



Title	Proteolytic degradation of sardine ( <i>Sardinella gibbosa</i> ) proteins by trypsin from skipjack tuna ( <i>Katsuwonus pelamis</i> ) spleen
Author(s)	Klomklao, Sappasith; Benjakul, Soottawat; Visessanguan, Wonnop; Kishimura, Hideki; Simpson, Benjamin K.
Citation	Food Chemistry, 98(1), 14-22 <a href="https://doi.org/10.1016/j.foodchem.2005.05.047">https://doi.org/10.1016/j.foodchem.2005.05.047</a>
Issue Date	2006
Doc URL	<a href="http://hdl.handle.net/2115/8509">http://hdl.handle.net/2115/8509</a>
Type	article (author version)
File Information	proteinhydrolysismanuscript(Foodchemistry).pdf



[Instructions for use](#)

1                   **Proteolytic Degradation of Sardine (*Sardinella gibbosa*) Proteins**  
2                   **by Trypsin from Skipjack Tuna (*Katsuwonus pelamis*) Spleen**

3  
4  
5                   To be submitted to  
6                   Food Chemistry

7  
8  
9  
10                Sappasith Klomklao<sup>1</sup>, Soottawat Benjakul<sup>1\*</sup>, Wonnop Visessanguan<sup>2</sup>,  
11                Hideki Kishimura<sup>3</sup> and Benjamin K. Simpson<sup>4</sup>

12  
13  
14  
15                <sup>1</sup>*Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla*  
16                *University, Hat Yai, Songkhla, 90112, Thailand.*

17                <sup>2</sup>*National Center for Genetic Engineering and Biotechnology, National Science and*  
18                *Technology Development Agency, 113 Paholayothin Rd., Klong 1, Klong Luang,*  
19                *Pathumthani, 12120, Thailand.*

20                <sup>3</sup>*Graduate School of Fisheries Sciences, Hokkaido University,*  
21                *Hakodate, Hokkaido, 041-8611, Japan*

22                <sup>4</sup>*Department of Food Science and Agricultural Chemistry, McGill University,*  
23                *Macdonald campus, 21111 Lakeshore Road, Ste. Anne de Bellevue,*  
24                *Quebec, Canada H9X 3V9.*

25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35                \*To whom correspondence should be addressed. Tel: 66-7428-6334.  
36                Fax: 66-7421-2889, e-mail: [soottawat.b@psu.ac.th](mailto:soottawat.b@psu.ac.th)

1 **Abstract**

2           Trypsin from skipjack tuna (*Katsuwonus pelamis*) spleen was purified by  
3 ammonium sulfate precipitation and a series of chromatographies including Sephacryl  
4 S-100 and Benzamidine Sepharose 4 Fast Flow (high sub). The enzyme was purified  
5 to 22.3 folds with a yield of 51.6%. The molecular weight of trypsin was estimated to  
6 be 42 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified  
7 trypsin was able to hydrolyze natural actomyosin (NAM) and myosin, but rarely  
8 hydrolyzed collagen. Myosin heavy chain was most susceptible to hydrolysis by  
9 trypsin as evidenced by the lowest band intensity remained. The effect of NaCl on  
10 proteolytic activity was also studied. The band intensity of myosin heavy chain  
11 slightly increased as NaCl concentration increased, suggesting the inhibitory activity  
12 of NaCl. When hydrolytic activities of skipjack tuna spleen and bovine pancreas  
13 trypsins on sardine proteins, including NAM, myosin and collagen were compared, it  
14 was found that trypsin from bovine pancreas showed the greater activity towards  
15 NAM and myosin than that from skipjack tuna spleen. However, both enzymes could  
16 not degrade collagen.

