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Identification of halophilic strains and its proteolytic degradation of fish protein

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Aims: The research was carried out to study the isolation, identification of protease-producing halophilic strains and investigation of hydrolysis of various anchovy muscle proteins by a halophilic protease from strain SR5-3.
Methodology and results: Seven protease-producing halophilic strains were isolated from Thai fermented food. On the basis of phenotypic and chemotaxonomic characteristics including high DNA-DNA relatedness (70.0-77.3%), six strains were identified as *Virgibacillus halodenitrificans* and a halophilic bacterium, the highest protease producer, SR5-3, was named as *Halobacillus*. The hydrolysis of different fish proteins by a halophilic protease from strain SR5-3 was determined. The halophilic protease completely digested fish collagen and myosin heavy chain (MHC), but partially hydrolysed actin. In order to study the effect of NaCl on proteolytic activity, the degree of hydrolysis of purified protease and commercial proteases towards anchovy protein powder was compared. The halophilic protease showed a greater degree of hydrolysis towards anchovy substrate than that from commercial proteases in the presence of 25% NaCl.
Conclusion, significance and impact study: This study revealed that six *V. halodenitrificans* strains and a *Halobacillus* strain SR5-3 secreted halophilic proteases. Upon digestion of fish protein, a halophilic protease showed higher protease activity and stability in heavily NaCl concentration, suggesting its potential application in acceleration of fish sauce production.

Keywords: Halophilic bacteria, *Virgibacillus*, *Halobacillus*, halophilic protease, fish protein

INTRODUCTION

Halobacteria have developed an efficient metabolism for the utilization of proteins, peptides, and amino acids. They produce enzymes that usually require high salt concentrations for their activity and stability (Lanyi, 1974). Many strains secrete caseinolytic and gelatinolytic enzymes into the medium (Norberg and Hofsten, 1969; Cowan *et al.*, 1987; Markus and Volker, 1998). These are extracellular proteases of serine type (Studdert *et al.*, 1997). In Thailand, there are many types of traditionally fermented products with high concentration of salt such as fermented shrimp paste (*ka-pi*), a dark-colored strong smelling paste made from shrimp containing high salt and fish sauce, a clear brown liquid produced by fermentation of heavily salted fish material for 12 to 18 months (Lopetcharat *et al*., 2001. These salty environments occurred to thrive halophilic bacteria such as *Haloarcula tradensis*, *Lentibacillus salicampi*, *Halobacillus* sp. SR5-3, *Halobacterium salinarum*, and *Halobacillus thailandensis*, *Filobacillus* sp. RF2-5 and *Virgibacillus* sp. SK33. Of the reported halophilic bacteria, *H. salinarum*, and *H. thailandensis*, *Halobacillus* sp. SR5-3, *Filobacillus* sp. RF2-5 and *Virgibacillus* sp. SK33 were reported as protease producers (Choorit and Prasertsan, 1992; Thongthai *et al.*, 1992; Chaiyanan *et al.*, 1999; Tanasupawat and Komagata, 2001, Namwong *et al*., 2005, Hiraga *et al*., 2005; Namwong *et al*., 2006; Sinsuwan *et al*., 2010; Namwong *et al*., 2011). The specific characteristic of halophilic enzymes is highly stabilized by NaCl (20-30%) and their activities are increased about 2-3 folds by addition of 20-30% NaCl (Hiraga *et al*., 2005; Okamoto *et al*., 2010). Based on the fermentation period of fish sauce up to 18 months, the growth of this industry is limited. It would be more advantageous if these halophilic enzymes are applicable

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in producing fish sauce in a shorter time with the premium quality. Therefore, in this study, we aimed to isolate and identify protease-producing halophilic bacteria isolated from Thai fermented food and investigate the hydrolysis of various anchovy muscle proteins by a purified halophilic protease of strain.

