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2. Yongsawatdigul, J., **Rodtong, S.**, and Raksakulthai, N. 2007. Acceleration of Thai fish sauce fermentation using proteinases and bacterial starter cultures. *Journal of Food Science*. 72(9): M382-M390.

NaCl-Activated Extracellular Proteinase from *Virgibacillus* sp. SK37 Isolated from Fish Sauce Fermentation

S. Sinsuwan, S. Rodtong, and J. Yongsawatdigul

ABSTRACT: *Virgibacillus* sp. SK37 exhibited high extracellular proteolytic activity in skim milk broth containing 10% NaCl. Optimum conditions of the crude proteinase were at pH 8.0 and 65 °C. The proteinase was strongly inhibited by phenylmethanesulfonyl fluoride (PMSF) and preferably hydrolyzed Suc-Ala-Ala-Pro-Phe-AMC, suggesting the serine proteinase with a subtilisin-like characteristic. Proteolytic activity increased with NaCl concentration up to 20%. Ca^{2+} activated the enzyme activity but reduced enzyme stability at 65 °C. Several proteinases with dominant molecular mass (MW) of 81, 67, 63, 50, 38, and 18 kDa were detected on native-polyacrylamide gel electrophoresis (native-PAGE) activity staining in the absence and presence of 25% NaCl. These results demonstrated that *Virgibacillus* sp. SK37 produced salt-activated extracellular proteinases. *Virgibacillus* sp. SK37 could be a promising strain for starter culture development used in fish sauce fermentation.

Keywords: bacterial proteinase, fish sauce, moderately halophilic bacterium, NaCl-activated proteinase, *Virgibacillus* sp.

Introduction

Moderately halophilic bacteria can grow well at relatively high salt concentration (3% to 14.5% NaCl), and show a reduced growth rate at higher NaCl (Kushner 1992). Several proteinases from moderate halophiles have been characterized from various sources. Activity of chymotrypsin-like proteinase from marine psychrophile PA-43 isolated from sea urchin was activated by NaCl (Irwin and others 2001), whereas activity of serine proteinase from *Salinivibrio costicola* 18AG isolated from salt spring decreased above 2% NaCl (Lama and others 2005). *Pseudoalteromonas* sp. CP76 and *Salinivibrio* sp. AF-2004 isolated from saline soil and saline lake, respectively, secreted metalloproteinases (Sánchez-Porro and others 2003; Karbalaei-Heidari and others 2007). Reports of proteinases secreted by halophilic bacteria isolated from food are rather limited.

Fish sauce is a popular condiment consumed in Southeast Asia. It is produced by a natural fermentation involving addition of salt to uneviscerated fish at a ratio of approximately 1:3. Since fermentation takes place under high salt content (approximately 30% NaCl), proteolytic activity derived from fish muscle and digestive tracts inevitably decreased. Therefore, an increase in total nitrogen and amino acid content was extremely slow, requiring about 12 to 18 mo for completing the fermentation process. Acceleration of protein hydrolysis of fish sauce fermentation has been a challenge. A variety of moderately halophilic bacteria was found during fish sauce fermentation, such as *Filobacillus* sp., *Lentibacillus salicampi*, *L. juripiscarius*, and *Halobacillus* sp. (Hiraga and others 2005; Namwong and others 2005, 2006). Application of starter culture producing high proteinase activity could be a plausible means to speed up protein hydrolysis and subsequently reduce fermentation time.

MS20070033 Submitted 1/12/2007, Accepted 4/12/2007. *Authors Sinsuwan and Yongsawatdigul are with School of Food Technology, Suranaree Univ. of Technology, Nakhon Ratchasima 30000, Thailand. Author Rodtong is with School of Microbiology, Suranaree Univ. of Technology, Nakhon Ratchasima 30000, Thailand. Direct inquiries to author Yongsawatdigul (E-mail: jirawat@sut.ac.th).*

We have recently isolated several strains of moderate halophiles from 1-mo-old fish sauce mashes. Strain SK37 showed the highest proteinase-producing ability among 165 strains studied. It was identified to be *Virgibacillus* sp. according to 16S rRNA gene sequence with the GenBank/NCBI accession number of DQ910840. *Virgibacillus* sp. SK37 was a Gram positive/variable, long rod with 0.6 to 0.7 × 3.0 to 6.6 μm, nonmotility and terminal or subterminal ellipsoidal spores. It can grow at a wide pH range of 4 to 11 and 20 to 45 °C. Preliminary results showed that *Virgibacillus* sp. SK37 grew and hydrolyzed anchovy at 25% NaCl, suggesting its potential to be developed as a starter culture for fish sauce fermentation. It is necessary to fully understand biochemical characteristics of extracellular proteinase produced by *Virgibacillus* sp. SK37 in order to successfully utilize the strain for starter culture development. Thus, our objective was to elucidate biochemical characteristics of extracellular proteinase secreted from *Virgibacillus* sp. SK37.

