Cloning and Expression of a *Burkholderia pseudomallei* Putative Peptidase M23B

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Abstract

Burkholderia pseudomallei is a Gram negative bacillus that causes melioidosis, an infectious disease endemic to Southeast Asia and Northern Australia. Identification and verification of the various virulence factors implicated in pathogenicity is vital in developing recombinant proteins as effective vaccine candidates. Previously, the *peptidase M23B* gene sequence was successfully identified through screening of a *B. pseudomallei* small insert library with a melioidosis patient serum. Here we report the cloning and expression of the *peptidase M23B* gene in an *Escherichia coli* expression system. The *peptidase M23B* gene sequence was amplified, cloned into the pET 200/D-TOPO vector and transformed into *E. coli* One Shot TOP10 cells. Recombinant clones were confirmed by restriction analysis with *Nhe*I and *Sac*I and insert DNA sequencing. The digestion profile indicated that the gene was inserted in the correct orientation within the vector. Sequence analysis demonstrated a high similarity to the *B. pseudomallei* K96243 *peptidase M23B* gene as well as the *B. mallei* ATCC 23344 *NlpD lipoprotein* gene. Expression of a selected clone in *E. coli* BL21 Star™ (DE3) was carried out. The expressed recombinant protein was analyzed through SDS-PAGE and western blotting. A protein band of 36 kDa was visible within the inclusion body fraction. Protein refolding and proteolytic activity analysis on the inclusion body fraction was performed. No proteolytic activity was observed for the recombinant protein, suggesting that the protein was probably not a peptidase enzyme but more likely a lipoprotein. Nevertheless, the cloning and expression of this gene which encodes an immunogenic protein, was successful and the recombinant protein obtained can be further characterized to determine its identity and effectiveness as a melioidosis vaccine candidate.

Keywords: *Burkholderia pseudomallei,* peptidase M23B, vaccine candidate

Introduction

Melioidosis is an infection caused by the bacterium *Burkholderia pseudomallei* [1]. Over the past decade, melioidosis has been identified as one of the major causes of human mortality and morbidity especially in the tropics [2]. This infection is endemic in Southeast Asia and Northern Australia and has also been reported in South Pacific, Africa, India, Middle East, China and South America [2, 3]. The clinical manifestation of melioidosis covers a wide range of complications, from acute septicemia to chronic and deteriorating localized infections [4]. This disease is characterized by the formation of abscesses on critical organs [5]. Melioidosis patients often require long periods of antibiotic treatment which is vital to eliminate *B. pseudomallei* and to prevent relapse of melioidosis [6, 7].

B. pseudomallei is a Gram negative, aerobic, motile and non-sporing bacterium [1]. The complete genome of this bacterium consists of two circular replicons of 4.07 Mb and 3.17 Mb each with a high G + C content [8]. *B. pseudomallei* is capable of expressing various virulence factors, both cell-associated and secreted antigens that have been implicated in the bacterium's pathogenicity and virulence. Cell-associated antigens include polysaccharide capsule, lipopolysaccharide, flagella and exopolysaccharide [5]. The secreted antigens include protease, hemolysin, lecithinase and lipase [9]. One of the major secreted enzymes in pathogenic bacteria is peptidase. Peptidase plays a major role in modulating bacterial infection by inducing direct destruction of host cells, assisting microbe infection and inducing cytotoxic effects on host cells [10, 11]. One of the well studied peptidases is *Pseudomonas aeruginosa* LasA. LasA is a zinc metallopeptidase that is capable of invading corneal membrane cells to assist bacterial spread through the tissue. *lasA* mutants are known to reduce *P. aeruginosa's* ability to invade rabbit corneal epithelial cells, suggesting that LasA functions in modulating *P. aeruginosa* infection in corneal keratitis [12]. There is very little information on the role of peptidases in *B. pseudomallei* virulence. Ashdown and Koehler [9] discovered that 94% of *B. pseudomallei* clinical isolates produced peptidases in addition to other active compounds. Sexton *et al.* [13] successfully identified a 36 kDa zinc metallopeptidase that is stable at pH 8.0 and active at 60ºC. This metallopeptidase is secreted by all clinical isolates and is able to digest the C3 component of complement and all classes of immunoglobulin. Strains lacking this enzyme produce less lung destruction compared to parental strains, suggesting the need for protease in *B. pseudomallei* infection.