MATERIALS AND METHODS

Screening of proteolytic producing isolates and their protease production

Halophilic bacteria were isolated from fermented shrimp paste and fish sauce products by spread plate technique on halophilic medium JCM No. 377 containing (g/L) 5 g, yeast extract (Difco); 5 g, casamino acids (Difco); 1 g, sodium glutamate; 3 g, trisodium citrate; 2 g, KCl; 20 g, $MgSO_4 \cdot 7H_2O$; 36 mg, $FeCl_2 \cdot 4H_2O$; 0.36 mg, $MnCl_2 \cdot 7H_2O$; 100 g, NaCl (pH 7.0-7.2); 20 g, agar, and incubated at 37 °C for 1-2 weeks.

All isolates were plated on JCM No. 377 agar medium containing 1% skim milk and 10% NaCl incubated for 3 days at 37 °C (Namwong *et al*., 2006). The protease-producing colonies surrounded by a clear zone indicating hydrolysis of casein were selected for further study. The effect of culture condition on protease production was determined by cultivation in 5 mL JCM No. 377 broth supplemented with nitrogen source (1% of soybean, casein, and polypeptone) and carbon source (1% starch, galactose and maltose) on a shaker (200 rpm) for 3 days at 37 °C. The protease activity was determined by Lowry *et al*. (1951). Unit of protease was defined as the amount of the enzyme yielding the equivalent of 1 µmol of tyrosine per minute under the defined assay conditions.

Identification methods

Cell form, cell size, cell arrangement, and colonial appearance were examined for the cell grown on JCM No. 377 medium at 37 °C for 5 days. Gram stain was conducted according to the procedure of Hucker and Conn (1923). Critical-point dried cells and spores were observed under a scanning electron microscope. Flagella were stained by the method described by Forbes *et al*. (1981). The biochemical characteristics were tested by the methods of Barrow and Feltham (1993) and Leifson (1963). Growth at 45 °C and NaCl requirement were determined in the medium containing various NaCl concentrations (0-30%). At lower NaCl range (0-2.0 % NaCl), the tested medium omitted $MgSO_4 \cdot 7H_2O$, KCl and trisodium citrate was used. Growth was monitored by measuring culture turbidity at 660 nm. Diaminopimelic acid (*meso*-DAP) in the peptidoglycan and menaquinone were analyzed as described previously (Komagata and Suzuki, 1987). Polar lipids were determined according to the method of Minnikin *et al*. (1984). The quantitative analysis of cellular fatty acids was employed with the Microbial Identification System (MIDI). DNAs were isolated from cells grown in JCM No. 377 broth incubated on a rotary shaker (150 rpm) at 37 °C for 24 h and purified by the method of Saito and Miura (1963). The DNA G+C content was determined by the method of Tamaoka and Komagata (1984) using a reversed-phase HPLC. DNA-DNA hybridization was conducted as reported previously (Ezaki *et al*., 1989) and detected by the colorimetric method (Tanasupawat *et. al*., 2000). The 16S rDNA fragments were amplified and the sequence was determined as described by Tanasupawat *et al*. (2006). The 16S rDNA sequence was multialigned with the CLUSTAL W program version 1.81 (Thompson *et al*., 1994), then the alignment was manually verified and edited prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) in the MEGA program version 2.1 (Kumar *et al*., 2001). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resampling.

Hydrolysis of different protein substrates by a halophilic protease

Natural actomyosin (NAM) and collagen were prepared according to the method of Benjakul *et al*. (1997) and Benjakul *et al*. (2003). Fresh anchovy were caught from Trang Province and transported in ice to food biotechnology research, BIOTECH, Pathumthani and kept frozen at –20 °C. The frozen fish were put in the blender and powderized in liquid nitrogen. The medium speed was selected for blending the anchovy fish. The anchovy protein powder was immediately kept at –20 °C before used. A halophilic protease from strain SR5-3 was purified according to the method of Namwong *et al*. (2006). One relative fluorescence unit of enzyme activity was defined as the enzyme quantity that liberates 1 µmole of AMC per mL of the reaction mixture per minute. The purified enzyme (0.175 U) was added to the reaction mixture containing 2 mg protein substrates and, including NAM, anchovy protein powder and collagen, and 247.5 µL of 25 mM phosphate buffer pH 6.0 and 10.0 containing 25% NaCl. The control was performed by incubating the reaction mixture at 50 °C for 60 min without the addition of purified protease. The reaction was terminated by adding preheated solution containing 2% SDS, 8 M urea and 2% βME incubated at 80 °C for 30 min. The solution was centrifuged at 8500 rpm for 10 min at room temperature (Klomklao *et al*., 2006). The supernatant was then subjected to SDS-PAGE analysis. SDS-PAGE was performed according to the method of Laemmli *et al*. (1970). The samples (15 µg) were loaded onto the gel made of 4% stacking gel and 7.5% separating gel for the collagen sample and 10% separating gel for NAM and anchovy protein samples.