Materials and Methods

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. (Bubendorf, 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., U.S.A.). Ethylenediaminetetraacetic acid (EDTA), L-cysteine, and L-histidine were purchased from Fluka (Buchs, Switzerland).

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Preparation of crude proteinase

Virgibacillus sp. SK37 was cultivated in neopeptone broth (NEO, 0.5% neopeptone, 1% $MgSO_4 \cdot 7H_2O$, 0.2% KNO_3, 0.0005% ferric citrate, and 1% glycerol) containing 10% NaCl at 35 °C, 100 rpm, for 3 d. Crude extracellular proteinase was collected by centrifugation at 8000 × *g* for 30 min, at 4 °C (RC 28S, Sorvall Co., Newtown, Conn., U.S.A.). Subsequently, it was dialyzed against 100 volumes of 20 mM Tris-maleate (pH 7.0) twice using the dialysis membrane with molecular weight cut-off (MWCO) 10 kDa (Pierce Chemical Co., Rockford, Ill., U.S.A.) at 4 °C overnight.

Proteinase activity assay

Proteinase activity was assayed using azocasein (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as a substrate according to the method of An and others (1994). The enzyme (500 μL) was added to the preincubated reaction mixture containing 2 mg azocasein and 200 mM Tris-HCl (pH 8.0) in a final volume of 1 mL. The reaction was incubated at the optimal temperature for 30 min, then stopped by adding 500 μL cold 50% trichloroacetic acid (TCA). Unhydrolyzed protein was precipitated at 4 °C for 15 min and centrifuged at 10000 × *g* for 10 min (Eppendorf AG 22331, Hamburg, Germany). The supernatant (1 mL) was added to 100 μL of 10 N NaOH and determined at 450 nm (GBC UV/VIS 916, GBC Scientific Equipment PTY, Ltd., Melbourne, Victoria, Australia). Blank determinations were conducted in the same manner except that the enzyme solution was added after TCA solution. Proteolytic activity was calculated as the difference in absorbance at 450 nm between sample and blank (ΔA_{450}).

Effect of temperature, pH, inhibitors, and ions

Effect of temperature on proteinase activity was carried out at 4 to 80 °C in 50 mM Tris-maleate (pH 7.0) by the method of An and others (1994), using azocasein as a substrate as described above. pH profile was measured at 65 °C at various pHs: pH 5, 5.5, 6 using 100 mM sodium acetate; pH 6.5, 7 using 50 mM Tris-maleate; pH 7.5, 8, 8.5, 9 using 200 mM Tris-HCl; and pH 9.5, 10, 11 using 200 mM glycine-NaOH.

The effects of various groups of proteinase inhibitors and metal ions were also investigated. The remaining activity was calculated using the activity of the control (without inhibitor/ions) as 100%.

Substrate specificity

Proteinase activity was assayed by the modified method of Barrett and Kirschke (1981) using various synthetic substrates. The reaction mixture (1 mL) contained 200 μL crude proteinase, 1 μM synthetic substrate, and 200 mM Tris-HCl (pH 8.0) and incubated at 65 °C for 10 min. The reaction was terminated by adding 1.5 mL of the stopping solution (30% butanol, 35% methanol, and 35% deionized water). Fluorescence intensity was measured at excitation wavelength of 380 nm and emission wavelength of 460 nm (RF-1501, Shimadzu Co., Kyoto, Japan). 7-Amino-4-methylcoumarin (AMC) was used as a standard. Unit activity (U) was defined as 1 μmol of AMC released per minute.

Effect of $CaCl_2$ and NaCl on proteinase activity

The effect of $CaCl_2$ (10 to 100 mM) on proteinase activity was determined by the modified method of Barrett and Kirschke (1981) as described above, using Suc-Ala-Ala-Pro-Phe-AMC as a substrate.

Effect of NaCl on proteinase activity was determined by incubating the proteinase in 200 mM Tris-HCl (pH 8.0) containing various NaCl concentrations (0% to 20%). In the presence of NaCl, deionized water was used instead of butanol to avoid phase separation. Then sample was heated at 90 °C for 5 min to end the activity.

