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Epitope mapping of *Burkholderia pseudomallei* serine metalloprotease: identification of serine protease epitope mimics

Shzu-Wei Chan [1], Sheila Nathan *

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

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Abstract

Filamentous phage random peptide libraries were used to identify the epitopes of *Burkholderia pseudomallei* protease by panning against IgG polyclonal sera that exhibited protease neutralizing properties. The isolated fusion peptides presented a consensus peptide sequence, **TKSMALSG**, which closely resembles part of the active site sequence, 435G**TSMAT**PHVA**G**445, of *B. pseudomallei* serine metalloprotease. By comparing the consensus sequence, TKSMALSG, with the predicted three-dimensional molecular model of *B. pseudomallei* serine metalloprotease, it appears that the potential antibody binding epitope was buried within the molecule. This active site was conformational whereby one continuous sub-region (SMA) was located between two discontinuous sub-regions, supplied by the flanking residues in the same polypeptide. All phages selected from the biopanning with IgG polyclonal sera showed good binding towards the polyclonal antibodies when compared to the negative control. In addition, these peptide-bearing phages showed competitive inhibition of *B. pseudomallei* serine metalloprotease binding to the polyclonal IgG.

Keywords: Epitope mapping; Phage display; *Burkholderia pseudomallei*; Molecular modeling

1. Introduction

Melioidosis is a tropical disease in humans and animals caused by *Burkholderia pseudomallei* that is endemic in Southeast Asia and Northern Australia [1]. The ability of *B. pseudomallei* to produce virulence factors such as exotoxin, phosphatase, hemolysin, lecithinase, lipase and protease [2–4] has been investigated by several groups, but the role of these factors remains unclear. Percheron et al. [5] have identified an exoprotease in *B. pseudomallei* that requires Zn^{2+} for optimal production. Sexton et al. [6] purified and characterized a protease with a molecular mass of 36000 Da from *B. pseudomallei* and the gene encoding this Zn-dependent metalloprotease (ZmpA) has recently been cloned [7]. A novel serine metalloprotease (MprA) from *B. pseudomallei* was also recently reported [8] whereby the protease production is modulated by the PmlI–PmlR quorum sensing system [9]. Sexton et al. [6] also reported that melioidosis patients produce antibodies to *B. pseudomallei* protease indicating that the protease is produced in vivo with the strain lacking the protease producing significantly less lung damage than the parental strain although Valade et al. [9] claim that the MprA protease only plays a minor role in *B. pseudomallei* virulence. Based on these numerous reports on the secretion of one or more proteases from *B. pseudomallei*,

* Corresponding author. Tel.: +60-3-89213862/89267446; fax: +60-3-89252698.

E-mail address: sheila@pkrisc.cc.ukm.my (S. Nathan).

[1] Present address: Animal Health Biotechnology, Temasek Life Sciences Laboratory, 1 Research link, National University of Singapore, Singapore 117604, Singapore.

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our group has undertaken to study the structure–function relationship of this protease. We have utilized anti-protease polyclonal antibodies as a tool for studying protease–antibody interactions.

Antibodies play a crucial role in the immune system, especially in defense against extracellular pathogens (such as encapsulated bacteria) and pathogens in extracellular phases of their life cycle (such as viral particles), and as such are a major immune component elicited by all vaccines currently in use for humans. Antibodies also have a variety of uses as biochemical and cellular markers, affinity reagents, diagnostics and therapeutics. Thus, our understanding of the principles governing antibody–antigen interactions is of great significance to a wide spectrum of research and applications.

Antibodies to protein antigens have been widely used in structural and functional analysis. Analyses of antibody–antigen complexes by X-ray crystallography [10,11], chemical modification [12] or deuterium exchange analysis by nuclear magnetic resonance [13] have provided a detailed picture of the amino acids that comprise a given epitope. Nevertheless, to date, few antibody–antigen complexes have been described in great detail because these methods require substantial effort and are technically difficult. Alternative techniques such as phage display have proven to be successful in the determination of continuous and discontinuous antigenic epitopes [14,15]. A library of random peptides displayed on the surface of filamentous phages can be screened for phages that bind to an antibody by a process known as biopanning [16]. This technique has been used in the mapping of critical residues involved at the interface of protein–protein complexes [17] although with limited structural information on the epitopes within the antigen and to identify short peptide mimetics of antigenic epitopes [14].

