of septicaemic melioidosis. This lethal pathogenic factor manifests its cytotoxic effects by the inhibition of cellular protein synthesis via ADP-ribosylation of the elongation factor-2 (EF-2), and hence attenuates the host immune system during *B. pseudomallei* infection (Rahmah *et al*., 1989).

Diagnosis of melioidosis is currently limited to techniques that are based on clinical features (Yabuuchi and Arakawa, 1993), serological examination, and bacteriological methods (Puthucheary, 1994). These are time consuming and generally too late to be useful. The ability to detect exotoxin in the serum at a concentration of 16 ng/ml by an anti-exotoxin monoclonal antibody through the enzyme-linked immunosorbent assay (ELISA) technique led to the utilization of the monoclonal antibody in a laboratory diagnosis of animal and human melioidosis (Ismail *et al*., 1987a; Ismail *et al*., 1991).

We previously described the production and characterization of monoclonal antibodies against the exotoxin (Nathan *et al*., 2000). These antibodies are useful for

*To whom correspondence should be addressed.
Tel: 603-8921-3862; Fax: 603-8925-2698
E-mail: sheila@pkrisc.cc.ukm.my

analysis of the structure-function relationship of the exotoxin to understand its role in the pathogenesis of melioidosis. This could be approached by the use of epitope mapping of the antigen by the monoclonal antibodies and the subsequent purification of the epitope. Nevertheless, the hybridoma cell line that secrets the monoclonal antibodies, 6E6A8F3B, was not very stable in culture, and the secreted antibodies also demonstrated non-specific binding to other *B. pseudomallei* extracellular pathogenic products. As a result of the low stability and low specificity of the hybridoma line, we cloned the single chain variable fragment (scFv) of the monoclonal antibody by phage display (Nathan *et al*., 2002). An scFv antibody with a molecular size of 25 kDa is the smallest antibody structure that retains the binding properties of the parent antibody, and it has a better penetration of solid tissue and rapid clearance of unbound antibody from the circulation (George, 1995). To construct the scFv antibody, the heavy and light chain variable domains of the monoclonal antibody 6E6A8F3B (Nathan *et al*., 2000) were cloned, and use of the phage display enabled the specific selection and improvement of binding affinities of the anti-exotoxin antibody toward the exotoxin. The scFv gene was subsequently subcloned from the original expression vector pComb3H to pComb3X (Lim *et al*., manuscript in preparation), because the latter vector allows better detection and purification of the expressed products (Rader and Barbas, 1997). The vast number of vectors available for the expression and manipulation of cloned genes, and complete information of the *Escherichia coli* genome have encouraged the use of *E. coli* as a protein expression host (Karu *et al*., 1995). In this study, we describe the optimization of the scFv expression towards *B. pseudomallei* exotoxin by utilizing various *E. coli* host cell strains and different concentrations of carbon sources. In our quest to produce a vaccine and diagnostic tool for melioidosis, the optimal scFv expression will permit the large scale expression of the anti-*B. pseudomallei* exotoxin scFv that is necessary for exotoxin purification by affinity chromatography.

Materials and Methods

Materials

Vector and clone The pComb3X vector (4.8 kb) (Barbas *et al*., 2001) and Clone C4X (3.95 kb), a recombinant antibody clone consisting of the anti-*B. pseudomallei* exotoxin scFv gene (750 bp) (Nathan *et al*., 2002), were cloned into the expression vector pComb3X (3.2 kb) (Lim *et al*., manuscript in preparation). Both pComb3X and Clone C4X were carried within the *E. coli* strain ER2537 (New England Biolabs, Beverly, USA).

Methods

***Burkholderia pseudomallei* exotoxin** *B. pseudomallei* exotoxin was prepared as previously described (Ismail *et al*., 1987a; Nathan *et al*., 2000).

