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Proteolytic degradation of sardine (*Sardinella gibbosa*) proteins by trypsin from skipjack tuna (*Katsuwonus pelamis*) spleen

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Abstract

Trypsin from the spleen of skipjack tuna (*Katsuwonus pelamis*) was purified by ammonium sulfate precipitation and a series of chromatographies, including Sephacryl S-100 and benzamidine-Sepharose 4 fast flow (high sub). The enzyme was purified 22.3-fold with a yield of 51.6%. The molecular weight of trypsin was estimated to be 42 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified trypsin was able to hydrolyse natural actomyosin (NAM) and myosin, but scarcely hydrolysed collagen. Myosin heavy chain was most susceptible to hydrolysis by trypsin as evidenced by the lowest band intensity remaining. The effect of NaCl on proteolytic activity was also studied. The band intensity of myosin heavy chain slightly increased as the NaCl concentration was increased, suggesting the inhibitory activity of NaCl. When hydrolytic activities of skipjack tuna spleen and bovine pancreas trypsins on sardine proteins, including NAM, myosin and collagen, were compared, it was found that trypsin from bovine pancreas showed a greater activity towards NAM and myosin than that from skipjack tuna spleen. However, neither enzymes could degrade collagen.

Keywords: Trypsin; Proteinase; Hydrolysis; Degradation; Myosin heavy chain; Muscle; Purification; Spleen; Tuna

1. Introduction

Protein hydrolysis plays an essential role in producing value-added products from under-utilised fish species, particularly fish sauces. Fish sauce is a clear brown liquid hydrolysate from salted fish, such as anchovy, sardine and mackerel. It is commonly used as a flavour enhancer or salt replacement in various food preparations (Lopetcharat, Choi, Park, & Daeschel, 2001). During fermentation, proteins are hydrolysed, mainly as a result of autolytic action by the digestive proteinases in fish (Orejana & Liston, 1981). Trypsin was reported to be involved in protein hydrolysis during the fermentation of fish sauce (Gildberg & Shi, 1994). Apart from trypsin, chymotrypsin and other digestive enzymes are principally responsible for autolysis (Lopetcharat et al., 2001). Internal organs are the important sources of fish proteases. The most important digestive enzymes are pepsin, secreted from gastric mucosa, trypsin and chymotrypsin secreted from the pancreas, pyloric caeca and intestine (Simpson, 2000). Recently, tuna

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spleen has been reported as one of the organs possessing high proteolytic activity (Klomklao, Benjakul, & Visessanguan, 2004). Klomklao et al. (2004) reported that major proteinases in spleen from skipjack tuna were trypsin-like serine proteinases and optimal activity was observed at pH 9.0 and 55 °C.

In Thailand, fish sauce is manufactured through fermentation up to 18 months (Lopetcharat & Park, 2002), leading to a limited expansion of the fish sauce industry. Therefore, it would be more advantageous if the fermentation period could be shortened without undesirable spoilage. Chaveesuk, Smith, and Simpson (1993) reported that the addition of trypsin and chymotrypsin (0.3% w/w) can accelerate the fermentation of fish sauce from herring and reduce the fermentation time to 2 months. Fish sauce from minced capelin was obtained after 6 months of fermentation with the addition of 5–10% enzyme-rich (trypsin and chymotrypsin) cod intestines (Gildberg, 2001). Due to the high proteolytic activity in skipjack tuna spleen, the addition of spleen to salted sardine could accelerate the protein hydrolysis during fermentation (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2006). However, there is no information regarding the hydrolytic activity of tuna spleen towards muscle proteins, especially from fish commonly used for fish sauce fermentation. Therefore, this study aimed to investigate the hydrolysis of various sardine muscle proteins by a trypsin-like proteinase from skipjack tuna spleen.

2. Materials and methods

2.1. Chemicals

Ethyleneglycol-bis(β-aminoethylether) *N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol (βME), L-tyrosine, bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), 1-(L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), high-molecular-weight markers and low-molecular-weight markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Sodium chloride, tris (hydroxymethyl) aminomethane and Folin–Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Fish sample preparation

The internal organs of skipjack tuna (*Katsuwonus pelamis*) were obtained from Chotiwat Industrial Co. (Thailand) Ltd., Songkhla. Those samples (5 kg) were packed in polyethylene bags, kept in ice and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 30 min. Pooled internal organs were then excised and separated into individual organs. Only spleen was collected, immediately frozen and stored at −20 °C until used.

