Purification and Characterisation of a *Burkholderia pseudomallei* Protease Expressed in Recombinant *E. coli*

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A genomic DNA fragment that contains the gene, which codes for a novel extracellular serine protease in *Burkholderia pseudomallei*, was cloned by using pQE40 as a vector. It was maintained in *Escherichia coli* JM109. The expression of the gene(s) resulted in the production of a 52 kDa protease. The recombinant protease was purified from the culture filtrate via ammonium sulfate fractionation, gel filtration, and anion-exchange chromatography. The purified protease had an optimum pH and temperature of pH 8.9 and 38°C, respectively. The protease activity was inhibited by EGTA, EDTA, and PMSF, but not 1,10-phenanthroline. The first 11 amino acid residues from the N-terminus of the purified protease were identified as LAPNDPYYYGY. PNDPYY was found to show homology to the *Bacillus cereus* microbial serine protease and *B. subtilis* PD498 serine protease. These results indicate that the protease that was purified in this study is an extracellular calcium-dependent serine protease. The purified protease was able to digest the human serum IgA, IgG, albumin, and transferrin, as well as bovine muscle actin and myosin. Furthermore, it was able to promote or cause dermonecrosis in experimental rabbits. These results propose the possible role of a novel *B. pseudomallei* extracellular calcium-dependent serine protease in the virulence of the pathogen.

Keywords: Purification, Recombinant protease, *B. pseudomallei*

Introduction

Burkholderia pseudomallei is the causative agent of human and livestock melioidosis (Choy *et al.*, 2000, Dance, 2000). Although, this Gram-negative pathogenic bacteria is endemic to Southeast Asia and Northern Australia, melioidosis and *B. pseudomallei* have been reported in India, Africa, Europe, Latin America, and Southern China (Yang *et al.*, 1995; Yang *et al.*, 1998; Dance, 2000; Leelarasamee, 2000). The pathogenicity of the microorganism may involve the participation of several extracellular enzymes. These include haemolysin, siderophore, exotoxin, and proteases (Ashdown and Koehler, 1990; Yang *et al.*, 1991; Haase *et al.*, 1997).

Proteases that were previously isolated from a *B. pseudomallei* culture filtrate were believed to play important roles in the virulence of the bacteria. These proteases were classified as zinc metalloproteases in lieu of the requirement of zinc ions during the production of the proteases and inhibition by 1,10-phenanthroline (a zinc chelator) (Sexton *et al.*, 1994; Percheron *et al.*, 1995). Sexton *et al.* (1994) also reported the purification of a 36 kDa metalloprotease from *B. pseudomallei*. The protease was capable of digesting biologically important proteins, such as the C3 component of the complement system, and all classes of immunoglobulins, including secretory IgA. These findings suggest a possible role of the protease in assisting the bacteria to evade the host's immune system. Furthermore, a 39 kDa *B. pseudomallei* Zn-metalloprotease was linked to soft tissue necrosis, when injected into the intradermal space of a guinea pig (Tumwasorn *et al.*, 1994) and a rabbit's cornea (unpublished). This suggests the involvement of the protease in promoting lesions within affected organs during localized infection. Recently, Lee and Liu (2000) cloned and sequenced a 50 kDa extracellular serine metalloprotease, which shares an amino acid sequence homology with the active residue sites of the subtilisin family of serine proteases. Furthermore, a 19 amino acid sequence at positions 161 to 180 was similar to the N-terminal amino acid sequence of the 36 kDa extracellular metalloprotease that was reported by Sexton *et al.* (1994).

In this study, we report the purification and characterization of a recombinant extracellular calcium-dependent serine protease from *B. pseudomallei*. The recombinant protease, under the direction of its own promoter, was expressed into the culture medium and purified by ammonium sulfate precipitation, gel filtration followed by anion-exchange chromatography. Characterization of the purified protease is described.

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Materials And Methods

Bacterial strains and culture conditions A clinical isolate of *B. pseudomallei* was obtained from the Pathogen Laboratory, Universiti Kebangsaan Malaysia. *E. coli* JM109 [*end*A1, *gyr*A96, *hsd*R17($r_k^-m_k^+$), *mcr*B$^+$, *rec*A1, *sup*E44, *thi*-1, Δ(*lac-pro*AB), F'(*tra*D36, *pro*AB, *lac*I^{q}ZΔM15)] was used for the cloning of the *B. pseudomallei* genomic fragments. A clone, designated QB3, that contained the recombinant plasmid, which consisted of the vector pQE40 and a 2.6 kb fragment of *B. pseudomallei* DNA, was selected for further analysis. QB3 was cultured in four batches of a 200 ml LB broth. Each contained 50 µg/ml of ampicillin for 20 h at 37°C and 250 rpm agitation.