Bacterial host strains Both the vector and clone C4X were transformed and expressed in the *E. coli* strain ER2537 [λ^- F' *lac* I^q*Δ(lacZ)M15 pro* A^+B^+*/fhuA2 supEΔ(lac-proAB) thiΔ(hsdMS-merB)5*($r_k^-m_k^-$McrBC$^-$) K12], TG1 (Stratagene) [supE thi 1-Δ *(lac-proAB)Δ(mcrB-hsdSM)5*($r_k^-m_k^-$) [F*traD36 proAB lacI*^{q}*Z ΔM15*]K12, XL1-Blue (Stratagene, La Jolla, USA) [*rec1 endA1 gyrA96 thi-1hsd R17 SupE44 rec1 lac* [F' *proAB lacI*^{q} *Z ΔM15* Tn*10* (Tet^r) K12] and HB2151 (Stratagene) [*araD (lac pro AB), lacI*^{q} *Δ(lacZ) M15*, *thi*/F' *proA*$^+$*B*$^+$*/fhua2Δ(thiΔ(hsdMSmerB)* ($^-r_k^-m_k^-$ *McrBC*$^-$) K12].

ER2537, TG1, and XL1-Blue are amber codon (UAG) suppressor strains, while HB2151 is an amber codon non-suppressor strain.

Bacterial growth Stock cells of *E. coli* ER2537 that carry either pComb3X vector (ER-3X) or Clone C4X (ER-C4X) were respectively streaked onto Luria-Bertani (LB) agar that contained 50 μg/ml carbenicilin and incubated overnight at 37°C. A single colony was used to inoculate 10 ml Superbroth (SB) medium that contained 50 μg/ml carbenicilin. The inoculums were grown overnight at 37°C with agitation. These cultures were then used for the phagemid DNA extraction and production of the infectious phage that carried the pComb3X vector and Clone C4X.

Phagemid DNA extraction The pComb3X and Clone C4X phagemid DNA were extracted with a Qiagen Miniprep Kit (Hilden, Germany), according to the manufacturers protocol. The extracted DNA were subsequently digested with *Sfi* I (15 U) (Promega, Madison, USA) at 50°C for 5 h to confirm the presence of the scFv gene in Clone C4X, prior to performing the protein expression. The digested products were analysed by 0.9% agarose gel electrophoresis.

Amplification of infectious phage carrying pComb3X and Clone C4X Overnight cultures of ER-C4X and ER-3X were used to produce phage. Five ml of each overnight culture were added to 45 ml of the SB medium that contained 50 μg/ml carbenisilin. They were then incubated at 37°C for 1 h with agitation. This was followed by adding 2 ml of the VCSM13 helper phage (titer 10^{11} ml^{-1}; Stratagene) and incubating for a further 2 h at 37°C. Kanamycin (0.5 mg/ml) was then added and the mixture was incubated overnight at 37°C. On the following day, the overnight cultures were pelleted by centrifugation at 6,000 × *g* for 15 min at 4°C. The phage was precipitated with 12 ml PEG8000/NaCl 5X, filtered through a Minisart filter (pore size 0.45 μm), and stored at 20°C.

Transfection of host cell strains TG1, XL1-Blue, and HB2151 Clone C4X and pComb3X DNA were directly introduced into the log phase TG1, XL1-Blue, and HB2151 host cells by transfection of the infectious phage that carried the DNA. The transfectants were incubated at room temperature for 30 min and plated onto LB agar containing carbenisilin overnight at 37°C.

Recombinant scFv expression Prior to the induction of the scFv expression 0.2% (v/v) glycerol or 0.2% (w/v) glucose was added to the ER2537, TG1, XL1- Blue, and HB2151 cells that carried C4X. The cultures were then incubated for 4-5 h at 30°C until the log phase (optical density (OD) at 600 nm was approximately 0.5).

To initiate the expression of the recombinant antibody genes, the cells in the log phase were induced by adding 0.05 mM isopropyl-β-D-thio-galactopiranoside (IPTG) (Promega) and followed by incubation for a further 5 h at 30ºC. Appropriate negative controls that were utilised included host cells, host cells carrying pComb3X, and the absence of IPTG and carbon sources.

Cell harvesting and extract preparation The cultures were pelleted by centrifugation at 2,600 × *g* for 20 min at 4ºC. The supernatants were stored at −20ºC. The cell suspensions were disrupted by freeze-thawing and the periplasmic products were stored at −20ºC.

Detection of scFv antibody fragment by Western blotting The production of the scFv fragment was detected by Western blotting (Towbin *et al*., 1979) and quantified by a qualitative comparison.Twenty µg of the expression product for each culture was initially separated on a 12% discontinuous SDS-polyacrylamide gel (Laemmli, 1970).

