Food Chemistry 119 (2010) 573–579

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

A NaCl-stable serine proteinase from *Virgibacillus* sp. SK33 isolated from Thai fish sauce

Sornchai Sinsuwan [a], Sureelak Rodtong [b], Jirawat Yongsawatdigul [a,*]

[a] School of Food Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[b] School of Microbiology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

ARTICLE INFO

Article history:
Received 30 December 2008
Received in revised form 24 June 2009
Accepted 24 June 2009

Keywords:
Virgibacillus sp.
NaCl-stable proteinase
Subtilisin-like proteinase
Serine proteinase
Fish sauce

ABSTRACT

An extracellular proteinase from *Virgibacillus* sp. SK33, isolated from 1 month-old fish sauce, was purified to electrophoretic homogeneity, using hydrophobic interaction chromatography and hydroxyapatite with purification fold of 2.5 and 7% yield. The anomalous molecular weight (MW) of 19 kDa was obtained from SDS–PAGE, whereas a MW of 33.7 kDa was determined by MALDI-TOF. Optimum conditions for catalytic activity were 55 °C and pH 7.5. The proteinase was strongly inhibited by phenylmethanesulfonyl fluoride (PMSF) and preferentially hydrolysed Suc-Ala-Ala-Pro-Phe-AMC, indicating a serine proteinase with subtilisin-like characteristics. K_m and k_{cat} of the purified proteinase were 27 μM and 12 s^{-1}, respectively. Proteinase activity, toward both synthetic and anchovy substrates, increased with NaCl up to 25%. The proteinase exhibited high stability in both the absence and presence of NaCl up to 25%. Approximately 2.5-fold increase in activity was observed in the presence of divalent cations, including Ca^{2+}, Mg^{2+} and Sr^{2+} at 100 mM. MALDI-TOF MS and LC–ESI-MS/MS analyses, as well as N-terminal sequences, revealed that the purified enzyme did not match microbial proteinases in the database, indicating it to be a novel proteinase.

1. Introduction

Most commercial proteinases are derived from mesophilic microorganisms and exhibit activity in the neutral to alkaline pH range, mild temperature and low ionic strength (Petersen, 1981). Low thermal stability of these enzymes is a major limitation in their application (Anwar & Saleemuddin, 1998). A proteinase from *B. amyloliquefaciens* could not function at >50 °C, while *Bacillus licheniformis* proteinase was readily inactivated near pH 4 (Adler-Nissen, 1993). In addition, activity of *Bacillus* proteinases decreased with increasing NaCl concentration (Gupta et al., 2005). Proteinases from mesophilic bacteria show relatively poor activity in extreme conditions.

Extremophilic microorganisms are considered to be an important source of enzymes that are able to function under harsh conditions (Kumar & Takagi, 1999). Extremophiles are organisms thriving in extreme conditions, which include thermophiles, acidophiles, alkalophiles, psychrophiles and halophiles. Archaebacteria can produce proteinases that are able to perform a catalytic reaction at relatively high NaCl concentration (5.8–30%). A chymotrypsin-like proteinase was purified from arhaeon *Natrialba asiatica* 172 P1 and showed optimum activity at 30% NaCl (Kamekura & Seno, 1990), whereas optimum activity of the purified chymotrypsin-like proteinase from arhaeon *Natrialba magadii* was at 1–1.5 M NaCl (5.8–8.8%) (Giménez, Studdert, Sánchez, & De Castro, 2000).

Moderate halophiles are microorganisms growing well at 3–15% NaCl and proteinase activity also increases with NaCl concentration (Ventosa, Nieto, & Oren, 1998). Activity of crude proteinases from *Virgibacillus* sp. SK33 isolated from fish sauce fermentation was first reported to increase with NaCl concentration up to 25% and showed higher proteolytic activity toward anchovy than did Alcalase and Protamex (Sinsuwan, Rodtong, & Yongsawatdigul, 2008). *Virgibacillus* sp. SK33 could be a good source of proteinases hydrolysing protein at high NaCl condition. To effectively utilise proteinases produced from *Virgibacillus* sp. SK33, their biochemical characteristics must be elucidated. Therefore, our objective was to purify and to biochemically characterise the predominant extracellular proteinase from *Virgibacillus* sp. SK33.

2. Materials and methods

2.1. Chemicals

t-Butyloxycarbonyl(Boc)–Asp(oBzl)-Pro-Arg-7-amino-4-methylcoumarin (AMC), succinyl(Suc)-Ala-Ala-Pro-Phe-AMC and carbobenzoxy(Z)-Phe-Arg-AMC were purchased from Bachem A.G.

* Corresponding author. Tel.: +66 44 22 4359; fax: +66 44 22 4387.
E-mail address: jirawat@sut.ac.th (J. Yongsawatdigul).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.06.064

(Dubendorf, Switzerland). Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Z-Arg-Arg-AMC, leupeptin, trypsin inhibitor I (soybean), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride (PMSF), bestatin, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidine)-butane (E-64), *N*-ethylmaleimide (NEM), iodoacetic acid (IAA), dithiothreitol (DTT), 2-mercaptoethanol (β-ME), bovine serum albumin, L-tyrosine and casein were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), L-cysteine, imidazole and L-histidine were purchased from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade.

2.2. Strain and growth condition

Strain SK33 was isolated from fish sauce fermentation and identified to be *Virgibacillus* sp. according to the 16S rRNA gene sequence (Accession No. DQ910838). *Virgibacillus* sp. SK33 was a Gram-positive/variable, long rod of 0.6–0.7 × 3.0–6.6 μm, non-motility and terminal or subterminal ellipsoidal spores. It grew over a wide pH range of 4–11 and 20–45 °C (Sinsuwan et al., 2008). The purified culture of *Virgibacillus* sp. SK33 was cultivated in a halobacterium salt broth supplemented with yeast extract (1% yeast extract, 0.3% trisodium citrate, 0.2% potassium chloride, 2.5% magnesium sulphate and 5% NaCl) at 40 °C with a shaking speed of 100 rpm for 3 days. Crude proteinase was collected by centrifugation at 8000*g* for 30 min at 4 °C.