Genomic library construction Genomic DNA was extracted from *B. pseudomallei* (Meada and Molla, 1989), digested with *Kpn* I (20U/µg, Promega, Madison, USA), and ligated to enzyme digested pQE40 for 1 h at 37°C. The ligated products were subsequently transformed into competent JM109 cells. The transformants were screened on LB agar with 3% skim milk.

Protease purification All of the steps were carried out at 4°C, unless stated otherwise. The culture filtrate was collected by centrifugation at 10,000 × g for 10 min. The supernatant was treated with solid ammonium sulfate to 65% saturation. After 16 h with gentle agitation, the precipitates were collected by centrifugation at 11,500 × g for 30 min. The protein pellet was dissolved in 5 ml of 5 mM ammonium bicarbonate (pH 8.0) and dialyzed against the same buffer prior to freeze-drying. The freeze-dried product was dissolved in 2 ml of 50 mM Tris-hydrochloride (HCl) (pH 8.0) that contained 5% v/v glycerol and 0.02% w/v sodium azide (Buffer A). This preparation was subjected to gel filtration on a Sephadex G-100 column (1.8 cm by 100 cm, AP Biotech, Sweden) with Buffer A. The fractions that contained the proteolytic activity were pooled and applied to a DEAE-cellulose DE 52 column (3 by 5 cm, Whatman, UK). They were then eluted with a stepwise increase of NaCl concentration in Buffer A. The purification steps were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

Protease activity assay The protease activity assay was determined with azocasein as the substrate in a modified method of Percheron *et al.* (1995). A 100 µl enzyme solution and a substrate (0.5% azocasein in water and 250 mM Tris- HCl pH 8.0) were pre-incubated at 37°C for 5 min prior to mixing and incubation at 37°C for 30 min. The reaction was halted by the addition of 150 µl of 10% trichloroacetic acid, incubated for 5 min at room temperature, and centrifuged at 9,000 × g for 5 min. Then 120 µl of the supernatant was transferred to a microtiter plate that contained 150 µl of 1 N NaOH. The resulting color development was measured at A_{405} on the MRX ELISA Reader. One unit of protease activity was defined as the amount of enzyme per ml that is required to cause an increase in adsorbance of 0.001 at 405 nm.

Molecular weight determination Determination of the molecular weight of the purified protease under native conditions involved gel filtration on a Sephadex G-100 column (1.8 by 100 cm) (Andrews, 1964). The protein markers that were used were

1 mg/ml Dextran Blue (2,000 kDa), 10 mg/ml bovine serum albumin (66 kDa), 3 mg/ml carbonic anhydrase (29 kDa), and 2 mg/ml cytochrome C (12.4 kDa) (Phamacia Biotech AB, Sweden). Then, 2 ml of the protein and enzyme preparations was used in the procedure. The protein elution was monitored through adsorbance at 280 nm.

Optimal conditions for activity For the determination of optimum pH, freeze-dried preparations of the recombinant protease were dissolved at 4°C in a 0.1 M phosphate buffer saline (PBS) for the pH ranges between 6-8 and 0.1 M carbonate/bicarbonate buffer for the pH ranges between 9-11. The protease preparation (150 µg/ml) was assayed according to the method described previously. The optimal temperature was determined in 50 mM Tris-HCl pH 8.0, according to the protease assay described previously, and incubated at varying temperatures.

Enzyme inhibition The purified recombinant protease was pre-incubated with selected inhibitors for 15 min at 37°C. Following pre-incubation, 100 µl aliquots were added to the azocasein-buffer solution. The inhibitors that were used were: Bestatin (10 mM in methanol), E64 (20 mM in 50 mM Tris-HCl pH 8.0 buffer), Pepstatin A (100 µg/ml in dimethyl sulfoxide (DMSO)), PMSF (100 mM in DMSO), E64 (20 mM in 50 mM Tris-HCl pH 8.0), EDTA and EGTA (0.5 M in 50 mM Tris-HCl pH 8.0), and 1, 10-phenanthroline (100 mM in DMSO). Appropriate controls in Tris-HCl, methanol, and DMSO were also assayed.

N-terminal amino acid sequencing N-terminal amino acid sequencing of the purified recombinant protease was performed on a Applied Biosystems Model 476A Protein Sequencer at Massey University, New Zealand. The sequence obtained was then subjected to BLASTp and a Protein Database analysis.

Degenerate PCR and dot blot A polymerase chain reaction (PCR) was performed with degenerate primers on 500 ng/µl *B. pseudomallei* genomic DNA and 30 ng/µl of the digested fragment of pQB3 that contained the 2.6 kb insert. The forward primer was designed based on the N-terminal amino acid sequence of the recombinant protease, PNDPYY; whereas the reverse primers were designed based on the conserved active histidine site of bacterial serine proteases (NGHGTHVAG). An annealing temperature of 72°C was used. The reaction was cycled 50 times (GeneAmp 9600, Perkin Elmer USA). Digoxygenin (DIG, Boehringer Mannheim, Mannheim, Germany) -labeled probes were generated by PCR using the 3 kb pQB3 fragment as a template. The resultant probe was used to hybridize against *B. pseudomallei* DNA, according the DIG Nuclei Acid Detection Kit manual.