Sardine (*Sardinella gibbosa*), with an average weight of 55–60 g, were caught from Songkhla-Pattani Coast along the Gulf of Thailand and off-loaded approximately 12 h after capture. Fish were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 2 h. The fish were filleted and the flesh was used for protein extraction.

2.3. Preparation of spleen extract

Frozen spleens were thawed using running water (26–28 °C) until the core temperature reached −2 to 0 °C. The samples were cut into pieces with a thickness of 1–1.5 cm and ground into powder in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan) according to the method of Klomklao et al. (2004). To prepare the extract, spleen powder was suspended in 20 mM Tris–HCl, pH 7.5, referred to as starting buffer (SB) at a ratio of 1:3 (w/v) and stirred continuously at 4 °C for 15 min. The suspension was centrifuged for 15 min at 4 °C at 5000*g*, using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA) to remove the tissue debris. The supernatant was collected and referred to as "splenic extract". All preparation procedures were carried out at 4°C.

2.4. Purification of trypsin from spleen

Spleen extract was subjected to ammonium sulfate precipitation at 30–70% saturation. The mixture was left at 4 °C for 2 h and centrifuged at 10,000*g* for 15 min at 4 °C. The pellet was collected and redissolved in SB. The dissolved pellet was dialysed against SB overnight at 4 °C prior to size exclusion chromatography. The sample was chromatographed on Sephacryl S-100 column (26 × 700 mm), which was equilibrated with approximately two bed volumes of SB. The sample was loaded onto a column at room temperature and then eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected and those with BAPNA activity were pooled. Absorbance at 280 nm (A_{280}) was also measured. The pooled fractions were mixed with NaCl to obtain a final concentration of 0.5 M prior to loading to benzamidine-Sepharose 4 fast flow (high sub), which was equilibrated with 0.5 M NaCl in SB. The sample was loaded at a flow rate of 1 ml/min at room temperature. The column was then washed with 0.5 M NaCl in SB until A_{280} was less than 0.05 and then eluted with 0.05 M glycine, pH 3, at a flow rate of 5 ml/

min. Fractions of 2.5 ml were collected and the fractions with BAPNA activity were pooled and used for further study.

2.5. Trypsin activity assay

Trypsin activity was measured by the method of Benjakul, Visessanguan, and Thummaratwasik (2000) with a slight modification using BAPNA as substrate. To initiate the reaction, 200 μl of diluted splenic extract were added to the preincubated reaction mixture containing 1000 μl of 0.5 mM of BAPNA in reaction buffer (0.1 M glycine–NaOH, pH 9.0) and 200 μl of distilled water. The mixture was incubated at 55 °C for precisely 15 min. The enzymatic reaction was terminated by adding 200 μl of 30% (v/v) acetic acid. The reaction mixture was centrifuged at 8000*g* for 3 min at room temperature (Hettich zentrifugen, Berlin, Germany). Trypsin activity was measured by the absorbance at 410 nm due to *p*-nitroaniline released. One BAPNA unit of activity was defined as $[\Delta A_{410\ nm} \times 1600 \times 1000/\text{min}/8800]$, where 8800 $M^{-1}\ cm^{-1}$ is the extinction coefficient of *p*-nitroaniline and 1600 is total volume of reaction assay (μl). The activity was expressed as U/ml.

2.6. Protein preparation

2.6.1. Natural actomyosin

Natural actomyosin (NAM) was prepared according to the method of Benjakul, Seymour, Morrissey, and An (1997) with a slight modification. Sardine muscle (10 g) was homogenised in 1000 ml of chilled (4 °C) 0.6 M KCl, pH 7.0, for 4 min using an IKA Labortechnik homogeniser (Selanger, Malaysia). 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* for 30 min at 4 °C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). 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.