Polyclonal and monoclonal antibodies would allow for analysis of the structure–function relationship to understand the role of the protease in the pathogenesis of melioidosis. One way to achieve this would be by using the antibodies to epitope map the antigen. In our laboratory, we have generated polyclonal antibodies towards *B. pseudomallei* protease by rabbit immunization and armed with these antibodies, the current study was designed to define and localize the antigenic determinants of the *B. pseudomallei* protease. Determining its epitope(s) should lead to definition of the region within the protease molecule that is involved in the association with antibodies and this will allow for the design of peptides as diagnostic reagents or potential vaccine candidates.

As the three-dimensional (3-D) molecular structure of the *B. pseudomallei* protease has not been determined experimentally, we combined phage-display technology and molecular modeling in an attempt to characterize the molecular surface of the protease that interacts with antibodies. We screened a 12-mer linear peptide library displayed on filamentous phage with anti-protease polyclonal antibodies and three families of peptides that bind to polyclonal antibodies were identified.

2. Materials and methods

2.1. Preparation of antigens and antibodies

B. pseudomallei protease and IgG fractions of polyclonal antibodies specific against *B. pseudomallei* protease used in this study were prepared as described previously [18]. Briefly, protease was purified from brain–heart infusion (BHI, HispanLab, Cuba) broth cultures grown at 37 °C for seven days, following which, cultures were subjected to ammonium sulphate precipitation (70% saturation), DEAE-Cellulose Ion Exchange chromatography (pH 8) and CM-Cellulose Ion Exchange chromatography (pH 6). Chromatographic fractions demonstrating protease activity were pooled and dialyzed. Purified *B. pseudomallei* protease (200 μg) was mixed with 0.5 ml incomplete Freund's Adjuvant. Over a period of two to three months, two New Zealand White rabbits were treated with four subcutaneous injections of 1 ml emulsion samples and administered on days 0, 14, 28 and 42. Antibody titres were monitored by an enzyme-linked immunosorbent assay (ELISA) with peroxidase-conjugated goat anti-rabbit immunoglobulins as the secondary antibody.

2.2. Inhibition of protease activity by polyclonal antibodies

Inhibition of *B. pseudomallei* protease activity was performed according to Percheron et al. [5] with modifications. Sera (1 μg) was added to 1 μg protease and incubated for 30 min at 37 °C. Azocasein (2%) and Tris–HCl (87.5 mM) were added to the incubated mix and further incubated for 5-min intervals up to 30 min. The reactivity was terminated with the addition of tricholoroacetic acid and centrifuged (9000*g*/5 min). The supernatant (120 μl) was added to a microtiter plate well and incubated with 1 N NaOH briefly followed by measuring absorbency at 405 nm. Commercial proteases and protease inhibitors were utilized as controls.

2.3. Biopanning with polyclonal antibodies

The phage 12-mer linear peptide library was obtained from New England BioLabs Inc (USA) and biopanning was conducted essentially as described in the manufacturer's manual. In this library, random peptides are fused to the minor coat protein (pIII) of M13 phage and expressed at the N-terminus of pIII followed by the GGGS spacer. To identify the *B. pseudomallei*