Digestion of physiological proteins The proteins that were used were human serum albumin, immunoglobulin A and G, and human placental transferrin that were obtained from the Pierce Co. (Rockford, USA), as well as bovine muscle myosin and actin from the Sigma Chemical Co. (St. Louis, USA). Stock solutions of these substrates were prepared at the concentration of 2 mg/ml in Buffer A. Each reaction mixture consisted of a 10 µl substrate, a 10 µl enzyme solution (150 µg/ml in Buffer A), and a 5 µl DTT: Azide solution (5 mM DTT, 0.1% wt./vol. sodium azide). After an

incubation of 3 h at 37°C, the reaction was stopped by the addition of a 5 μl SDS-PAGE sample buffer. It was heated at 90°C for 5 min. The protein digestion was viewed by SDS-PAGE (Laemmli, 1970).

Results

Construction of genomic library and selection of clones A *Burkholderia pseudomallei* genomic library was constructed by shot-gun cloning. The transformants were screened for the production of a halo on milk agar plates. The production of a halo indicates the hydrolysis of casein within the milk by a protease enzyme. No halos were visible for the clones of JM109 alone, or JM109 carrying only the pQE40 vector (data not shown). The lack of a clear halo around the colonies of the *E. coli* JM109 transformant that contained pQE40 denotes the inability of the vector to produce extracellular protease. Similar observations were reported for the detection of extracellular protease production of *Pseudomonas aeruginosa* (Sokol *et al.*, 1979), *Staphylococcus hyicus* (Ayora and Götz, 1994), and recombinant *E. coli* that contained a *P. aeruginosa* alkaline protease gene (Guzzo *et al.*, 1990) and *Bacillus subtilis* neutral protease (Tran *et al.*, 1991). The presence of inserts within the protease positive clones was confirmed by a restriction enzyme analysis (data not shown). The clone, QB3, was selected for expression and further characterization.

Expression of recombinant protease The recombinant clone, QB3, contains the recombinant plasmid pQB3 that consists of the vector pQE40 (4 kb) and a 2.6 kb *B. pseudomallei* DNA fragment that was transformed and maintained in *E. coli* JM109. This clone demonstrated extracellular protease production, which was visualized as a clear halo, due to the proteolytic digestion of casein when grown on LB agar that contained ampicillin (50 μg/ml) and 3% w/v skim milk. Proteolytic activity in the culture filtrate of QB3 was also demonstrated. This indicates the production of extracellular protease. However, proteolytic activity was
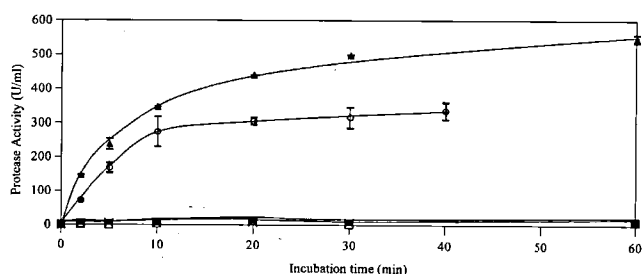
undetectable in the culture filtrates of *E. coli* JM109 and *E. coli* JM109 that contained pQE40 alone (Fig. 1). These results clearly suggest that the 2.6 kb *B. pseudomallei* DNA fragment contains a structural gene/s for proteolytic activity. This includes the factors that are necessary for the secretion of the protease/s.

Although pQE40 is an expression vector that contains a *lac* promoter, QB3 was found to produce recombinant proteases that are independent of the induction by IPTG. As advised by the manufacturer, 5 mM IPTG was added to the culture at the early log phase of the growth curve. A time-point assay for specific activity of proteolytic activity in the culture filtrate of QB3 in the presence or absence of IPTG showed no significant difference (Fig. 2). Furthermore, the production of the recombinant protease increases with the number of cells, which is monitored by the adsorbance at 600 nm. These results strongly suggest that a promoter is present in the 2.6kb *B. pseudomallei* DNA fragment, and it is capable of constitutive expression of the protease gene/s.