Effect of $CaCl_2$ on proteinase stability

Effect of $CaCl_2$ on proteinase stability was investigated by preincubating the enzyme in 200 mM Tris-HCl (pH 8.0) containing various $CaCl_2$ concentrations (0 to 100 mM) at 65 °C for 2 h. When incubation time was reached, samples were immediately cooled in iced water and subsequently were determined for residual activity. The relative residual activity was calculated using activity of sample without incubation at each $CaCl_2$ concentration as 100%.

Proteinase stability was also monitored by preincubating the crude proteinase in 200 mM Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$ or in the absence of $CaCl_2$. Samples were incubated at either 30 °C for 24 h or 65 °C for 4 h. Samples were rapidly cooled and assayed by the modified method of Barrett and Kirschke (1981) as described above.

Activity staining

Activity staining was determined using native polyacrylamide gel electrophoresis (native-PAGE) activity staining according to García-Carreno and others (1993). Crude proteinase containing 0.02 mU, 10% glycerol, 0.063 mM Tris-HCl (pH 6.8), 0.05% bromophenol blue, and either 1 mM PMSF, 10 mM EDTA, or 10 mM L-histidine was loaded into polyacrylamide gel (12.5% T) and ran at 100 V. Subsequently, it was immersed in 2% casein, 100 mM Tris-HCl, pH 8.0 at 4 °C for 30 min and it was washed twice with 100 mM Tris-HCl (pH 8.0). Proteolytic reaction was carried out in 100 mM Tris-HCl, pH 8.0, containing either no salt or 25% NaCl and incubated at 65 °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 it was destained in 25% ethanol and 10% acetic acid. Clear zone indicated the caseinolytic activity.

Molecular weight estimation

Molecular weight (MW) of crude proteinase was estimated using native-PAGE activity staining (García-Carreno and others 1993) and Ferguson plot analysis (Bollag and others 1996). A semilogarithmic plot between the relative migration value (R_f) and acrylamide concentrations (6, 7, 8, 9, 10 and 12.5% T) was constructed and the regression was analyzed. The negative slopes were plotted against the MW value of protein standard (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and the MW of proteinase was estimated.

Statistical analysis

Crude proteinase was prepared in 3 replications. Each analysis was done in duplicate and mean values were presented. The effect of Ca^{2+} and incubation time on enzyme activity was analyzed using completely randomized design (CRD). Analysis of variance (ANOVA) was conducted using SAS (SAS Inst. Inc., Carry, N.C., U.S.A.). Differences among mean values were established using Duncan multiple range test (DMRT) at $P < 0.05$.

Results and Discussion

Temperature and pH optimum

Proteinase activity of crude proteinase from *Virgibacillus* sp. SK37 increased with temperature and reached maximum activity at 65 °C (Figure 1), indicating that crude enzyme was activated at relatively high temperature. Proteinase from moderately halophilic bacteria, *Pseudoalteromonas* sp. CP76, *Salinivibrio costicola* 18AG, marine psychrophile PA-43, and *Salinivibrio* sp. AF-2004 showed optimum temperature at 55 to 65 °C (Irwin and others 2001; Sánchez-Porro and others 2003; Lama and others 2005; Karbalaei-Heidari and others 2007). Optimum pH of crude proteinase from *Virgibacillus* sp. SK37 was found at pH 8 (Figure 2). Proteinase from

Pseudoalteromonas sp. CP76, *Salinivibrio costicala* 18AG, marine psychrophile PA-43, and *Salinivibrio* sp. AF-2004 showed optimum pH of 8 to 8.5 (Irwin and others 2001; Sánchez-Porro and others 2003; Lama and others 2005; Karbalaei-Heidari and others 2007).

Effect of inhibitors and ions on proteinase activity

Proteinase activity of *Virgibacillus* sp. SK37 was strongly inhibited by PMSF (Table 1). A chymotrypsin-like proteinase inhibitor, TPCK, did not inhibit proteolytic activity. Trypsin-like proteinase inhibitors (leupeptin, soybean inhibitor, and TLCK) and cysteine inhibitors (E-64, NEM, and IAA) did not affect proteinase activity. Metalloproteinase inhibitors showed moderate inhibition. Dithiothreitol (DTT), 2-mercaptoethanol (2-BME), and L-cysteine, which are known as cysteine proteinase activators, showed no activation effect, confirming that cysteine proteinases were not involved. Based on the inhibitor and activator study, crude proteinase from *Virgibacillus* sp. SK37 was likely to be serine proteinase. It was reported that *Bacillus subtilis* CN2, *Halobacillus* sp. SR5-3, and *Filobacillus* sp. RF2-5 isolated from fish sauce mash secreted serine proteinase, while *Bacillus subtilis* JM-3 and *Halobacillus thailandensis* produced acid- and metallo-proteinase, respectively (Chaiyanan and others 1999; Uchida and others 2004; Hiraga and others 2005; Kim and Kim 2005; Namwong and others 2006).