protease epitope(s), the 12-mer linear peptide library was utilized for phage affinity selection against *B. pseudomallei* protease specific polyclonal sera. The library carried approximately 1.9×10^9 electroporated sequences which were amplified to ensure the presence of at least 20 copies of each sequence. Briefly, microtitre plate wells were coated with antibody (10 μg/ml in 0.1 M $Na_2CO_3/NaHCO_3$, pH 9.6) and then blocked with 5 mg/ml bovine serum albumin (BSA) for 1 hour. Streptavidin and BSA were used as positive and negative controls respectively as recommended by the manufacturer [19]. The experiment was carried out at room temperature (~25 °C). The peptide library (~10^{11} phage particles) was added into each coated well and incubated for 30 min. The wells were washed with TBST (TBS + 0.1% Tween-20) and bound phage were eluted with 0.2 M glycine–HCl, pH 2.2 containing 1 mg/ml BSA. Eluates were neutralized with Tris–HCl (1 M, pH 9) and titrated as plaque forming units (pfu). *Escherichia coli* ER2738 cells were infected with the remaining phage and grown for 4.5 h at 37 °C with strong agitation. Cells were harvested and phage particles were isolated by polyethylene glycol precipitation and resuspended in TBS (50 mM Tris–HCl, pH 7.5 containing 150 mM NaCl). The panning process was repeated for a further three rounds.

2.4. Plaque amplification and DNA sequencing

Plaque amplification was carried out by transferring a random blue plaque into a 1 ml diluted *E. coli* ER2738 overnight culture. The culture was incubated for 5 h at 37 °C with shaking and harvested by centrifugation at 10000*g* for 30 s. The upper 80% of the aqueous phase was transferred to a fresh tube and either stored with 50% glycerol at −20 °C or used for phage DNA purification. Single-stranded DNA was extracted essentially as described by Sambrook et al. [17] and sequencing of DNA inserts was done on the ABI 377 automated sequencer using fluorescent dye terminators (Perkin–Elmer).

2.5. Phage ELISA

Microtitre plates (Nunc, Denmark) were coated overnight at 4 °C with polyclonal antibodies against *B. pseudomallei* protease (1 μg/25 μl in 1 M $Na_2CO_3/NaHCO_3$, pH 9.6). The coated plates were blocked with 5% skimmed milk for 1 h at 4 °C. Serial dilutions of phage-bearing peptides were added and incubated for 2 h at 37 °C. The plates were then washed with 0.5% PBST prior to the addition of peroxidase conjugated sheep anti-M13 IgG (Pharmacia) (1:1000). After 1 h at 37 °C and 10 washes with 0.5% PBST, 50 μl ABTS:peroxidase B (1:1) substrate was added. The optical density was measured at 405 nm.

2.6. Peptide–protease inhibition assays

Competition assays between selected phage displayed peptides and *B. pseudomallei* protease for binding to polyclonal antibodies against *B. pseudomallei* protease were carried out as follows. Microtitre plates were coated overnight at 4 °C with polyclonal antibodies against *B. pseudomallei* protease (1 μg/25 μl in 0.1 M sodium bicarbonate, pH 8.6). The coated plates were blocked with 5% skimmed milk for 1 h at 4 °C. Phage-displayed peptides (10^7–10^{11} pfu) and 1 μg heat-treated *B. pseudomallei* protease (95 °C, 10 min) were added and incubated for 2 h at 37 °C with agitation. The plates were then washed with 0.5% PBST (10X) prior to the addition of pre-incubated polyclonal antibody (1:1000)-peroxidase conjugated IgG anti-rabbit (Pharmacia) (1:30000) complex. After 1 h at 37 °C and washing (10X) with 0.5% PBST, 50 μl ABTS: peroxidase B (1:1) substrate was added. The optical density was measured at 405 nm. The 50% inhibition relative constant, IC_{50}^{rel} was expressed as the concentration of phage-displayed peptides that inhibited 50% of the maximal ELISA response.

2.7. Molecular modelling

The model for *B. pseudomallei* MprA serine metalloprotease was derived using the serine metalloprotease gene sequence (GenBank Accession No. AAG32166) available at the NCBI database. This serine metalloprotease sequence served as the template to construct a 3-D molecular model of the serine metalloprotease using the SWISS-MODEL software in the ExPasy database (www.expasy.com).