Purification of recombinant protease The recombinant protease was purified by gel filtration and ion-exchange column chromatography from the culture filtrate of QB3. The extracellular protease was produced at the end of the exponential phase of growth in the LB broth that contained 50 μg/ml of ampicillin. The initial fractionation was performed with ammonium sulfate at 0-65% saturation on the culture
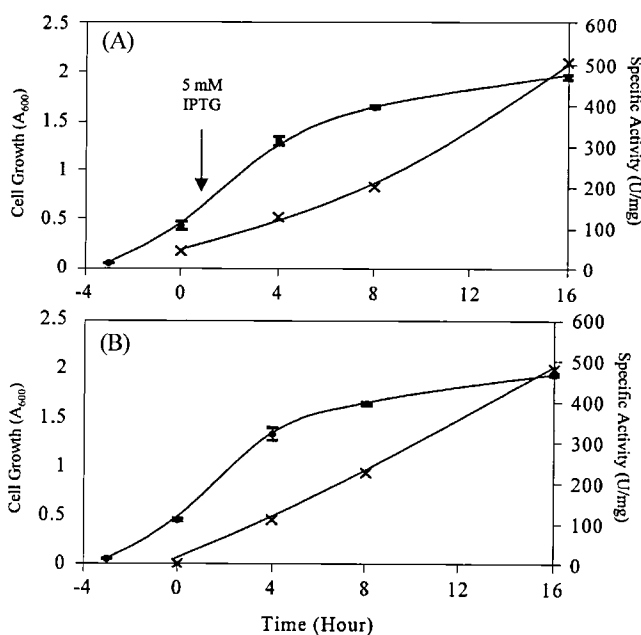

Fig. 1. Protease activity in the culture filtrate of QB3, *B. pseudomallei*, *E. coli* JM109 and *E. coli* JM109 containing the vector pQE40. The *E. coli* cells were grown in LB medium for 20 h at 37°C and 250 rpm agitation, whereas *B. pseudomallei* was grown in BHIB medium in a 7-day static culture at 32°C. Protease activity was determined by an azocasein test as described in the text. △ QB3, O *B. pseudomallei*, *E. coli* JM109 and, × *E. coli* JM109 containing pQE40.

Fig. 2. Increase of protease production and secretion into the culture medium by QB3 in the presence and absence of IPTG. The specific activity of the recombinant protease was determined every 4 hours post-induction with 5 mM IPTG (time 0), when the cells were in the early exponential growth phase. B-Uninduced culture, A- Culture induced by IPTG. - × - Specific Activity, - ● - Adsorbance at 600 nm.

Table 1. Summary of the purification of recombinant protease from the culture filtrate of QB3

Stage	Volume (ml)	Total Protein (mg)	Total Activity ($\times 10^3$ U)[a]	Specific Activity ($\times 10^3$ U/mg)	Percentage Recovery (%)	Purification (Fold)
Culture filtrate	370	60.31	22.20	0.36	100	1
$(NH_4)_2SO_4$ 0-65% saturation	16	10.24	19.01	1.86	85.6	5.0
Sephadex G-100	45	3.69	17.46	10.78	78.6	13.1
DEAE-Cellulose pH 8.0	54	1.30	14.47	11.13	65.2	30.9

[a]One unit of activity is the amount of enzyme required to produce a 0.001 change in adsorbance at 405 nm per min under standard assay conditions.
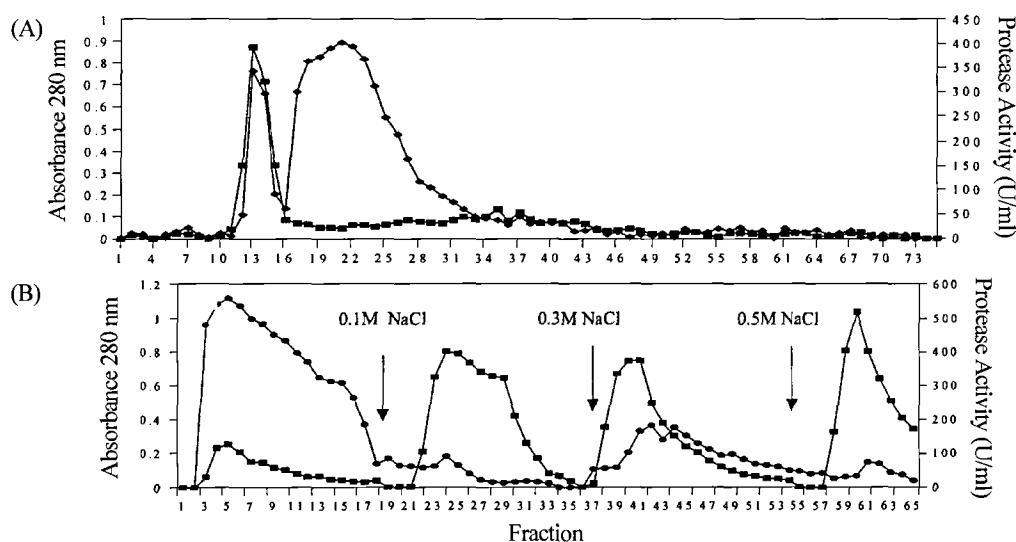

