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Recombinant Production and Characterization of an Extracellular Subtilisin-Like Serine Protease from *Acinetobacter baumannii* of Fermented Food Origin

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Abstract

Acinetobacter baumannii is a ubiquitous bacteria that is increasingly becoming a formidable nosocomial pathogen. Due to its clinical relevance, studies on the bacteria's secretory molecules especially extracellular proteases are of interest primarily in relation to the enzyme's role in virulence. Besides, favorable properties that extracellular proteases possess may be exploited for commercial use thus there is a need to investigate extracellular proteases from *Acinetobacter baumannii* to gain insights into their catalytic properties. In this study, an extracellular subtilisin-like serine protease from *Acinetobacter baumannii* designated as SPSFQ that was isolated from fermented food was recombinantly expressed and characterized. The mature catalytically active form of SPSFQ shared a high percentage sequence identity of 99% to extracellular proteases from clinical isolates of *Acinetobacter baumannii* and *Klebsiella pneumoniae* as well as a moderately high percentage identity to other bacterial proteases with known keratinolytic and collagenolytic activity. The homology model of mature SPSFQ revealed its structure is composed of 10 β-strands, 8 α-helices, and connecting loops resembling a typical architecture of subtilisin-like α/β motif. SPSFQ is catalytically active at an optimum temperature of 40 °C and pH 9. Its activity is stimulated in the presence of Ca^{2+} and severely inhibited in the presence of PMSF. SPSFQ also displayed the ability to degrade several tissue-associated protein substrates such as keratin, collagen, and fibrin. Accordingly, our study shed light on the catalytic properties of a previously uncharacterized extracellular serine protease from *Acinetobacter baumannii* that warrants further investigations into its potential role as a virulence factor in pathogenicity and commercial applications.

Keywords *Acinetobacter baumannii* · Subtilisin-like serine protease · S8 peptidase · substrate specificity

1 Introduction

Acinetobacter species consist of gram-negative coccobacilli bacteria that are widely occurring in nature with studies showing that the genus can be grown from virtually all soil and surface water samples [1, 2]. *Acinetobacter* species

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have also been detected in food samples of animal and plant origins and have been found to be a natural part of human skin flora [3–5]. However, the most medically significant species, *Acinetobacter baumannii* was found to rarely occur on human skin under normal circumstances [6, 7]. Despite this, *Acinetobacter baumannii* is increasingly reported to cause nosocomial skin and soft tissue infections which can be fatal [8–10]. Besides that, *Acinetobacter baumannii* also infects high-risk, immune-compromised patients in intensive care units (ICU) to cause catheter-related bacteremia (bloodstream infection) and ventilator-associated pneumonia [11, 12]. The ubiquitous nature of *Acinetobacter* species provides multiple routes by which bacteria from this genus can be introduced into hospital settings [13]. In the past, members from the *Acinetobacter* genus are considered to be relatively low-grade saprophytes with little clinical significance because up until the early 1970s, nosocomial infections caused by *Acinetobacter spp*., especially *Acinetobacter baumannii* could be controlled either with a single or a combination of several commercial antimicrobial drugs [2, 14]. Unfortunately, the impact of uncontrolled usage of antimicrobial drugs caused the emergence of an ever-increasing number of antibiotic resistance pools from numerous countries around the world [15].

Proteases play diverse roles in living organisms and many proteases have the potential for biotechnological applications. Their applications are in the area of food processing, detergent, nutraceutical, and medical industries [16]. Bacterial extracellular proteases especially extracellular serine proteases or ESPs are among the most widely studied of all bacterial enzymes. This is mainly due to their important roles in biological systems thus studying them provides insights into the mechanisms of enzyme action and protein structure–function relationship [17–19]. Furthermore, ESPs have broad substrate specificity which is reflective of their role as scavenging enzymes, and coupled with their relative stability in harsh extracellular environments, they make attractive candidates for potential commercial application. Structurally, ESPs consist of an N terminal secretion signal sequence which is followed by a pro-domain sequence. The signal sequence function to mediate the secretion of the enzyme whilst the pro-domain sequence is autocatalytically cleaved to form the enzyme's mature active conformation [19, 20]. Aside from their beneficial scientific and commercial roles, extracellular subtilisin proteases can also act as virulence factors in pathogenicity thus, making them viable drug targets for therapeutic agents against diseases [21, 22].

There is currently widespread interest in the study of secretory molecules from the *Acinetobacter* genus especially from *Acinetobacter baumannii* due to its clinical relevance [23, 24]. One of the main molecules is extracellular secretory proteases that play diverse essential roles in the interactions of *Acinetobacter baumannii* with the environment and host tissues. *Acinetobacter* genus was previously reported to have no extracellular protease activity [25], however recently several clinical isolates have been shown to secrete active proteases that may contribute to virulence [26–30]. On the other hand, proteases from environmental isolates of *Acinetobacter* can be harnessed for industrial applications but studies on this are limited with only 2 recent significant reports from the past 5 years on proteases from the *Acinetobacter* genus with potential biotechnological applications [31, 32]. Due to the clinical significance of *Acinetobacter baumannii* and the ability of ESPs to degrade a wide range of proteinaceous substrates, it is expected that ESPs from *Acinetobacter baumannii* may display favorable catalytic activities with the potential to be exploited for commercial use. Thus there is a need to characterize the structure and function of new ESPs from *Acinetobacter baumannii* in order to gain insights into their catalytic activity that may be associated with pathogenicity or have the potential for commercial exploitation.

2 Materials and Methods

2.1 Microorganism and Culture Condition

The bacterial strain *Acinetobacter baumannii* TU04 (GenBank: KP204010.1) was isolated from *Tapai Ubi,* a Malaysian traditional cassava-fermented food [33, 34]. *Escherichia coli* DH5α and BL21 (DE3) were employed as hosts for cloning and expression of protease gene, respectively. *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) medium supplemented with ampicillin (100 µg/ml) for plasmid selection.

2.2 Isolation and Identification of *SPSFQ* Gene

Genomic DNA was purified from *Acinetobacter baumannii* TU4 using a Genomic DNA Extraction kit (Promega, USA). A pair of degenerate oligonucleotide primer Ser*AciB*_F: (5′- GGGTTTCATATGATGGCAAATCCTGTAAATG–3′) and Ser*AciB*_R: (5′-GGCCTCGAGTTAAGGAGATTTATATAACAAGCG–3′) corresponding to the extracellular serine protease were designed based on the available genomic database of *Acinetobacter* strains at the National Centre for Biotechnology Information (NCBI). The PCR reaction solution was prepared by mixing 100–200 ng of bacterial genome DNA as template, 10 µM of gene-specific primers each, 2.5 mM dNTPs, 1 × TransTaq HiFi Buffer I, 5U of TransTaq DNA Polymerase High Fidelity (HiFi) (TransGen Biotech, China) and final reaction volume was made to 50µL using the nuclease-free water. The PCR reaction was conducted in an MJ Mini 48-Well Personal Thermal Cycler (Bio-Rad Laboratories, USA) using the following

thermal profile: an initial denaturation of 5 min at 94 °C, 35 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 45 s) and a final extension of 5 min at 72 °C. The amplicon was recovered using NucleoSpin® Gel kit (Macherey–Nagel, Germany) prior to ligation into pGEM-T easy vector system (Promega, USA) and transformed into chemically competent *E. coli* DH5α cells. The positive clones were confirmed by colony PCR and sent for nucleotide sequencing service provided by First Base Laboratories, Malaysia. The complete nucleotide sequence was assembled by aligning the forward and reverse strands using BioEdit software before subjected for identification using the nucleotide BLAST algorithm of NCBI [35].

2.3 Amino Acid Sequence, Phylogenetic and Structural Model Analysis

Several bioinformatics tools were employed to analyze the nucleotide of the gene encoding SPSFQ. The amino acid translation was done by the Expert Protein Analysis System(ExPASy) Translate tool (http://www.expasy.org) and the active site residues, conserved domains, as well as possible calcium-binding sites, were analyzed by the NCBI's Conserved Domain Database (CDD) https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [36, 37]. The phylogenetic tree was built using the protein sequences from the NCBI database bootstrapped with 500 replications in the MEGA version 5.0 software [38]. The information on molecular weight and amino acid composition of SPSFQ was calculated by ExPAsy ProtParam tool (https://web.expasy.org/protparam/) and potential disulfide linkage was predicted by DiAmino acid Neural Network Application (DiANNA) webserver (http://clavius.bc.edu/~clotelab/DiANNA/) [36, 37, 39]. The Modeller version 9.19 program was employed for homology modeling of SPSFQ protein structure based on the keratinase MtaKer from *Meiothermus taiwanensis* WR-220 (PDB code: 5WSL_A) and PROCHECK was performed to evaluate the stereochemical quality of the 3D model structure [40–42]. All graphical images of the SPSFQ model structure were generated using the PyMOL Molecular Graphic System (Schrodinger) [43]. Electrostatic surface analysis of subtilisin S8 proteases was calculated using APBS (Adaptive Poisson-Boltzmann Solver) Electrostatic plugin in Pymol software [43].