3. Results

3.1. Inhibition of protease activity by the B. pseudomallei specific polyclonal antibodies

The polyclonal antibodies harvested following completion of the immunization protocol were used in a modified Azocasein assay and inhibition of *B. pseudomallei* protease was monitored. The polyclonal antibodies were able to inhibit up to 85% of the original proteolytic activity (Table 1). Inhibition was also observed for protease treated with commercial protease inhibitors, EDTA and PMSF indicating that the protease had both metallo- and serine protease characteristics.

3.2. Selection of phage-displayed peptides binding B. pseudomallei protease specific IgG polyclonal antibody

To identify the major epitope of *B. pseudomallei* protease, the phage display method was used whereby random 12-mer peptides were screened for affinity binding

Table 1
Percentage inhibition of *B. pseudomallei* serine metalloprotease activity by anti-*B. pseudomallei* protease specific polyclonal antibodies

Sample	% Inhibition of protease activity
PBS + protease	0
Polyclonal antibodies + protease	85
EDTA + protease	71
PMSF + protease	50

EDTA and PMSF (Phenylmethylsulfonyl fluoride) are known protease inhibitors.

of peptides to *B. pseudomallei* protease specific IgG polyclonal antibodies. The antibodies were protein-A affinity-purified [18], immobilized on microtitre wells and bound phage were selected and amplified through 3–4 biopanning cycles. The eluted phage population from the first cycle of selection was amplified and used for the successive round of selection and amplification. For each cycle, $\sim 10^{18}$ phage (input) were added to the wells and the total amount of phage recovered (output) was determined whereupon an output–input ratio was calculated. The output–input ratio increased through successive selections, indicating that the phage population was successfully enriched for IgG binders. After four rounds of selection, a 795-fold enrichment was obtained (data not shown) and the peptide sequences displayed by individual phages were determined by DNA sequencing.

Thirty-five phages from the final round of biopanning were selected at random, sequenced and based on the presence of common amino acids at similar positions, were grouped into three families presenting consensus motifs (Table 2). The first family, characterized by the sequence TKSMALSG, contained 24 clones (68.7%), with a small variation in sequence for clones 4–29 and 4–12. The second family was characterized by the consensus sequence IPRWN(F/L)MS (17.2%). The third family had no consensus sequence. Biopanning of the phage library against streptavidin (the positive control) selected for the consensus sequence of HPQFEAL (data not shown) in agreement with the sequence identified by Devlin et al. [20].

Table 2
Amino acid sequences of 12-mer peptides obtained by biopanning

Clone	Peptide sequence[a]	Frequency	Percentage
Group 1			
4–5	LTAN**TKSMALSG**	20	57.1
4–21	VPPN**TKSMALSG**	1	2.9
4–41	**LTTHTKSMALSG**	1	2.9
4–9	**LTTDHSTTVING**	2	5.7
4–29	VPANI**KSMALSG**	1	2.9
4–12	VA**PSMA**k**S**IPAK	1	2.9
Group 2			
4–1	AQLP**IPRWN**F**MS**	4	11.4
4–30	AHLH**IPRWN**L**MS**	1	2.9
4–7	KTNNSY**IP**PLT**W**	1	2.9
Group 3			
4–4	STLAKHAPAGYR	2	5.7
4–17	ISQSLMPLSPWR	1	2.9

[a] Residues selected two or more times at the same position are in bold.