Fig. 3. Sephadex G100 and DEAE-selulose purification profile of the Recombinant Protease A. Ammonium sulfat precipitated protein was loaded into a Sephadex G-100 column (1.8 cm × 100 cm) and eluted with Buffer A. The void was collected in fractions 1-14. B. The eluted protease fractions from the Sephadex G-100 were loaded onto a DEAE-selulose column (3 cm × 3 cm; pH 8.0). Protein was eluted by fractionating with Buffer A containing 0.1 M, 0.2 M dan 0.5 M NaCl. ◆ Protease activity, U/ml; ■ Adsorbtion at 280 nm

filtrate. A 16 h precipitation time was found to yield a four-fold recovery, compared to a 1.5 h precipitation time (21.1 ± 3.2)%. Furthermore, this fractionation step resulted in a five-fold increase in specific activity (Table 1).

Two peaks with protease activity were eluted from the gel filtration column (Fig. 3). The activity eluted at the void volume contained 16.4% of the total protease activity and 52.5% of total protein. The majority of the eluted protease was purified by a factor of 13.1 (Table 1). The protease in the void volume is believed to be aggregated protein, possibly due to the high concentration of the initial sample (5 mg/ml), as well as the low ionic strength (50 mM). Nonetheless, the possibility of activity in this peak that was contributed in total or in-part by the host *E. coli* soluble protease has not been investigated. The protease from the elution fractions was pooled and further purified by ion exchange chromatography. The enzyme preparation passed through the anion-exchange column DEAE-cellulose and protease activity was observed in the flow-through (Fig. 3B). This profile corresponds to the results that were reported by Sexton *et al.* (1994), where the *B. pseudomallei* extracellular metalloprotease did not bind to a

DEAE-cellulose column in 10 mM Tris-HCl pH 8. Nevertheless, this chromatography step eliminated approximately 65% of the total protein that was loaded into the column and achieved a 31-fold purification with a final yield of 65.2% (Table 1). SDS-PAGE of the flow-through fractions with a 12.5% gel, using a standard Laemmli (1970) protocol, revealed a single protein band when stained in 0.1% Coomassie Blue (Fig. 4).

The molecular weight of the recombinant protease was approximately 32 kDa by gel filtration and 52 kDa from the SDS-PAGE profile. The discrepancy in the protease molecular weight may have been the result of conformational differences between the native enzyme and the reduced, denatured form of the protein. Such molecular weight differences have been reported in the purification of a number of Gram negative extracellular proteases. These include *S. marcescens* extracellular serine protease and metalloprotease, (Yanagida *et al.*, 1986), *Aeromonas hydrophila* heat-stable metalloprotease (Leung and Stevenson, 1988), and *B. subtilis* extracellular metalloprotease (Rufo *et al.*, 1990).
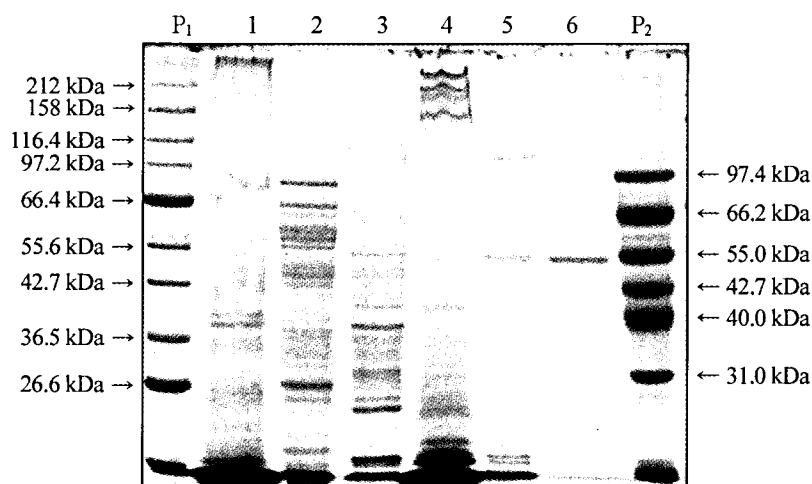
Fig. 4. SDS-PAGE (12.5%) of purified recombinant protease from the culture filtrate of QB3 25 µg protein were loaded into wells 2 4, whilst wells 5 and 6 contained 1.5 µg protein. P_1- Protein standards from New England Biolabs, 1- *B. pseudomallei* culture filtrate, 2- *E. coli* JM109 pQE40 culture filtrate, 3- QB3 culture filtrate, 4- Ammonium sulfat precipitated product, 5- Protease fraction from Sephadex G-100, 6- Protease fraction from DEAE-selulose and P_2- Protein standards from Promega Co.