2.4 Expression of Recombinant Protease SPSFQ

The verified pGEM-*SPSFQ* sequence was sub-cloned into the expression plasmid, pET-22b (+) using a pair of primers: forward primer (5′-CGC GGA TCC GAT GGC AAA TCC TGT AAA TG-3′) reverse primer (5′-CTG CGT CGA CAG GAG ATT TAT ATA ACA AGC G-3′) with *Bam*H1 and *Sal*1 restriction sites incorporated (underlined). The resulting construct was designated as pET-*SPSFQ*. Confirmation of the positive clones was carried out via colony PCR and nucleotide sequencing. The expression construct pET-*SPSFQ* with the correct nucleotide sequence and reading frame was then transformed into the expression host, *E. coli* BL21 (DE3) for recombinant SPSFQ expression analysis.

A single colony of *E. coli* BL21(DE3) harboring the expression plasmid pET-*spsfq* was grown in LB broth and incubated at 37 °C for approximately18 hours with a shaking speed of 200 rpm. The overnight starter culture was diluted 100-fold into 50 mL LB broth containing carbenicillin (100 µg/mL) agitated at 37 °C. Preliminary expression analysis of SPSFQ was induced using a range of isopropyl β-D-thiogalactoside (IPTG) concentrations: 0.05, 0.1, and 0.2 mmol/L at OD_{600} value of 0.6 and incubated at 37 °C. After four hours post-induction, cells were harvested by centrifugation at 10,000×*g* and resuspended in cold lysis buffer (50 mM Tris–HCl, pH 8.0, and 250 mM NaCl). The cells were disrupted by the sonication method and the resulting extract was centrifuged at 12,000×g for 20 min under the cold condition to separate soluble cell fraction from the cell pellet. The cell lysates were analyzed on 12% SDS-PAGE and further confirmation using anti-6x-His mouse monoclonal antibodies (Thermo Scientific, #MA1-21315) in the Western blotting.

2.5 Protein Purification

Cell-free crude extract from 6 L culture was applied to a HiTrap HP (GE Healthcare) and eluted using 20 mM Tris–HCl pH 9.0, 300 mM Imidazole, NaCl 150 mM at a constantly increasing concentration of imidazole gradient (flow rate of 1.0 mL/min). Fractions containing positive proteolytic activity were pooled for ultrafiltration and buffer exchanged to a low-salt buffer (20 mM Tris–HCl pH 9.0) using a regenerated cellulose membrane with a 10 kDa pore size (Amicon Ultra, Millipore) before purification using a HiTrap Q HP anion exchange column. Recombinant SPSFQ was eluted from the HiTrap Q HP column using 20 mM Tris–HCl pH 9.0, 1 M NaCl at a constant flow rate of 1.0 mL/min. Fractions exhibiting protease activities were pooled to be concentrated and buffer-exchanged against low-salt buffer. 100% glycerol was added into the buffer containing concentrated protease to a final concentration of 20% glycerol to stabilize the purified recombinant SPSFQ. The sample was then kept at − 20 °C prior to protease characterization.

3 Enzyme Assay and Characterization

3.1 Caseinolytic Activity Assay

Five millilitre of 0.65% (w/v) casein powder dissolved in 50 mM potassium phosphate buffer (pH 8.0) and 1 mL

of purified recombinant SPSFQ (0.4 µM) was incubated at 37 °C for 30 min. The reaction was stopped with the addition of 5 mL of 15% (w/v) trichloroacetic acid for 30 min at 37 °C. The reaction mixture was centrifuged at 11,000 g for 1 min and 2 mL of the reaction mixture was transferred into new containers to be incubated with 5 mL 0.5 M Na_2CO_3 and 1 mL 50 mM Folic Ciocalteau reagent (Sigma Aldrich; St Louis, MO, USA) for 30 min at 37 °C. The color change was measured using a spectrophotometer at 660 nm against blank prepared in the same manner. One unit of proteolytic activity was defined as the amount of enzyme needed to release 1 µmol of tyrosine per min under the assay conditions.

3.2 Effects of Temperature, pH, Metal Ions, Protease Inhibitor, Surfactants, Denaturants and Organic Solvent on Recombinant SPSFQ Activity

The optimum temperature for SPSFQ protease activity was determined by calculating relative activity after assaying ~ 0.4 µM mL^{-1} of the enzyme at various temperatures ranging from 20 to 60 °C. The relative activity of the enzyme was determined by measuring the amount of tyrosine released underspecified assay conditions and the data were expressed in percentage (%). A temperature profile of the relative activity versus temperature was constructed by taking enzyme activity at the optimum temperature as 100%. To evaluate the optimum pH, the enzyme activity of the purified protease SPSFQ (~ 0.4 µM mL^{-1}) was measured at 37 °C under a pH range of 4.0–11.0. Casein solutions were prepared using the following buffer system: 50 mM of sodium acetate buffer, pH 4.0–5.0; Tris–HCl buffer, pH 6.0–7.0; potassium phosphate, pH 8.0–9.0 and Glycine–NaOH, pH 10.0–11.0, respectively.

The effects of metal ions on SPSFQ enzyme activity were studied by pre-incubating the enzyme in the presence of various monovalent (Na^+ and K^+) and divalent (Mg^{2+}, Ca^{2+}, Zn^{2+}, and Cu^{2+}) metal ions at a concentration of 5 mM for 30 min at 37 °C. Recombinant SPSFQ activity was assayed in the presence of protease inhibitors (phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and β-mercaptoethanol) at concentrations of 1 and 5 mM; surfactants, and denaturant (urea, sodium dodecyl sulfate (SDS)) at concentrations of 1 and 5 mM, Tween-20 at concentrations of 0.5% and 1.0%, v/vas well as organic solvents (methanol, ethanol, and isopropanol) at a concentration of 5%. The purified SPSFQ enzyme was briefly pre-incubated with selected reagents as mentioned above for 30 min at 37 °C. The assay was carried out using the caseinolytic assay protocol as described previously. Their relative activities were recorded and expressed in percentage (%).

4 Substrate Specificity Assay

4.1 Proteolytic Activity on Different Protein Substrates

The catalytic activity of purified SPSFQ was assayed with various protein substrates: casein, gelatin, fibrin (0.65% w/v) following standard protease assay as explained in *Caseinolytic Activity Assay*. The catalytic activity towards the simplest substrate, casein was used as control (100%).

4.2 Keratinolytic Activity Assay

Keratinolytic activity of purified SPSFQ was assayed using keratin azure K 8500 as a substrate and by following standard keratinase assay protocol (Sigma Aldrich). The reaction mixture for keratin degradation assay consisted of 20 mg of keratin azure, 4 mL of 50 mM sodium phosphate solution, and 1 mL of 1 µM of the purified SPSFQ sample. The keratinolytic reaction was incubated at 37 °C for 1 h to allow color development. The degradation of the modified substrate was recorded quantitatively by measuring the absorbance at 595 nm, using Proteinase K as a standard. One unit of keratinolytic activity was defined as the amount of enzyme required to increase absorbance by 0.01 at 595 nm, within 1 h at 37 °C.

4.3 Collagenolytic Activity Assay

Recombinant SPSFQ was assayed for collagenolytic activity with azo dye-impregnated collagen (Sigma # A4341) as substrate. The reaction mixture for collagen degradation assay consisted of 25 mg of azo dye impregnated collagen, 4 mL of 0.1 M potassium phosphate solution, and 1 mL of 1 µM purified SPSFQ. The reaction mixture was incubated for 1 h at 37 °C under constant shaking. Colour change was recorded quantitatively by measuring the absorbance at 520 nm, using trypsin as a standard. One unit of collagenolytic activity was defined as the amount of enzyme required to increase absorbance by 0.01 at 520 nm, within 1 h at 37 °C.

5 Results

5.1 Cloning and Sequencing of *SPSFQ* Gene

Sequencing of a gene isolated from *Acinetobacter baumannii* TU04 revealed an 1104-bp nucleotide sequence encoding an open reading frame of 368 amino acid residues (Fig. 1). Sequence analysis using the Blast program against the NCBI

database indicated that the protein has a high percentage identity of 97–99.5% to serine protease, an extracellular serine protease, subtilase family protein, and subtilisin-like serine protease of different *Acinetobacter* strains (Table 1). The phylogenetic analysis clustered the SPSFQ protein in the *Acinetobacter* clade of subtilisin-like serine proteases group with *Acinetobacter baumanni* ACICU which is a multidrug-resistant clinical strain as its closest neighbor (Fig. 2) [44].