3.3. Comparison of the selected consensus sequences motifs with the B. pseudomallei serine metalloprotease gene

As the *B. pseudomallei* protease purified in this study was inhibited by both EDTA and PMSF, we concluded that the purified protease was most likely the MprA serine metalloprotease previously described by Lee and Liu [8]. The primary amino acid sequence of this protein was obtained from GenBank (Accession No. AAG32166) and ScanProsite predicted seven characteristic motifs (data not shown). These motifs were aligned with the consensus sequences obtained from the three different consensus peptide groups (Fig. 1). A motif homolog to the major consensus sequence TKSMALSG of group 1 was found at the serine active site at residues 435–445 with 100% similarity for amino acids Ser–Met–Ala (SMA). The other minor consensus sequences were homologous to *N*-myristoylation sites on the *B. pseudomallei* serine metalloprotease, 370-GSVSTT-375 homologous to phage clone 4–9 (amino acids STT); 273-GAVTNN-278 homologous to phage clone 4–7 (amino acids TNN); 413-GILSTL-418 homologous to phage clone 4–4 (amino acids STL). Another major consensus sequence IPRWN(F/L)MS from group 2 was found at residues 117–124 of *B. pseudomallei* serine metalloprotease with three amino acids homolog, IXXXXMS. The phage clone 4–17 peptide sequence was located at residues 451–460 of the *B. pseudomallei* serine metalloprotease gene with a three amino acid homolog, LSP. In addition, we selected seven alkaline serine proteinases from *Altermonas* sp. (JC4908), *Vibrio cholerae* (NP_229814), *Thermus* sp. (P80146), *Bacillus licheniformis* (CAA62666), *B. subtilis* (P04189), *Bacillus* sp. (AAC43581), *Paecilomyces lilainus* (AAA91584) to align with the *B. pseudomallei* serine metalloprotease amino acid sequence. Interestingly, the serine active site (GTSMA(A/S/T)PH(V/I)(A/V/T)G) is highly conserved in all the serine proteinases (data not shown). The major consensus sequence with three amino acids homolog, SMA from group 1 was selected from this highly conserved sequence of the serine protease.

To localize these identified motifs within the *B. pseudomallei* serine metalloprotease structure, the *B. pseudomallei* serine metalloprotease amino acid sequence (GenBank Accession No. AAG32166) was used to gen-

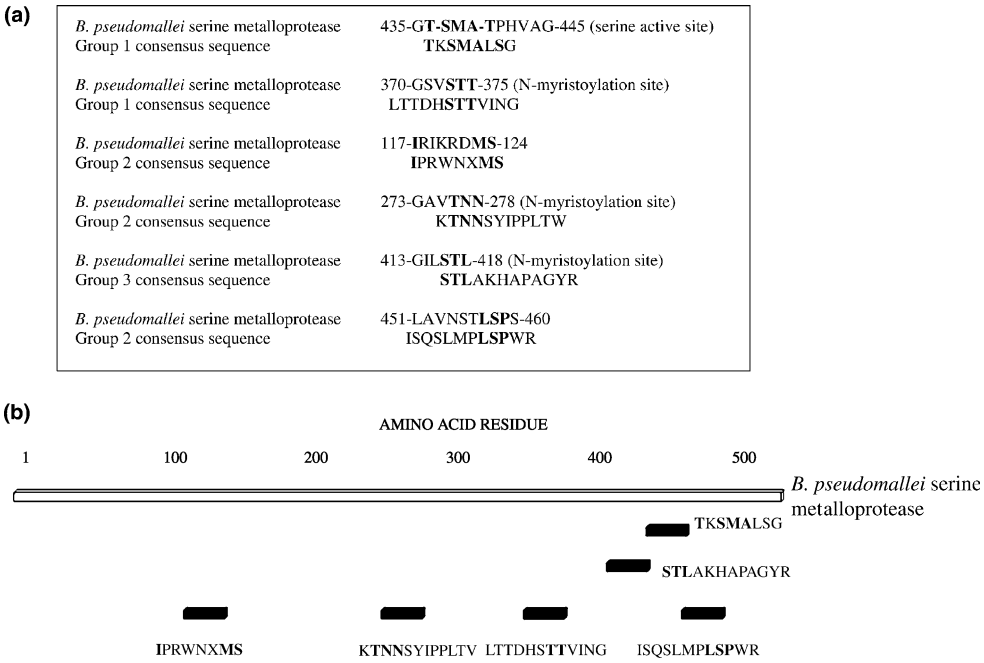

Fig. 1. (a) Sequence homology between selected peptides and *B. pseudomallei* serine metalloprotease. (b) Alignment of selected phage displayed peptide sequences with the *B. pseudomallei* serine metalloprotease amino acid sequence.