2.3. Purification

All purification steps were carried out at ~4 °C, using a Purifier 10 (ÄKTA, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Ammonium sulphate was slowly added to the crude proteinase to attain a final concentration of 1 M. The crude proteinase was subsequently centrifuged at 10,000*g* for 30 min and filtered through a 0.45 μm-membrane filter. The filtrate was loaded onto a phenyl-Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) column (2.6 × 6.5 cm) equilibrated with 1 M $(NH_4)_2SO_4$, 50 mM Tris–HCl, pH 8.0 and washed with the same buffer. Elution was performed with a linear gradient from 1 to 0 M $(NH_4)_2SO_4$, 50 mM Tris–HCl, pH 8.0. Fractions of 5 ml were collected at a flow rate of 1 ml/min. Active fractions were pooled and diafiltrated against 50 mM Tris–HCl (pH 8.0), using a membrane with molecular weight cut-off (MWCO) of 10 kDa (Vivaspin, Sartorius AG, Goettingen, Germany).

Diafiltrated sample was applied to a hydroxyapatite column (5 ml) (Econo-Pac® CHT-II Cartridge, Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with 50 mM Tris–HCl (pH 8.0). The column was washed with equilibrating buffer and eluted with a linear gradient of 0–0.3 M $(NH_4)_2SO_4$, 50 mM Tris–HCl, pH 8.0. Fractions of 2.5 ml were collected at a flow rate of 0.5 ml/min. Active fractions were pooled. Protein content was monitored at 280 nm and proteinase activity was determined as described below.

2.4. Activity staining

Activity staining was determined according to García-Carreño, Dimes, and Haard (1993). The purified proteinase was mixed with an equal volume of a sample buffer containing β-ME (125 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS and 10% β-ME) and in the absence of β-ME. The gel containing 12.5% acrylamide was run at 100 V (Laemmli, 1970). Subsequently, it was immersed in 2% casein, 100 mM Tris–HCl, pH 8.0, at 4 °C for 30 min, and washed twice with 100 mM Tris–HCl (pH 8.0). Proteolytic reaction was carried out in 100 mM Tris–HCl (pH 7.5) at 55 °C for 30 min. Gel was stained in 0.1% Coomassie brilliant blue R-250, 40% methanol and 10% acetic acid for 1 h and destained in 25% ethanol and 10% acetic acid. A clear zone indicated the presence of proteinase.

2.5. Enzymatic activity assay

Proteinase activity was assayed by the method of Barrett and Kirschke (1981) with some modifications. A fluorogenic substrate, Suc-Ala-Ala-Pro-Phe-AMC, was used as a substrate. The reaction mixture (1 ml) contained 50 μl of the purified enzyme, 1 μM synthetic substrate, 200 mM Tris–HCl (pH 7.5) and was incubated at 55 °C for 5 min. The reaction was stopped by adding 1.5 ml of the stopping solution (30% butanol, 35% methanol and 35% deionised water). Fluorescence intensity was measured at the excitation and emission wavelengths of 380 and 460 nm, respectively (RF-1501, Shimadzu Co., Kyoto, Japan). Units of enzymatic activity were expressed as katal.

2.6. Biochemical characteristics

2.6.1. Estimation of molecular weight by gel filtration

The molecular weight (MW) of the native proteinase was determined using a Superose 6 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) column (1.6 × 85 cm) equilibrated and eluted with 0.15 M NaCl, 50 mM Tris–HCl, pH 8.0. Fractions of 0.85 ml were collected at a flow rate of 0.85 ml/min. Void volume (V_o) was determined using blue dextran (Sigma Chemical Co., St. Louis, MO, USA). The ratio of elution volume (V_e) to V_o was calculated. The column was calibrated using a protein standard consisting of β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (13 kDa) (Sigma Chemical Co., St. Louis, MO, USA).

2.6.2. Estimation of molecular weight by MALDI-TOF

The purified proteinase was dialysed twice against 100 volumes of deionised water, using a dialysis membrane with MWCO 10 kDa (Pierce Chemical Company, Rockford, IL, USA). The dialyzed sample was mixed with an equal volume of matrix solution (10 mg/ml α-cyano-4-hydroxy-cinnamic acid (4-HCCA) in acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) (1:2)) and subsequently the mixture was spotted onto the MALDI holder. Mass measurement was carried out using the MALDI-TOF (MS model reflex V, Bruker Daltonik GmbH, Bremen, Germany) with a 2 GHz LeCroy digitiser and 337 nm nitrogen laser.

2.6.3. Isoelectric point determination

Isoelectric point (pI) of the purified proteinase was determined by PhastGel isoelectric focusing (IEF) with a PhastGel IEF 3–9 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The operating condition was performed by prefocusing, sample application and focusing steps at 2000, 100 and 2000 V, respectively. The temperature was controlled at 15 °C. A broad range pI standard (3–10) was used (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The protein band was detected by silver staining.

2.6.4. Effect of temperature/pH and thermal stability

The optimal temperature of proteinase activity was measured at 4, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 °C in 200 mM Tris–HCl (pH 8.0), which was the optimal pH of crude proteinase. pH profile was measured at 55 °C at various pHs: pH 5.0, 5.5, 6.0, using 100 mM sodium acetate; pH 6.5, 7.0, using 100 mM Tris-maleate; pH 7.5, 8.0, 8.5, 9.0, using 200 mM Tris–HCl; pH 9.5, 10.0, using 100 mM borate buffer; pH 11.0, using 200 mM carbonate buffer, and pH 12.0, using 200 mM phosphate buffer.