Effects of enzyme types and concentration to the hydrolysis of anchovy protein powder

The anchovy protein powder as protease substrate was dissolved in 25 mM phosphate buffer (pH 6.0) containing 25% NaCl. Commercial enzymes [Flavourzyme® 500 mg

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(5-50 U), α-Chymotrypsin (400-4000 U), Novozyme® FM 2.0L (0.24-2.4U) and Neutrase 0.8L (0.1-1.0 U)] at 0.01, 0.05 and 0.1% and 0.35-1.05 U of the halophilic protease were added into the total protein solution with the protein concentration of 2% as determined by the Lowry *et al*. (1951). To initiate the reaction, the enzymes were added and incubated at 40 °C for 60 min. The enzyme was inactivated in boiling water for 3 min, followed by rapid cooling in iced water, The hydrolysates obtained were subjected to the determinations of degree of hydrolysis. (Klomklao *et al*., 2006).

Determination of degree of hydrolysis

The degree of hydrolysis (DH) was determined according to the method of Benjakul *et al*. (1995). DH was calculated as follows:

$$DH\ (\%) = [(L_t-L_0)/(L_{total}-L_0)X100]$$

where L_t was the amount of a-amino acid released at time t. L_0 was the amount of a-amino acid before hydrolysis. L_{total} was total a-amino group after acid hydrolysis. To prepare the completely hydrolyzed sample, (1 mL) was added with 1.5 mL of 10 M HCl. The mixture was heated in a heating box at 100 °C for 24 h. After cooling, the pH was adjusted to a neutral pH before the determination of free amino acid group content.

RESULTS AND DISCUSSION

Screening of proteolytic producing isolates and their protease production

A total of 35 bacterial strains were isolated from fermented food produced in Thailand using the spread plate technique on JCM No. 377 medium containing 1% skim milk and 10% NaCl. Seven strains, SSK3-2, SR5-3, SSK10-5, SSK9-1, PN2-3, PN2-10, PN5-1 showed the highest caseinolytic halo-forming colonies on the plate containing 10% NaCl. However, the clear zone around colonies on milk-agar plates did not assess the level of protease activity quantitatively. Therefore, protease activities were assayed using casein as substrate in the presence of 10% NaCl (w/v). The halophilic strains were cultivated in the JCM No. 377 medium with the specified nitrogen and carbon sources supplementation. The supernatant was determined for the protease activity. Of the strains, strain SR5-3 had the maximum protease production (0.6 U/mL) in the medium containing 1% casein as shown in Figure 1. The strain, SR5-3 was selected for the further study. It was identified as *Halobacillus* and its protease properties were characterized by Namwong *et al*. (2006). Briefly, the purified 43 kDa protease showed optimal activity at 50 °C and pH 9-10 in 20% NaCl. The proteolytic activity was enhanced about 2.5-fold by the addition of 20-35% NaCl, and a purified halophilic protease was highly stabilized by NaCl.