Table 1 BLAST analysis of SPSFQ serine protease from *Acinetobacter baumannii* TU04

Identification	Protease type	Amino acid identities	Identity (%)
Serine protease	Serine	367/368	99.5
Extracellular serine proteinase	Serine	364/368	98.9
Subtilase family protein	Serine	363/368	98.6
Subtilisin-like serine protease	Serine	363/368	98.6
Alkaline serine exoprotease A	Serine	360/368	97.8

5.2 SPSFQ Primary Sequence and Domain Analysis

The NCBI CDD domain analysis divided the deduced SPSFQ amino acid sequence into two major regions, an Inhibitor I9 domain (Q18-D90) and Peptidase S8 domain (A91-P368) (Fig. 1 and 3). These two domains are unique features of serine peptidases grouped under the S8A subfamily of S8 or often known as the subtilase family in clan SB (subtilisin-like serine protease) based on the classification in the MEROPS database [45]. The inhibitor I9 region or usually termed as pro-domain was found to carry a specific inhibitory activity against the adjacent peptidase S8 domain until it is auto-catalytically cleaved during enzyme maturation [46, 47]. This domain was also found to act as a molecular chaperon to assist the proper folding of mature serine peptidases into their active conformation [48–50]. As the inhibitor I9 domain has an only inhibitory function, consequently in this study, amino acid multiple sequence alignment analysis and homology modeling studies were focused mainly on the catalytically relevant Peptidase S8 domain. Multiple sequence alignment of SPSFQ amino acid was performed against a protease from a clinical isolate, *Acinetobacter baumannii* strain 1656-2, and closely related serine proteases from other bacteria with known Inhibitor I9 and Peptidase S8 domains and percentage amino acid identity range of between 38 and 99% to SPSFQ (Table 2).