Activity of *Virgibacillus* sp. SK37 proteinase was not affected by various metal ions, except Hg^{2+} (Table 1). Proteinase from γ-Proteobacterium DGII and *Brevibacterium linens* ATCC 9174 was inhibited by Cu^{2+}, Co^{2+}, Mn^{2+}, Hg^{2+}, and Zn^{2+} (Rattray and others 1995; Sana and others 2006), whereas *Salinivibrio costicala* 18AG proteinase was activated by Cu^{2+} and Mn^{2+} (Lama and others 2005). Monovalent cations (Li^+, Na^+, and K^+) did not reduce proteinase activity, but divalent cations (Mg^{2+} and Ca^{2+}) showed moderate activation (Table 1). The effect of divalent cations on bacterial proteinases varied with strains. Proteinase activity of *Bacillus pumilus*, *Bacillus* sp. NCDC 180, and *Brevibacterium linens* ATCC 9174 slightly increased with Ca^{2+} and Mg^{2+} concentration (Rattray and others 1995; Kumar and others 1999; Huang and others 2003), whereas activity of *Bacillus* sp. Vel proteinase decreased at 5 mM Ca^{2+} and Mg^{2+} (Gupta and others 2005). Activity of *Bacillus mojavensis* and *B. licheniformis* AP-1 proteinases were not affected by Mg^{2+} and Ca^{2+} (Beg and Gupta 2003; Tang and others 2004). Typically, efficiency of catalytic activity depends upon conformational flexibility (Daniel 1996). Divalent salt might induce structural changes by salting in effect, leading to a more flexibility of substrate-binding flaps; consequently, enzyme activity increases (Szeltner and Polgár 1996).

Substrate specificity

Crude proteinase of *Virgibacillus* sp. SK37 only cleaved Suc-Ala-Ala-Pro-Phe-AMC, suggesting that P_1 of the proteinase preferred aromatic amino acid residue (Table 2). Suc-Ala-Ala-Pro-Phe-chromogenic is a typical substrate for subtilisin (Graycar and others 2004). According to the effect of inhibitor and substrate specificity,

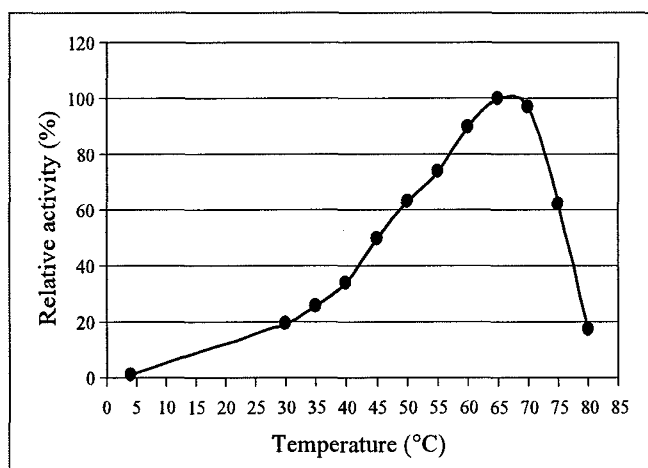

Figure 1 – Temperature optimum of proteinase from *Virgibacillus* sp. SK37

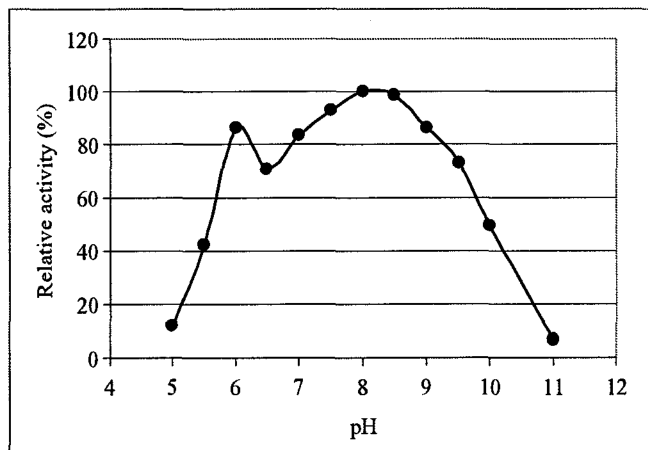

Figure 2 – pH optimum of proteinase from *Virgibacillus* sp. SK37

Table 1 – Effect of various inhibitors and ions on enzyme activity of extracellular proteinase from *Virgibacillus* sp. SK37