Identification of halophilic strains

Six halophilic isolates, SSK3-2, SSK10-5, SSK9-1, PN2-3, PN2-10 and PN5-1, were taxonomically studied. They were Gram positive rods, measuring approximately 0.5-0.8 x 2.0-6.0 µm. Cells were motile. Terminal or subterminal ellipsoidal spores were observed in swollen sporangia (Figure 2). Colonies on JCM No. 377 agar plate were white cream-colored and low convex or raised, smooth, and circular. They produced catalase and oxidase but not urease. Optimal growth temperature was 30-37 °C. Growth occurred at 40-45 °C but not at 50 °C. The phenotypic characteristics and other properties of halophilic strains were summarized in Table 1. They contained *meso*-DAP as the diagnostic diamino acid in the cell wall peptidoglycan. The major fatty acids were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The major menaquinone was MK-7. Predominant polar lipids were phosphatidylglycerol, diphosphatidylglycerol. DNA G+C contents ranged from 37.3-37.7 mol% as shown in Tables 1 and 2. These characteristics seem to agree with those of the genus *Virgibacillus.* (Heyndrickx *et al*., 1998; Heyrman *et al*. 2003; Yoon *et al*., 2004; Lee *et al*., 2012; Zhang *et al*., 2013).

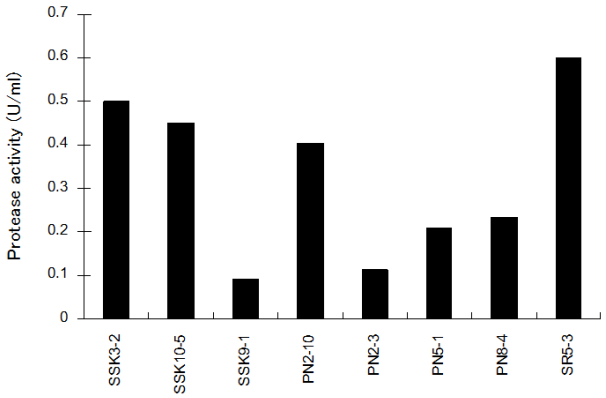

Figure 1: Protease activity of halophilic isolates.

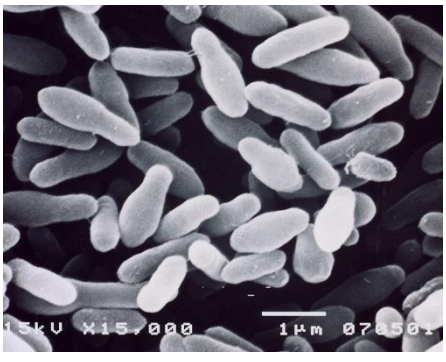

Figure 2: Scanning electron micrograph of strain SSK3-2 grown JCM no. 377 medium at 37 °C for 3 days. Bar, 1 µm.