erate a predicted 3-D molecular model using the SWISS-MODEL software. The SWISS-MODEL software searches for homologous target sequences of known structures in a database of annotated 3-D comparative protein structure models to generate a predicted 3-D model by the fully automated homology-modelling pipeline. Nevertheless, the software failed to predict the structure for residues 1-251 of the *B. pseudomallei* serine metalloprotease as these residues exhibited no homology with any sequence in the database of known structures. Thus, the 3-D molecular model generated for *B. pseudomallei* serine metalloprotease represents residues 251–500, which contain several α-helices and β-chains linked by loops (Fig. 2(a)). The consensus sequences identified from the selected phages that were identical to the *B. pseudomallei* serine metalloprotease primary sequence motifs were morphed onto the predicted 3-D structure and shown to be mostly located on the surface except for the consensus sequence, TKSMALSG (Fig. 2(b)). It appears that the major potential antibody binding epitope was buried within the molecule. This active site was conformational with the SMA continuous sub-region located between two discontinuous sub-regions supplied by the flanking residues in the same polypeptide, TK and LSG. The ST(T/L) and TNN residues that can be represented as myristoylation sites are most likely involved in the partitioning of protease to host cellular membranes and can serve to promote protease–substrate/receptor interactions.

3.4. ELISA phage and inhibition assay of selected peptides

To test the specificity of the 11 phage clones selected from the final round of biopanning with *B. pseudomallei* protease-specific IgG, an enzyme-linked immunosorbent assay (ELISA) was performed with the selected phage clones and purified IgG. 2.5×10^{11} pfu were added to IgG coated microtitre wells and the absorption reading of the wild-type M13 phage was used as the cut-off value for a positive reaction between peptide and IgG. All the phage clones tested demonstrated good specificity towards the specific IgG (Fig. 3(a)). The best three peptide binders (clones 4–7, 4–12 and 4–17) were clones representing the three groups of consensus sequences.

An inhibition assay for individual phage clones was also carried out to determine the inhibitory effect of the consensus sequences towards the binding of *B. pseudomallei* serine metalloprotease to protease-specific IgG antibodies. All the selected clones were able to competitively inhibit binding of *B. pseudomallei* serine metalloprotease to the IgG polyclonal antibodies. Fig. 3(b) is a representation of the competitive inhibition for clones 4-1, 4-4, 4–5 and 4–12. These results confirm that the peptides borne by the phages mimic particular regions on the protease molecule that are involved in binding to IgG polyclonal antibodies. These peptides should be further evaluated for their ability to induce the immune system and neutralize the effects of the protease in animal models of melioidosis.