2.6.2. Myosin

Myosin was extracted by the method described by Martone, Busconi, Folco, Trucco, and Sanchez (1986), as modified by Vissessanguan, Ogawa, Nakai, and An (2000). All steps were performed at 4 °C to minimise proteolysis and protein denaturation. Fish fillets were finely chopped and treated with 10 volumes of buffer A (0.10 M KCl, 1 mM PMSF, 10 μM E-64, 0.02 NaN_3 and 20 mM Tris–HCl, pH 7.5). After incubation on ice for 10 min with occasional stirring, the washed muscle was recovered by centrifugation at 1000*g* for 10 min. The pellet was suspended in 5 volumes of buffer B (0.45 M KCl, 5 mM βME, 0.2 M $Mg(CH_3COO)_2$, 1 mM EGTA, and 20 mM Tris–maleate, pH 6.8), and ATP was added to obtain a final concentration of 10 mM. The mixture was kept on ice for 1 h with occasional stirring and centrifuged at 10,000*g* for 15 min. Supernatant was collected and treated slowly with 25 volumes of 1 mM $NaHCO_3$, followed by incubation for 15 min on ice. Precipitated myosin was collected by centrifugation at 12,000*g*, resuspended gently with 5 volumes of buffer C (0.50 M KCl, 5 mM βME and 20 mM Tris–HCl, pH 7.5), and treated with 3 volumes of 1 mM $NaHCO_3$. $MgCl_2$ was also added to obtain a final concentration of 10 mM. The mixture was kept overnight on ice prior to centrifugation at 22,000*g* for 15 min. Myosin, recovered as a pellet, was used immediately or stored at −20 °C in 50% glycerol.

2.6.3. Collagen

Collagen was prepared according to the method of Kittiphattanabawon, Benjakul, Visessanguan, Nagai, and Tanaka (2005) 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 soaking in 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 20,000*g* 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.

2.7. Hydrolysis of different protein substrates by purified proteinase

Purified enzyme (0.25 U) was added to the reaction mixture containing 4 mg protein substrates, including NAM, myosin or collagen, and 825 ml of 0.1 M glycine–NaOH, pH 9.0. The hydrolysis was conducted by incubating the mixture at 55 °C for 0, 5, 10, 20, 30 and 60 min. The control was performed by incubating the reaction mixture at 55 °C for 60 min without the addition of purified proteinase. The reaction was terminated by adding preheated solution containing 2% SDS, 8 M urea and 2% βME (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 (Hettich zentrifugen, Berlin, Germany) to remove the debris. The supernatant was then subjected to SDS–PAGE analysis.

2.8. Effect of NaCl on proteolytic activity

The reaction mixture containing NAM and various NaCl concentrations (0%, 5%, 10%, 15%, 20% and 25% (w/v)) was mixed with purified proteinase. Hydrolysis was performed for 10 min at 55 °C and the hydrolysis was monitored as previously described.

2.9. Peptide mapping

Peptide mappings of protein substrates hydrolysed by different enzymes were performed according to the method of Saito, Kunisaki, Urano, and Kimura (2002) with a slight modification. Protein substrates were suspended in 0.1 M glycine–NaOH, pH 9.0 at 4 °C. After the addition of 0.25 U of purified enzyme and trypsin, from bovine pancreas, to the reaction mixture, consisting of 4 mg protein substrate, the mixture was incubated at 55 °C for 10, 20 and 60 min for NAM, myosin and collagen, respectively. The reaction was terminated and proteins were solubilised as previously described. Samples were then subjected to SDS–PAGE analysis. The peptide patterns of protein substrates hydrolysed by the two proteases were compared.

2.10. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (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 (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 myosin samples. Electrophoresis was run at a constant current of 15 mA per gel, using a Mini-Protean II Cell apparatus. After electrophoresis, the gels were stained with 0.2% Coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 10% acetic acid.

2.11. Protein determination

Protein concentration was measured by the method of Lowry, Rosebrough, Fan, and Randall (1951), using bovine serum albumin as a standard.

3. Results and discussion

3.1. Purification of trypsin from the spleen of skipjack tuna

Purification of trypsin from the spleen of skipjack tuna is summarised in Table 1. The specific activity and purification fold were 132 U/mg protein and 4.4-fold, respectively, when 30–70% ammonium sulfate was used. From the result, activity loss of 30% was noted after ammonium sulfate precipitation. This might be due to the denaturation of proteinases caused by the "salting out" effect (Klomklao et al., 2004). Salting out is a simple method and generally used as an initial step in trypsin purification (Bezerra et al., 2001; Heu, Kim, & Pyeun, 1995; Simpson & Haard, 1984). Krisjansson (1991) found that ammonium sulfate precipitation of trypsin from the pyloric caeca of rainbow trout at 30–70% saturation resulted in an increase in specific activity by 4.9-fold.