Thermal stability of the enzyme was investigated by pre-incubating the purified enzyme (5 μg) in 200 mM Tris–HCl (pH 7.5)

at 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C for 30 min. When incubation time was reached, samples were immediately cooled in iced water. The residual activity was determined at the optimal condition obtained from the above experiments.

2.6.5. Effect of NaCl on activity and stability

Effect of NaCl on proteinase activity was determined by incubating the purified proteinase in 200 mM Tris–HCl (pH 7.5) at various NaCl concentrations (0–25%) at 55 °C for 5 min. Deionised water (1.5 ml) was used instead of the stopping reagent. The reactions were terminated by heating at 90 °C for 5 min. The residual activity was calculated as the percentage of sample without NaCl as 100%.

NaCl stability of the purified proteinase was monitored by pre-incubating the purified enzyme (5 μg) in 5%, 10%, 15%, 20% and 25% NaCl, in 200 mM Tris–HCl, pH 7.5 at 55 °C for 60 min. The sample were rapidly cooled and assayed at the optimum condition, 200 mM Tris–HCl (pH 7.5), 20% NaCl, at 55 °C.

2.6.6. Effect of inhibitors and other substances

The effects of various inhibitors and other substances on proteinase activity were determined. Reaction without substances was taken as 100% activity. In addition, effects of divalent ions, Ca^{2+}, Sr^{2+} and Mg^{2+}, on proteinase activity were determined at various concentrations ranging from 0–100 mM.

2.6.7. Substrate specificity and kinetic constant

Activity of the purified proteinase was determined using various synthetic substrates at 1 μM, including Boc-Asp(oBzl)-Pro-Arg-AMC, Boc-Gln-Ala-Arg-AMC, Boc-Val-Leu-Lys-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Z-Arg-Arg-AMC and Z-Phe-Arg-AMC. Activity was determined under the standard assay condition. Specific activity was expressed as kat/mg protein.

K_m and k_{cat} value of the purified proteinase were determined in 200 mM Tris–HCl (pH 7.5) at 55 °C. Suc-Ala-Ala-Pro-Phe-AMC, at concentrations ranging from 10 to 200 μM, was used. Kinetic parameters were determined using linear regression of the Hanes-Wolff plot (Copeland, 2000).

2.7. Mass spectrometry for protein identification

The purified proteinase was applied on SDS–PAGE (Laemmli, 1970) and visualised by staining with Coomassie brilliant blue R-250. The protein band was excised, placed in 96-well plates and washed with water. Tryptic digestions were performed on an Ettan™Spot Handing Workstation robot (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's specification. The peptide sample was mixed with an equal volume of matrix solution (10 mg/ml 4-HCCA in ACN and 0.1% TFA (1:2)), applied onto a sample holder and dried at room temperature. The peptide mass fingerprint (PMF) of tryptic-digested peptides was determined by MALDI-TOF (MS model reflex V, Bruker Daltonik GmbH, Bremen, Germany) equipped with a 2 GHz LeCroy digitiser and 337 nm nitrogen laser. The spectrum of digested peptides was obtained by the positive ion mode at an acceleration voltage of 20 kV and 400 ns extraction delay. Mass data were compared with the non-redundant protein sequence database (NCBInr) of National Center for Biotechnology Information using the MASCOT search engine (Matrix Science, London, UK).

Further protein identification was also carried out using liquid chromatography–electrospray ionisation tandem mass spectrometry (LC–ESI-MS/MS) equipped with a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Co., Waltham, MA, USA). Tryptic digested peptides were acidified with 0.1% TFA in 30% ACN and separated using a Zorbax 300SB-C18 column (5 μm, 5 × 0.3 mm) (Agilent Technologies, Inc., Palo Alto, CA, USA) with elution gradient 2–60% buffer B (buffer A, 0.1% formic acid in deionised water; buffer B, 0.1% formic acid in ACN) at a flow rate of 100 μl/min. The tryptic-digested peptide ions were detected in a survey scan from 400 to 1600 amu. LC-ESI-MS/MS data were examined using a SEQUEST search algorithm with NCBInr. Amino acid sequences of some peptides from *de novo* peptide sequencing were searched using the Basic Local Alignment Search Tool (BLAST).

2.8. N-terminal amino acid sequence analysis

The purified proteinase was dialyzed twice against 100 volumes of deionised water, using a dialysis membrane with MWCO 10 kDa, at 4 °C overnight (Pierce Chemical Company, Rockford, IL, USA). The sample was concentrated by ultrafiltration with a membrane of MWCO 10 kDa (Vivaspin, Sartorius AG, Goettingen, Germany). The N-terminal amino acid sequence of the enzyme was determined, using a protein sequencer (Applied Biosystems Procise 492 HT, Applied Biosystems, Foster City, CA, USA) connected to a reverse-phase high performance liquid chromatography (RP-HPLC) apparatus for phenylthiohydantoin-derivative identification.

2.9. Hydrolysis of anchovy proteins

Whole anchovy (*Stolephorus indicus*) was homogenised in cooled 50 mM Tris-maleate, pH 7.0, at various NaCl concentrations (0%, 5%, 10%, 15%, 20% and 25% NaCl) at a ratio of fish to buffer of 1:5. The homogenate was stirred at 4 °C for 30 min. The homogenate was centrifuged at 13,000*g* for 30 min. The supernatants were used as a substrate of the purified enzyme. Protein content was determined by a dye binding method (Bradford, 1976), using bovine serum albumin as a standard.