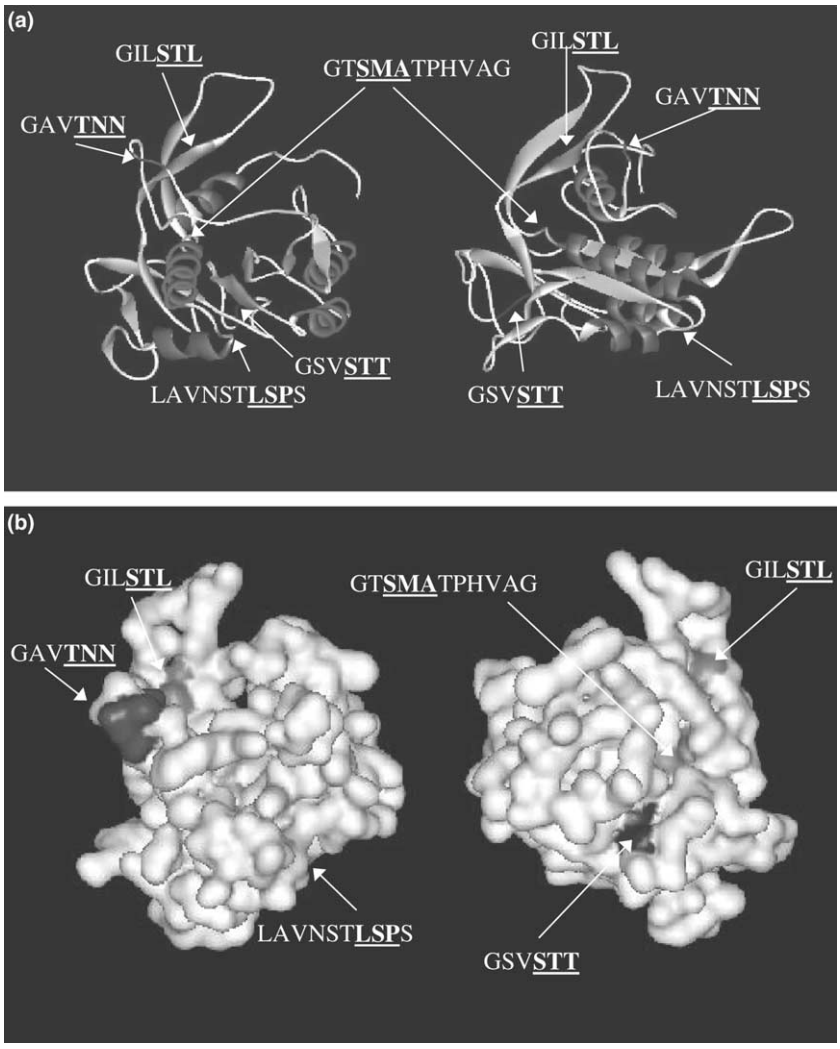

Fig. 2. Molecular modeling of *B. pseudomallei* serine metalloprotease. (a) Ribbon chain representation of *B. pseudomallei* serine metalloprotease and rotated about 90° around vertical axis. Epitopes identified by the selected peptide phages were labelled. (b) Molecular surface representation of *B. pseudomallei* serine metalloprotease and rotated about 90° around the vertical axis. Epitopes identified by the selected peptide phages are labeled.

4. Discussion

The aim of this study was to determine the residues composing the functional epitope(s) of the *B. pseudomallei* serine metalloprotease MprA by peptide display technology and to locate these residues on a homology-modelled 3-D molecular model of *B. pseudomallei* serine metalloprotease.

Phage peptide display allowed for the identification of three families of peptides binding specifically to protease-specific polyclonal antibodies. The alignment of the first family of phagotopes with the *B. pseudomallei* serine metalloprotease sequence was able to identify the presence of the consensus –SMA- sequence located at the active site, 435-GT**SMA**TPHVAG-445, of *B. pseudomallei* serine metalloprotease. It is possible that the proteolytic activity neutralization properties seen above with the polyclonal antibodies is because the IgG molecules within the pool of anti-protease sera can bind to the SMA (active site) region of the serine metalloprotease. The binding of IgG on the myristoylation (ST(T/L) and TNN) regions of the serine metalloprotease could also concomitantly prevent protease–protein (substrate) interactions. A second alignment of significance was found between the second family of phagotopes with the consensus sequences IXXXXMS aligning to the *B. pseudomallei* serine metalloprotease sequence location at residues 117–124. However, as these residues lie within the region of the serine metalloprotease that could not be modeled, we are unable to postulate the possible role and exact location relative to the 3-D structure of the native bacterial serine metalloprotease. No significant alignment between the consensus of the selected phagotopes of the third family and *B. pseudomallei* serine metalloprotease was possible. Nevertheless, phages bearing this consensus sequence