Enzymatic properties of recombinant protease The purified protease migrated as a single band when partially denatured in SDS and subjected to non-reducing SDS-PAGE. When overlaid with a gelatin-containing polyacrylamide gel, the resulting zymogram displayed a single activity band of approximately 52 kDa. This confirms the presence of one protease that resulted from the purification procedure (Ling *et al.*, submitted). The optimum pH of the purified protease is pH 9. The protease is active between pH 6-9, but activity falls beyond pH 9 (data not shown). The recombinant protease has an optimum temperature of 38°C. It is intolerant to a change in temperature. It rapidly loses its activity at temperatures beyond 45°C.

The protease is inhibited by more than one class of protease inhibitors. It was inhibited by 1 mM phenylmethylsulfonyl fluoride (PMSF), a known serine protease inhibitor, as well as by the metal chelators, EGTA (1 mM) and EDTA (5 mM). However, it was not inhibited by the zinc ion chelator, 1,10-

phenanthroline (1 mM) (Table 2). These results suggest that the recombinant protease is a serine protease that is heavily dependent on calcium ions for its activity. Lee and Liu (2000) reported a 50 kDa *B. pseudomallei* serine metalloprotease that displayed similar inhibition by PMSF and EDTA. Nevertheless, the purified protease in this study differs from the protease that was reported by Lee and Liu (2000), which is stable at 60°C.

N-terminal amino acid sequencing The N-terminal amino acid sequence of the purified recombinant protease was determined, and the sequence LAPNDPYYYGY was obtained. The amino acids, PNDPYY, correspond to the amino acid sequence of serine proteases of the subtilisin family, such as *B. subtilis* PD498 serine protease, *B. cereus* microbial serine protease, *Thermoactinomyces vulgaris* thermitase, and the *Dichelobacter nodosus* extracellular subtilisin-like protease precursor (Table 3). The sequence also

Table 2. The effect of protease inhibitors towards activity of the B3 recombinant protease

Protease Inhibitor	% Protease Activity		
	Protease QB3 35 U/ml	Trypsin 10 mg/ml	Thermolisin 26 U/ml
0.1 mM Bestatin	91.0 ± 6.8	-	-
5 mM EDTA	18.5 ± 5.2	103.8 ± 3.0	4.0 ± 0.4
1 mM EGTA	23.1 ± 7.5	108.6 ± 2.3	6.1 ± 0.9
0.1 mM E64	100.0 ± 13.6	93.8 ± 3.2	101.3 ± 6.4
1 µg/ml Pepstatin A	95.0 ± 2.7	-	-
1 mM 1,10-Phenantrolin	96.9 ± 18.5	103.0 ± 1.4	12.4 ± 3.1
1 mM PMSF	21.8 ± 8.0	37.6 ± 1.8	104.7 ± 1.6

Protease inihibition was assayed according to Dunn (1989). The assay was performed in triplicate whereby protease was incubated in the presence of the inhibitor for 10 min at 37°C. The percentage of inhibition was calculated by comparing protease activities in the presence and absence of inhibitors. Trypsin and Thermolisin (Sigma Co.) were used as controls.

Table 3. Sequence comparison of N-terminal amino acid sequence of B3 with deposited sequences

Source/Organism (GeneBank, NCBI)	Consensus Sequence		
B3 sequence	1	LAPNDPYYYGY	11
Protease [Bacillus sp. PD498]	119	SPNDPYYSAY	128
Serine Protease Bacillus sp.	124	PNDPYYQGY	132
Thermoactinomyces vulgaris Thermitase	3	PNDPY	7
Yflo [Bacillus subtilis]	293	LMPNDAYFYGV	303
Protease II (oligopeptidase B) E. coli	583	PQDPQYYEY	591
Serine Protease [B. cereus]	3	PNDPYYKN	10
Serine Carboxypeptidase-like Protein [Sorghum bicolor]	526	SDPLYYNW	533
V5 Acidic extracellular Protease [Dichelobacter nodosus]	2	APNDPFYND	10

The numbers indicate the positions of the amino acids within the gene that demonstrate similarity to the B3 sequence

displays some, though incomplete, similarity to the serine metalloprotease that was reported by Lee and Liu (2000).

Degenerate primers were designed based on the N'-terminal amino acid sequence of the recombinant protease (PNDPYY), and the conserved active histidine site of the subtilisin family of serine proteases (NGHGTHVAG). Amplification of the pQB3 insert fragment and B. pseudomallei genomic DNA, using these primers, produced a single PCR product with a molecular mass of 170 bp (data not shown). The amplified product of the pQB3 insert fragment, when used as a probe, hybridized to the amplified product of the B. pseudomallei genomic DNA template, as well as to the B. pseudomallei genomic DNA. These results further suggest that the recombinant protease is an extracellular B. pseudomallei serine protease.