The pellet obtained from the previous step was dissolved in 20 mM Tris–HCl, pH 7.5, and dialysed against the same buffer for 24 h at 4 °C. The dialysed enzymes were further purified by gel filtration on Sephacryl S-100. Purification of 15.6-fold with a yield of 66.7% was obtained. Kishimura and Hayashi (2002) found that the use of gel filtration on Sephadex G-50 in the purification process of trypsin from starfish pancreas led to an increase in activity by 34-fold.

Pooled active Sephacryl S-100 fractions were further purified by affinity chromatography on a benzamidine-Sepharose 4 fast flow (high sub) column. Purification of 22.3-fold with a yield of 51.6% was obtained after this step. The use of affinity chromatography on benzamidine-Sepharose in the final step of the purification process of trypsin from rainbow trout pyloric caeca

Table 1
Purification of trypsin from the spleen of skipjack tuna

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude extract	20,378	674	30.2	1.0	100
$(NH_4)_2SO_4$ (30–70%)	14,459	45.8	132	4.4	70.9
Sephacryl S-100	13,585	30.8	470	15.6	66.7
Benzamidine-Sepharose 4 fast flow	10,514	8.2	675	22.3	51.6

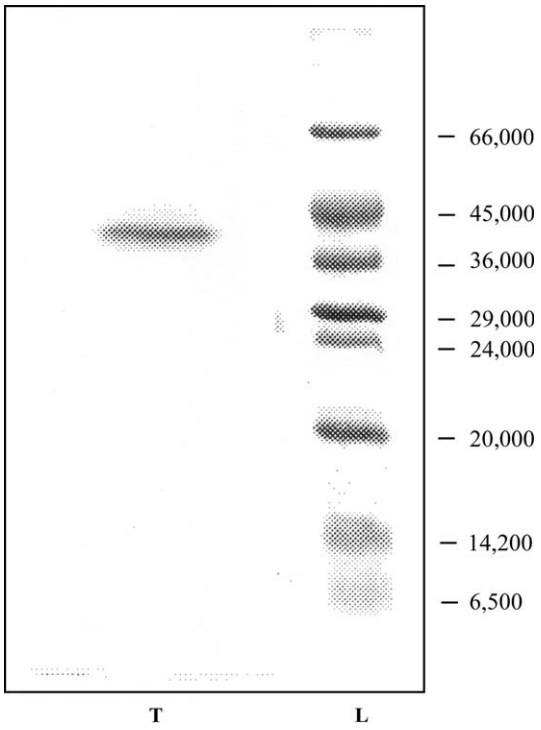

Fig. 1. SDS–PAGE pattern of purified trypsin from the spleen of skipjack tuna: T; purified trypsin, L; low-molecular-weight standard.

resulted in an increase in activity by 70.4-fold (Krisjansson, 1991).

3.2. Protein pattern of trypsin from the spleen of skipjack tuna

Protein pattern of trypsin obtained from the purification process is shown in Fig. 1. Crude extract contained a variety of proteins of different molecular weights (data not shown). When the benzamidine-Sepharose 4 fast flow fraction was analysed by SDS–PAGE, a single protein band with an estimated molecular mass of 42 kDa was found. Molecular masses of 23.5–28 kDa have been reported for trypsins isolated from various fish species (Hjelmeland & Raa, 1982; Simpson & Haard, 1984; Simpson, Simpson, & Haard, 1989). The differences in molecular mass between skipjack tuna spleen trypsin and other trypsins might be due to the different habitat or climate where fish live as well as the genetic variation among species (Klomklao et al., 2004; Torrissen, 1984). Electrophoresis results indicated that a large amount of contaminating proteins was removed during purification. Subsequently, increased purity of trypsin was observed, as shown in Table 1.