Substances	Targeted enzyme	Final concentration	Relative activity (%)
Leupeptin	Trypsin-like and some cysteine proteinases	100 μM	80
Trypsin inhibitor I (soybean)	Trypsin-like proteinase	0.02 mg/mL	78
TLCK	Trypsin-like proteinase	100 μM	89
TPCK	Chymotrypsin-like proteinase	100 μM	87
PMSF	Serine proteinase	1 mM	13
EDTA	Metallo proteinase	10 mM	76
L-Histidine	Metallo proteinase	10 mM	87
Bestatin	Aminopeptidase	10 μM	109
Pepstatin A	Acid proteinase	10 μM	100
E-64	Cysteine proteinase	10 μM	100
N-Ethylmaleimide	Cysteine proteinase	1 mM	100
Iodoacetic acid	Cysteine proteinase	1 mM	103
Dithiothreitol		10 mM	90
2-Mercaptoethanol		10 mM	100
L-Cysteine		10 mM	96
Mono- and di-valent cations			
Li^+		10 mM	104
Na^+		10 mM	96
K^+		10 mM	93
Mg^{2+}		10 mM	116
Ca^{2+}		10 mM	118
Metal ions			
Cu^{2+}		1 mM	93
Cd^{2+}		1 mM	113
Co^{2+}		1 mM	95
Mn^{2+}		1 mM	105
Hg^{2+}		1 mM	72
Zn^{2+}		1 mM	92

the crude proteinase from *Virgibacillus* sp. SK37 showed subtilisin-like characteristics. *Filobacillus* sp. RF2-5 proteinase preferably cleaved phenylalanine, methionine, and threonine at P_1 (Hiraga and others 2005), while *Halobacillus* sp. SR5-3 proteinase efficiently hydrolyzed leucine, glutamine, and alanine at P_1 (Namwong and others 2006). Proteinase from *Bacillus licheniformis* SMI 4.C.1 efficiently hydrolyzed substrate containing phenylalanine and leucine at P_1 (Manachini and Fortina 1998), while proteinase from *Bacillus thermoruber* was found to hydrolyze Z-Ala-Ala-Leu-*p* nitroanilide (*p*NA), indicating a subtilisin-like characteristic (Manachini and others 1988). In addition, *Bacillus* sp. PS719 proteinase cleaved arginine at P_1, suggesting a trypsin-like proteinase (Hutadilok-Towatana and others 1999).

Effect of $CaCl_2$ on proteinase activity and stability

Proteinase from *Virgibacillus* sp. SK37 was activated by Ca^{2+} for about 2 folds at 100 mM Ca^{2+} (Figure 3A). The effect of Ca^{2+} on activity of bacterial proteinases varies with strains. Activity of *Bacillus* sp. PS719 proteinase increased with Ca^{2+} up to 2 mM (Hutadilok-Towatana and others 1999). Activity of proteinase from *Salinivibrio costicala* 18AG was increased 2.4 times in the presence of 2 mM Ca^{2+} (Lama and others 2005), while activity of *Halobacterium halobium* S9 proteinase increased 2 times at 1 mM Ca^{2+} (Capiralla and others 2002). However, proteinase from moderately halophilic bacteria, *Pseudoalteromonas* sp. CP76, was slightly inhibited by 5 and 10 mM Ca^{2+} (Sánchez-Porro and others 2003). Proteinases from some genera, *Salinivibrio* sp. AF-2004 and *Bacillus subtilis* Y-108, were not affected by Ca^{2+} (Yang and others 2000; Karbalaei-Heidari and others 2007).