The amino acids of the peptidase S8 region of SPSFQ contained conserved features as highlighted in the multiple

```
       -10        -20        -30 +1                  18          29
MKYLLPTAAA GLLLLAAQPA MAMDIGINSD PMANPVNDSS QAKGIIKNQY IVILNKDAGP

        39         49         59         69         79          89
SKDFAQNIAK QHAGKVLQSY DTVLKGFAIY LPDTAGTAFI EAMKKNPHVL SVESDTIVNI

        99        109        119        129        139         149
DATTQNNPDW GLDRIDQKAL PLNSAYSYLQ TGSGTTAYIV DTGILSSHQE FSGRVLSGYT

       159        169        179        189        199         209
AISDGNGTTD CNGHGTHVAG TVGGTTYGVA KNVNLVPIRI LGCDGSGASS NVIAGLDWIL

       219        229        239        249        259         269
KNGKKPAVVN MSLGGATSSS LDSAVENLYN NGYVMVAAAG NSNTDACTSS PARVSKAITV

       279        289        299        309        319         329
AATDNTDTRA SYSNYGSCVD IFAPGSQINS SWIGSNTATK ILNGTSMATP HVAGVVAEML

       339        349        359        369        379
QSTPTASPQT ISTNLLNQAS SNVVKNPSGS PNRLLYKSPV DKLAAALEHH HHH
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Fig. 1 The amino acid sequence of the cloned SPSFQ from *Acinetobacter baumannii* TU04. The predicted inhibitor I9 domain (Q18-D90) and adjacent Peptidase_S8 domain (A91-P368) are labeled as underlined and in italic, respectively. The N-terminal and C-terminal expression vector cassette are labeled in bold

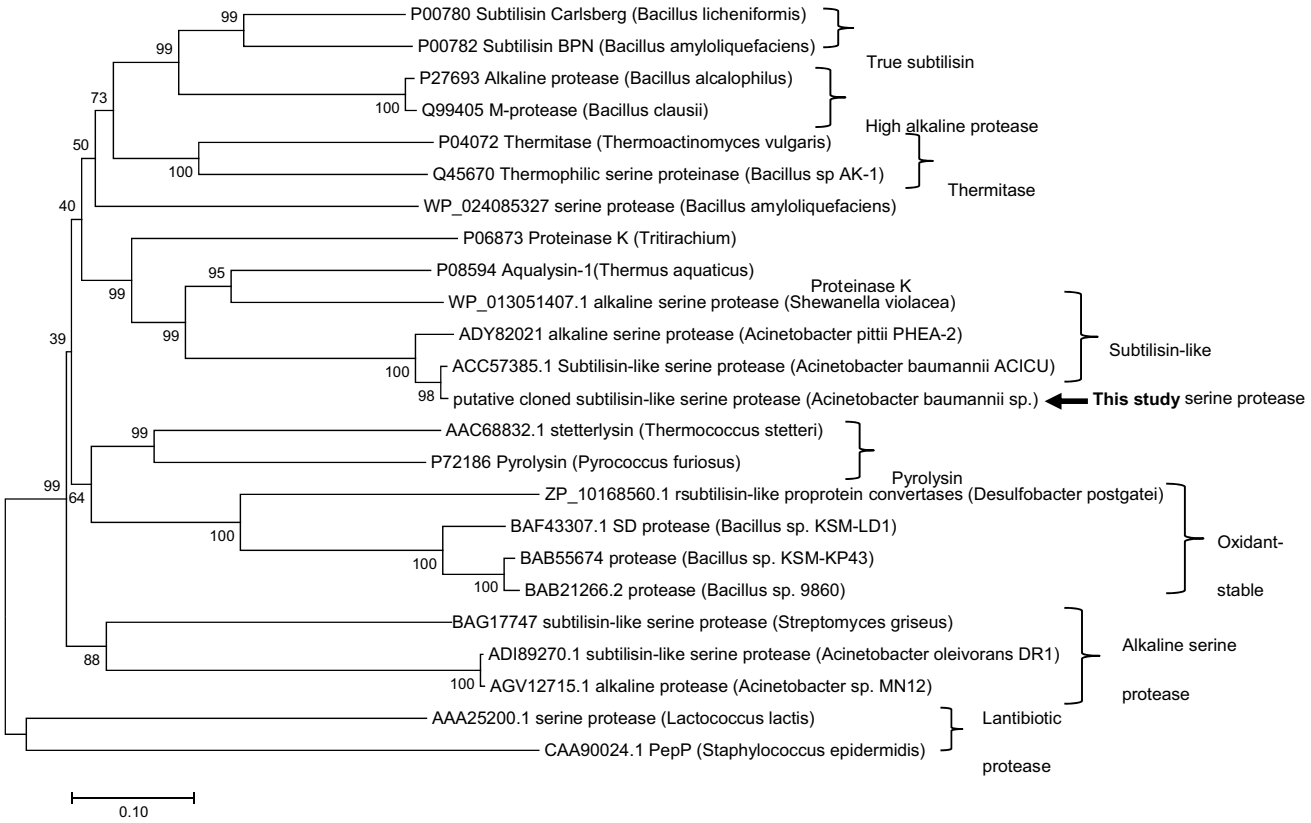

Fig. 2 Phylogenetic tree of SPSFQ from *Acinetobacter baumannii* TU04 based on protein sequence representing different clusters of protease family. The evolutionary analysis was conducted by using MEGA version 5.0 software

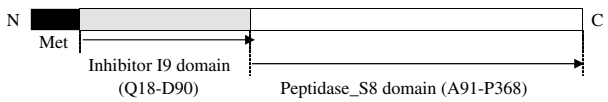

Fig. 3 Schematic diagram of predictive domain structure of SPSFQ represents the position of N-terminal Inhibitor I9 and Peptidase_S8

sequence alignment (Fig. 4). The catalytic triad consisting of Asp40, His73, and Ser225 and a catalytic subsite, Asn160 termed as an oxyanion hole of SPSFQ are well aligned with other proteases. Based on the catalytic mechanism of serine peptidases, the hydroxyl group of catalytic Ser225 serves as a catalytic nucleophile to attack the scissile peptide bond of the substrate while His73 acts as proton donor and acceptor, and Asp40 is believed to assist in the proper orientation of His imidazole ring during the process [51, 52]. The

Table 2 Comparison of amino acid sequence of mature SPSFQ with other serine proteases from bacteria and fungi are generated using Clustal Omega identity matrix analysis

	Identity (%)							
	1	2	3	4	5	6	7	8
1. SPSFQ *Acinetobacter baumannii* TU4	100	99.3	99.3	59.9	47.3	42.3	40.5	38.2
2. Extracellular serine protease *Acinetobacter baumannii* 1656-2 (ADX04172)		100	100	59.9	48.0	42.4	40.9	38.6
3. Extracellular serine protease *Klebsiella pneumoniae* (SSW74921)			100	59.9	48.0	42.4	40.9	38.6
4. Keratinase *Meiothermus taiwanensis* WR-220				100	49.8	47.1	45.9	44.4
5. Destructin-1 *Pseudogymnoascus destructans*					100	53.7	51.8	54.7
6. Proteinase K *Tritirachium album Limber*						100	62.0	65.1
7. Cuticle-degrading PL646 *Paecilomyces Lilacinus*							100	72.0
8. Cuticle-degrading Ver112 *Verticillium Psalliotae*								100

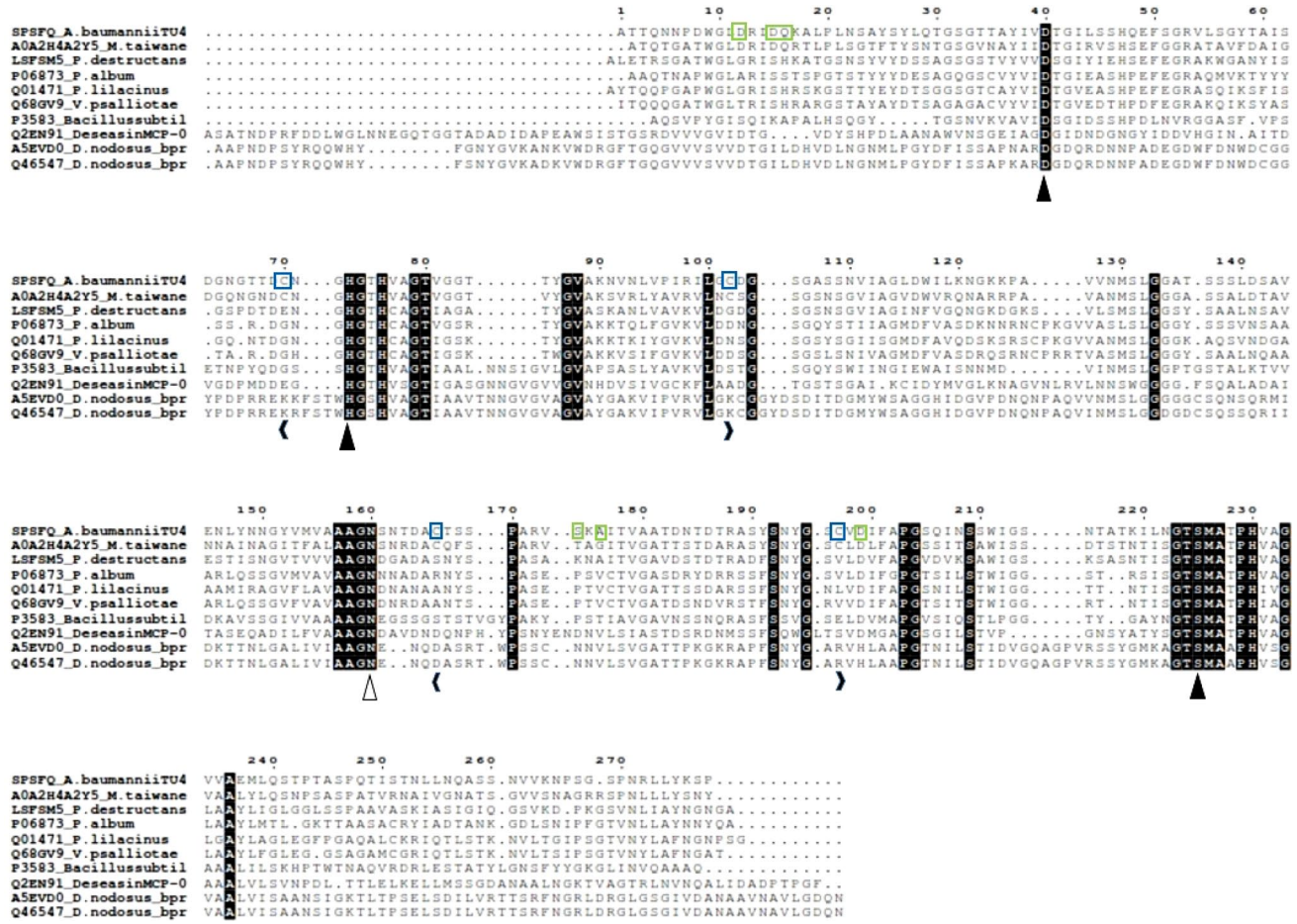

Fig. 4 Comparative amino acid alignment of SPSFQ with subtilisin-like serine proteases from different organisms: *Meiothermus taiwanensis* Wr-220 (A0A2H4A2Y5); *Pseudogymnoascus destructans* (LSFSM5); *Parengyodontium album* (P06873); *Paecilomyces lilacinus*, PL646 (Q01471), *Verticillium psalliotae*, Ver112 (Q68GV9); fibrin-degrading nattokinase from *Bacillus subtilis* (P3583); marine *Pseudoalteromonas sp* (Q2EN91); *Dichelobacter nodosus* (A5EVD0 and Q46547). The regions of amino acids with black background and white foreground are completely conserved.The catalytic triad Asp40, His73, Ser225 and oxyanion hole Asn160 (SPSFQ numbering) are highlighted with black and open circle, respectively. The cysteine pairs participated in the disulphide bridge are marked with < and > (< Cys70-Cys102 > and < Cys166-Cys197 >). The predicted calcium-binding sites for SPSFQ are also highlighted in green boxes. The illustration is generated by ESPript (http://espript.ibcp.fr) [84]

stabilization of tetrahedral intermediate generated during the reaction steps is contributed by Asn160 by forming a structural component of oxyanion hole with catalytic serine [52]. The presence of catalytic triad residues in the order of Asp-His-Ser and Asn to form the oxyanion hole is conserved within the S8 subfamily yet different from the S53 subfamily although they are grouped under the same clan SB of serine peptidases [45, 52]. The SPSFQ sequence is likely to contain two calcium-coordination sites at positions Asp12, Asp15, Gln16, and Ser174, Ala176, Asp199 respectively (Fig. 4). In the peptidases S8 subfamily, the binding of calcium ion to one or more calcium-binding sites in the enzyme molecule is essential for correct folding and structure stability besides enhancing the thermal stability of the proteases which contribute to their resistance against proteolysis, either by itself or by other proteases [42, 53, 54].

5.3 3D Structure of SPSFQ Model

In order to further understand the catalytic properties of the active form of SPSFQ, we generated a structural model of SPSFQ without the Inhibitor I9 region. Based on known 3D structures extracted from Protein Databank Database (PDB), SPSFQ (A91-P368) showed a percentage sequence identity of 61% to a thermophilic keratinase from *M. taiwanensis* WR-220, MtKer (PDB code: 5WSL) that was then selected as the template for homology modeling [42].

The resulting homology model of SPSFQ was evaluated for its quality utilizing several model evaluation servers (Table 3). First, the Phi/Psi Ramachandran plot of the PROCHECK tool was used to assess the backbone phi and psi dihedral angles of the model [55]. PROCHECK analysis results showed that 97.8% of residues from the model

Table 3 Model evaluation summary using different tools

Model evaluation tool	Evaluation scheme	Score result (%)	Normal range of the score (%)
