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# Proteolytic Dedradation of Anchovy (*Spolephorus* spp.) Proteins by Halophilic Proteinase from *Halobacillus* sp. SR5-3

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***Abstract*****—**The halophilic proteinase showed a maximal activity at 50°C and pH 9~10, in 20% NaCl and was highly stabilized by NaCl. It was able to hydrolyse natural actomyosin (NAM), collagen and anchovy protein. For NAM hydrolysis, the myosin heavy chain was completely digested by halophilic proteinase as evidenced by the lowest band intensity remaining, but partially hydrolysed actin. The SR5-3 proteinase was also capable hydrolyzing two major components of collagen, β- and α-compounds, effectively. The degree of hydrolysis (DH) of the halophilic proteinase and commercial proteinases (Novozyme, Neutrase, chymotrypsin and Flavourzyme) on the anchovy protein, were compared, and it was found that the proteinase showed a greater degree of hydrolysis towards anchovy protein than that from commercial proteinases. DH of halophilic proteinase was sharply enhanced according to the increase in the concentration of enzyme from 0.035 U to 0.105 U. The results warranting that the acceleration of the production of fish sauce with higher quality, may be achieved by adding of the halophilic proteinase from this bacterium.

***Keywords*****—**Halophilic proteinase, *Halobacillus* sp. SR5-3, anchovy (*Spolephorus* spp.) proteins, fish sauce

## I. Introduction

NAM-PLA (fish sauce) is a clear brown liquid developed during fermentation of heavily salted fish material in closed tanks at tropical temperatures. It is commonly used as a flavour enhancer or salt replacement in various food preparation [1], [2]. During fermentation, fish proteins are hydrolysed, mainly as a result of autolytic action from the digestive proteinases in fish. The most important digestive sources of enzymes are pepsin, secreted from gastric mucosa, trypsin and chymotrypsin secreted from pancreas [3], [4]. Recently, Trypsin-like serine proteinase from the spleen of skijack tuna (*Katsuwonus pelamis*) was reported. The hydrolytic activity of enzyme was inactivated, when the NaCl concentration was increased [5], [6]. Apart from trypsin, chymotrypsin and other digestive enzymes were unavaiable to cleave the protein in the presence of high NaCl concentration.

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Therefore, the remains of fish protein might be digested by halophilic proteinases due to fish sauce contained heavily NaCl that occurred to thrive proteinase-producing halophilic bacteria such as *Filobacillus* sp. RF2-5 and *Halobacillus* sp. SR5-3 [7], [8]. The first specific characteristic of halophilic enzymes was highly stabilized by NaCl (20-30%). The second, their activity were increased about 2-3 folds by addition of 20-30% NaCl and also showed the maximum at the same condition. Untill now, non-halophilic proteinases, trypsin and chymotrypsin (0.3% w/v) have been used to speed up the fermentation of fish sauce [9]. Unfortunately, a knowledge of halophilic proteinases from Thai fish sauce have been proved for 5 years. There is no information regarding the hydrolytic activity of halophilic proteinase towards muscle proteins, especially from fish commonly used for fish sauce fermentation. Therefore, this study aimed to investigate the hydrolysis of various anchovy muscle proteins by a purified proteinase from *Halobacillus* sp. SR5-3.

## II. Material And methods

### *1. Purification of Proteinase from Halobacillus sp. SR5-3*

The purified proteinase produced from *Halobacillus* sp. SR5-3 was prepared according to the method of Namwong [8]. Briefly, SR5-3 strain was cultured in JCM No. 377 medium supplemented with ami ami, glutamic acid and aspartic acid. A proteinase was obtained by using a single step of chromatography, bacitracin-Sepharose column. The purity of the purified enzyme was analyzed by SDS-PAGE and native-PAGE [10]. Proteinase activity was assayed by the peptidyl-7-amino-4-methylcoumarin method [11], and the peptidase activity was assayed in the presence of optimal NaCl concentration of 20% NaCl. One relative fluorescence unit (U) of enzyme activity was defined as the enzyme quantity that liberates 1 μmole of AMC (amino methyl cumarin**)** per ml of the reaction mixture per minute. Protein concentration was estimated by Lowry [12] with crystalline bovine serum albumin (Sigma Chemical) as a standard.