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We have undertaken to identify as many *B. pseudomallei* immunogenic proteins as possible [Su *et al.,* in prep]. This identification is vital in developing effective vaccine and diagnostic tools for melioidosis. In this study, the gene sequence encoding peptidase M23B was successfully identified through screening of a *B. pseudomallei* small insert library with a melioidosis patient serum. Here, we report on the cloning, expression and characterization of the *B. pseudomallei* peptidase.

Materials and Methods

Bacterial DNA genome

B. pseudomallei clinical isolate D286 genomic DNA was obtained from the Pathogen Laboratory, Centre for Gene Analysis and Technology, Universiti Kebangsaan Malaysia.

Primers for polymerase chain reaction and automated sequencing

Primers for PCR (M23BF1: 5'-CACCATGAGCAAGA GCGAGATC-3' and reverse primer, M23BR1: 5'-CTAGCCCTGCTGCCGGCTCG-3') were designed based on the *B. pseudomallei peptidase M23B* gene sequence (Wellcome Trust Sanger Institute Database on *B. pseudomallei* K96342) using Primer Premier 4.0. Primers used for sequencing were the T7 forward and reverse primers. Primer sequences used for primer walking were BpF1: 5'-CGGCGCAGCATCCGGAGCAC-3' and BpF2:5'-CTGCAGGCTTGGAACCGGAT-3'.

Polymerase chain reaction

Amplification of the *B. pseudomallei peptidase M23B* gene was carried out using the Expand High Fidelity PCR System (Roche, Germany) in a final volume of 50 µl containing 32.25 µl ddH$_2$O, 250 ng template, 40 mM dNTP, 25 pmol forward and reverse primer, 1X PCR buffer, 25 mM MgCl$_2$, 6% dimethyl sulfoxide (DMSO) and 1U Expand High Fidelity *Taq* Polymerase. The PCR programme used was: 95°C for 5 min, 10 cycles of 95°C for 1 min, 64.6°C for 1 min and 72°C for 1 min, 20 cycles of 95°C for 1 min, 64.6°C for 1 min and 72°C for 1 min plus 5 sec per cycle followed by a final 10 min extension at 72°C. The PCR product was subjected to 1% agarose gel electrophoresis. Appropriate positive and negative controls were included.

Cloning of the peptidase M23B gene

The PCR product was purified with the QIAquick Gen Extraction Kit according to the manufacturer's instructions. The purified PCR product was ligated into the pET 200/D-TOPO expression vector (2:1 molar ratio). The ligation mixture was transformed into One Shot TOP10 competent cells by heat shock. The transformation mixture was spread on LB agar with 50 µg/ml kanamycin and incubated overnight at 37°C.

Analysis of selected recombinant clones

Twenty transformed colonies were randomly selected and plasmids were extracted using the QIAprep Spin Miniprep kit (QIAGEN, USA). The recombinant clones were digested with *Sac*I and *Nhe*I and also subjected to automated DNA sequencing. The sequence obtained was analysed against the Wellcome Trust Sanger Institute database (http://sanger.ac.uk/) by BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Recombinant protein expression, analysis and characterization

One selected recombinant clone was transformed into *E. coli* BL21 Star™ (DE3) and grown overnight on LB agar containing 50 µg/ml kanamycin at 37°C. A single colony was inoculated into 5 ml LB-kan and grown overnight at 37°C and 250 rpm. The overnight culture (100 µl) was sub-cultured into 10 ml LB-kan to achieve a 1:100 dilution and incubated at 30°C and 250 rpm. When the culture achieved a density of 0.6 to 0.8 absorption value at 600 nm, 1 mM IPTG was added and incubation was continued for five hours. Supernatant and inclusion body fractions were obtained by freeze-thawing and sonification in the Vibracell (Sonics and Materials, USA) and analyzed through sodium dodesyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Recombinant protein solubilized in 6M urea was refolded through serial dilution and washes to gradually remove the urea content. The skimmed milk agar assay and zymography were performed to analyze peptidolytic activity [14].