Following the SDS-gel electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane (Osmonics, Downers Grove, USA) and blocked overnight in 5% (w/v) milk powder in PBS at 4ºC, followed by four 10 min-washes in 0.1% (v/v) Tween 20 in PBS (PBST). The membrane was then incubated in peroxidase-conjugated mouse secondary anti-hemagglutinin (anti-HA) (1 : 500), and 500 µl of HRP Supersignal substrate was added. The reaction was detected by exposing the membrane to X-ray film.

Indirect enzyme-linked immunosorbent assay (ELISA) Ninety six-well plates (Nunc, Roskilde, Denmark) were coated overnight with 100 µl *B. pseudomallei* exotoxin (1 mg/ml) in a coating buffer (0.1 M $NaHCO_3$, pH 8.6). The plate was blocked for 1 h at 37ºC with 5% (w/v) milk powder and 100 µl of each sample (primary antibody) was added to the wells. Peroxidase-conjugated anti-HA (1 : 500) was used as the secondary antibody, while the ABTS : peroxidase (1 : 1) solution was used as the substrate. The reaction was monitored at OD 405 nm on Dynex MRX ELISA Reader.

Results and Discussion

The aim of this study was to optimize the scFv anti-*B. pseudomallei* exotoxin expression through selection of a suitable host strain and carbon source. All of the expression products were analysed by Western blotting and indirect ELISA.

Transfection The phagemid DNA that is carried by all four host strains was extracted and digested with *Sfi* I to confirm the presence of the insert prior to protein expression. Digestion of Clone C4X confirmed the presence of the 750 bp scFv gene which had previously been inserted into pComb3X (Fig. 1). Digested pComb3X produced the non-coding stuffer of 1.6 kb which was used as a control insert to preclude the gene III expression in the pComb3X vector by destroying the reading frame of gene III (Amstrong *et al*., 1995). The reading frame was restored within the clone when the stuffer fragment

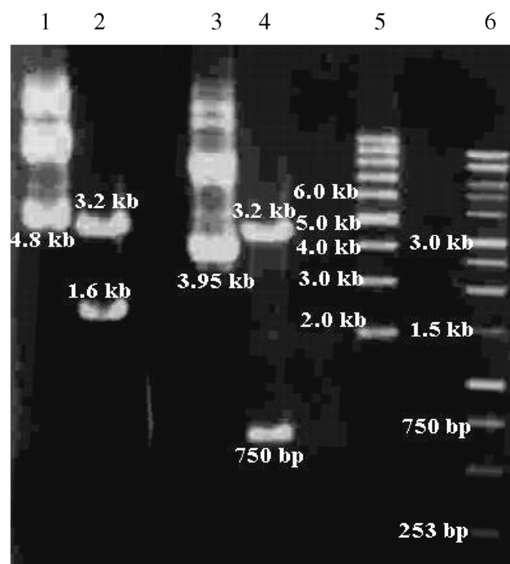

Fig. 1. Phagemid DNA extraction and *Sfi* I digestion profile. Lane 1: Undigested pComb3X vector (4.8 kb). Lane 2: Digested pComb3X (3.2 kb vector and 1.6 kb stuffer fragment). Lane 3: Undigested Clone C4X (3.95 kb). Lane 4: Digested Clone C4X (750 bp scFv gene and 3.2 kb vector). Lane 5: Supercoil DNA ladder. Lane 6: 1 kb DNA ladder.

was replaced with the scFv gene (Andris-Widhopf *et al*., 2000).

Recombinant scFv expression The scFv gene was inserted downstream of the *lac* promoter between the leader sequence OmpA and gene III to construct Clone C4X (Lim *et al*., manuscript in preparation). The expression of the 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 that were used have the *lac* I^q genotype to limit the background expression from the *lac* promoter. This also ensures that the expression only occurs upon the induction by IPTG as constitutive expression, which would almost certainly be detrimental and debilitating to the host (Carrier *et al*., 1993). Overproduction of foreign proteins that are encoded within the DNA that is introduced into the host cell will trigger the metabolic burden phenomenon (Glick, 1995); therefore, the IPTG concentration that was used in this study was limited to 0.05 mM (Donovan *et al*., 2000).