17

18 **Keywords:** Trypsin, Proteinase, Hydrolysis, Degradation, Myosin heavy chain,  
19 Muscle, Purification, Spleen, Tuna

20

21

22

23

24

25

## 1 **1. Introduction**

2 Protein hydrolysis plays an essential role in producing value-added products  
3 from underutilized fish species, particularly fish sauce. Fish sauce is a clear brown  
4 liquid hydrolysate from salted fish such as anchovy, sardine, mackerel etc. It is  
5 commonly used as a flavor enhancer or salt replacement in various food preparation  
6 (Lopetcharat, Choi, Park & Daeschel, 2001). During fermentation, proteins are  
7 hydrolyzed, mainly as a result of autolytic action by the digestive proteinases in fish  
8 (Orejana & Liston, 1981). Trypsin was reported to involve in protein hydrolysis  
9 during fermentation of fish sauce (Gildberg & Shi, 1994). Apart from trypsin,  
10 chymotrypsin and other digestive enzymes are principally responsible for autolysis  
11 (Lopetcharat et al., 2001). Internal organs are the important sources of fish proteases.  
12 The most important digestive enzymes are pepsin, secreted from gastric mucosa,  
13 trypsin, and chymotrypsin secreted from the pancreas, pyloric caeca and intestine  
14 (Simpson 2000). Recently, Tuna spleen is one of organs possessing the high  
15 proteolytic activity (Klomklao, Benjakul & Visessanguan, 2004). Klomklao et al.  
16 (2004) reported that major proteinases in spleen from skipjack tuna were trypsin-like  
17 serine proteinases and optimal activity was observed at pH 9.0 and 55°C.

18 In Thailand, fish sauce is manufactured through fermentation up to 18 months  
19 (Lopetcharat & Park, 2002), leading to the limited expansion of fish sauce industry.  
20 Therefore, it would be more advantageous if the fermentation period could be  
21 shortened without undersirable spoilage. Chaveesuk, Smith and Simpson (1993)  
22 reported that the addition of trypsin and chymotrypsin (0.3% w/w) can accelerate the  
23 fermentation of fish sauce from herring and reduce the fermentation time to 2 months.  
24 The fish sauce from minced capelin was obtained after 6 months of fermentation with  
25 the addition of 5 to 10% enzyme-rich (trypsin and chymotrypsin) cod intestines

1 (Gildberg, 2001). Due to high proteolytic activity in skipjack tuna spleen, the addition  
2 of spleen into salted sardine could accelerate the protein hydrolysis during  
3 fermentation (Klomklao, Benjakul, Visessanguan, Kishimura & Simpson 2005).  
4 However, no information regarding the hydrolytic activity of tuna spleen towards  
5 muscle proteins, especially from fish commonly used for fish sauce fermentation.  
6 Therefore, this study aimed to investigate the hydrolysis of various sardine muscle  
7 proteins by skipjack tuna spleen trypsin-like proteinase.

8

## 9 **2. Materials and Methods**

### 10 *2.1 Chemicals*

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

21

### 22 *2.2 Fish sample preparation*

23 Internal organs from skipjack tuna (*Katsuwonus pelamis*) were obtained from  
24 Chotiwat Industrial Co. (Thailand) Ltd., Songkhla. Those samples (5 kg) were packed  
25 in polyethylene bag, kept in ice and transported to the Department of Food

1 Technology, Prince of Songkla University, Hat Yai within 30 min. Pooled internal  
2 organs were then excised and separated into individual organs. Only spleen was  
3 collected, immediately frozen and stored at  $-20^{\circ}\text{C}$  until used.

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

10

### 11 *2.3 Preparation of spleen extract*

12 Frozen spleens were thawed using a running water ( $26-28^{\circ}\text{C}$ ) until the core  
13 temperature reached  $-2$  to  $0^{\circ}\text{C}$ . The samples were cut into pieces with a thickness of  
14 1-1.5 cm and ground into powder in the liquid nitrogen using a National Model MX-  
15 T2GN blender (Taipei, Taiwan) according to the method of Klomklao et al. (2004).  
16 To prepare the extract, spleen powder was suspended in 20 mM Tris-HCl, pH 7.5  
17 referred to as starting buffer (SB) at a ratio of 1:3 (w/v) and stirred continuously at  
18  $4^{\circ}\text{C}$  for 15 min. The suspension was centrifuged for 15 min at  $4^{\circ}\text{C}$  at  $5,000\times g$  using a  
19 Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA) to remove the tissue  
20 debris. The supernatant was collected and referred to as “splenic extract”. All  
21 preparation procedures were carried out at  $4^{\circ}\text{C}$ .