### *2. Protein Preparation*

Natural actomyosin (NAM) was prepared according to the method of Benjakul [13] with a slight modification. Anchovy (*Spolephorus* spp.) muscle (50 g) was homogenised in 250 ml of chilled (4 °C) 0.6 M KCl, pH 7.0, for 4 min. The sample was placed in ice and each 20 s of blending was followed by a 20 s rest interval to avoid overheating during the extraction. The extract was centrifuged at 5000 g (relative centrifugal force) for 30 min at 4 °C. Three volumes of chilled distilled water were added to precipitate NAM.

NAM (the pellet) was collected by centrifuging at 5000 g for 20 min at 4 °C. The sample were kept in 40% glycerol at -20°C.

Collagen was prepared according to the method of Benjakul [14] with a slight modification. All preparation procedures were performed at 4 °C. To remove non-collagenous proteins, the sardine fillets containing skin were ground and mixed with 0.1 N NaOH at a sample/alkali solution ratio of 1:10 (w/v). The mixture was stirred for 6 h. The alkali solution was changed every 2 h. Then, the deproteinised samples were washed with cold distilled water until neutral or faintly basic pHs of wash water were obtained. Deproteinised samples were defatted with 10% butyl alcohol, with a solid/solvent ratio of 1:10 (w/v) for 18 h and the solvent was changed every 6 h. Defatted samples were washed with cold water, followed by soakingin 0.5 M acetic acid with a solid/solvent ratio of 1:30 (w/v) for 24 h. The mixture was filtered through two layers of cheese cloth. The residue was re-extracted under the same conditions. Both filtrates were combined. The collagen was precipitated by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M Tris–HCl, pH 7.0. The resultant precipitate was collected by centrifugation at 20,000g for 60 min. The pellet was dissolved in 0.5 M acetic acid, dialysed against 0.1 M acetic acid and distilled water, respectively, and then freeze-dried.

Anchovy protein was prepared according to the method of Benjakul [15]. Anchovy fish were freezed in -20°C for 12 h. The frozen fish were put in the blender and pour liquid nitrogen. The medium speed was selected for blending the anchovy fish. The total proteins were immediately kept at -20°C before using.

*3. Hydrolysis of Different Protein Substrates by Purified Proteinase*

Purified enzyme (0.035 U) was added to the reaction mixture containing 2 mg protein substrates and, including NAM, anchovy protein and collagen, and 247.5 µl of 25 mM phosphate buffer pH 6.0 and 10.0 containing 25.25% NaCl. The hydrolysis was conducted by incubating the mixture at 50 °C for 0, 10, 20, 40 and 60 min. The control was performed by incubating the reaction mixture at 50 °C for 60 min without the addition of purified proteinase. The reaction was terminated by adding 250 µl of preheated solution containing 2% SDS, 8 M urea and 2% βME (β-mercaptoethanol) (80 °C). The mixture was further incubated at 80 °C for 30 min to solubilise total proteins. The solution was centrifuged at 8500 rpm for 10 min at room temperature to remove the debris. The supernatant was then subjected to SDS–PAGE analysis. SDS–PAGE was performed according to the method of Laemmli [16]. Protein solutions were mixed at 3:1 (v/v) ratio with the SDS–PAGE sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol,10% β -mercaptoethanol) and boiled for 3 min. The samples (1-10 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. Electrophoresis was run at a constant current of 20 mA per gel, the gels were stained with 0.2% Coomassie brilliant blue R-250 in 45% methanol.

*4. Effects of Enzyme Types and Concentration on the Hydrolysis of Anchovy protein*

The anchovy protein was dissolved in 25 mM phosphate buffer (pH 6.0) containing 25% NaCl. Commercial enzymes [α-chymotrypsin (400-4,000U), Novozyme® FM 2.0L (0.024-0.24U), Neutrase 0.8L (0.01-0.1U) and Flavourzyme® 500MG (5-50U)] at 0.01, 0.05 and 0.1% (w/w) and the purified proteinase (0.035-0.105 U) were added into the total protein solution with the protein concentration of 2% determined by the Lowry [12]. Prior to the reaction, total protein solution was pre-incubated at reaction temperature for 15 min. 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.