Many factors can affect the efficiency of the expression (e.g. 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 amounts of host cell resources, which places a metabolic burden on the host. It leads to a decrease in growth and a reduction of the protein expression. Therefore, to enhance the protein expression in this study, a concentration of 0.2% glucose (w/v) or glycerol (v/v) was added to the growth medium (Donovan *et al*., 2000). Growth was

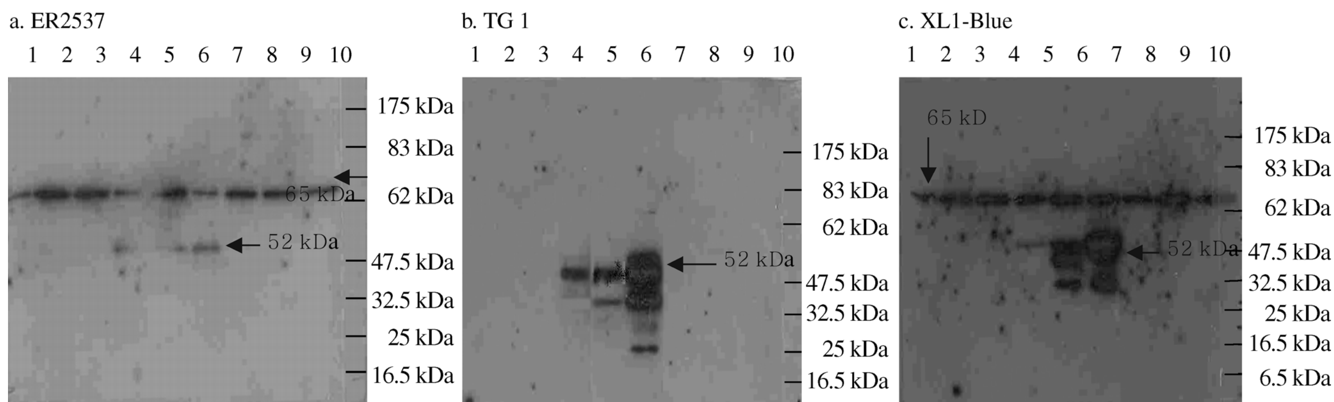

Fig. 2. Western blot profile of proteins expressed in the periplasm under induction of 0.05 mM IPTG. Glycerol feeding significantly increased the scFv expression with the optimal expression occuring in XL1-Blue Clone C4X. Lane 1: Host cell without additional carbon source. Lane 2: Host cell with 0.2% glucose. Lane 3: Host cell with 0.2% glycerol. Lane 4: Host cell Clone C4X without additional carbon source. Lane 5: Host cell Clone C4X with 0.2% glucose. Lane 6: Host cell Clone C4X with 0.2% glycerol. Lane 7: Host cell pComb3X without additional carbon source. Lane 8: Host cell pComb3X with 0.2% glucose. Lane 9: Host cell pComb3X with 0.2% glycerol. Lane 10: Protein marker.

maintained at 30ºC to enhance the production of correctly-folded functional scFv antibodies (Glick, 1995).

The proteins that were expressed in the extracellular culture medium and periplasm were separated by SDS-PAGE to retain the scFv protein as a single chain polypeptide and prevent the formation of diabodies. The scFv that was expressed in this study was fused to a nine amino acid peptide hemaglutinin (HA) at the carboxyl terminal, which enabled the detection of the scFv by secondary-conjugated anti-HA via Western blotting. The scFv proteins were only detected in the host cells that carried C4X, and not in the controls (host cells as well as host cells containing pComb3X alone) (Fig. 2 and 3).

In the absence of the inducing agent (IPTG), the scFv proteins were detected only in the periplasm of ER2537, TG1, and XL1-Blue cells that carried C4X (data not shown). This implied that in the absence of an inducer and in the presence of the *lac* repressor, there was a basal level expression from the *lac* promoter. No scFv protein was detected in the extracellular medium of all of the hosts for C4X (data not shown). This included the non-suppressor HB2151 which was expected to show secretion of soluble scFv into the growth medium.