Results and Discussion

Construction of recombinant peptidase M23B clone

Analysis of the *peptidase M23B* gene amplification product by agarose gel electrophoresis demonstrated a band of 950 bp, similar to the expected *peptidase M23B* gene size of 948 bp (data not shown, indicating the successful amplification of the *peptidase M23B* gene. Figure 1 is a representation of the constructed *peptidase M23B* clone within the pET expression vector. Amplification of the *B. pseudomallei peptidase M23B* gene was performed using the Expand High Fidelity PCR System enzyme. This system contains two types of enzymes, DNA *Taq* polymerase and DNA *Tgo*
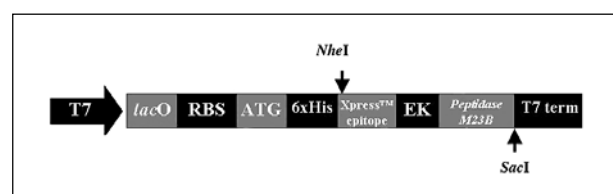

Figure 1: The recombinant pET 200D/Topo – *Peptidase M23B* Construct. RBS, ribosome binding site; 6x HIS, N-terminal Hisitidine tag; X-press epitope, for detection with the anti X-express antibody; EK, Enterokinase

polymerase. The combination of these enzymes helps to produce higher, specific and accurate PCR yields [15]. DMSO is usually added as an additive to reduce the denaturation temperature of GC rich templates to improve the PCR reaction [16].

Analysis of recombinant clones

Recombinant clone analysis was carried out by agarose gel electrophoresis, restriction digestion and DNA sequencing. Twenty clones (BpP1-BpP20) were selected at random and plasmids were extracted and subjected to size determination (Figure 2). From the electrophoresis profile, only five recombinant plasmids (BpP7, BpP9, BpP12, BpP15 and BpP16) were of the expected size of 6689 bp. Recombinant plasmid BpP12 and the pET 200/ D-TOTP vector were subjected to single and double
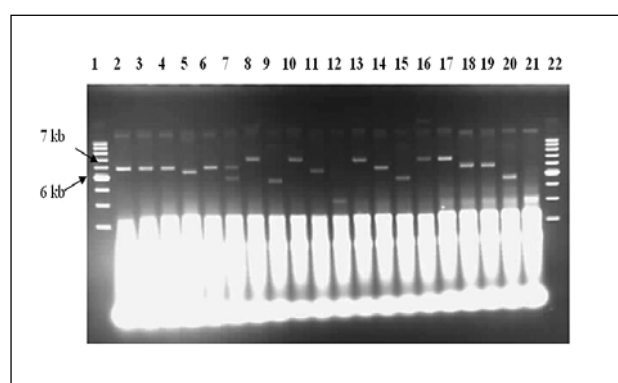
with *Nhe*I and *Sac*I produced two linearized DNA fragments of 5.7 kb and 1 kb each, as expected indicating that the gene was indeed inserted within the vector. Double digestion of the pET 200/D-TOPO vector produced only one linearized DNA fragment of 5.7 kb (Figure 3). Automated DNA sequencing of the insert was performed to verify the identity of the cloned gene. In this study, the T7 forward and reverse primers were used for DNA sequencing. Primer walking with the BpF1 and BpF2 primers was carried out to enable complete sequencing of the 950 bp insert. The BpP12 insert sequence obtained was then compared with the *B. pseudomallei* strain K96243 *peptidase M23*B gene sequence from The Wellcome Trust Sanger Institute database (www.sanger.ac.uk). Alignment of both sequences confirmed the identity of the cloned gene as
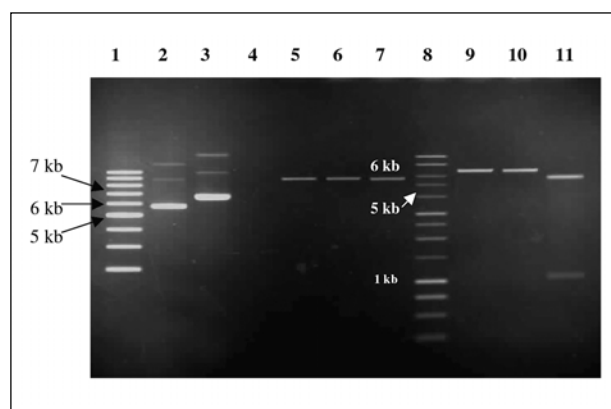

Figure 2: Electrophoresis profile of recombinant plasmids. Lanes 1 & 22: DNA super-coiled marker; Lanes 2 – 21: BpP1 – BpP20