The reaction mixture (1 ml) contained 0.5 nkat purified proteinase, 200 mM Tris–HCl (pH 7.5), 100 μM leupeptin and 1.5 mg/ml of anchovy protein solubilised at various NaCl concentrations. Concentration of NaCl in the reaction was adjusted with respect to NaCl concentration of the substrate. The mixture was incubated at 55 °C for 30 min. The reaction was stopped by adding trichloroacetic acid (TCA) to a final concentration of 10%. The samples were cooled at 4 °C for 1 h to allow complete protein precipitation and then centrifuged at 10,000*g* for 10 min. TCA-soluble oligopeptide contents were determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), using tyrosine as a standard. Blanks were prepared in the same manner except that the heated proteinase (90 °C for 5 min) was used.

3. Results

3.1. Purification and biochemical characteristics

Purity of proteinase from *Virgibacillus* sp. SK33 increased about 2.5-fold with a yield of 7% (Table 1). The purified proteinase showed a single band in SDS–PAGE in the absence and presence of β-ME, with an apparent MW of ~19 kDa (Fig. 1A). In addition, zymograms showed a single band with high activity (Fig. 1A). MW of the purified proteinase, under native conditions as determined by gel filtration chromatography, was ~43 kDa. The MALDI-TOF spectra showed two peaks at *m/z* of 16,881 and 33,724 (Fig. 1B). The small intensity at *m/z* 16,881 was likely the doubly charged ion. Hence, the absolute MW of the proteinase was 33.7 kDa. The MW of proteinase, estimated using MALDI-TOF, was about 2 times greater than that observed by SDS–PAGE.

pI of the purified proteinase was ~4.28 (Fig. 2). The proteinase activity was gradually increased with temperature and reached the maximum activity at 55 °C (Fig. 3). The proteinase also showed

Table 1
Purification of the purified proteinase from *Virgibacillus* sp. SK33.

Step	Total unit activity (nkat)	Total protein (mg)	Specific activity (nkat/mg protein)	Purification (fold)	Yield (%)
Crude in 1 M $(NH_4)_2SO_4$	9.4	3.7	2.5	1	100
Phenyl-sepharose	1.6	0.5	3.3	1.3	16
Hydroxyapatite	0.7	0.1	6.4	2.5	7

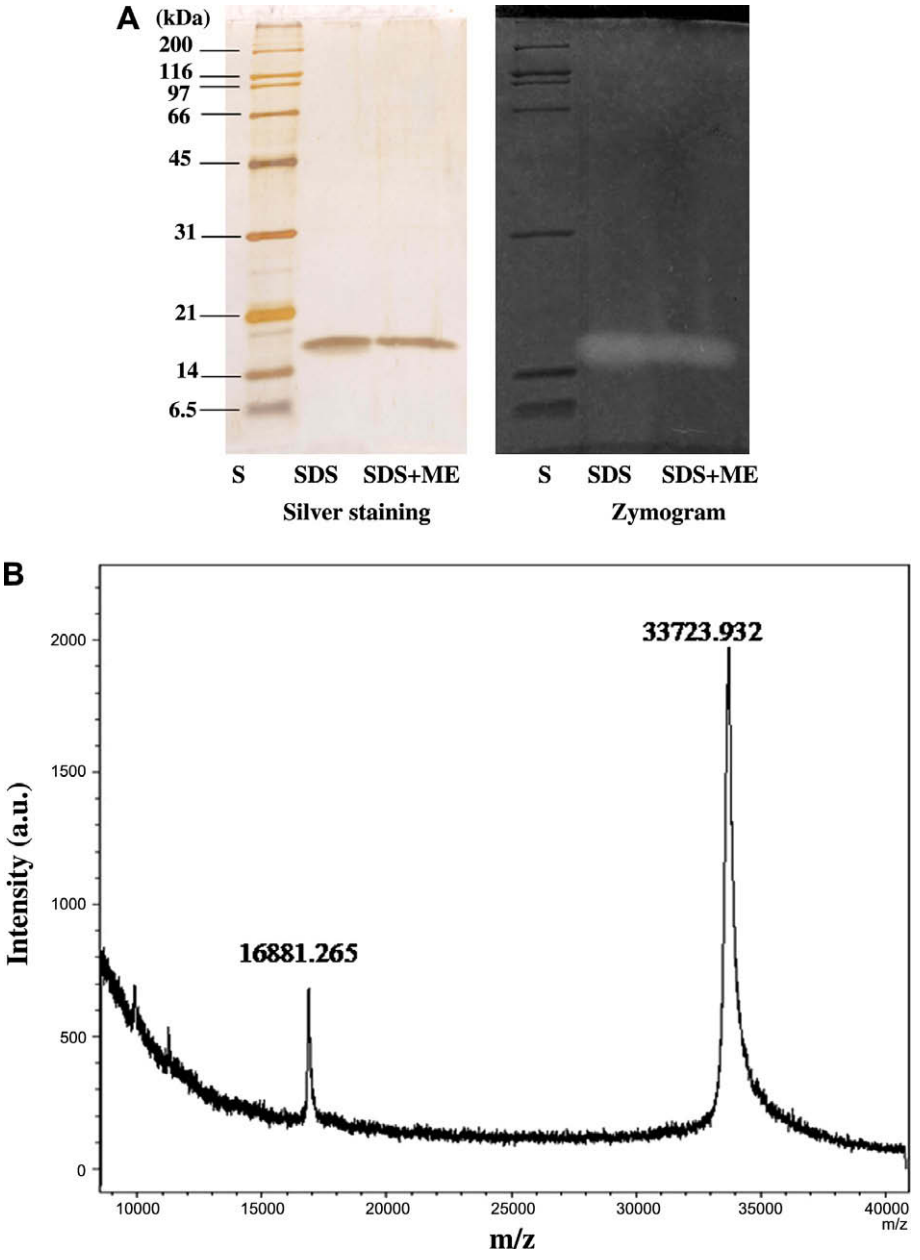

Fig. 1. Estimation of molecular weight of the purified proteinase from *Virgibacillus* sp. SK33. (A) SDS–PAGE (12.5% T) of the purified proteinase visualised by silver staining and activity staining (zymogram). S = standard molecular weight; SDS = treatment buffer containing SDS and SDS + ME = treatment buffer containing SDS and 2-mercaptoethanol; (B) MALDI-TOF spectra.

high thermal stability at its optimal temperature, 55 °C (Fig. 3). The purified proteinase showed high activity over a wide pH range of 6.5–8.5, with an optimum pH of 7.5 (Fig. 4).