*5. Determination of Free Amino Group and Degree of Hydrolysis*

The degree of hydrolysis (DH) was determined according to the method of Benjakul [13] with slight modification. Protein solutions and hydrolysates (125 µl) were mixed with 2 ml of 0.2 M sodium phosphate buffer, pH 8.2, 1 ml of 0.01% TNBS. The solution was mixed thoroughly and placed in a temperature-controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and a-amino acid was expressed in terms of leucine. DH was calculated as follows Benjakul [13].

$$DH\ (\%) = [(L_t-L_0)/(L_{total}-L_0)X100] \quad (1)$$

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

## III. Results And Discussions

### A. Characteristics of the Purified Proteinase from *Halobacillus sp. SR5-3*

The 43 kDa-proteinase was stable in a broad pH range from pH 5.0-8.0 (optimally at pH 10.0), for 120 min when heated up to 50 °C (optimally at 50°C) in the presence of 20% NaCl. The peptidase activity increased about 2.5-fold in the presence of 20-35% NaCl compared with that of control. The stability of the proteinase was drastically increased in the presence of 20-35% of NaCl, but it was completely lost under the conditions of low concentration of NaCl. The summarized characteristics of the purified proteinase showed in Table 1.These results related the activity of the halophilic proteinases from *Halobacterium halobium*, *Haloferax mediterranei* and *Filobacillus* sp. RF2-5 [7], [16]–[19] .Thus, this proteinase is a halophilic proteinase or is salt activated. We focus this study on the proteinase was salt-activated activity that might be involved in the degradation of fish protein during fish sauce production.

TABLE I
THE PROPERTIES OF PROTEINASE FROM *HALOBACILLUS* SP. SR5-3

| | |
|---|---|
| Molecular weight | 43 kDa (SDS-PAGE) |
| Optimum conc. of NaCl | 20-30% (pH 7.5, 37 °C, 20 min) |
| Optimum pH | 9-10 (37 °C, 20 min) |
| pH stability | 7 (37 °C, 180 min) |
| Optimum temp | 50 °C (pH 7.5, 20 min) |
| Thermal stabiliy | 30-50 °C (pH 7.5, 120 min) |
| Inhibitors | Inhibition (%) |
| PMSF (1 mM) | 99 |
| Chymostatin (100 μM) | 93 |
| α- MAPI[a] (50 μg/ml) | 87 |
| Leupeptin (10 mM) | 13 |
| E-64 (10 μM) | 15 |
| EDTA (1 mM) | 0 |
| EGTA (1 mM) | 0 |
| Phosphoramidon (10 μM) | 0 |

[a]MAPI, α-microbial alkaline-proteinase inhibiotor.
The number in parenthesis following proteinase inhibitors show final concentrations.

### *B. Hydrolysis of Different Protein Substrates by Purified Proteinase from Halobacillus sp. SR5-3*

#### *1. Natural Actomyosin (NAM)*

The major constituents of natural actomyosin (NAM) was actin but, Myosin heavy chain (MHC) was found as minor components (Fig. 1). Among all proteins, MHC was completely hydrolyzed in a reaction mixtures containing 25% NaCl at pH 6.0 and pH 10.0 within 10 min by halophilic proteinase. For actin, the degradation was rapidly increased as the time of hydrolysis increased. However, there is slightly original band observed based on the concentration of enzyme and time of hydrolysis might not be excess for hydrolysis the high intensity of original band. Both of pH, there is no different digestion of actin by enzyme. During incubation at 50 °C, autolysis of sample (without purified proteinase addition) was occurred. This possibly indicated the presence of a myofibrillar associated proteinase that bound tightly with NAM and could not be removed during the extraction process [20], [21]. From the result, the purified proteinase from *Halobacillus* sp. SR5-3 hydrolysed NAM effectively, particularly MHC and actin which are the minor and dominant protein in anchovy muscle, respectively.