The expression of the scFv protein by the *lac* promoter was activated in the presence of IPTG (Fig. 2 and 3). The scFv protein (52 kDa) were clearly detected from C4X in all of the hosts periplasm with the exception of HB2151 (Fig. 2). Several bands of low molecular weight were also detected by Western blotting. These could be due to either endogenous protease digestion or an internal start codon, ATG, that was preceded by a suitably-spaced ribosome binding site (RBS). The scFv protein in the extracellular medium was detected only in the HB2151 Clone C4X, as a band of 30 kDa (Fig. 3). This was a result of the secretion of soluble scFv from the periplasm into the extracellular growth medium. The scFv anti-*B. pseudomallei* exotoxin that was expressed in the amber codon (UAG) suppressor strains (ER2537, TG1, and XL1-

Fig. 3. Western blot profile of proteins expressed into the extracellular medium of HB2151 upon induction by 0.05 mM IPTG. Lane 1: HB2151 without additional carbon source. Lane 2: HB2151 with 0.2% glucose. Lane 3: HB2151 with 0.2% glycerol. Lane 4: HB2151 Clone C4X without additional carbon source. Lane 5: HB2151 Clone C4X with 0.2% glucose. Lane 6: HB2151 Clone C4X with 0.2% glycerol. Lane 7: HB2151 pComb3X without additional carbon source. Lane 8: HB2151 pComb3X with 0.2% glucose. Lane 9: HB2151 pComb3X with 0.2% glycerol. Lane 10: Protein marker.

Blue) was in the form of scFv (27.5 kDa) that fused to the linked His_6 tag (0.66 kDa), HA tag (0.99 kDa), and pIII protein (22.44 kDa) at the carboxyl terminal. The translational stop codon (UAG) was placed at the junction between the antibody and gIII protein sequences (Andris-Widhopf *et al.*, 2000). These stop codon suppressor strains do not recognize the stop codon on the transcript and allow the scFv-pIII fusion protein to be produced. This scFv-pIII fusion protein is insoluble and tends to accumulate in the host cells periplasmic compartment. The size expected for this insoluble scFv protein is approximately 52 kDa. On the other hand, the scFv protein that was expressed in the strain HB2151 (Fig. 3), a non-amber codon suppressor strain, is in the form of scFv that is fused to the linked His_6 tag and HA tag without pIII. This strain is able to recognize the stop codon at the 3 end of the scFv gene, and the synthesis of scFv will be halted upstream of gIII. The 30 kDa scFv fusion that is produced is soluble and able to be secreted from the periplasmic space into the

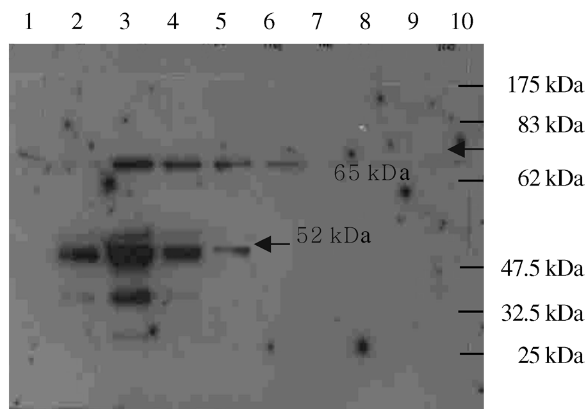

Fig. 4. Western blot profile of proteins expressed in XL1-Blue periplasm at different concentrations of glycerol fed under induction of 0.05 mM IPTG. Expression of scFv is optimal at 0.05% glycerol. Lane 1: XL1-Blue without additional carbon source. Lanes 2-5: XL1-Blue Clone C4X with 0% glycerol (Lane 2), 0.05% glycerol (Lane 3), 0.2% glycerol (Lane 4), 1.0% glycerol (Lane 5). Lanes 6-9: XL1-Blue pComb3X vector with 0% glycerol (Lane 6), 0.05% glycerol (Lane 7), 0.2% glycerol (Lane 8) and 1.0% glycerol (Lane 9). Lane 10: Protein marker.