Activity of the purified proteinase increased with NaCl and reached a maximum at 10% NaCl and remained constant up to 25% NaCl (Fig. 5). Approximately 2.5-fold increase in activity was observed at 25% NaCl. In addition, activity of the purified proteinase towards anchovy protein increased with increasing NaCl concentrations and attained the maximum at 20–25% NaCl ($p < 0.05$) (Fig. 6). The purified proteinase activity was completely inhibited by PMSF, whereas metallo proteinase inhibitors did not affect the activity (Table 2). The studied metal ions had no effect on proteinase activity (Table 2). Divalent cations, namely Ca^{2+}, Sr^{2+} and Mg^{2+}, showed activation effects up to 100 mM (Table 2 and Fig. 7). Among various synthetic substrates tested, the purified proteinase hydrolysed only Suc-Ala-Ala-Pro-Phe-AMC, with an activity of

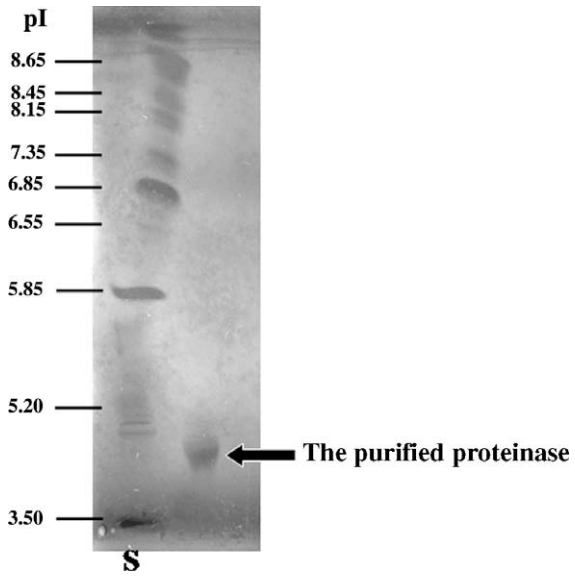

Fig. 2. Isoelectric focusing of the purified proteinase. Applied voltages at 2000, 100 and 2000 V were used for prefocusing, sample application and focusing steps, respectively. Temperature was kept at 15 °C. Silver staining was performed. S = isoelectric focusing standard.

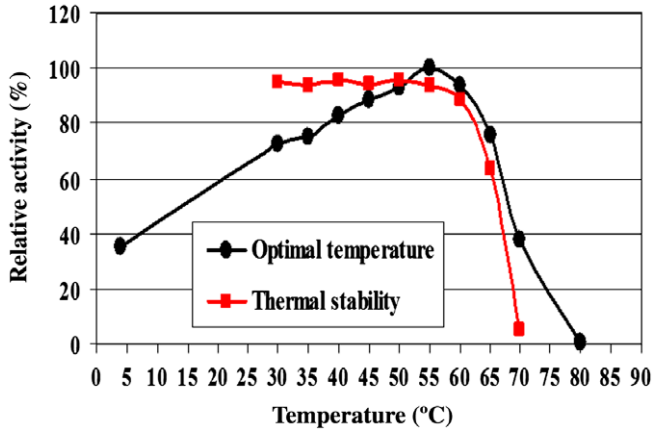

Fig. 3. Temperature profile and thermal stability of the purified proteinase. Thermal stability was determined at pH 7.5 for 30 min.

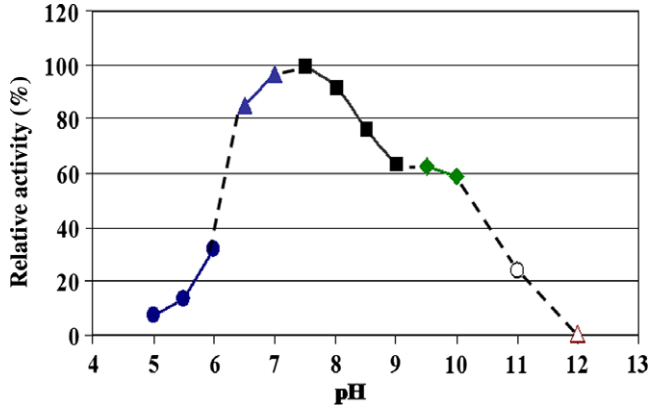

Fig. 4. pH profile of the purified proteinase. = sodium acetate; = Tris–maleate; = Tris–HCl; = borate buffer; = glycine–NaOH and = phosphate buffer. All samples were incubated at 55 °C.

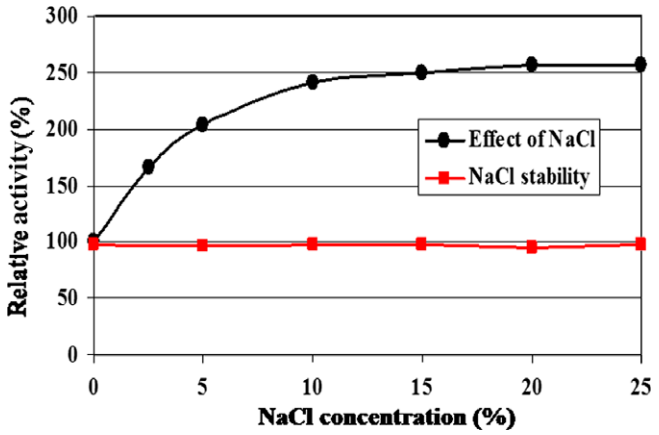

Fig. 5. Effects of NaCl on activity and stability of the purified *Virgibacillus* sp. SK33 proteinase. NaCl stability was determined at 55 °C for 60 min.