#### *2. Collagen*

The hydrolytic degradation of collagen was observed when collagen was incubated with the halophilic proteinase referred as chymotrypsin-like serine proteinase or subtilisin-like serine proteinase at pH 6.0 and 10.0 in the presence of 25% NaCl (Fig. 2). As a result, the protein pattern of major components was changed when analysed by SDS–PAGE revealed that the peptide bonds in the β- or α- components were cleaved by the halophilic enzyme. The degradation of collagen, the major component, β- and α- compounds were hydrolyzed efficeincy at pH 6.0 as similar as pH 10.0. Among three components, the $\alpha_2$- components were the most susceptible to hydrolysis, followed β- and $\alpha_1$- components. Previous reports, non-halophilic proteinases, trypsin and pepsin generally undergo limited cleavage in the non-helical region of collagen molecules [6], [22]. For the control, hydrolysis of collagen was observed even omitted the halophilic proteinase.

Aoki and Ueno [22] reported that collagens were degraded by cathepsin L in white muscle of mackerel but not by cathepsin B. Thus, the degradation of collagen depends upon the source of collagen as well as on the types of proteinase. These data suggested that collagen is a good substrate for chymotrypsin-like serine proteinase or subtilisin-like serine proteinase from halophilic bacteria, *Halobacillus* sp. SR5-3. Therefore, the purified halophilic proteinase is more suitable for degradation of fish protein than non-halophilic proteinases (trypsin and pepsin) due to the halophilic proteinase can hydrolyze almost completely the major component of either NAM and collagen.

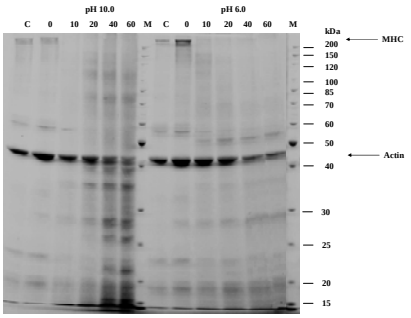


Fig. 1 Hydrolysis of NAM by purified proteinas from the *Halobacillus* sp. SR5-3 at 50 °C. H, high-molecular-weight standard; C, control (incubated without enzyme addition for 60 min at 50 °C); MHC, myosin heavy chain. Numbers designate the incubation time (min) at 50 °C

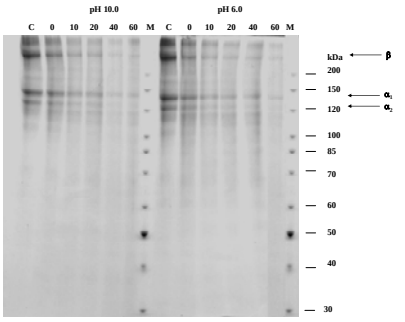


Fig. 2 Hydrolysis of collagen by purified proteinas from the *Halobacillus* sp. SR5-3 at 50 °C. H, high-molecular-weight standard; C, control (incubated without enzyme addition for 60 min at 50 °C); MHC, myosin heavy chain. Numbers designate the incubation time (min) at 50 °C

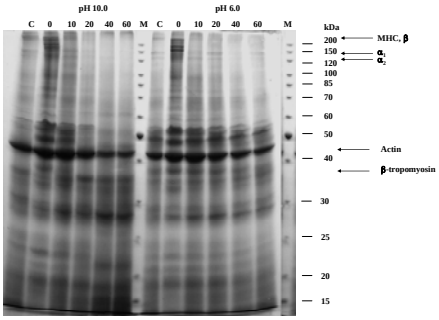


Fig. 3 Hydrolysis of anchovy protein by purified proteinas from the *Halobacillus* sp. SR5-3 at 50 °C. H, high-molecular-weight standard; C, control (incubated without enzyme addition for 60 min at 50 °C); MHC, myosin heavy chain. Numbers designate the incubation time (min) at 50 °C