3.3. Hydrolysis of different protein substrates by purified trypsin from the spleen of skipjack tuna

3.3.1. Natural actomyosin (NAM)

NAM, extracted from sardine, contained myosin heavy chain (MHC) and actin as major constituents. β-tropomyosin and myosin light chain (MLC) were found as minor components (Fig. 2). Among all proteins, MHC was the most susceptible to hydrolysis, followed by actin. MHC was degraded rapidly within 5 min by the purified trypsin (Fig. 2). Total disappearance of MHC was observed after 10 min of incubation at 55 °C. For actin, the degradation increased as the incubation time increased. However, the degradation rate was lower than that of MHC. From the result, it was noted that autolysis of sample (without purified trypsin addition) occurred to some extent during incubation at 55 °C. This possibly indicated the presence of a myofibrillar associated proteinase that bound tightly with NAM and could not be removed during the extraction process. Fish muscle was reported to contain myofi-

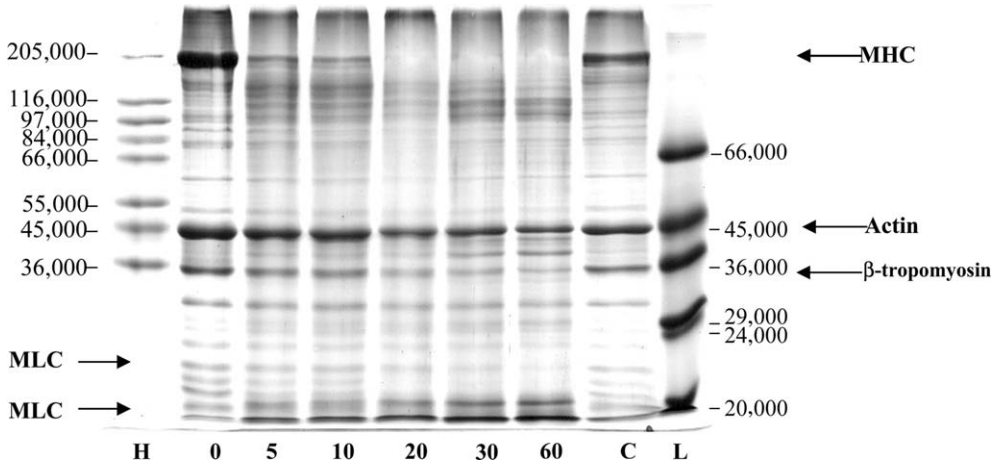

Fig. 2. Hydrolysis of NAM by purified trypsin from the spleen of skipjack tuna at 55 °C. H, high-molecular-weight standard; L, low-molecular-weight standard; C, control (incubated without enzyme addition for 60 min at 55 °C); MHC, myosin heavy chain; MLC, myosin light chain. Numbers designate the incubation time (min) at 55 °C.

bril-bound proteinases (Benjakul, Visessanguan, & Leelapongwattana, 2003; Cao, Osatomi, Hara, & Ishihara, 2000; Osatomi, Sasai, Cao, Hara, & Ishihara, 1997). Generally, myofibrillar proteins are susceptible to degradation by lysosomal enzymes and calcium-activated neutral proteinases (Ouali & Valin, 1981). Yamashita and Konagaya (1991) also reported that three myofibrillar components (α-actinin and troponin-T and -I) were markedly degraded by salmon cathepsin B and L, along with the disappearance of myosin heavy and light chains. From the result, trypsin from skipjack tuna spleen hydrolysed myofibrillar proteins effectively, particularly MHC which is the dominant protein in fish muscle.

3.3.2. Myosin

The proteolytic degradation pattern of sardine myosin, analysed by SDS–PAGE, revealed that MHC was hydrolysed continuously throughout the incubation time of 60 min (Fig. 3). MHC was degraded markedly within 5 min with the appearance of hydrolysis products having M_r ranges of 116,000–66,000. MHC decreased by 90% of the original content within 30 min of incubation at 55 °C. However, no change in the myosin light chain (MLC) was observed, even with an extended incubation time. At 60 min, no protein with M_r of 36, 22 and 20 kDa were found. An, Seymour, Wu, and Morriessey (1994) reported that, among the Pacific whiting proteins, MHC was the most extensively hydrolysed by cathepsin L, followed by troponin-T and α- and β-tropomyosin. For the control (without purified proteinase), a slight degradation of myosin heavy chain was observed (lane C) (Fig. 3). The result suggested the existence of a myofibril-bound proteinase in partially purified MHC.