Figure 3: Electrophoresis profile of recombinant plasmid BpP12 and pET 200/D-TOPO vector digested with *Nhe*I and *Sac*I. Lane 1: DNA super-coiled marker; 2: pET 200/D-TOPO vector; 3: BpP12; 4: empty; 5: pET 200/D-TOPO vector + *Nhe*I; 6: pET 200/ D-TOPO vector + *Sac*I; 7: pET 200/D-TOPO vector + *Nhe*I + *Sac*I; 8: 1 kb DNA ladder; 9: BpP12 + *Nhe*I; 10: BpP12 + *Sac*I; 11: BpP12 + *Nhe*I + *Sac*I

digestion (Figure 3). Restriction digestion was carried out to ensure that the gene sequence within the clone was ligated in the correct orientation within the pET 200/D-TOPO vector. *Sac*I and *Nhe*I were chosen for ease of analysis. Double digestion of recombinant plasmid BpP12

Table 1:   DNA sequence analysis with BLAST towards the Genbank bacterial non-redundant database

Database reference no.	Organism	Similarity	Score	E value	Protein function
BX571966.1	*Burkholderia pseudomallei* K96243	99%	932	0	Peptidase M23B
CP000125.1	*Burkholderia pseudomallei* 1710b	99%	932	0	Peptidase M23B
CP000011.2	*Burkholderia mallei* ATCC 23344	99%	924	0	Putative NlpD lipoprotein

B. pseudomallei peptidase M23B with 99% similarity to the K96243 sequence (Table 1). Four base changes were noted; T294C, C666T, T398C and C530T. The first two changes did not affect the encoded amino acid ie glycine, whilst the latter two changes caused a change in the encoded amino acid, from alanine to valine. These differences were probably due to natural variants amongst

B. pseudomallei strains. The sequence analysis also demonstrated a high similarity of the cloned gene sequence to the *B. pseudomallei* 1710b *peptidase M23B* (Table 1). A significant similarity was also shown towards the *B. mallei* ATCC 23344 putative *NlpD lipoprotein* gene due to the presence of a lipoprotein domain within the BpP12 gene sequence.

Expression and characterization of recombinant peptidase M23B

Expression of the *B. pseudomallei peptidase M23B* gene was performed in *E. coli* BL21 Star™ (DE3) at 30ºC with 1 mM IPTG induction. Expression was carried out at a lower temperature to avoid formation of inclusion bodies [17]. The growth of *E. coli* is slower at temperatures below 37ºC [18], thus, an induction time of 3 and 5 hours was performed to determine the optimal induction period. Expression of the *peptidase M23B* gene was expected to produce a protein of 36 kDa.

The expressed recombinant protein was analyzed through SDS-PAGE and western blotting. No protein band of 36 kDa was visible for the supernatant fraction (Figure 4a). For the inclusion body fraction, a band of 36 kDa was obtained for cultures induced with IPTG for 3 and 5 hours (Figure 4b). For the uninduced clone, no protein band of 36 kDa was observed, suggesting that induction with IPTG is required for *peptidase M23B* gene expression to take place. The positive control, *lacZ* produced a thick band of 120 kDa as expected. Comparison of intensity of the bands for the 3 and 5 hour induction time showed that the 5-hour induction period produced more recombinant protein. Thus, all further expression was conducted with 5 hour induction. For the western analysis, anti-HisGly antibody w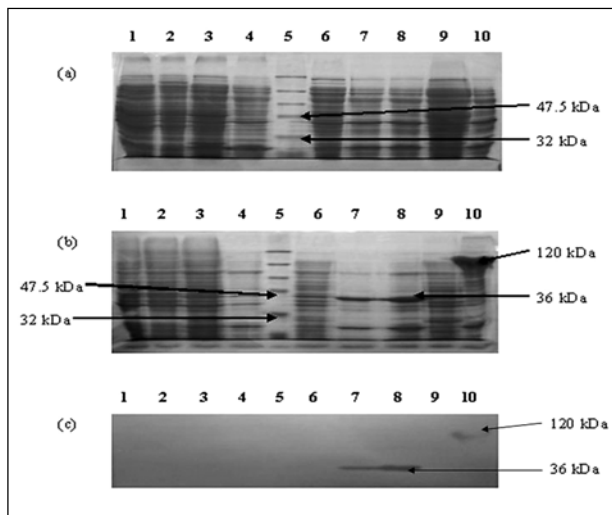as used to detect expressed recombinant protein as the recombinant protein is fused to a polyhistidine tag at the N-terminal. From the representative autoradiogram, a protein band of 36 kDa was detected in the IPTG induced samples of both 3 and 5 hour induction (Figure 4c). The expressed recombinant protein obtained was insoluble eventhough expression was carried out at the lower temperature. This situation might occur in instances where bacterial cells are cultured in rich medium like LB medium whereupon, their growth rate becomes faster and this disrupts proper protein folding leading to formation of insoluble protein in inclusion bodies [19].