PROCHECK	The number of amino acids in the allowed region based on Psi/Phi Ramachandran Plot	97.8	> 90
VERIFY3D	The number of amino acids having an average 3D-1D score above 0.2	100	> 80
ERRAT	The overall quality for nonbonded atomic interactions	90.1	> 50

were scored in the favored and allowed region indicating a good stereo-chemical quality of the model. Similar results were achieved when the SPSFQ model was analyzed using VERIFY3D which showed that 100% of residues displayed an average 3D-1D score above 0.2, beyond the satisfactory score of 80% [56, 57]. The overall quality for nonbonded atomic interactions of the SPSFQ model was also evaluated by the ERRAT program by comparing the statistics of the SPSFQ model with that of highly refined structures [58]. An ERRAT score of 90.1% was achieved which is greater than the acceptable score of 50%. These structure validation analyses indicated that the SPSFQ homology model is of good quality and reliable, meeting the standard protein structure stereo-chemical geometry requirements.

The structural features of the model are composed of 10 β-strands, 8 α-helices and connecting loops resembling a typical architecture of subtilisin-like α/β domain with parallel β sheets occupying the central region of the structure, sandwiched between two layers of α helices (Fig. 5) [59, 60]. Two calcium ions, as well as two disulfide bridges were found associated with the structure of the model template, MtKer (5WSL) [42] and this correlated with the positions of amino acids predicted to occupy the calcium-binding sites and amino acids predicted to form disulfide bridges in the SPSFQ model (Fig. 5). The superposition between SPSFQ and 5WSL with a close-up view of the catalytic triad and oxyanion hole residues is illustrated in Fig. 6. This highly identical geometrical arrangement of catalytic triad Asp-His-Ser in the α/β-elements of the protein structure served as a distinct feature of SPSFQ as a new member that falls under the S8 subfamily (subtilase) of the subtilisin-like serine protease superfamily [45, 51].

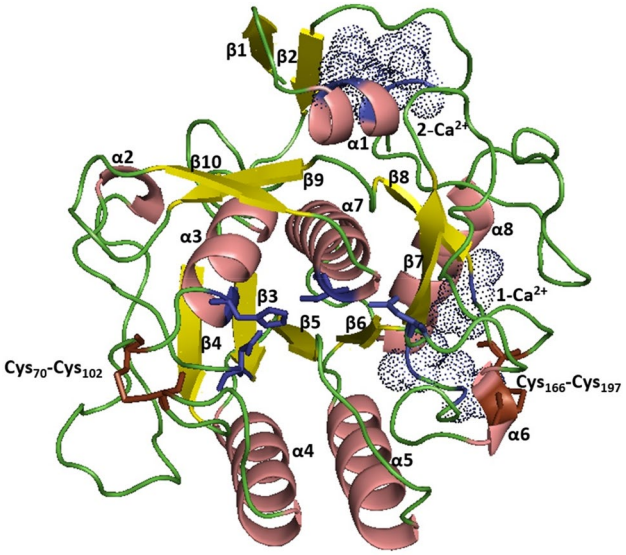

Fig. 5 Ribbon-plot representation of the secondary elements and theoretical tertiary structure of the S8 domain of SPSFQ. The α helices, β strands and loops are colored salmon red, yellow and green, respectively. Side chains of the catalytic triad (Asp40-His73-Ser225) and Asn160 are shown as blue *sticks*. The predicted primary and secondary Ca2 + binding site are rendered in blue dotted surface. The potential cysteine pairs for disulphide bridge are shown as brown *sticks*

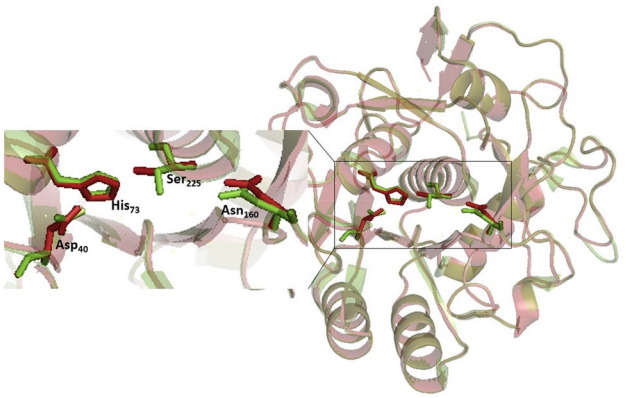

Fig. 6 Superposition of SPSFQ (red) and its template, 5WSL (green), and a close-up view of the catalytic triad and oxyanion hole (SPSFQ numbering) (color figure online)

5.4 Recombinant Expression and Purification of Recombinant SPSFQ

Bioinformatics analysis indicated that the SPSFQ sequence consists of two major domains; the N-terminal inhibitor I9 (pro-domain) and peptidase S8 domain carrying the conserved catalytic triad of Asp-His-Ser that belongs to the S8A subfamily peptidases. To investigate whether these domains exhibited functional characteristics of a serine protease, an expression plasmid of SPSFQ was constructed in a pET22b

vector containing both N-terminal pro-domain and peptidase S8 domain linked by a C-terminal 6xHis-tag for recombinant expression in *E. coli* system with a calculated molecular weight at 42,897 Da.

During preliminary expression analysis of IPTG-induced crude lysates, the apparent molecular weight of recombinant SPSFQ was determined to be approximately 30 kDa when immunoblotted with 6xHis tagged mouse monoclonal antibodies (Fig. 7a). Compared to uninduced cell lysates, only the IPTG-induced samples showed the proteolytic ability by digesting the 1% casein and skimmed milk substrates (Fig. 7b and c) which indicated the presence of recombinant protease activity. The discrepancy of the size observed here might due to the occurrence of autocatalytic processing of N-terminal inhibitor I9 domain with an approximate size of 12.8 kDa (based on amino acid sequence prediction) that was cloned together during the construction of SPSFQ expression plasmid (Fig. 1). The subtilisin family members of bacterial extracellular serine proteases shared similar N-terminal primary structure characteristics containing the pro-domain which on cleavage, assist in the folding of the adjacent catalytic peptidase domain to its mature active conformation [17, 18, 61].

5.5 Purification of Recombinant SPSFQ

Crude extract from twelve gram of cell biomass was initially purified on a HiTrap HP column and SPSFQ eluted at an imidazole concentration of 100–150 mM. Further purification was successfully carried out on a Q-sepharose column and SPSFQ was eluted at NaCl concentrations of 579 to 683 mM. The purified SPSFQ was concentrated further using a Vivaspin centrifugal filter with a molecular weight cut-off of 10 kDa and this yielded pure SPSFQ at a final concentration of 1.18 mg/mL. A typical purification table for purification of SPSFQ is presented in Table 4 and the SDS-PAGE image of the purified SPSFQ is shown in Fig. 8.

5.6 Characterization of Purified SPSFQ

In order to further understand the catalytic properties of SPSFQ, physicochemical parameters for SPSFQ activity were characterized. SPSFQ remained catalytically active over a range of temperatures varying from 20 to 70 °C, at pH 8. The optimum reaction temperature for SPSFQ was observed at 40 °C, above which the activity began to decline sharply where it almost halved at 50 °C, further decreased to almost 15% at 60 °C and was almost completely abolished at 70 °C (Fig. 9). There was a steady increase in SPSFQ activity from pH 4–8 and the activity peaked at pH 9 (100%) however, a further increase in pH beyond 9 decreased the enzymatic activity (Fig. 10). The influence of metal ions on protease activity is summarized in Fig. 11. Some metal ions are believed to have a stimulatory or inhibitory effect on enzyme activity. The metal ions tested did not significantly show any appreciable inhibitory effects as they caused only a minimal decrease in SPSFQ enzyme activity.