22

### 23 *2.4 Purification of trypsin from spleen*

24 Spleen extract was subjected to ammonium sulfate precipitation at 30-70%  
25 saturation. The mixture was left at  $4^{\circ}\text{C}$  for 2 h and centrifuged at  $10,000\times g$  for 15 min

1 at 4°C. The pellet was collected and redissolved in SB. The dissolved pellet was  
2 dialyzed against SB overnight at 4°C prior to size exclusion chromatography. The  
3 sample was chromatographed on Sephacryl S-100 column (26×700 mm), which was  
4 equilibrated with approximately two bed volumes of SB. Sample was loaded to  
5 column at room temperature and then eluted with the same buffer at a flow rate of 0.5  
6 ml/min. Fractions of 3 ml were collected and those with BAPNA activity were pooled.  
7 Absorbance at 280 nm ( $A_{280}$ ) was also measured. The pooled fractions were mixed  
8 with NaCl to obtain a final concentration of 0.5 M prior to loading to Benzamidine-  
9 Sepharose 4 Fast Flow (high sub), which was equilibrated with 0.5 M NaCl in SB.  
10 The sample was loaded at a flow rate of 1 ml/min at room temperature. The column  
11 was then washed with 0.5 M NaCl in SB until  $A_{280}$  was less than 0.05 and then eluted  
12 with 0.05 M glycine, pH 3, at a flow rate of 5 ml/min. Fraction of 2.5 ml were  
13 collected and the fractions with BAPNA activity were pooled and used for further  
14 study.

15

### 16 *2.5 Trypsin activity assay*

17 Trypsin activity was measured by the method of Benjakul, Visessanguan and  
18 Thummaratwasik (2000) with a slight modification using BAPNA as substrate. To  
19 initiate the reaction, 200 µl of diluted splenic extract was added to the preincubated  
20 reaction mixture containing 1000 µl of 0.5 mM of BAPNA in reaction buffer (0.1 M  
21 glycine-NaOH, pH 9.0) and 200 µl of distilled water. The mixture was incubated at  
22 55°C for precisely 15 min. The enzymatic reaction was terminated by adding 200 µl  
23 of 30% acetic acid (v/v). The reaction mixture was centrifuged at 8,000×g for 3 min at  
24 room temperature (Hettich zentrifugen, Berlin, Germany). Trypsin activity was  
25 measured by the absorbance at 410 nm due to *p*-nitroaniline released. One BAPNA

1 unit of activity was defined as  $[\Delta A_{410\text{nm}} \times 1600 \times 1000 / \text{min} / 8800]$ , where  $8800 \text{ M}^{-1} \text{cm}^{-1}$   
2 is the extinction coefficient of *p*-nitroaniline and 1600 is total volume of reaction  
3 assay ( $\mu\text{l}$ ). The activity was expressed as units/ml.

4

## 5 *2.6 Protein preparation*

### 6 *2.6.1 Natural actomyosin*

7 Natural actomyosin (NAM) was prepared according to the method of Benjakul,  
8 Seymour, Morrissey and An (1997) with a slight modification. Sardine muscle (10g)  
9 was homogenised in 1000 ml of chilled ( $4^{\circ}\text{C}$ ) 0.6 M KCl, pH 7.0 for 4 min using an  
10 IKA Labortechnik homogenizer (Selanger, Malaysia). The sample was placed in ice  
11 and each 20 sec of blending was followed by a 20 sec rest interval to avoid  
12 overheating during extraction. The extract was centrifuged at  $5,000 \times g$  for 30 min at  
13  $4^{\circ}\text{C}$  using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). Three  
14 volumes of chilled distilled water were added to precipitate NAM. NAM (the pellet)  
15 was collected by centrifuging at  $5,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ .

16

### 17 *2.6.2 Myosin*

18 Myosin was extracted by the method described by Martone, Busconi, Folco,  
19 Trucco and Sanchez (1986) as modified by Vissessanguan, Ogawa, Nakai and An  
20 (2000). All steps were performed at  $4^{\circ}\text{C}$  to minimize proteolysis and protein  
21 denaturation. Fish fillets were finely chopped and added with 10 volumes of Buffer A  
22 (0.10 M KCl, 1 mM PMSF, 10  $\mu\text{M}$  E-64, 0.02  $\text{NaN}_3$  and 20 mM Tris-HCl, pH 7.5).  
23 After incubation on ice for 10 min with occasional stirring, the washed muscle was  
24 recovered by centrifugation at  $1,000 \times g$  for 10 min. The pellet was suspended in 5  
25 volumes of Buffer B (0.45 M KCl, 5 mM  $\beta\text{ME}$ , 0.2 M  $\text{Mg}(\text{CH}_3\text{COO})_2$ , 1 mM EGTA,