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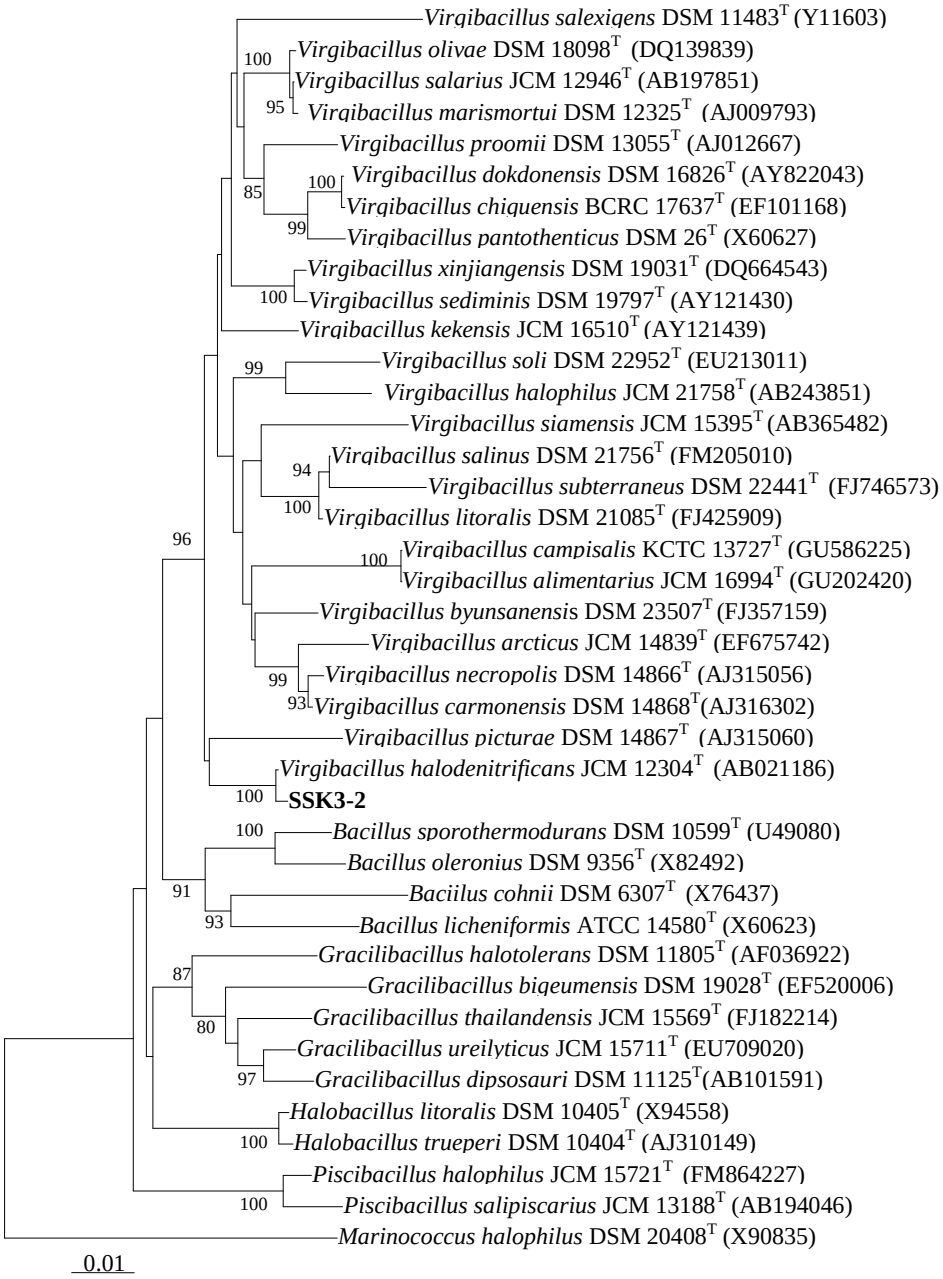

Figure 3: Neighbor-joining tree showing the relationships between strain SSK3-2 and related bacterial species based on 16S rRNA gene sequences. The numbers on the branches indicate the percentage bootstrap values above 80%, based on 1000 replications. Bar, 1 substitution per 100 nucleotide positions.

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The 16S rRNA-based phylogenetic analysis clearly indicated that the representative strain SSK3-2 belonged to the genus *Virgibacillus* as shown in Figure 3. The representative strain was related most closely to *V. halodenitrificans* JCM 12304^T with 99.7 % similarity and a high bootstrap value (100%). The result of the DNA-DNA hybridization study indicated that the halophilic strains were closely related among themselves with 90.5-96.9 % of DNA-DNA relatedness, suggesting that they belong to the same species (Table 2). They showed high levels of DNA-DNA relatedness with *V. halodenitrificans* JCM 12304^T more than 70 % and in the reciprocal analysis, the relatedness between the representative strain, SSK3-2, and *V. halodenitrificans* JCM 12304^T was 70 %, warranting that halophilic strains being included in *V. halodenitrificans* JCM 12304^T (Wayne *et al*., 1987). On the basis of polyphasic taxonomic approach, the tested strains shared almost identical phenotypic and chemotaxonomic properties and exhibited high DNA-DNA relatedness to *V. halodenitrificans* JCM 12304^T, accordingly, they were identified as *Virgibacillus halodenitrificans* (Heyndrickx *et al*., 1998; Heyrman *et al*. 2003; Yoon *et al*., 2004; Lee *et al*., 2012; Zhang *et al*., 2013).