Table 2—Substrate specificity of extracellular proteinase from *Virgibacillus* sp. SK37

Synthetic substrates	Specificity	Relative activity (%)
Boc-Asp(oBzl)-Pro-Arg-AMC	Trypsin-like, α-thrombin-like	2
Boc-Gln-Ala-Arg-AMC	Trypsin-like	0
Boc-Val-Leu-Lys-AMC	Plasmin-like	5
Suc-Ala-Ala-Pro-Phe-AMC	Chymotrypsin-like, subtilisin-like	100
Z-Phe-Arg-AMC	Cathepsin L-like	5
Z-Arg-Arg-AMC	Cathepsin B-like	0

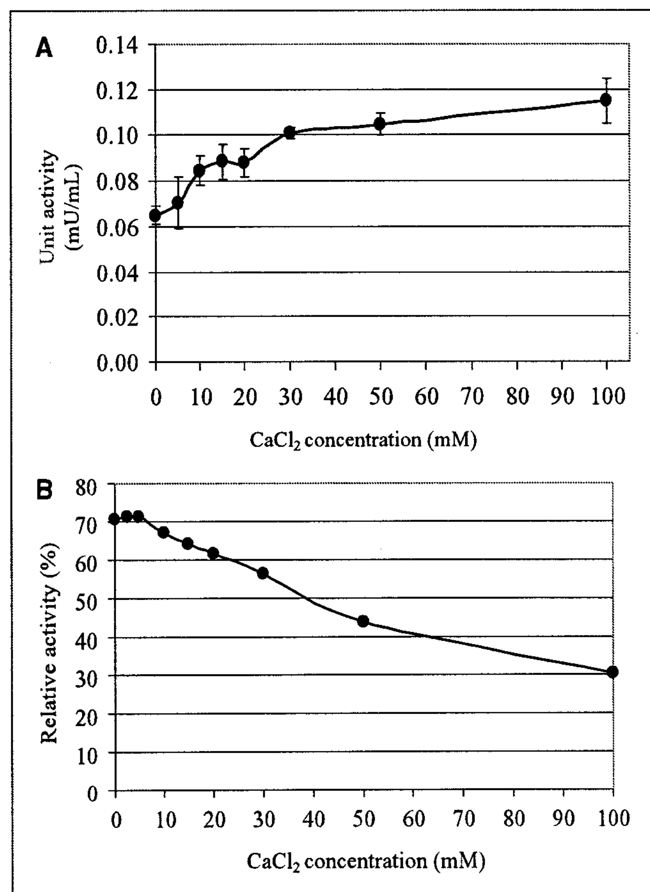

Figure 3—Effect of $CaCl_2$ concentration on activity (A) and stability at 65 °C, 2 h (B) of proteinase from *Virgibacillus* sp. SK37

In spite of the activation effect, Ca^{2+} appeared to decrease thermal stability at concentrations >5 mM (Figure 3B). The activity of crude proteinase at 100 mM Ca^{2+} was only 30% of the control, whereas 70% residual activity was noticed in the absence of Ca^{2+} (Figure 3B). In addition, proteinase from *Virgibacillus* sp. SK37 was stable at 30 °C at either 0 or 10 mM Ca^{2+}, but the activity decreased almost 60% at its optimum temperature (65 °C) in the presence of 10 mM Ca^{2+} (Figure 4). Although Ca^{2+} increased activity of the proteinase, it appeared to destabilize the enzyme at concentration as low as 10 mM. High Ca^{2+} concentration could also induce autolysis. Thus, in the application where the reaction time is extended longer than 4 h, Ca^{2+} content should be limited to be <10 mM to maintain enzyme stability. However, higher concentration for up to 30 mM Ca^{2+} can be added to increase proteinase activity when the reaction time is controlled within 2 h. The traditional fish sauce fermentation typically takes place outdoors at temperature ranging from 30 to 40 °C with relatively low soluble Ca^{2+} content. Our results indicated that *Virgibacillus* sp. SK37 proteinase showed good stability at the typical fermentation condition. This could be a desirable feature of the enzyme to be applied in fish sauce fermentation.

The effect of Ca^{2+} on proteinase stability usually varies with strains. Thermal stability of *Bacillus mojavensis* increased with Ca^{2+} up to 10 mM (Beg and Gupta 2003). Thermal stability of proteinase from *Bacillus stearothermophilus* F1 increased with Ca^{2+} concentration up to 20 mM (Rahman and others 1994). Stability of proteinase from *Bacillus* sp. KSM-K16 was also increased between 50 and 70 °C at 5 mM Ca^{2+} (Kobayashi and others 1996). Despite the destabilization caused by Ca^{2+}, proteinase from *Virgibacillus* sp. SK37 was more stable when compared to other moderately halophilic bacteria. About 80% of the original activity was retained at 65 °C, its optimal temperature, for 1 h (Figure 4). In contrast, activity of