1 and 20 mM Tris-maleate, pH 6.8), and ATP was added to obtain a final concentration  
2 of 10 mM. The mixture was kept on ice for 1 h with occasional stirring and  
3 centrifuged at 10,000×g for 15 min. Supernatant was collected and added slowly with  
4 25 volumes of 1 mM NaHCO<sub>3</sub>, followed by incubation for 15 min on ice. Precipitated  
5 myosin was collected by centrifugation at 12,000×g, resuspended gently with 5  
6 volumes of Buffer C (0.50 M KCl, 5 mM βME and 20 mM Tris-HCl, pH 7.5), and  
7 added with 3 volumes of 1 mM NaHCO<sub>3</sub>. MgCl<sub>2</sub> was also added to obtain a final  
8 concentration of 10 mM. The mixture was kept overnight on ice prior to  
9 centrifugation at 22,000×g for 15 min. Myosin recovered as pellet was used  
10 immediately or stored at -20 °C in 50% glycerol.

11

### 12 2.6.3 Collagen

13 Collagen was prepared according to the method of Kittiphattanabawon,  
14 Benjakul, Visessanguan, Nagai and Tanaka (2005) with a slight modification. All  
15 preparation procedures were performed at 4°C. To remove non-collagenous proteins,  
16 the sardine fillets containing skin were ground and mixed with 0.1 N NaOH at a  
17 sample/alkali solution ratio of 1:10 (w/v). The mixture was stirred for 6 h. The alkali  
18 solution was changed every 2 h. Then, the deproteinised samples were washed with  
19 cold distilled water until neutral or faintly basic pHs of wash water were obtained.

20 Deproteinised samples were defatted with 10% butyl alcohol with a  
21 solid/solvent ratio of 1:10 (w/v) for 18 h and the solvent was changed every 6 h.  
22 Defatted samples were washed with cold water, followed by soaking in 0.5 M acetic  
23 acid with a solid/solvent ratio of 1:30 (w/v) for 24 h. The mixture was filtered with  
24 two layers of cheese cloth. The residue was re-extracted under the same condition.  
25 Both filtrates were combined. The collagen was precipitated by adding NaCl to a final

1 concentration of 2.6 M in the presence of 0.05 M Tris-HCl, pH 7.0. The resultant  
2 precipitate was collected by centrifugation at 20,000×g for 60 min. The pellet was  
3 dissolved in 0.5 M acetic acid, dialysed against 0.1 M acetic acid and distilled water,  
4 respectively, and then freeze-dried.

5

#### 6 *2.7 Hydrolysis of different protein substrates by purified proteinase*

7 Purified enzyme (0.25 unit) was added to the reaction mixture containing 4 mg  
8 protein substrates including NAM, myosin or collagen, and 825 ml of 0.1 M glycine-  
9 NaOH, pH 9.0. The hydrolysis was conducted by incubating the mixture at 55°C for 0,  
10 5, 10, 20, 30 and 60 min. The control was performed by incubating the reaction  
11 mixture at 55°C for 60 min without the addition of purified proteinase. Reaction was  
12 terminated by adding preheated solution containing 2% SDS, 8M urea and 2% βME  
13 (80°C). The mixture was further incubated at 80°C for 30 min to solubilise total  
14 proteins. The solution was centrifuged at 8,500 rpm for 10 min at room temperature  
15 (Hettich zentrifugen, Berlin, Germany) to remove the debris. Supernatant was then  
16 subjected to SDS-PAGE analysis.

17

#### 18 *2.8 Effect of NaCl on proteolytic activity*

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

22

23

24

25

## 1 2.9 Peptide mapping

2 Peptide mappings of protein substrates hydrolysed by different enzymes were  
3 performed according to the method of Saito, Kunisaki, Urano and Kimura (2002) with  
4 a slight modification. Protein substrates were suspended in 0.1 M glycine-NaOH, pH  
5 9.0 at 4°C. After the addition of 0.25 unit of purified enzyme and trypsin from bovine  
6 pancreas to reaction mixture consisting of 4 mg protein substrate, the mixture was  
7 incubated at 55°C for 10, 20 and 60 min for NAM, myosin and collagen, respectively.  
8 Reaction was terminated and proteins were solubilised as previously described.  
9 Samples were then subjected to SDS-PAGE analysis. Peptide patterns of protein  
10 substrates hydrolysed by two proteases were compared.