Table 1: Characteristics of *Virgibacillus* strains and type strain.

Characteristics	*Virgibacillus* sp. (6 strains)	*V. halodenitrificans* JCM 12304^T
Spore shape	E	E
Gram strain	V	V
Spore position	T/ST	T/ST
Nitrate reduction	+	+
Hydrolysis of casein	+	+
Hydrolysis of gelatin	+	+
H_2S production	-	-
Growth at 45 °C	+	+
Growth in 0 %NaCl	+(-1)	+
Acid production from:		
D-Fructose	+(-1)	+
Galactose	+(-3)	+
Glucose	+	+
Mannitol	+(-1)	+
Mannose	+(-3)	+
Rhamnose	-	-
Trehalose	-	+
Polar lipids	PG,DPG	PG,DPG
Major Menaguinone	MK-7	MK-7
DNA G+C content (mol(%	37.7-37.3	38.0
Major menaquinone	MK-7	MK-7
Major polar lipids	PG, DPG	PG, DPG
Fatty acid profiles	Anteiso C15:0, Anteiso C17:0	Anteiso C15:0, Anteiso C17:0

+, positive; -, negative. E, ellipsoidal; ST, subterminal; T, terminal; V, variable; DPG, Diphosphatidylglycerol; PG, Phosphatidylglycerol. Number in parentheses indicates number of isolate which shows positive or negative reaction.

Table 2: DNA-DNA relatedness and DNA G +C contents of *Virgibacillus* strains.

Strains	G+C content (mol%)	DNA-DNA relatedness with Labeled strain (%)	
		SSK3-2	JCM 12304^T
SSK3-2	37.6	100	70.0
SSK9-1	37.5	94.3	77.3
SSK10-5	37.6	90.5	70.0
PN2-3	37.3	92.0	74.4
PN2-10	37.7	90.6	73.9
PN5-1	37.7	96.9	74.7
V. halodenitrificans JCM 12304^T	38.0	70.0	100

Hydrolysis of different protein substrates by purified halophilic protease

Anchovy protein powder contained NAM, collagen and other protein components representing total proteins present in anchovy which are important substrates in fermentation of fish sauce (Figure 4). According to SDS-PAGE pattern, muscle proteins with high molecular weight, such as MHC, β- and α-subunits of fish collagen were degraded more extensively after adding of the purified protease from strain SR5-3 in the presence of 25% NaCl at both pH 6.0 and 10.0, as shown by a concomitant increase in the lower MW peptide fragments. Changes in actin band were also observed, although it was hydrolysed at slower rate than MHC.

Similar results were observed when NAM was used as substrate (Figure 5), although autolysis of NAM (without purified protease addition) was unexpectedly observed during incubation at 50 °C. This possibly suggested the presence of a myofibrillar associated protease that bound tightly with NAM and could not be removed during the extraction process (Osatomi *et al*., 1997; Cao *et al*., 2000). However, a larger extent of degradation was observed when halophilic protease was added. Among all proteins in NAM, MHC was rapidly and completely hydrolyzed within 10 min by the halophilic protease whilst degradation of actin increased as the time of hydrolysis increased.

The hydrolytic degradation of collagen is depicted in Figure 6. SDS-PAGE pattern revealed that β- or α-chains of collagen were cleaved by the halophilic enzyme at pH 6.0 as similar as pH 10.0. Among three components, α_2-chain was likely to be the most susceptible to hydrolysis, followed by β- and α_1-chains, respectively. Klomkao *et al*. (2006) and Yamashita and Konagaya (1991) reported that collagen was not a good substrate for non-halophilic proteinases, trypsin and pepsin.

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For NAM, the degradation of actin and MHC by trypsin from skipjack tuna was not observed at 25% NaCl (Klomkao *et al*., 2006). These data suggested that collagen and actin are the good substrates for protease from *Halobacillus* strain SR5-3. Therefore, the purified halophilic protease is more suitable for degradation of fish protein than non-halophilic proteases (trypsin and pepsin) in the presence of 25% NaCl.