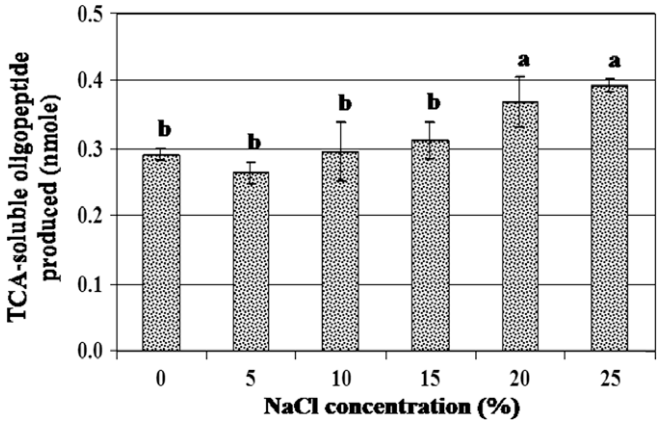

Fig. 6. Effects of NaCl on proteolytic activity of the purified *Virgibacillus* sp. SK33 proteinase (0.5 nkat) using anchovy proteins as a substrate. Letters indicate significant differences at $p < 0.05$.

Table 2
Effect of various substances on the activity of the purified proteinase.

Substances	Final concentration (mM)	Relative activity (%)
Control		100
EDTA	10	94
Imidazole	10	88
Dithiothreitol	10	95
2-Mercaptoethanol	10	100
L-cysteine	10	97
Metal ions		
$CuCl_2$	1	106
$CdCl_2$	1	113
$CoCl_2$	1	108
$FeCl_3$	1	119
$MnCl_2$	1	108
$HgCl_2$	1	96
$ZnSO_4$	1	127
Mono and divalent cations		
$LiCl_2$	10	102
NaCl	10	105
KCl	10	99
$MgCl_2$	10	125
$CaCl_2$	10	122
$SrCi_2$	10	119
$BaCl_2$	10	65

39 nkat/mg protein. K_m and k_{cat} value of the purified proteinase from *Virgibacillus* sp. SK33 were 27 μM and 12 s^{-1}, respectively.

3.2. Protein identification by mass spectrometry

PMF of the *Virgibacillus* sp. SK33 proteinase showed 19 peptides that did not match any proteins from microorganisms in the database. Insignificant protein scores were obtained when compared with the small-conductance mechano-sensitive channel from *Pseudomonas entomophila* L48 and the hypothetical protein L8106_10252 from *Lyngbya* sp. PCC 8106. LC-ESI-MS/MS spectra also confirmed the nonexistence of peptides derived from the *Virgibacillus* sp. SK33 proteinase. In addition, amino acid sequences obtained from the *de novo* sequencing, namely LTVLNWR, VDVLGGAFR and RMDVDEVKDPLSAPRARR, showed no significant similarities to the known microbial proteinases. The N-terminal sequence of the enzyme was distinctively different from other bacterial proteinases (Table 3). Based on these results, the predominant proteinase from *Virgibacillus* sp. SK 33 seemed to be a novel proteinase, exhibiting high activity and stability at high salt content.

4. Discussion

The purified proteinase, in the presence and absence of β-ME, showed the same protein pattern on SDS–PAGE (Fig. 1A). In addition, a reducing agent, DTT, had no effect on enzyme activity (Table 2). It was, therefore, postulated that the disulphide bond was unlikely to be the main force stabilizing the enzyme structure. The discrepancy in molecular weight determination between MALDI-TOF and SDS–PAGE suggested an anomalous electrophoretic mobility of the enzyme. Such a phenomenon was also reported in ribonuclease U2, a very acidic protein with the pI of 2.8–3.3 (García-Ortega et al., 2005). Part of the peptides from elastase digestion of a streptococcal inhibitor protein also showed irregular electrophoretic behaviour under SDS–PAGE, with nearly twice its true molecular weight (Fernie-King, Seilly, & Lachmann, 2004). Apparent molecular weight determination based on SDS–PAGE is rather erratic, especially when a protein contains abnormally high charges and/or amino acids impeding SDS binding.

Archaebacteria, *Halobacterium mediterranei* and *Natrialba magadii*, produced proteinases with maximum activity at 55–60 °C (Giménez et al., 2000; Stepanov et al., 1992), which was similar to that of the purified proteinase from *Virgibacillus* sp. SK33. Another distinct characteristic of the enzyme was its high thermal stability at the optimal temperature, 55 °C, in contrast to several microbial proteinases, which were unstable in such a condition (Kim & Kim, 2005). Optimum activity, at neutral pH (7.5), of *Virgibacillus* sp. SK33 proteinase could be advantageous for food application. It should be noted that a small shoulder at around pH 9.5–10 was observed. This could be caused by the effect of borate buffer used at these pH values.