11

## 12 2.10 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

13 SDS-PAGE was performed according to the method of Laemmli (1970).  
14 Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer  
15 (0.125M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10%  $\beta$ -mercaptoethanol) and  
16 boiled for 3 min. The samples (15  $\mu$ g) were loaded on the gel made of 4% stacking  
17 gel and 7.5% separating gel for collagen sample and 10% separating gel for NAM and  
18 myosin samples. Electrophoresis was run at a constant current of 15 mA per gel using  
19 a Mini-Protean II Cell apparatus. After electrophoresis, the gels were stained with  
20 0.2% Coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid and  
21 destained with 30% methanol and 10% acetic acid.

22

## 23 2.11 Protein determination

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

### 1 **3. Results and Discussion**

#### 2 *3.1 Purification of trypsin from skipjack tuna spleen*

3 Purification of trypsin from skipjack tuna spleen is summarized in Table 1.  
4 The specific activity and purification fold were 131.9 U/mg protein and 4.4,  
5 respectively, when 30-70% ammonium sulfate was used. From the result, activity loss  
6 of 30% was noted after ammonium sulfate precipitation. This might be due to the  
7 denaturation of proteinases caused by the “Salting out” effect (Klomklao et al., 2004).  
8 Salting out is a simple method and generally used as an initial step in trypsin  
9 purification (Simpson & Haard, 1984; Heu, Kim & Pyeun, 1995; Bezerra et al., 2001).  
10 Kristjansson (1991) found that ammonium sulfate precipitation of trypsin from the  
11 pyloric caeca of rainbow trout at 30-70% saturation resulted in the increase in specific  
12 activity by 4.9 folds.

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

19 Pooled active Sephacryl S-100 fractions were further purified by affinity  
20 chromatography on Benzamidine-Sepharose 4 Fast Flow (high sub) column.  
21 Purification fold of 22.3 with a yield of 51.6% was obtained after this step. The use of  
22 affinity chromatography on Benzamidine-Sephasose in the final step of purification  
23 process of trypsin from rainbow trout pyloric caeca resulted in an increase in activity  
24 by 70.4 folds (Kistjansson, 1991).

25

### 1 3.2 Protein pattern of trypsin from skipjack tuna spleen

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

14

### 15 3.3 Hydrolysis of different protein substrates by purified trypsin from skipjack tuna 16 spleen

#### 17 3.31 Natural actomyosin (NAM)

18 NAM extracted from sardine contained myosin heavy chain (MHC) and actin  
19 as major constituents.  $\beta$ -tropomyosin and myosin light chain (MLC) were found as  
20 minor components (Fig. 2). Among all proteins, MHC was the most susceptible to  
21 hydrolysis followed by actin. MHC was degraded rapidly within 5 min by the purified  
22 trypsin (Fig. 2). Total disappearance of MHC was observed after 10 min of incubation  
23 at 55°C. For actin, the degradation increased as the incubation time increased.  
24 However the degradation rate was lower than that of MHC. From the result, it was  
25 noted that autolysis of sample (without purified trypsin addition) occurred to some

1 extent during incubation at 55°C. This possibly indicated the presence of myofibrillar  
2 associated proteinase that bound tightly with NAM and could not be removed during  
3 extraction process. Fish muscle was reported to contained myofibril bound proteinases  
4 (Benjakul, Visessanguan & Leelapongwattana, 2003; Osatomi, Sasai, Cao, Hara &  
5 Ishihara, 1997; Cao, Osatomi, Hara & Ishihara, 2000). Generally, myofibrillar  
6 proteins are susceptible to degradation by lysosomal enzymes and calcium-activated  
7 neutral proteinases (Ouali and Valin, 1981). Yamashita and Konagaya (1991) also  
8 reported that three myofibrillar components ( $\alpha$ -actinin and troponin-T and -I) were  
9 markedly degraded by salmon cathepsin B and L, along with the disappearance of  
10 myosin heavy and light chains. From the result, trypsin from skipjack tuna spleen  
11 hydrolysed myofibrillar proteins effectively, particularly MHC which is the dominant  
12 protein in the fish muscle.