3.3.3. Collagen

No hydrolytic degradation of collagen was observed when collagen was incubated at 55 °C for up to 60 min

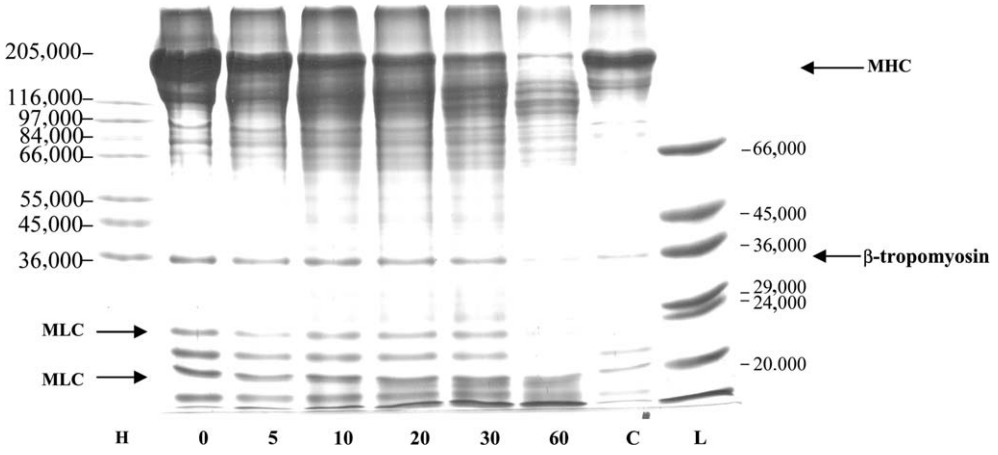

Fig. 3. Hydrolysis of myosin by purified trypsin from the spleen of skipjack tuna at 55 °C. H, high-molecular-weight standard; L, low-molecular-weight standard; C, control (incubated without enzyme addition for 60 min at 55 °C); MHC, myosin heavy chain; MLC, myosin light chain. Numbers designate the incubation time (min) at 55 °C.

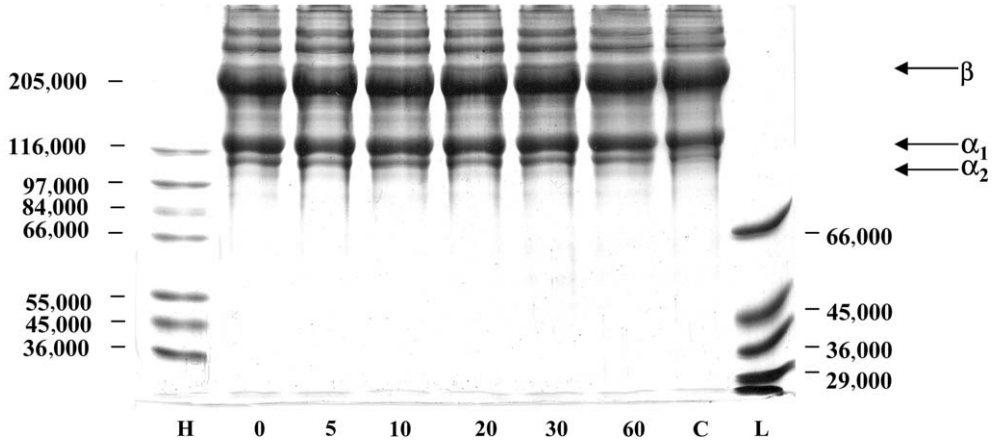

Fig. 4. Hydrolysis of collagen by purified trypsin from the spleen of skipjack tuna at 55 °C. H, high-molecular-weight standard; L, low-molecular-weight standard; C, control (incubated without enzyme addition for 60 min at 55 °C). Numbers designate the incubation time (min) at 55 °C.

in the presence of purified trypsin from skipjack tuna spleen (Fig. 4). Neither the β- nor the α-compounds were hydrolysed by added trypsin. Collagen type I, consisting of two α_1 chains and one α_2 chain, was found in the skin of bigeye snapper (Kittiphattanabawon et al., 2005). The β-component is also present in collagen from many fish species (Ciarlo, Paredi, & Fraga, 1997; Nagai & Suzuki, 2000). This suggests that collagen was not a good substrate for trypsin from the spleen of skipjack tuna. Yamashita and Konagaya (1991) reported that native collagens were degraded at 20 °C by chum salmon cathepsin L but not by cathepsin B. Thus, the degradation of collagen depends upon the source of collagen as well as on the types of proteinase. Collagen molecules in the connective tissue generally undergo limited cleavage in the non-helical region by the various proteases, such as pepsin and trypsin (Yamashita & Konagaya, 1991). For the control (without purified proteinase), the degradation of collagen was not observed. From the result, trypsin might hydrolyse the extracted collagen only at the non-helical region but could not cleave the peptide bonds in the α or β components. As a result, the protein pattern of major components was not changed when analysed by SDS–PAGE.