We assayed the recombinant protein for protease activity via skimmed milk hydrolysis. When sufficient activity is present, the formation of a clear halo surrounding the colonies on agar caused by hydrolisis of casein substrate in the skimmed milk is usually observed [16]. However, for the *peptidase M23B* bearing clones, no clear halo formation was observed (data not shown). In addition to the skimmed milk agar assay, M23B peptidase activity was also monitored by zymography. Zymogram is a fast and reliable method to analyze proteolytic activity after seperation in non-reducing SDS-PAGE [20, 21]. In zymography, SDS-PAGE is carried out under non-reducing conditions and at cold temperatures to preserve the native state of the protein. Proteolytic activity is confirmed when an translucent band against a dark background is obtained after staining of the substrate gel. Zymography on the peptidase M23 recombinant protein also showed no proteolytic activity, suggesting that the protein encoded by the *peptidase M23B* gene lacked proteolytic activity. Problems in refolding of the purified protein may be a contributory factor, as could be the base differences observed in the sequence when compared to the K96243 M23B peptidase sequence. The change from Ala to Val may have resulted in perturbing the active site region of the peptidase.

Although the recombinant protein was expected to be proteolytically active, but this study showed that protein encoded by the *B. pseudomallei peptidase M23B* gene did not exhibit any proteolytic activity and was probably not a peptidase enzyme. From the sequence analysis (Table 1), we have shown that the gene sequence was highly similar to that of *B. mallei* ATCC 23344 putative NlpD lipoprotein. There are known lipoproteins that have previously been classified in the peptidase M23B family such as *E. coli* NlpD which did not contain proteolytic activity [22]. A study by Padmalayam et al. [23] showed that *Bartonella bacilliformis* lipoprotein, which is also homologous to *E. coli* NlpD is immunogenic and involved in this bacterium's virulence. Lipoprotein B (LppB) from *Haemophilus somnus* has been proven to be involved in causing hemophiliosis in cattle [24]. This suggests that the protein encoded by the *B. pseudomallei peptidase M23B/NlpD lipoprotein* gene may act as a virulence factor for this bacterium.

Figure 4: SDS-PAGE and western blot analysis of expressed recombinant protein BpP12 at 30ºC with 1 mM IPTG induction for 3 and 5 hours. (a) SDS-PAGE profile for the supernatant fraction, (b) SDS-PAGE profile for the inclusion body fraction, (c) Autoradiogram of the inclusion body fraction protein sample detected with anti-HisGly antibody. Lane 1: BL21 Star™ (DE3) – IPTG; 2: BL21 Star™ (DE3) + IPTG; 3: pET 200/D-TOPO vector – IPTG; 4: pET 200/D-TOPO vector + IPTG; 5: broad range standard protein marker; 6: recombinant clone BpP12 – IPTG; 7: recombinant clone BpP12 + IPTG (3 hr); 8: recombinant clone BpP12 + IPTG (5 hr); 9: pET 200/D/lacZ – IPTG; 10: pET 200/D/lacZ + IPTG

Conclusion

In this study, a recombinant clone carrying the gene sequence encoding the *B. pseudomallei* peptidase M23B was successfully constructed. DNA sequence analyses indicated 99% similarity between the recombinant clone gene sequence and *B. pseudomallei* K96243 ***peptidase M23B*** sequence. A high similarity was also shown towards *B. mallei* ATCC 23344 putative NlpD lipoprotein. The recombinant protein was successfully expressed in *E. coli* Star™ (DE3). Protein expression at 30ºC and induced with 1 mM IPTG for 3 and 5 hours produced inactive recombinant protein of 36 kDa in the inclusion body fractions. The refolded recombinant protein exhibited no proteolytic activity both on skimmed milk agar and zymogram. This suggests that the expressed recombinant protein was probably not a peptidase enzyme but could be a lipoprotein. Further characterization is needed to determine the identity and role of this recombinant protein in *B. pseudomallei* pathogenicity and its effectiveness as a melioidosis candidate vaccine.

Acknowledgement

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