13

#### 14 3.3.2 *Myosin*

15 The proteolytic degradation pattern of sardine myosin analysed by SDS-PAGE  
16 revealed that MHC was hydrolysed continuously throughout the incubation time of 60  
17 min (Fig. 3). MHC was degraded markedly within 5 min with the appearance of  
18 hydrolysis products having  $M_r$  ranges of 116,000- 66,000. MHC decreased by 90% of  
19 the original content within 30 min of incubation at 55°C. However, no change in  
20 myosin light chain (MLC) was observed even with an extended incubation time. At  
21 60 min, no protein with  $M_r$  of 36, 22 and 20 kDa were found. An, Seymour, Wu and  
22 Morrissey (1994) reported that among the Pacific whiting proteins, MHC was the  
23 most extensively hydrolysed by cathepsin L, followed by troponin-T and  $\alpha$ -and  $\beta$ -  
24 tropomyosin. For the control (without purified proteinase), a slight degradation of

1 myosin heavy chain was observed (lane C) (Fig. 3). The result suggested the existence  
2 of a myofibril-bound proteinase in partially purified MHC.

3

#### 4 *3.3.2 Collagen*

5 No hydrolytic degradation of collagen were observed when collagen was  
6 incubated at 55°C up to 60 min in the presence of purified skipjack tuna spleen trypsin  
7 (Fig. 4). Both  $\beta$  and  $\alpha$ -compounds were not hydrolysed by added trypsin. Collagen  
8 type I consisting of two  $\alpha_1$  chain and one  $\alpha_2$  chain was found in the skin of bigeye  
9 snapper (Kittiphattanabawon et al., 2005).  $\beta$ -component was also present in collagen  
10 from many fish species (Ciarlo, Paredi & Frage, 1997, Nagai & Suzuki, 2000). This  
11 suggests that collagen was not a good substrate for trypsin from skipjack tuna spleen.  
12 Yamashita et al. (1991) reported that native collagens were degraded at 20°C by chum  
13 salmon cathepsin L but not by cathepsin B. Thus, the degradation of collagen  
14 depended on the source of collagen as well as types of proteinases. Collagen  
15 molecules in the connective tissue generally undergo limited cleavage in the non-  
16 helical region by the various protease, such as pepsin, trypsin (Yamashita et al., 1991).  
17 For the control (without purified proteinase), the degradation of collagen was not  
18 observed. From the result, trypsin might hydrolyse the extracted collagen only at the  
19 non-helical region but could not cleave the peptide bonds in the  $\alpha$  or  $\beta$  components.  
20 As a result, protein pattern of major components were not changed when analysed by  
21 SDS-PAGE.

22

23

24

25

### 1 *3.4 Effect of NaCl on proteolytic activity*

2       The effect of NaCl on hydrolytic activity of purified trypsin on NAM is  
3 depicted in Fig. 5. The band intensity of MHC slightly increased with increasing NaCl  
4 concentration up to 25%. Some losses in activity occurred as NaCl concentration  
5 increased, probably owing to the partial denaturation of proteinases caused by the  
6 “Salting out” effect (Klomklao et al., 2004). Thermostable proteinase in salted  
7 anchovy muscle was still active and able to degrade myofibrillar protein in the  
8 commercial salted fillets containing 16-17% NaCl (Ishida, Niizelei & Nagayama,  
9 1994). Therefore, the uses of spleen proteinases might be possible to accelerate the  
10 protein hydrolysis in fish sauce production, in which salt at high level was used.