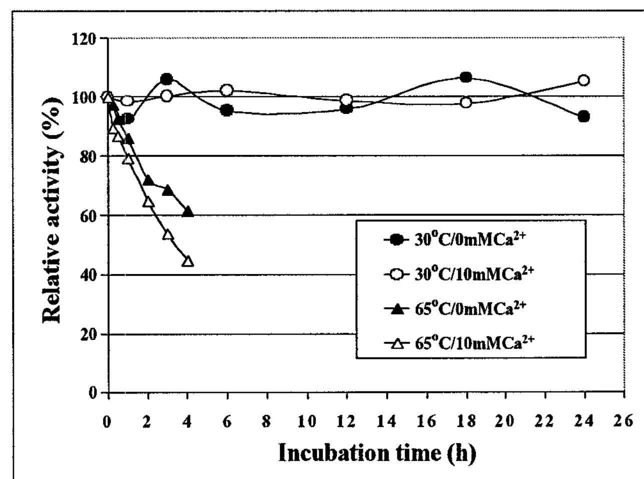

Figure 4—Thermal stability of *Virgibacillus* sp. SK37 proteinase in the absence of Ca^{2+} and 10 mM Ca^{2+} incubated at either 30 °C for 24 h or 65 °C for 4 h

Salinivibrio sp. AF-2004 proteinase remained only approximately 40% when incubated at the optimum temperature (65 °C) for 15 min (Karbalaei-Heidari and others 2007). Proteinase from *Filobacillus* sp. RF2-5 and *Salinivibrio costicala* 18AG was unstable at the optimum temperature (60 °C) (Hiraga and others 2005; Lama and others 2005). Higher thermal stability could be an advantageous characteristic as far as the application is concerned.

Effect of NaCl on proteinase activity

Activity of proteinase from *Virgibacillus* sp. SK37 gradually increased with NaCl concentration and reached maximum activity at 20% NaCl, which was about 5.5 times greater than at 0% NaCl (Figure 5). High NaCl concentration of fish sauce fermentation was a conducive condition for *Virgibacillus* sp. SK37 proteinase to be involved in protein hydrolysis. When compared to proteinases from other halophiles, the proteinase from *Virgibacillus* sp. SK37 exhibited activity at higher NaCl concentration. Proteinase from *Pseudoalteromonas* sp. CP76 showed maximum activity at only 0 to 1 M NaCl (0% to 5.8%), whereas activity of *Salinivibrio* sp. AF-2004 proteinase decreased at >0.5 M NaCl (2.9%) (Karbalaei-Heidari and others 2007). Halophilic enzymes uniquely contain positively charged amino acids exhibiting relatively low pI value (Rao and Argos 1981; Inouye and others 1997; Irwin and others 2001). At neutral pH, they possess charges on the surface, which can interact with hydrated NaCl through electrostatic interaction, preventing the enzyme from aggregation and inactivation (Rao and Argos 1981; Irwin and others 2001). This was an important feature rendering the halophilic enzyme to be active at high salt content.

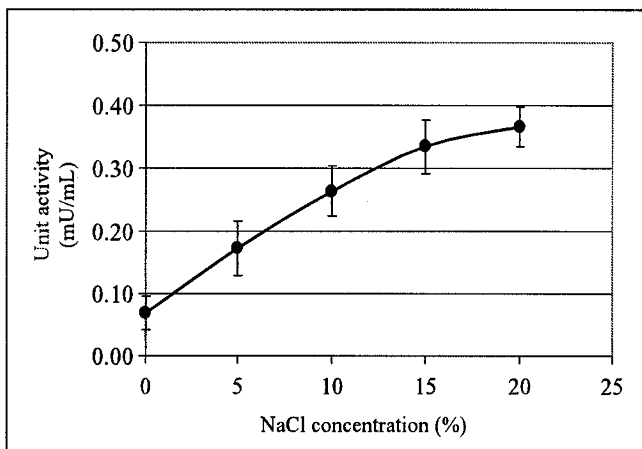

Figure 5 – Effect of NaCl on proteinase activity using Suc-Ala-Ala-Pro-Phe-AMC as a substrate