The purified proteinase from a moderate halophile, *Virgibacillus* sp. SK33, showed activity in either the absence or presence of NaCl. In addition, activity increased with increasing NaCl concentration with the highest 2.5-fold increase at 25% NaCl. The enhancing effect of NaCl on proteinase activity was observed in both a synthetic and fish protein substrate (Figs. 5 and 6). Another distinct feature of this enzyme was its high stability over a wide range of NaCl concentration, 0–25%. Archaebacterium proteinase from *Halobacterium halobium* was completely and irreversibly inactivated at <2 M NaCl (11.7% NaCl) (Izotova et al., 1983), whereas *H. halobium* ATCC 43214 proteinase was irreversibly inactivated at <4 M NaCl (Kim & Dordick, 1997). On the other hand, activity of eubacterial proteinase was often unstable under high salt content. Activities of *B. subtilis* JM-3 and *Filobacillus* sp. RF2–5 proteinases decreased to ~10% of the original at 30% NaCl (Hiraga et al., 2005; Kim & Kim, 2005). The low pI value of *Virgibacillus* sp. SK33 proteinase implied that the surface of the enzyme contained many acidic amino acids, which is a unique characteristic of halophilic proteinases. A negatively charged surface would allow the enzyme to interact with hydrated salt ions and water, protecting the enzyme from the salting-out effect. Thus, high thermal and NaCl stability of *Virgibacillus* sp. SK33 proteinase would be a vital feature for application at high salt content, such as in fish sauce or soy sauce fermentation.

Activity of the purified proteinase increased almost ~2 times at 100 mM Ca^{2+}, Sr^{2+} and Mg^{2+} (Fig. 7). These divalent cations might induce structural changes, leading to more flexibility of the substrate-binding site. However, the effect of divalent ions varied with strain. Proteinase from *B. pumilus* was also activated by Mg^{2+} or Ca^{2+} (Kumar, 2002), whereas *Natrialba asiatica* 172 P1 proteinase activity was not affected by Ca^{2+}, Sr^{2+} and Mg^{2+} (Kamekura & Seno, 1990). Our results demonstrate that the purified proteinase from *Virgibacillus* sp. SK33 was activated not only by high NaCl content, but also by Ca^{2+}. Addition of Ca^{2+} could be a critical means to obtain maximum activity. The purified proteinase displayed specificity toward Suc-Ala-Ala-Pro-Phe-AMC, indicating that phenylalanine at the P_1 position and proline at the P_2 position, located on the cleaved peptide bond, were preferred. Likewise, a subtilisin preferentially hydrolyses large and non-β-branched hydrophobic residues at P_1 and small neutral side chains at P_2 (Graycar, Ballinger, & Wells, 2004). According to these results of synthetic substrates and inhibitors, the purified enzyme is classified as a subtilisin-like proteinase.

Recently, MS techniques have been used for protein identification. A 45-kDa purified proteinase from *Serratia rubidaea* was analysed by ESI-MS/MS and showed similar homology to a 50-kDa proteinase of *S. marcescens* (Doddapaneni et al., 2007). PMF, LC-ESI-MS/MS, and *de novo* sequencing results revealed that peptide pattern and amino acid sequences of the purified proteinase from *Virgibacillus* sp. SK33 did not match those in the database. In addition, N-terminal amino acid sequences of the purified enzyme showed no homology to any bacterial proteinases. These results suggest that the purified proteinase is a novel proteinase.

Table 3
Comparison of N-terminal amino acid sequences between the purified proteinase and proteinases from non- and halophiles.

Proteinase/strain	N-terminal amino acid sequence										Reference
The proteinase from *Virgibacillus* sp. SK33	S	Y	E	W	–	W	D	R	R	R	
Proteinases from extreme and moderate halophiles											
Natrialba asiatica 172P1	A	T	P	N	D	P	Q	Y	G	Q	Kamekura and Seno (1990)
Halobacterium mediterranei	D	T	A	N	D	P	K	Y	G	S	Stepanov et al. (1992)
Filobacillus sp. RF2–5	A	L	D	T	G	V	-	W	D	-	Hiraga et al. (2005)
Salinivibrio sp. AF-2004	A	T	A	G	G	T	G	P	G	G	Karbalaei-Heidari, Ziaee, Schaller, and Amoozegar (2007)
Expro-I (*B. subtilis* FP-133)	A	E	S	V	P	Y	G	V	S	E	Setyorini, Takenaka, Murakami, and Aoki (2006)
Expro-II (*B. subtilis* FP-133)	A	D	A	T	G	X	G	G	N	Q	Setyorini et al. (2006)

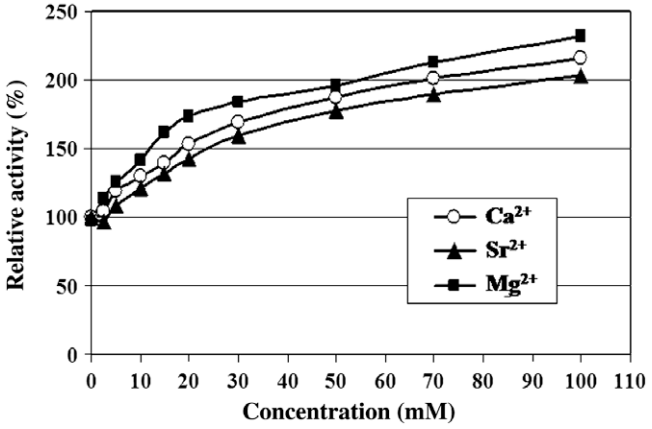

Fig. 7. Effects of divalent ions on the purified proteinase activity.

5. Conclusions

A novel serine proteinase from *Virgibacillus* sp. SK33 was purified and characterised. The unique characteristic of the subtilisin proteinase was the ability to hydrolyse fish protein in either the absence or presence of high NaCl content (25%). The proteinase showed high stability over a wide range of NaCl concentration of 0–25%. In addition, the enzyme was activated by Ca^{2+}. The enzyme can be applied to hydrolyze protein at a high salt concentration. This could be an important processing-aid agent for protein hydrolysis processes involving high salt content, such as fish sauce or soy sauce fermentation.