11

### 12 *3.5 Peptide mapping of protein substrates*

13       The peptide maps of sardine protein substrates hydrolysed by purified trypsin  
14 in comparison with trypsin from bovine pancreas are shown in Fig. 6 and 7. At the  
15 same level added, trypsin from bovine pancreas exhibited much higher hydrolytic  
16 activity on NAM and myosin than trypsin from skipjack tuna spleen. For NAM and  
17 myosin hydrolysed by trypsin from bovine pancreas (Fig. 6), all components of NAM  
18 were more hydrolysed, as shown by the lower original band intensity of each  
19 component remained with a concomitant increase in lower MW peptide fragments.  
20 For NAM, actin was still remained with addition of skipjack tuna spleen trypsin.  
21 Therefore, bovine pancreas trypsin was more effective in NAM and myosin  
22 hydrolysis. On the other hand, collagen could not be hydrolysed by both enzymes (Fig.  
23 7).  $\alpha$  and  $\beta$  components were still remained in presence of both enzymes. The result  
24 suggested that these components of collagen were tolerant to digestion by both  
25 trypsins. Therefore, skipjack tuna spleen trypsin might not cleave the connective



1 tissues in fish skin or muscle. Nevertheless, it effectively induced the liquefaction  
2 process via the hydrolysis of myofibrillar proteins.

3

#### 4 **4. Conclusion**

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

11

#### 12 **Acknowledgments**

13 Financial support from Thailand Research Fund under the Royal Golden  
14 Jubilee Ph.D. Program (Grant No. PHD/0216/2546) to Sappasith Klomklao.

15

#### 16 **References**

- 17 An, H., Seymour, T.A., Wu, J.W., & Morrissey, M.T. (1994). Assay systems and  
18 characterization of Pacific whiting (*Merluccius productus*) protease.  
19 *Journal of Food Science*, *59*, 277-289.
- 20 Benjakul, S., Seymour, T.A., Morrissey, M.T., & An, H. (1997). Physicochemical  
21 changes in Pacific whiting muscle proteins during ice storage. *Journal of Food*  
22 *Science*, *62*, 729-733.
- 23 Benjakul, S., Visessanguan, W., & Leelapongwattana, K. (2003). Purification and  
24 characterization of heat-stable alkaline proteinase from bigeye snapper

- 1           (*Priacanthus macracanthus*) muscle. *Comparative Biochemistry and*  
2           *Physiology Part B*, 24, 107-127.
- 3 Benjakul, S., Visessanguan, W., & Thummaratwasik, P. (2000). Isolation and  
4           characterization of trypsin inhibitors from some Thai legume seeds. *Journal of*  
5           *Food Biochemistry*, 24, 107-127.
- 6 Bezerra, R.S., Santo, J.F., Paiva, P.M.G., Correia, M.T.S., Coelho, L.C.B.B., Vieira,  
7           V.L.A., & Carvalho, L.B. (2001). Partial purification and characterization of  
8           a thermostable trypsin from pyloric caeca of tambaqui (*Colossoma*  
9           *macropomum*). *Journal of Food Biochemistry*, 25, 199-210.
- 10 Cao, M.J., Osatomi, K., Hara, K., & Ishihara, T. (2000). Identification of a myofibril-  
11           bound serine proteinase (MBSP) in the skeletal muscle of lizard fish *Saurida*  
12           *wanieso* which specifically cleaves the arginine site. *Comparative*  
13           *Biochemistry and Physiology Part B*, 125, 255-264.
- 14 Chaveesuk, R., Smith, J.P., & Simpson, B.K. (1993). Production of fish sauce and  
15           acceleration of sauce fermentation using proteolytic enzymes. *Journal of*  
16           *Aquatic Food Product Technology*, 2(3), 59-77.
- 17 Ciarlo, A.S., Paredi, M.E., & Fraga, A.N. (1997). Isolation of soluble collagen from  
18           hake skin (*Merluccius hubbsi*). *Journal of Aquatic Food Product Technology*,  
19           6(1), 65-77.
- 20 Gildberg, A. (2001). Utilization of male Arctic capelin and Atlantic cod intestines for  
21           fish sauce production-evaluation of fermentation conditions. *Bioresource*  
22           *Technology*, 76, 119- 123.
- 23 Gildberg, A., & Shi, X.Q. (1994). Recovery of tryptic enzymes from fish sauce.  
24           *Process Biochemistry*, 29, 151-155.