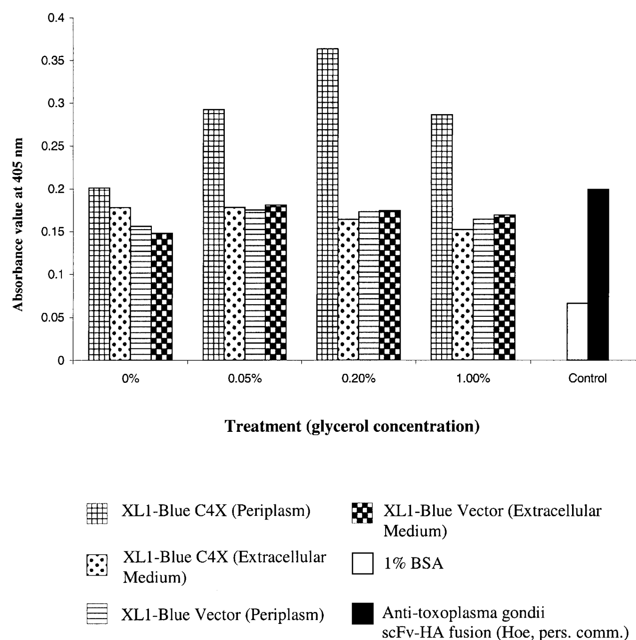

Fig. 5. Indirect ELISA of anti-*B. pseudomallei* exotoxin scFv expressed in XL1-Blue with different concentrations of glycerol supplementation.

extracellular medium. Secretion of soluble scFv into the growth medium eases the harvesting of scFv, especially for commercial production, while the accumulation of scFv within the oxydized periplasmic space permits a better and functional folding of scFv. Some host proteins (65 kDa) were also detected by Western blotting, due to the presence of the nine amino acids in certain *E. coli* proteins that can be detected by anti-HA (Roche Molecular Chemicals, 1999). The expression of pIII was precluded by the stuffer fragment in the pComb3X vector control cells, as previously described.

The scFv expression of Clone C4X in all of the host strains was optimal in the growth medium that contained an additional carbon source (i.e. glucose or glycerol) (Figs. 2 and 3). Therefore, there is a need to supply an additional carbon source for the enhanced protein expression. Glycerol at a concentration of 0.2% (v/v) was shown to increase the scFv protein expression level when compared to 0.2% (v/v) glucose. Since glucose catabolite repression occurs at concentrations greater than 1 g/l (De Bellis and Schwartz, 1990), glycerol was then selected as the carbon source candidate for the study that is described below. Among the host strains that were used for the scFv expression, XL1-Blue exhibited the highest level of the scFv expression (Fig. 2) and was selected for the study on the scFv expression at different concentrations of glycerol.

Under the induction of IPTG (Fig. 4), the scFv expression in the periplasm of XL1-Blue-C4X increased when the concentration of supplemented glycerol increased from 0% (v/v) to 0.05% (v/v) with an optimal expression at 0.05% (v/v) glycerol; it decreased when the glycerol concentrations increased from 0.2% (v/v) to 1.0% (v/v). This could be due to the over-feeding of glycerol to the host cells, which enhanced glycerol metabolism and consequently led to the fast accumulation of dihydroxyacetone phosphate in the host cell. Accumulation of this metabolite led to the formation of methylglyoxal which was detrimental to the host cell (Lin, 1987). No scFv protein was detected in the extracellular medium of the XL1-Blue Clone C4X (data not shown).

Anti-*B. pseudomallei* exotoxin scFv affinity by indirect ELISA An indirect ELISA demonstrated that scFv antibodies that were expressed in the presence of all of the glycerol concentrations reacted positively towards the exotoxin (Fig. 5). The scFv that was expressed in 0.2% (v/v) glycerol showed the highest affinity towards exotoxin in contrast to the scFv that was expressed in 0.05% (v/v) glycerol, as previously described. This could be due to the overproduction of the scFv protein in the periplasm that leads to the formation of inclusion bodies when the expression is conducted in 0.05% (v/v) glycerol (Carrier *et al*., 1993; Karu *et al*., 1995). The inclusion bodies resulted in the accumulation of misfolded and incorrect conformation of the scFv, hence decreasing the affinity of the antibodies towards its antigen (Carrier *et al*., 1993).

Conclusion

We successfully optimized the conditions for the expression of anti-*B. pseudomallei* exotoxin scFv by utilizing XL1-Blue as the host that is supplemented with a carbon glycerol source of

0.05%. The ability to induce the scFv gene expression by IPTG permits the separation of cell growth from product synthesis, which results in higher total yields of the scFv protein when compared to the constitutive expression. The functional protein expression plays an important role in producing scFv that is able to react positively towards the exotoxin.

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