Effects of enzyme types and concentration on the hydrolysis of anchovy protein powder

The degree of hydrolysis of various concentrations of purified halophilic protease from strain SR5-3 and commercial proteases was compared by using 2% anchovy protein powder as substrate at pH of 6.0 and 25% NaCl. Table 3 indicates that DH increased with the increase of concentration of commercial enzymes and the purified protease. However, a sharp increase in DH was observed as the concentration of purified enzyme was increased from 0.35 to 1.05 U at substrate pH 6.0 and in the presence of 25% NaCl. Hence, in high concentrations of NaCl, the enzyme takes advantage of the salting-out nature of the medium on the protein substrate, and this makes the partitioning of the substrate into the protease's active site more favorable (Norberg and Hofsten, 1969; Ryu *et al*., 1994). From the results, the halophilic protease is an excellent candidate as a catalyst for hydrolysis of fish protein in fish sauce production. However, DH slightly increased at commercial enzyme-to-substrate level of 0.1%. According to the previous studies, trypsin from skipjack tuna spleen as non-halophilic protease and the commercial proteases (Protease-P-Amano6, Alcalase®, Protex 7L®, and Neutrase®) were undesirable to digestion of sardine protein in the presence of NaCl. Some losses in activity of trypsin and commercial proteases occurred at the high concentration of NaCl, probably owing to the partial denaturation of proteinases caused by the ‘‘salting out’’ effect (Klomkao *et al*., 2004; 2006; Hathwar *et al*., 2011).

Table 3: Degree of hydrolysis of anchovy protein by halophilic SR5-3 protease and commercial proteases.

	Enzyme added	Degree of hydrolysis (%)[a]
Control	-	0.08 ± 0.15
SR5-3 protease	0.35 U	16.01 ± 0.70
	0.70 U	17.41 ± 0.63
	1.05 U	19.40 ± 0.26
Novozyme® FM 2.0L	0.01 %	1.61 ± 0.15
	0.05 %	1.53 ± 0.26
	0.10 %	2.04 ± 0.67
α-Chymotrypsin	0.01 %	2.38 ± 0.96
	0.05 %	2.33 ± 0.64
	0.10 %	2.46 ± 0.57
Flovourzyme® 0.8L	0.01 %	12.91 ± 1.53
	0.05 %	12.61 ± 0.79
	0.10 %	13.29 ± 0.07
Neutrase® 500MG	0.01 %	13.16 ± 1.69
	0.05 %	13.20 ± 0.70
	0.10 %	13.59 ± 0.90

[a] Hydrolysis was conducted at 40 °C, pH 6.0, 25% NaCl or 60 min. Control was incubated without enzyme addition for 60 min.

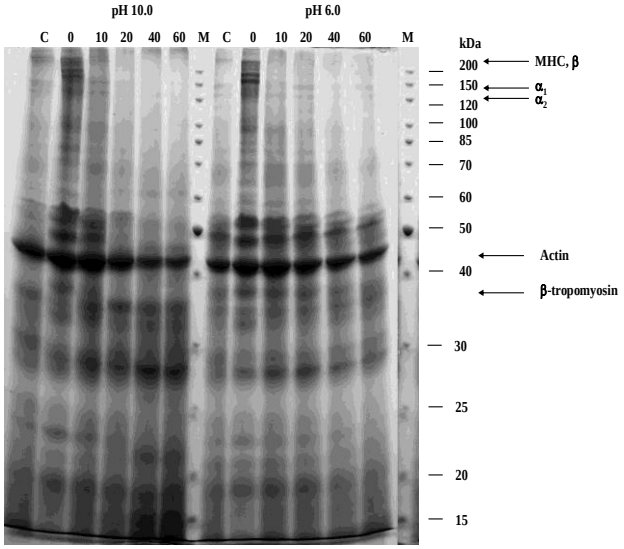

Figure 4: Hydrolysis of anchovy protein by purified protease. Hydrolysis was conducted using 0.175 U/2 mg protein at 50 °C, pH 6.0 and 10.0 containing 25% NaCl. C, control (incubated without enzyme addition for 60 min); MHC, myosin heavy chain. Numbers designate the incubation time (min).