Digestion of physiological proteins and dermonecrosis The purified recombinant protease was able to digest a number of physiologically important proteins. Fig. 5 demonstrates the complete digestion of the human serum IgA, albumin, and transferrin, as well as bovine muscle actin and myosin, in addition to the heavy chain of the human serum IgG. These proteins are involved in the host immune system, as well as the transportation of iron that enhances the bacteria's survival and growth in the host's hostile environment. Sexton et al. obtained similar results (1994) for the purified 36 kDa B. pseudomallei extracellular metalloprotease.

Furthermore, the purified recombinant protease was able to cause or promote dermonecrosis when it was injected into the rabbit intradermal space (results not shown). Similar results have also been reported by Tumwasorn et al. (1994), who applied a purified 49 kDa B. pseudomallei extracellular metalloprotease on a guinea pig. These results lead us to believe that the recombinant protease that was purified in this case may also play a role in the virulence of B. pseudomallei.

Discussion

Previous studies on the extracellular proteases from B. pseudomallei found that these proteases were metal-chelator-sensitive with a molecular mass of 36 kDa (Sexton et al.,

1994), 42 kDa (Paucod et al., 1994), and 49 kDa (Tumwasorn et al., 1994). A 50 kDa serine metalloprotease that is sensitive to EDTA and PMSF, but not 1,10-phenanthroline (a zinc ion chelator), has also been cloned from B. pseudomallei and sequenced (Lee and Liu, 2000). In our present study, we were able to purify a 52 kDa protease (determined by SDS-PAGE) from the extracellular products of an E. coli JM 109 that contained the recombinant plasmid pQB3, which consists of the vector pQE40 and a 2.6 kb genomic fragment from B. pseudomallei. The production and secretion of the protease into the culture medium was under the direction of its own promoter and unaffected by IPTG in the growth medium. It increased with the number of cells (Fig. 2). The purification procedure involved ammonium sulfate precipitation, gel filtration by Sephadex G-100, and anion-exchange chromatography by DEAE-cellulose at pH 8.0 (Table 1). The purified protease revealed homogeneity on SDS-PAGE when stained with a Coomassie staining solution (Fig. 5).

The purified recombinant protease exhibited optimum enzyme activities at pH 8.9 and 38°C. These results are different than the ones that were reported by Sexton et al. (1994), and Lee and Liu (2000). They reported a protease that was stable at 60°C and optimally active at pH 8.0. The recombinant protease showed a fairly wide range of optimum pH from 6 to 10. This indicates that the protease may present different functions in different environments. The recombinant protease was inactivated at temperatures above 60°C. Thus, it may be different than those that were previously reported.

The serine protease inhibitor, PMSF, inhibited the purified recombinant protease. This suggests that the protease is a serine protease. Moreover, the protease is inhibited by the divalent chelator, EDTA, and the calcium ion's chelator, EGTA, but not the zinc ion's chelator, 1,10-phenanthroline (Table 2). This indicates that the protease is not a metalloprotease, but rather a calcium-dependent serine protease. The N-terminal amino acid sequence of the recombinant protease further strengthens this suggestion with a homology of the PNDPYY sequence to members of the subtilisin family of serine proteases. Furthermore, PCR, which used degenerate primers that are designed based on PNDPYY and the histidine active site sequences of members of the
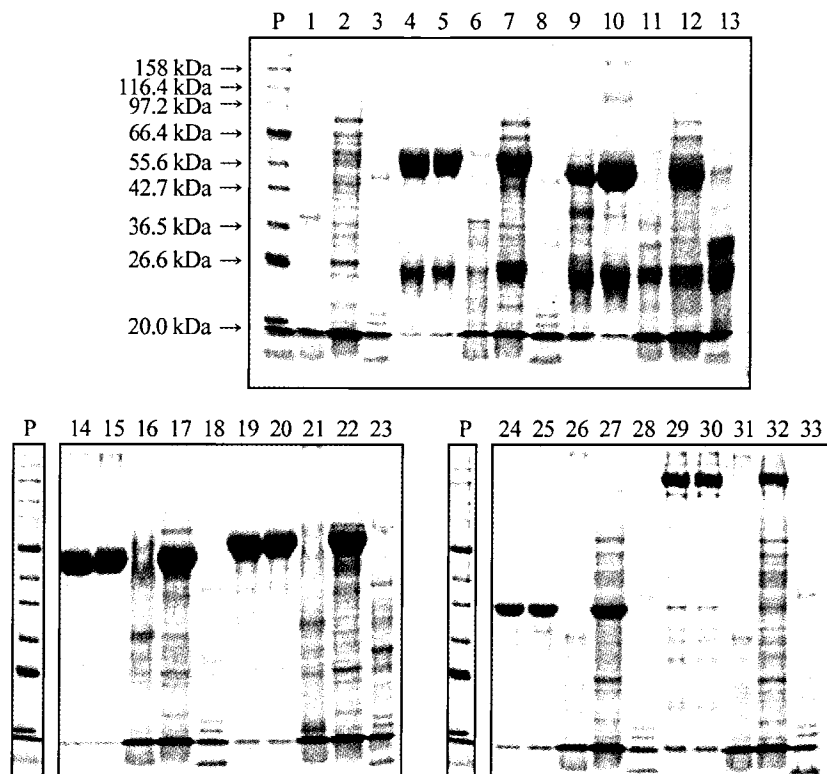
Fig. 5. Digestion of physiological proteins by the recombinant protease Protein substrates (2 mg/ml) were incubated with the recombinant protease (150 μg/ml) for 3 hours 37°C and observed SDS-PAGE (12.5%). P-Protein marker, 1- Partially purified *B. pseudomallei* (Bp) protease, 2- JM109 pQE40 (QE) culture filtrate, 3- Recombinant protease (B3), 4- IgA (not incubated), 5- IgA (incubated), 6- IgA+Bp, 7- IgA+QE, 8- IgA+B3, 9- IgG (not incubated), 10- IgG (incubated), 11- IgG+Bp, 12- IgG+QE, 13- IgG+B3, 14- Albumin (not incubated), 15- Albumin (incubated), 16- Albumin+Bp, 17- Albumin+QE, 18- Albumin+ B3, 19- Transferin (not incubated), 20- Transferin (incubated), 21- Transferin+Bp, 22- Transferin+QE, 23- Transferin+B3, 24- Actin (not incubated), 25- Actin (incubated), 26- Actin+Bp, 27- Actin+QE, 28- Actin+B3, 29- Myosin (not incubated), 30- Myosin (incubated), 31- Myosin+Bp, 32- Myosin+QE dan 33- Myosin+B3.