Activity staining

MW of crude proteinase from *Virgibacillus* sp. SK37 was estimated to be 81, 67, 63, 50, 38, and 18 kDa, based on native-PAGE activity staining. All proteinases showed caseinolytic activity at high salt content (Figure 6), confirming that the enzymes hydrolyzed protein substrate at 25% NaCl. Crude proteinase from archaeon *Natronococcus occultus* showed 7 distinct bands with MW ranging from 50 to 120 kDa (Studdert and others 1997), while proteinases from *Brevibacterium linens* F exhibited MW varying from 37 to 325 kDa (Hayashi and others 1990). In the presence of metalloproteinase inhibitors, namely EDTA and L-histidine, all proteinases showed similar activity staining pattern to that of control, but all proteinases were inactivated by PMSF (Figure 6). These results supported the assumption that proteinases from *Virgibacillus* sp. SK37 were serine proteinases. Crude proteinase from *Halobacillus thailandensis* sp. also contained serine proteinase with MW of 100 and 17 kDa, and 42-kDa metalloproteinase (Chaiyanan and others 1999). It should be noted that the clear zone at high salt content increased with reaction time (Figure 7), indicating that *Virgibacillus* sp. SK37 proteinase was able to hydrolyze protein at high salt content for up to 2 h.

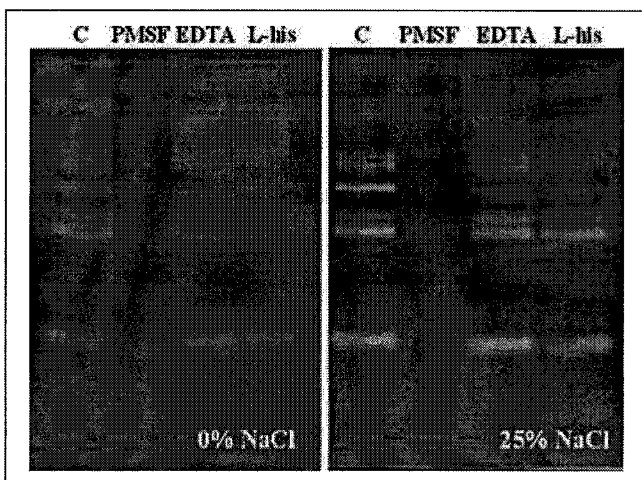

Figure 6 – Activity staining (native-PAGE, 12.5% T) of proteinase from *Virgibacillus* sp. SK37 in the presence and absence of 25% NaCl. C = control (without inhibitor); PMSF = 1 mM; EDTA = 10 mM; and L-his = 10 mM L-histidine.

Conclusions

Crude proteinase exhibited the optimum activity at 65 °C and pH 8.0, respectively. Several proteinases were observed with MW of 81, 67, 63, 50, 38, and 18 kDa. They showed subtilisin characteristics and caseinolytic activity at 25% NaCl. *Virgibacillus* sp. SK37 proteinase could be activated by NaCl and retained its activity at 20% to 25%NaCl. This is the 1st report of NaCl-activated proteinase from *Virgibacillus* sp. isolated from fish sauce. The enzyme appeared to have potential in hydrolyzing protein at high salt content, such as the fish sauce fermentation process. In addition, *Virgibacillus* sp.

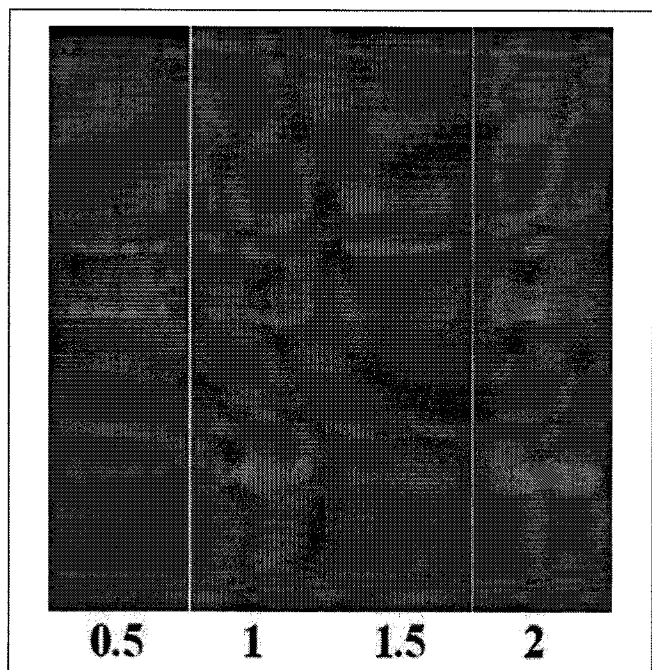

Figure 7 – Activity staining (native-PAGE, 12.5% T) of crude proteinase from *Virgibacillus* sp. SK37 at 25% NaCl. Numbers indicate incubation time in hours.

SK37 could be a promising strain for starter culture development used in fish sauce fermentation.

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