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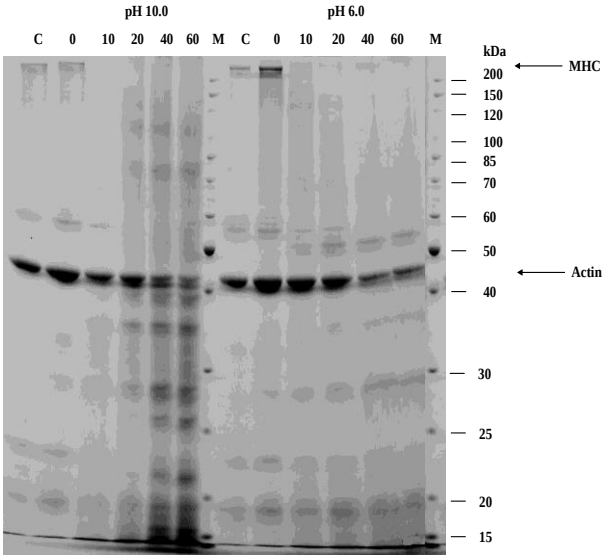

Figure 5: Hydrolysis of NAM by purified protease. Hydrolysis was conducted using 0.175 U/2 mg protein at 50 °C, pH 6.0 and 10.0 containing 25% NaCl. C, control (incubated without enzyme addition for 60 min); MHC, myosin heavy chain. Numbers designate the incubation time (min).

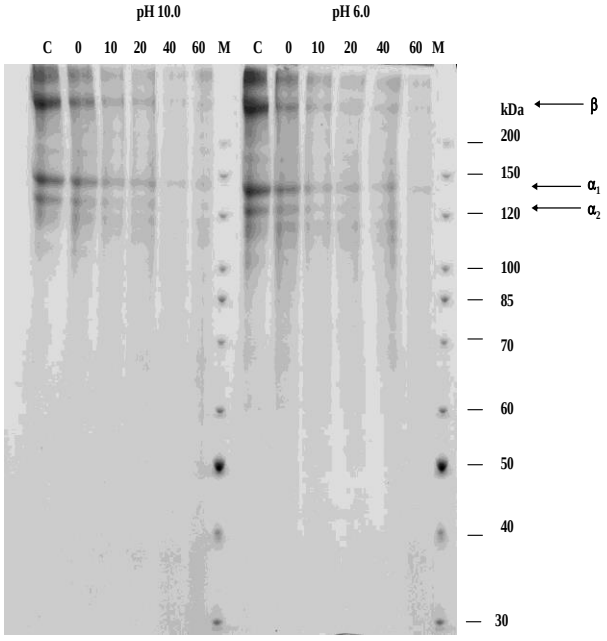

Figure 6: Hydrolysis of collagen by purified protease. Hydrolysis was conducted using 0.175 U/2 mg protein at 50 °C, pH 6.0 and 10.0 containing 25% NaCl. C, control (incubated without enzyme addition for 60 min at 50 °C). Numbers designate the incubation time (min).

CONCLUSION

According to the previous reports, shrimp paste and fish sauce contained a high concentration of NaCl which permit in a proliferation in the protease-producing halophilic bacteria of the genera *Virgibacillus* and *Halobacillus*. The halophilic serine protease from strain SR5-3 was capable of hydrolyzing mycosin heavy chain and the major component of collagen (β- and α-compounds) effectively. The halophilic protease showed a greater degree of hydrolysis than did commercial proteases toward anchovy protein at the condition of production of Thai fish sauce (25% NaCl and pH 6.0) suggesting that the purified protease may accelerate the protein hydrolysis during fermentation better than other commercial proteases Therefore, the fermentation period of fish sauce (12-18 months), might be shortened by the addition of halophilic protease, a potential novel enzyme for industrial applications.

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