The effects of protease inhibitors; PMSF, EDTA, urea, and 2-mercaptoethanol at concentrations of 1 and 5 mM on enzyme activity were also investigated. While EDTA (metalloprotease inhibitor) and 2-mercaptoethanol had no significant inhibitory effects on the proteolytic activity of SPSFQ,

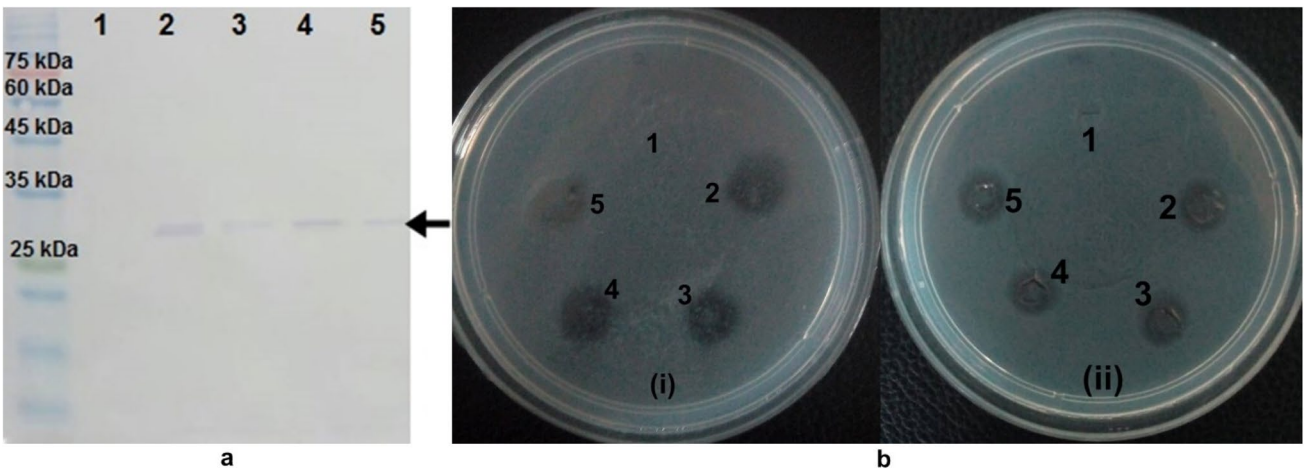

Fig. 7 Analysis of SPSFQ expression at 37 °C with different IPTG concentrations. **a** Confirmation of 6x-His tagged recombinant SPSFQ by immunoblotting analysis. Lanes 1- uninduced BL21 (DE3) host, 2- uninduced SPSFQ, 3 to 5- induced SPSFQ at 0.05, 0.1, 0.2 mM IPTG, respectively. **b** Paper disc-agar diffusion assay for protease activity. Disc filter papers were infused with equal volume (20 µl) of crude cell lysates and placed on agar plate containing 1% of different protein substrates (i) casein (ii) skim milk. For better demonstration of protease activities, the disc papers were removed after overnight incubation at 37 °C. The disc positions are labeled 1- uninduced BL21 (DE3) host, 2- uninduced SPSFQ, 3 to 5- induced SPSFQ at 0.05, 0.1, 0.2 mM IPTG, respectively (color figure online)

Table 4 Purification summary of recombinant SPSFQ from *Acinetobacter baumannii* TU04 (SPSFQ)

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude Lysate	4807.2	17,120.09	3.56	1	100
HiTrap HP	10.29	1105.62	107.45	30.170	6.458
Q Sepharose	2.43	461.52	189.93	53.330	2.696
Vivaspin 10 kDa	2.08	469.26	225.33	63.272	2.741

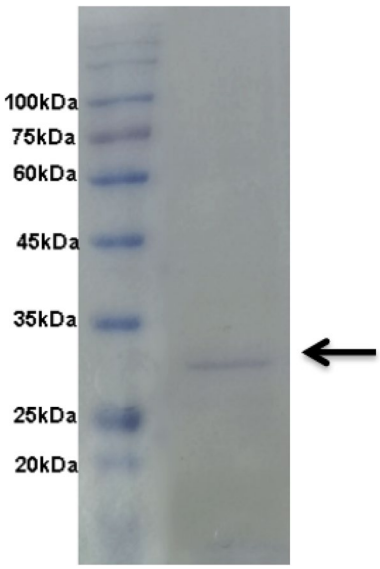

Fig. 8 12% SDS page gel of purified SPSFQ. Lane 1: Protein marker; Lane 2: SPSFQ protein band after anion exchange chromatography purification (indicated with arrow) (color figure online)

1 and 5 mM PMSF (serine protease inhibitor) caused a significant reduction to SPSFQ activity by approximately 74% and 99%, respectively (Fig. 12). The effect of 1 and 5 mM of the anionic detergent SDS and 0.5% and 1% of the non-ionic detergent Tween 20 on enzyme activity is shown in Fig. 12. Interestingly, a low concentration of urea (5 mM) is enough to distort the structural integrity of SPSFQ, thus exhibiting a significantly lower relative activity of 17.71%. Relative activities recorded by other inhibitors are also summarized in Fig. 12. Although ionic surfactants (cationic and anionic) were reported to disrupt the structural integrity of most proteases, SPSFQ was observed to be able to retain 90.25 and 87.78% activity after incubation with 1 mM and 5 mM of SDS respectively at 37℃ for 30 min. However, 0.5 and 1.0% Tween-20 (non-ionic surfactants) conferred mild inhibitory effects to the enzyme with a reduction of the enzyme activity to 79% and 86.34%, respectively.

One of the crucial features of secreted serine protease is that the enzymes have evolved to confer broad substrate preference in order to accommodate versatile biological roles in the external environment. Thus, it is expected for extracellular proteases to be able to hydrolyze more than one proteinaceous substrates albeit with varying degrees of specificity. Furthermore, primary sequence analysis showed that SPSFQ shared moderately high amino acid similarity to other reported bacterial proteases that conferred catalytic activity towards tissue-associated protein components. Therefore, the enzymatic activities of SPSFQ on casein and other tissue-associated proteinaceous substrates such as gelatin (denatured collagen), keratin, collagen, and fibrin were assayed. Results presented in Table 5 indicated that besides casein, purified SPSFQ was able to hydrolyze a

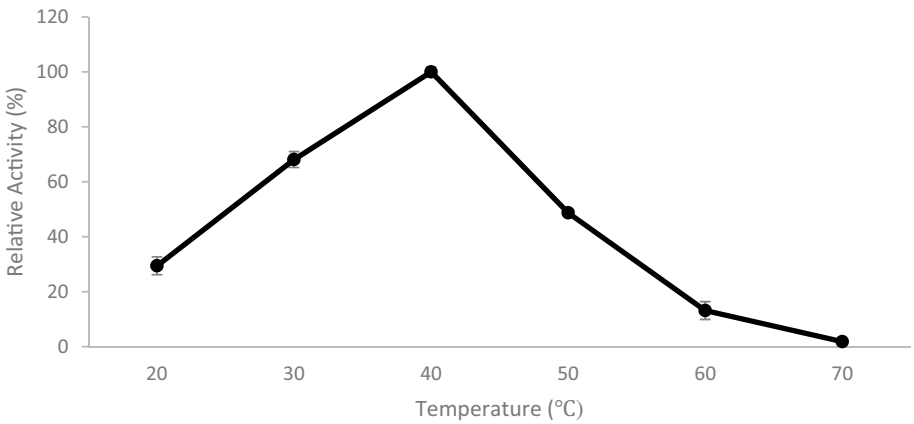

Fig. 9 Influence of temperature on the activity of purified SPSFQ from *Acinetobacter baumannii* TU04. Protease assay was carried out at various temperatures (20 to 70 °C) for 30 min at pH 8. Mean values are plotted with error bar representing the mean standard deviation of triplicates in each individual experiment (color figure online)

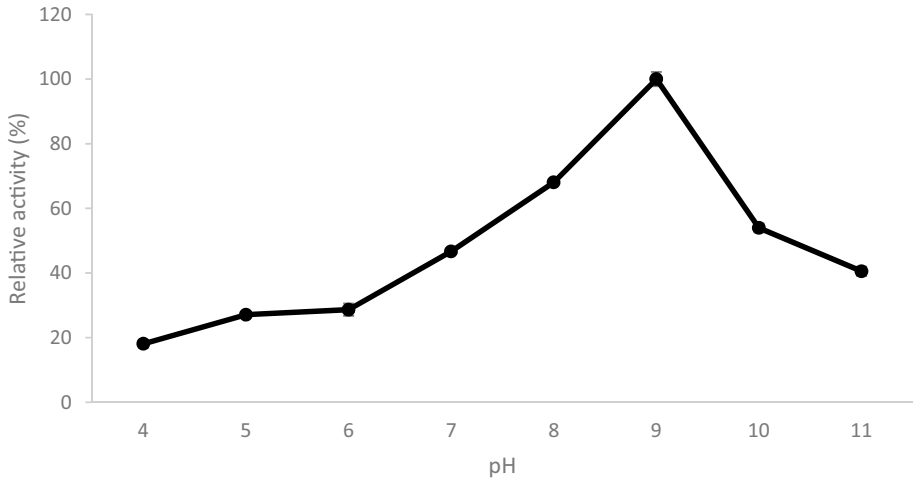

Fig. 10 Influence of pH on the activity of purified SPSFQ from *Acinetobacter baumannii* TU04. Protease assay was carried out at various pH (4–11), for 30 min. Mean values are plotted with error bar representing the mean standard deviation of triplicates in each individual experiment (color figure online)

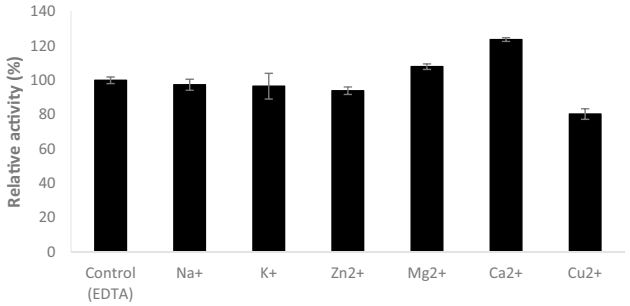

Fig. 11 Influence of metal ions on the activity of purified SPSFQ from *Acinetobacter baumannii* TU04. Protease assay was carried out by pre-incubated reaction mix with several metal ions for 30 min. Mean values are plotted with error bar representing the mean standard deviation of triplicates in each individual experiment (color figure online)

variety of natural/modified complex protein substrates i.e. denatured collagen (gelatin), fibrin, azure keratin, and azocoll (azo dye-impregnated collagen). The highest SPSFQ activity was observed for casein at 92 U/mL followed by to a much lesser degree; gelatin and azure keratin with activity values of15.871 U/mL and 9.375 U/mL respectively. SPSFQ showed low catalytic activity towards fibrin (1.089 U/mL) and Azocoll (0.037 U/mL).

6 Discussion

Amino acid sequence and domain analysis of SPSFQ indicated that SPSFQ is a serine protease grouped under the S8 subfamily of clan SB (subtilisin-like serine protease). This is further reinforced by the structural analysis of the SPSFQ model that showed identical amino acid and geometrical arrangement of important catalytic sites to its homology

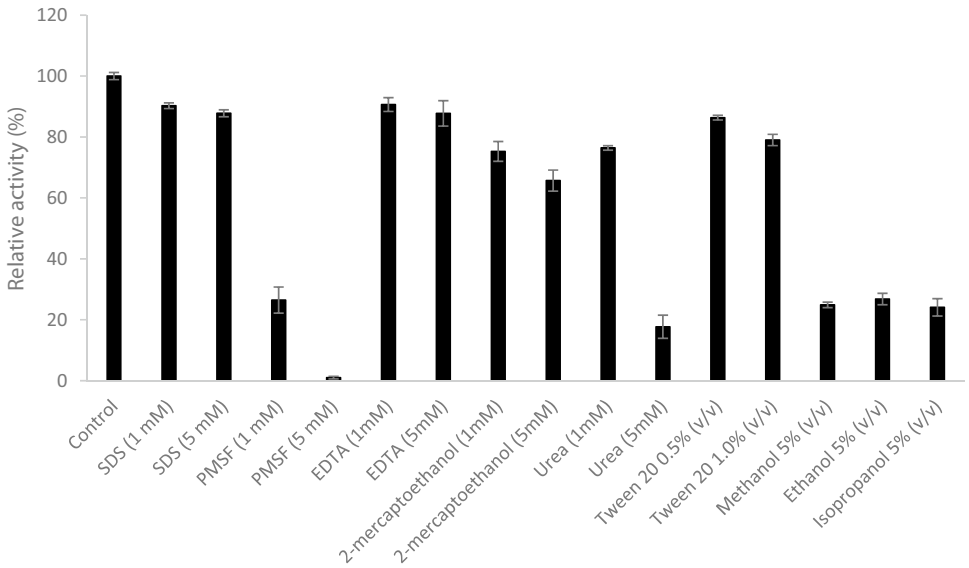

Fig. 12 Influence of various reagents on activity of the purified recombinant protease from *Acinetobacter baumannii* TU04. Mean values are plotted with error bar representing the mean standard deviation of triplicate in each individual experiment (color figure online)

Table 5 Substrate specificity profile of the purified SPSFQ from *Acinetobacter baumannii* TU04

Substrate	Concentration (w/v) (%)	Monitoring wavelength (nm)	Activity enzyme (U/mL)
Natural substrate			
Casein	0.65	660	92.304
Gelatin	0.65	660	15.871
Fibrin	0.65	660	1.089
Modified substrate			
Azure Keratine	0.44	595	9.375
Azocoll	0.49	520	0.037