subtilisin family, produced a single product of the same size respectively, when the *B. pseudomallei* DNA and the cloned fragment were used as templates. Although the N-terminal amino acid sequence of the recombinant protease shares a 4-residue homology to the amino acid sequence of the serine metalloprotease that was studied by Lee and Liu (2000), the 2 proteases are most likely closely related yet distinct with regards to their enzymatic properties.

Further characterization of the purified protease revealed that it is capable of completely digesting a number of physiologically important proteins, such as human serum IgA, IgG, and albumin, human placental transferrin, as well as bovine muscle myocin and actin (Fig. 5). The extracellular metalloprotease that was purified by Sexton *et al.* (1994) also displayed similar activity. This lack of protein substrate specificity of the *B. pseudomallei* extracellular protease/s may be important for its ability to evade the host's immune system. The ability of the recombinant protease to cause or promote dermonecrosis in rabbits is similar to that reported for the 39 kDa extracellular metalloprotease of *B. pseudomallei* (Tumwasorn *et al.*, 1994). These results strongly suggest that an extracellular calcium-dependent serine protease of *B.*

pseudomallei may play a similar role in the virulence and pathogenicity of the bacteria to those of reported metalloproteases.

Gram negative bacteria produce more than one extracellular protease. These are mostly of the metalloprotease and serine protease classes. This bacteria includes *Serratia marcescens* (Ohnishi and Horinouchi, 1996; Salamone and Wodzinski, 1997), *Vibrio sp.* (Kothary and Kreger, 1987; Chowdhury *et al.*, 1990; Marcello *et al.*, 1996), and *Aeromonas hydrophila* (Nieto and Ellis, 1986; Leung and Stevenson, 1988). Extracellular proteases were cloned from bacteria and expressed into the culture medium by *E. coli*. In general, when the protease gene is cloned together with the structural genes for secretion, the recombinant *E. coli* will be capable of exporting the protease into the growth medium (Makrides, 1996). In this study, the insert size of 2.6 kb is too small to contain genes that encode the accessory secretory proteins that are required for a type II secretion pathway, which is responsible for the secretion of *P. aeruginosa* elastase from a recombinant *E. coli* (Guzzo *et al.*, 1990). It seems likely that the protease may be secreted by the type IV autosecretors that are similar to the *Neisseria gonorrheae* IgA protease (Pohlner

et al., 1987), *S. marcescens* serine protease (Ohnishi and Horinouchi, 1996), and a novel protease of *Bacteriodes forsythus* ATCC43047 (Saito *et al.*, 1997). Secretion of these proteases does not require accessory secretory proteins, but the information that is necessary for secretion is encoded within the structural protease gene (Ohnishi *et al.*, 1997). However, the secretory pathway of this *B. pseudomallei* calcium-dependent serine protease has not yet been studied. The identification of this pathway could contribute to a generation of control measures to reduce the incidence of melioidosis.

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