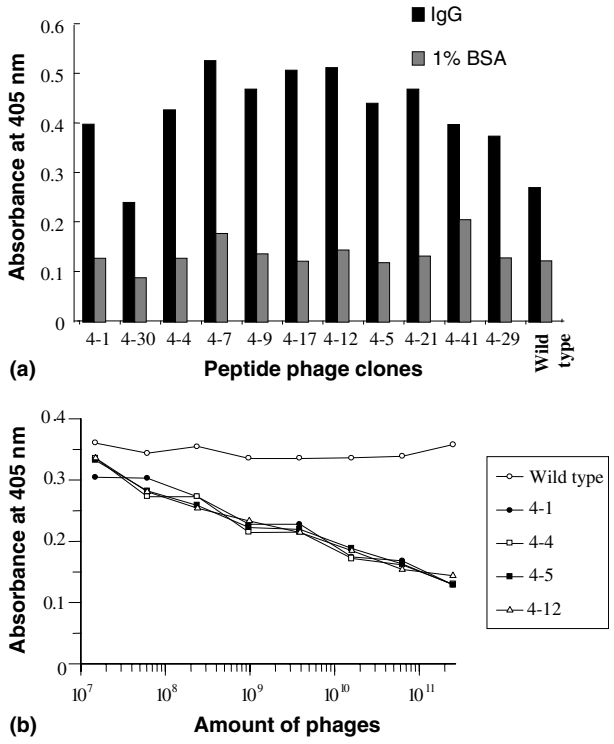

Fig. 3. (a) Specificity of selected phages towards IgG polyclonal antibodies specific *B. pseudomallei* (b) Representation of competitive inhibition for four selected phages clones to the binding of the *B. pseudomallei* serine metalloprotease to the IgG polyclonal antibodies specific *B. pseudomallei*.

were able to bind particularly well to the IgG molecules in the ELISA, suggesting that these phagotopes mimic discontinuous fragments of the natural epitope. Linear epitopes are thought to be rare or even non-existent in proteins [21]. However, antibodies are capable of selecting peptide mimetics containing only linear parts of discontinuous epitopes [22]. The revealed regions of the serine metalloprotease may also represent the linear part of a discontinuous epitope.

The 3-D structural model obtained for *B. pseudomallei* serine metalloprotease was only partially complete but we were still able to identify the residues critical for polyclonal antibody binding. Our model shows that residues of the first consensus family are either gathered in space in the folded molecule or buried within the molecule and constitute a compact region. Other minor consensus families are solvent exposed and located mostly on the surface of the molecule. This characteristic was previously reported for the 3-D structural model for β-lactoglobulin epitopes recognized by phage display technology [21].

As all the selected peptides and *B. pseudomallei* serine metalloprotease competed for binding to polyclonal antibodies, the putative epitope(s) of *B. pseudomallei* is expected to be similar in sequence as the selected peptides. We have determined the 50% relative inhibition constant (IC_{50}^{rel}) for individual selected phages of $\sim 10^{10}$ phages indicating that all the selected peptides play an important role in the interaction of IgG polyclonal antibodies and *B. pseudomallei* serine metalloprotease and have potential as peptide vaccines. Synthetic peptides are safer than subunits or attenuated microrganisms and thus have the potential to be developed into a new generation of vaccines [23]. Further characterization of the identified epitopes requires a more detailed molecular description and understanding of antibody induction. Touji et al. [24] and Sundaram et al. [25] have recently reported of the immunogenic and neutralizing properties of synthetic peptides where they emphasize the requirement of the antigenic epitopes to mimic the 3-D structure of the corresponsing region in the native protein. We are currently in the process of immunizing mice with selected peptide-bearing phage as well as synthetic free peptides and the ability of the resulting polyclonal antibodies to neutralize protease activity will be determined.

In conclusion, we have identified possible immunogenic epitopes of *B. pseudomallei* serine metalloprotease. The major epitope of *B. pseudomallei* serine metalloprotease was located at the serine active site, 435-GT**SMA**TPHVAG-445 whilst a minor epitope was found at 117-IRIKRDMS-124. Three-dimensional molecular modelling and mutant synthetic peptides derived from the 435-GTSMATPHVAG-445 region may assist further in the design of better peptides that mimic the interface of *B. pseudomallei* serine metalloprotease capable of triggering an antibody response. These peptides could become appropriate candidates for the conception of a vaccine used in the treatment of melioidosis.

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