modeling template, 5WSL which is also a protease from the peptidase S8 subfamily. The amino acid sequence and structural features observed in both SPSFQ and MtKer crystal structure (5WSL) were unique to the peptidase 8 subfamily. Both enzymes displayed two calcium ion binding sites which are crucial for the correct folding and structural stability of the enzymes. SPSFQ also displayed two predicted disulfide bridges at identical sites to that of MtKer structure (5WSL). Previous studies on peptidases have shown that disulfide bonds can increase thermal stability and breaking the disulfides with reducing agents lead to inactivation as well as a significant reduction in the thermostability of the enzymes [42, 62, 63].

Further analysis of SPSFQ amino acid sequence homology showed that it shared the highest percentage identity of 99% to extracellular serine proteases from clinical isolates, *Acinetobacter baumannii* 1656-2 (ADX04172) and *Klebsiella pneumoniae* (SSW74921); followed by 59.9% sequence identity to keratinase from *Meiothermus taiwanesis* (MtKer) and 47.3% to Destructin-1 from *Pseudogymnoascus destructans*. *Acinetobacter baumannii* 1656-2 is a medically relevant strain that was shown to produce sturdy biofilm and has multiple-drug resistance [64]. Interestingly, SPSFQ was also shown to be almost identical to an extracellular serine protease from another pathogenic bacteria, *Klebsiella pneumoniae*. *Acinetobacter baumannii* and *Klebsiella pnuemoniae* are both nosocomial pathogens that can cause ventilator-associated pneumonia and sepsis [65]. More interestingly, both bacteria were reported to cause dual infection in patients with multiple comorbidities where the two strains were reported to grow entwined in a colony presented as a single morphology that required repeated cultures to separate [66]. However, the functional properties of the two proteases could not be compared further as the protease from *Klebsiella pnuemoniae* has not been characterized.

MtKer and Desctructin-1 are both proteases with the ability to degrade mammalian structural proteins that are the main components of tissues and skin i.e.; keratin and collagen respectively. In particular, Destructin-1 is the enzyme implicated in the white-nose disease that affects bats in North America. The enzyme that is secreted by the psychrophilic fungus, *Pseudogymnoascus destructans* causes extensive damage in the subcutaneous tissues which results in ulcerative necrosis and tissue destruction [67]. The recombinant form of keratinase from *Meiothermus taiwanesis* (MtKer) possessed keratinolytic activities at temperatures ranging from 25 to 75 °C and pH from 4 to 11, with a maximum keratinolytic activity at 65 °C and pH 10 [42]. Wu and co-workers proposed that keratinase activity of a protease can be predicted based on overall structural similarity to the crystal structure of MtKer (5WSL) and went on to prove their hypothesis by successfully showing that two proteins with similar structural fold to MtKer, aqualysin I from *T. aquaticus* YT1 and peptidase from *D. radiodurans* R1 both possessed strong keratinase activity despite having only moderately high sequence identity of 59.7% and 53.7% respectively to MtKer [42].

Recombinant SPSFQ was expressed as an active 30 kDa protein which is smaller than the calculated molecular weight of 42,897 Da. This result was similar to that of a recombinant alkaline serine protease isolated from *Acinetobacter* sp. IHBB B 5011 (MN12) was expressed as a 35 kDa biologically active protease upon maturation of its larger precursor protein that had a molecular weight of 50 kDa [31]. It has been well-documented that extracellular subtilisin-like proteolytic enzymes of the S8A subfamily are initially synthesized as an intermediate precursor before undergoing activation by intramolecular auto-proteolysis of its pro-peptide to form the mature and active protease [42, 46, 51, 68, 69]. SPSFQ showed optimum activity at pH 9 in accordance with other previously reported serine proteases that mostly displayed optimum activity at neutral to alkaline pH [70]. The addition of Ca^{2+} and Mg^{2+} showed stimulatory effects on SPSFQ activity and this reflected similar results from other serine proteases from bacteria such as *Geobacillus toebii* and *Geobacillus sp*. YMTC 1049 [71]. Susceptibility to the inhibitory effect of PMSF displayed by SPSFQ also confirmed that it is a serine protease as PMSF is generally known to confer irreversible inhibition effects on serine proteases by sulphonating the reactive serine residue of a protease thus, suppressing the catalytic activity of the enzyme [72]. SPSFQ also showed catalytic stability in the presence of sodium dodecyl sulphate (SDS) which is a surfactant often used in detergents and cleaning agents. This coupled with its optimum activity under alkaline pH indicates the potential application in detergents like many other reported extracellular subtilisins [73, 74].

SPSFQ showed varying degrees of catalytic activities towards different protein substrates. The enzyme showed the highest activity towards casein but much lower activities for more complex substrates such as gelatin, azure keratin, fibrin, and azo-collagen. The varying degree to which