3.4. Effect of NaCl on proteolytic activity

The effect of NaCl on the hydrolytic activity of purified trypsin on NAM is depicted in Fig. 5. The band intensity of MHC slightly increased with increasing NaCl concentration up to 25%. Some losses in activity occurred as the NaCl concentration was increased, probably owing to the partial denaturation of proteinases caused by the "salting out" effect (Klomklao et al., 2004). A thermostable proteinase in salted anchovy muscle was still active and able to degrade myofibrillar proteins in commercial salted fillets containing 16–17% NaCl (Ishida, Niizelei, & Nagayama, 1994). Therefore, by using spleen proteinases, it might be possible to accelerate the protein hydrolysis in fish sauce production, in which salt at high level is used.

3.5. Peptide mapping of protein substrates

The peptide maps of sardine protein substrates hydrolysed by purified trypsin in comparison with trypsin from bovine pancreas are shown in Figs. 6 and 7. At the same level added, trypsin from bovine pancreas exhibited a much higher hydrolytic activity on NAM and myosin than did trypsin from the spleen of skipjack tuna. For NAM and myosin hydrolysis by trypsin from bovine pancreas (Fig. 6), all components of NAM were hydrolysed more extensively, as shown by the lower intensity of the original bands of each component remaining with a concomitant increase in the lower MW peptide fragments. For NAM, actin still remained with addition of trypsin from skipjack tuna spleen. Therefore, trypsin from bovine pancreas was more effective in NAM and myosin hydrolysis. On the other hand, collagen could not be hydrolysed by either enzymes (Fig. 7). α and β Components still remained in the presence of both enzymes. The result suggested that these components of collagen were tolerant to digestion by both trypsins. Therefore, trypsin from skipjack tuna spleen might not cleave the connective tissues in fish skin or muscle. Nevertheless, it effectively induced the liquefaction process via the hydrolysis of the myofibrillar proteins.

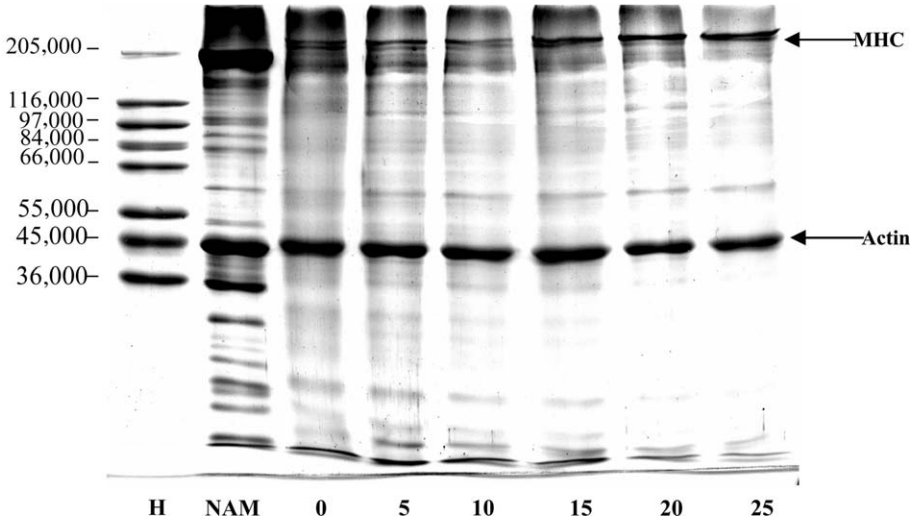

Fig. 5. Effect of NaCl concentrations on the hydrolysis of NAM by purified trypsin from the spleen of skipjack tuna. Hydrolysis was conducted using 0.25 U trypsin/4 mg protein at 55 °C for 10 min. H, high-molecular-weight standard; MHC, myosin heavy chain. Numbers designate the NaCl concentrations (% w/w).