*3. Anchovy Protein*

It was the representation of whole protein in anchovy fish containing NAM, collagen and other components (Fig. 3). As the results of SDS-PAGE profile, MHC or β-compound, $\alpha_2$-compound were degraded more extensively after adding of the purified proteinase at pH 6.0 and 10.0, as shown by the disappearence of original bands of each component remaining with a concomitant increase in the lower MW peptide fragments. On the other hand, $\alpha_1$- compound and actin were still remain the slightly and high intensity of original bands, respectively, with addition of halophilic proteinase. These data is similar with the hydrolysis of collagen, but distinguished with the degradation of NAM that showed low intensity of actin band. Because of many proteins including actin loose their conformation, when they exposed in the high concentration of NaCl or called 'Salting out' effect [5] except small molecules were still original conformation. Thus, the cleavage site of nomal protein is suitable to bind with active site of the halophilic proteinase than that of actin. From all results, the halophilic proteinase was preferable to degrade NAM, collagen and anchovy protein at optimal pH (pH 6.0) as well as stability pH (pH 10.0) in the presence of 25% NaCl. This condition is closely to the traditional process of fish sacue. The data suggesting that the proteinase from *Halobacillus* sp. SR5-3, involved in degradation of fish protein during fermentation at high salt concentrationeven under low pH as pH 6.

*C. Effects of Enzyme Types and Concentration on the Hydrolysis of Anchovy Protein*

The response graph for degree of hydrolysis (Fig. 4) of anchovy proteins as a function of various the concentration of enzyme of commercial proteinase form (α-chymotrypsin, Novozyme® FM 2.0L, Neutrase 0.8L and Flavourzyme® 500MG) and the purified proteinase, respectively, at time of hydrolysis of 60 min, the substrate pH of 6.0 and 25% NaCl, indicated that DH increased with the increase of concentration of commercial enzymes and the purified proteinase. A sharp increase in DH was observed as the concentration of purified enzyme was increased from 0.035 to 0.105U and the highest slope was observed (1.6981), at substrate pH 6.0 and in the presence of 25% NaCl. Hence, in high concentrations of NaC1, 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 [16], [17], [23]. From the results, the halophilic proteinase 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% (0.1-4,000U) thus, the slope was closely to zero (0.043-0.2123). Some losses in activity of commercial proteinases occurred at the high concentration of NaCl, probably owing to the partial denaturation of proteinases caused by the ''salting out'' effect [5]. In a study related to hydrolyzing sardine protein by trypsin from skipjack tuna spleen as non-halophilic proteinase and the commercial proteases (Protease-P-Amano6, Alcalase®, Protex 7L®, and Neutrase®) that was undesirable to digestion of protein in the presence of NaCl [5], [6], [24]. As seen in this study, a high DH at the condition of production of Thai fish sauce (25% NaCl and pH 6.0), the purified proteinase may accelerate the protein hydrolysis during fermentation better than other commercial proteases. Therefore, the fermentation period (18 months) could be shortened without undesirable spoilage by addition of the purified proteinase from halobphilic bacteria, *Halobacillus* sp. SR5-3.

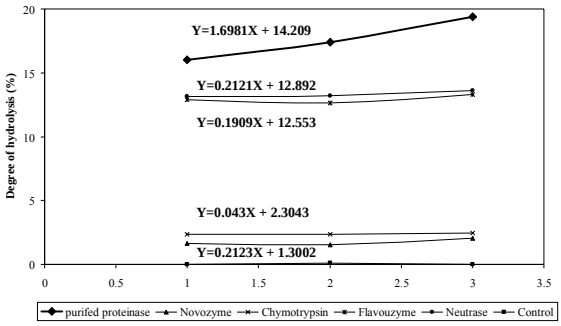


Fig. 4 The degree of hydrolysis of various contration of halophilic proteinase and commercial proteinases (α-chymotrypsin, Novozyme® FM 2.0L, Neutrase 0.8L and Flavourzyme® 500MG) against anchovy protein. Anchovy protein was dissolved in phosphate buffer containing 25% NaCl, pH 6.0. The reaction mixtures were inbubated at 40°C for 60 min.

## IV. Conclusion

The halophilic serine proteinase from *Halobacillus* sp. SR5-3 was capable of hydrolyzing myosin heavy chain, actin and the major component of collagen (β- and α-compounds) effectively. The purified proteinase showed greater degree of hydrolysis than did commercial proteinase (α-chymotrypsin, Novozyme® FM 2.0L, Neutrase 0.8L and Flavourzyme® 500MG) toward anchovy protein. Therefore, the halophilic serine proteinase can be a potential novel enzyme for further applications, especially to speed up the fish sauce production.

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