SPSFQ was able to degrade tissue-associated protein substrates may be explained by the properties of protein substrates, including hydrophobicity and surface charge which are two factors influential in determining the catalytic efficiency of proteases [54, 75]. Liu and co-workers described the association between electrostatic surface potential of cuticle-degrading proteases with diffusion and adsorption efficiency of the enzymes toward the negatively charged cuticles [54, 76]. In order to investigate this further, comparison of the electrostatic surface potential of the SPSFQ model with several other crystal structures and 3D models of subtilisin S8 enzymes that had been shown to have strong degrading capabilities for either keratin, fibrin, collagen, cuticle, or extracellular matrices (ECM) such as fibronectin, insoluble elastin, and hoof materials was conducted [42, 67, 75, 77, 78]. In Fig. 13a, the substrate-binding cleft of SPSFQ showed uniform distribution of positive and negative charges, with the less neutral region. This is different from the keratin degrader, MtKer (Fig. 13b) which showed a more neutral region with some patches of negative and positive charges at the substrate-binding cleft. Interestingly, a predominantly negative charge on substrate-binding cleft can be observed in increasing order for proteases degrading the following substrates: fibrin (Fig. 13c), ECM (Fig. 13i), cuticle (Fig. 13d and e) and collagen (Fig. 13g and h). Several reports also emphasized that the key properties of selectivity in proteolytic cleavage by subtilisin-like proteases are determined by the nature of residues occupying the S1 substrate binding cleft [54, 75, 77–80]. Sequence alignment of subtilisins in complex with either inhibitor, pro-peptide, or peptide fragments indicated that the small, neutral amino acid serine at position 161 in SPSFQ may compromise its ability to degrade collagen and fibrin because proteases that can degrade those substrates have charged amino acids Asp or Glu at the same positions in the S1 substrate binding cleft (Fig. 14).

Other subtilisin S8 proteases including keratinase from *Meiothermus taiwanensis* WR-220, proteinase K from *Tritirachium album*, cuticle-degrading enzymes from *P. lilacinus* and *L.psalliotae* were reported to exhibit high catalytic activity towards their respective proteinaceous substrates; keratin, cuticle extract, and collagen, but at a higher optimum temperature between 50 and 65 °C [42, 77]. These observations correlated with the enzymes' structures that

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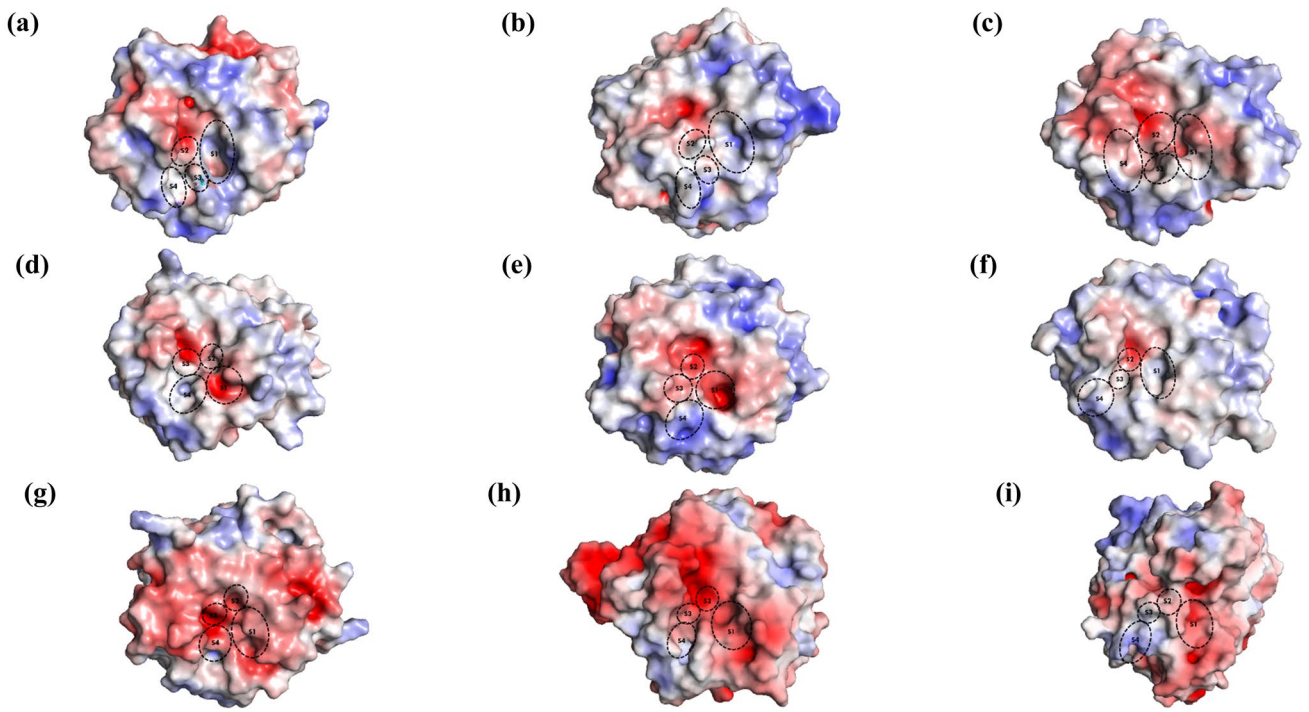

Fig. 13 Molecular electrostatic surface potentials of subtilisin-like serine proteases: **a** SPSFQ, **b** Keratin degrading -5WSL, **c** Fibrinolytic-5GL8, **d** Cuticle degrading-3F7M, **e** Cuticle degrading-3F7O, **f** Proteinase K-1IC6, **g** Collagen degrading-Destructin, **h** Collagen degrading-3VV3, **i** extracellular matrix degrading-3TI9. The neutral, positive and negative charged electrostatic surfaces are coloured white, blue and red, respectively. The approximate location of substrate binding cleft; S1, S2, S3, S4 are indicated (color figure online)

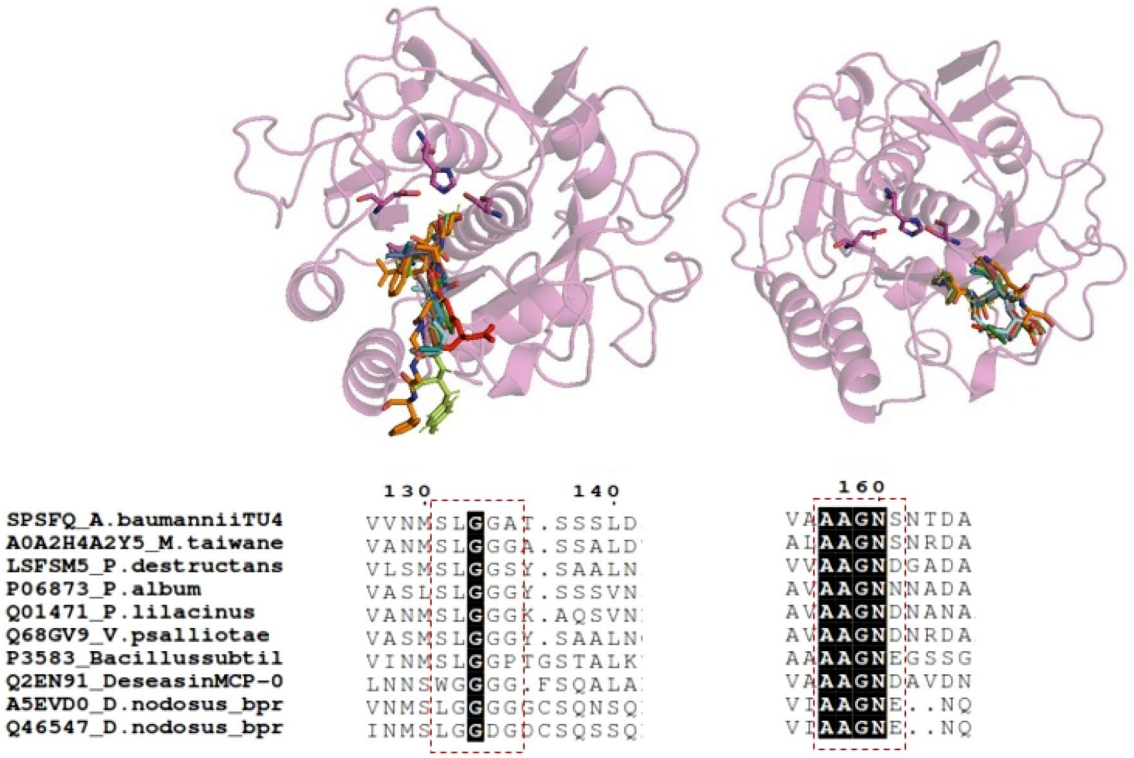

Fig. 14 S1 substrate binding pocket of SPSFQ with subtilisin-like serine proteases from different organisms: Keratinase from *Meiothermus taiwanensis* Wr-220 (A0A2H4A2Y5); Destructin-1 from *Pseudogymnoascus destructans* (LSFSM5); Proteinase K from *Parengyodontium album* (P06873); cuticle-degrading proteases from *Paecilomyces lilacinus*, PL646 (Q01471) and *Verticillium psalliotae*, Ver112 (Q68GV9); fibrin-degrading nattokinase from *Bacillus subtilis* (P3583); collagen-degrading Deseasin from marine *Pseudoalteromonas sp* (Q2EN91); extracellular matrix-degrading proteases from *Dichelobacter nodosus* (A5EVD0 and Q46547). The regions of amino acids with black background and white foreground are completely conserved. The dotted frame in red indicates amino acids that are involved in the formation of S1 site (color figure online)

exhibited conformational flexibility at elevated temperatures in the substrate-binding region, thus enhancing the substrate affinity and catalytic efficiency of these enzymes [54, 77, 81]. However, this is not observed in SPSFQ which is categorized as a mesophilic protease as it showed optimum catalytic activity at temperatures of not more than 40 °C. Highly electro-negative charge at the substrate-binding cleft of collagenolytic proteases from deep-sea bacterium *Pseudoalteromonas sp.* and psychrophilic fungus *Pseudogymnoascus destructans* contributed to the electrostatic attraction force with positively charged collagen substrate [75, 82]. In contrast, fungus-derived Proteinase K showed an overall more neutral charge on the substrate-binding cleft (Fig. 13f), yet was still able to degrade cuticle and collagen substrates at a similar degree to cuticle degrading protease, Ver112 [77]. The presence of acidic residues enhances the character of the S1 pocket to be more electro-negative hence increasing the flexibility and affinity of a protease to accommodate more complex substrates to catalyze [54, 75]. This observation may explain why SPSFQ showed less catalytic efficiency towards complex substrates such as collagen and fibrin compared to the simpler forms of proteinaceous substrates such as casein and gelatin (denatured collagen). Substrate specificity and promiscuity often observed in proteases may be contributed by a combination of electrostatic charge, flexibility, and shape complementarity of the substrate-binding cleft [83].

7 Conclusion

Results from this study showed that SPSFQ shares a very high amino acid sequence identity with extracellular proteases from clinically relevant strains of *Acinetobacter baumannii* and *Klebsiella pneumoniae*. SPSFQ may be part of the proteolytic secretome of *Acinetobacter baumannii* that contributes to the physiological health of the bacteria by providing peptidic nutrients through hydrolysis of protein substrates. This is further reinforced by the substrate specificity study which showed that SPSFQ was able to degrade a variety of tissue-associated protein substrates. Whether the activity of this enzyme contributes to the virulence of

pathogenic strains of the bacteria is still unknown however it warrants further investigation of SPSFQ related proteases in these clinical strains. In terms of commercial value, SPSFQ showed that it has favorable properties such as stable at alkaline pH and stable in the presence of surfactants, indicating that SPSFQ has the potential to be used as a proteolytic enzyme in detergent. SPSFQ showed varying degrees of promiscuity towards different protein substrates that may be attributed to the physico-chemical landscape of the enzyme's substrate binding site.

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Declaration

Conflict of interest All the authors declare that they have no conflict of interest.

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