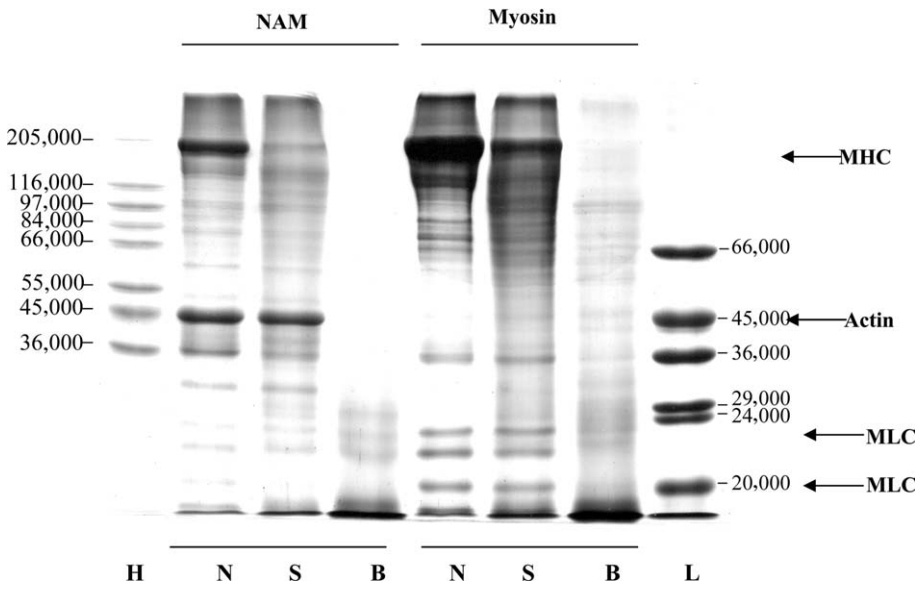

Fig. 6. Peptide mapping of sardine NAM and myosin hydrolysed by purified trypsin from the spleen of skipjack tuna and trypsin from bovine pancreas. H, high-molecular-weight standard; L, low-molecular-weight standard; MHC, myosin heavy chain; MLC, myosin light chain; N, without enzyme addition; S, treated with trypsin from the spleen of skipjack tuna (0.25 U/4 mg protein); B, treated with bovine pancreas trypsin (0.25 U/4 mg protein). Reaction was conducted at 55 °C for 10 min and 20 min for NAM and myosin, respectively.

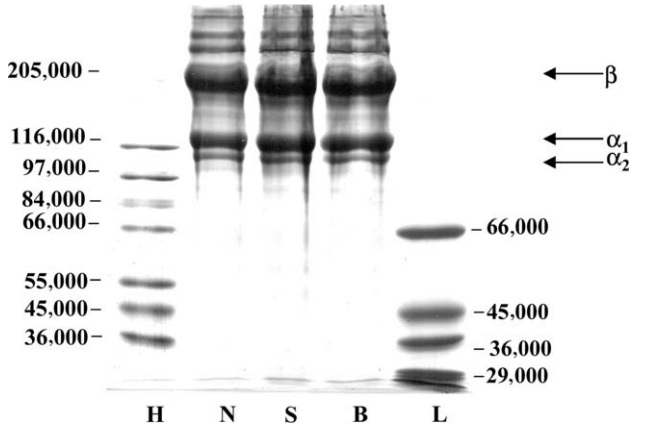

Fig. 7. Peptide mapping of sardine collagen hydrolysed by purified trypsin from the spleen of skipjack tuna and trypsin from bovine pancreas. H, high-molecular-weight standard; L, low-molecular-weight standard; N, without enzyme addition; S, treated with trypsin from the spleen of skipjack tuna (0.25 U/4 mg protein); B, treated with bovine pancreas trypsin (0.25 U/4 mg protein). Reaction was conducted at 55 °C for 60 min.

4. Conclusion

Skipjack tuna spleen trypsin was capable of hydrolysing myosin heavy chain effectively. However, this enzyme could not degrade collagen. The trypsin from bovine pancreas showed greater hydrolysis activity than did purified trypsin from the spleen of skipjack tuna toward myofibrillar proteins. Therefore, spleen proteinase can be a potential novel enzyme for further applications, especially for the acceleration of fish sauce production.

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