Acknowledgements

This work was financially supported by a research grant from the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand, under research agreement No. BT-B-06-FG-19-4603. We also thank Mr. Surasak Jiemsup, Genome Institute (GI), BIOTEC, Thailand, for technical assistance in mass spectrometry analyses.

References

Adler-Nissen, J. (1993). Proteases. In T. Nagodawithana & G. Reed (Eds.), *Enzymes in food processing* (3rd ed., pp. 159–203). San Diego: Academic Press.

Anwar, A., & Saleemuddin, M. (1998). Alkaline proteinases: A review. *Bioresource Technology, 64*, 175–183.

Barrett, A. J., & Kirschke, H. (1981). Cathepsin B, cathepsin H, and cathepsin L. In S. P. Colowick & N. O. Kaplan (Eds.). *Methods in enzymology* (Vol. 80, pp. 535–561). New York: Academic Press.

Bradford, M. (1976). A rapid sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry, 72*, 248–254.

Copeland, R. A. (2000). *Enzymes: A practical introduction to structure, mechanism, and data analysis* (2nd ed.). New York: Wiley-VCH.

Doddapaneni, K. K., Tatineni, R., Vellanki, R. N., Gandu, B., Panyala, N. R., Chakali, B., et al. (2007). Purification and characterization of two novel extra cellular proteases from *Serratia rubidaea*. *Process Biochemistry, 42*, 1229–1236.

Fernie-King, B. A., Seilly, D. J., & Lachmann, P. J. (2004). The interaction of streptococcal inhibitor of complement and its proteolytic fragments with human beta defensins. *Immunology, 111*, 444–452.

García-Carreño, F. L., Dimes, L. E., & Haard, N. F. (1993). Substrate–gel electrophoresis for composition and molecular weight of proteinase or proteinaceous proteinase inhibitors. *Analytical Biochemistry, 214*, 65–69.

García-Ortega, L., De los Ríos, V., Martínez-Ruiz, A., Oñaderra, M., Lacadena, J., Martínez del Pozo, A., et al. (2005). Anomalous electrophoretic behavior of a very acidic protein: Ribonuclease U2. *Electrophoresis, 26*, 3407–3413.

Giménez, M. I., Studdert, C. A., Sánchez, J. J., & De Castro, R. E. (2000). Extracellular protease of *Natrialba magadii*: Purification and biochemical characterization. *Extremophiles, 4*, 181–188.

Graycar, T. P., Ballinger, M. D., & Wells, J. A. (2004). Subtilisin (2nd ed.. In A. J. Barrett, N. D. Rawlings, & J. F. Woessner (Eds.). *Handbook of proteolytic enzymes* (Vol. 2, pp. 1786–1792). Amsterdam: Elsevier.

Gupta, A., Roy, I., Patel, R. K., Singh, S. P., Khare, S. K., & Gupta, M. N. (2005). One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. *Journal of Chromatography A, 1075*, 103–108.

Hiraga, K., Nishikata, Y., Namwong, S., Tanasupawat, S., Takada, K., & Oda, K. (2005). Purification and characterization of serine proteinase from a halophilic bacterium, *Filobacillus* sp. RF2–5. *Bioscience, Biotechnology, and Biochemistry, 69*, 8–44.

Izotova, L. S., Strongin, A. Y., Chekulaeva, L. N., Sterkin, V. E., Ostoslavskaya, V. I., Lyublinskaya, L. A., et al. (1983). Purification and properties of serine protease from *Halobacterium halobium*. *Journal of Bacteriology, 155*, 826–830.

Kamekura, M., & Seno, Y. (1990). A halophilic extracellular protease from a halophilic archaebacterium strain 172 P1. *Biochemistry and Cell Biology, 68*, 352–359.

Karbalaei-Heidari, H. R., Ziaee, A. A., Schaller, J., & Amoozegar, M. A. (2007). Purification and characterization of an extracellular haloalkaline protease produced by the moderately halophilic bacterium, *Salinivibrio* sp. strain AF-2004. *Enzyme Microbial Technology, 40*, 6–272.

Kim, J., & Dordick, J. S. (1997). Unusual salt and solvent dependence of a protease from an extreme halophile. *Biotechnology and Bioengineering, 55*, 471–479.

Kim, W. J., & Kim, S. M. (2005). Purification and characterization of *Bacillus subtilis* JM-3 protease from anchovy sauce. *Journal of Food Biochemistry, 29*, 591–610.

Kumar, C. G. (2002). Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. *Letters in Applied Microbiology, 34*, 13–17.

Kumar, C. G., & Takagi, H. (1999). Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnology Advance, 17*, 561–594.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature, 227*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry, 193*, 265–275.

Petersen, B. P. (1981). The impact of the enzymic hydrolysis process on recovery and use of proteins. In G. G. Birch, N. Blakebrough, & K. J. Parker (Eds.), *Enzymes and food processing* (pp. 149–175). London: Applied Science.

Setyorini, E., Takenaka, S., Murakami, S., & Aoki, K. (2006). Purification and characterization of two novel halotolerant extracellular proteases from *Bacillus subtilis* strain FP-133. *Bioscience Biotechnology Biochemistry, 70*, 433–440.

Sinsuwan, S., Rodtong, S., & Yongsawatdigul, J. (2008). Production and characterization of NaCl-activated proteinases from *Virgibacillus* sp. SK33 isolated from fish sauce fermentation. *Process Biochemistry, 43*, 5–192.

Stepanov, V. M., Rudenskaya, G. N., Revina, L. P., Gryaznova, Y. B., Lysogorskaya, E. N., Filippova, I. Y., et al. (1992). A serine proteinase of an archaebacterium, *Halobacterium mediterranei*: A homologue of eubacterial subtilisins. *The Biochemical Journal, 285*, 281–286.

Ventosa, A., Nieto, J. J., & Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiology and Molecular Biology Reviews, 62*, 504–544.