- 1 Hjelmeland, K., & Raa, J. (1982). Characteristics of two trypsin type isozymes  
2 isolated from the Arctic fish capelin (*Mallotus villosus*). *Comparative*  
3 *Biochemistry and Physiology Part B*, 71, 557-562.
- 4 Heu, M.S., Kim, H.R., & Pyeun, J.H. (1995). Comparison of trypsin and  
5 chymotrypsin from the viscera of anchovy (*Engraulis japonica*). *Comparative*  
6 *Biochemistry and Physiology Part B*, 112, 557-567.
- 7 Ishida, M., Niizelei, S., & Nagayama, F. (1994). Thermostable proteinase in salted  
8 anchovy muscle. *Journal of Food Science*, 59, 781-785.
- 9 Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T., & Tanaka, M.  
10 (2005). Characterisation of acid-soluble collagen from skin and bone of bigeye  
11 snapper (*Priacanthus tayenus*). *Food Chemistry*, 89, 363-372.
- 12 Klomklao, S., Benjakul, S., & Visessanguan, W. (2004). Comparative studies on  
13 proteolytic activity of spleen extracts from three tuna species commonly used  
14 in Thailand. *Journal of Food Biochemistry*, 28, 355-372.
- 15 Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., & Simpson, B.K.  
16 (2005). Effect of skipjack tuna spleen on the liquefaction and characteristics of  
17 sardine fish sauce. *Food Chemistry*, (in preparation).
- 18 Krisjansson, M. (1991). Purification and characterization of trypsin from the pyloric  
19 caeca of rainbow trout (*Oncorhynchus mykiss*). *Journal of Agricultural and*  
20 *Food Chemistry*, 39, 1738-1742.
- 21 Kishimura, H., & Hayashi, K. (2002). Isolation and characteristics of trypsin from  
22 pyloric ceca of the starfish *Asterina pectinifera*. *Comparative Biochemistry*  
23 *and Physiology Part B*, 132, 485-490.
- 24 Laemmli, U.K. (1970). Cleavage of structure proteins during the assembly of the head  
25 of bacteriophage T<sub>4</sub>. *Nature*, 227, 680-685.

- 1 Lopetcharat, K., & Park, J.W. (2002). Characteristics of fish sauce made from Pacific  
2 whiting and surimi by-products during fermentation storage. *Journal of Food*  
3 *Science*, *67*, 511-516.
- 4 Lopetcharat, K., Choi, Y.J., Park, J.W., & Daeschel, M.A. (2001). Fish sauce products  
5 and manufacturing: a review. *Food Review International*, *17*, 65-68.
- 6 Lowry, O.H., Rosebrough, N.J., Fan, A.L., & Randall, R.J. (1951). Protein  
7 measurement with Folin phenol reagent. *Journal of Biological Chemistry*,  
8 *193*, 256-275.
- 9 Martone, C.B., Busconi, L., Folco, E.J., Trucco, R.E. and Sanchez, J.J. (1986). A  
10 simplified myosin preparation from marine fish species. *Journal of Food*  
11 *Science*, *51*, 1554-1555.
- 12 Nagai, T., & Suzuki, N. (2000). Isolation of collagen from fish waste material-skin,  
13 bone and fins. *Food Chemistry*, *68*, 277-281.
- 14 Ouali, A., & Valin, C. (1981). Effect of muscle lysosomal enzymes and calcium  
15 activated neutral proteinase on myofibrillar ATPase activity: Relationship with  
16 ageing changes. *Meat Science*, *5*, 233-245.
- 17 Orejana, F.M., & Liston, J. (1981). Agents of proteolysis and its inhibition in Patis  
18 (fish sauce) fermentation. *Journal of Food Science*, *47*, 198-203.
- 19 Osatomi, K., Sasai, H., Cao, M., Hara, K., & Ishihara, T. (1997). Purification and  
20 characterization of myofibril-bound serine proteinase from carp, *Cyprinus*  
21 *carpio*, ordinary muscle. *Comparative Biochemistry and Physiology Part B*.  
22 *116*, 183-190.
- 23 Saito, M., Kunisaki, N., Urano, N., & Kimura, S. (2002). Collagen as the major  
24 edible component of sea cucumber (*Stichopus japonicus*). *Journal of Food*  
25 *Science*, *67*, 1319-1322.

- 1 Simpson, B.K. (2000). Digestive proteinases from marine animals. In N.F. Haard &  
2 B.K. Simpson (Eds.), *Seafood enzymes: Utilization and influence on*  
3 *postharvest seafood quality* (pp. 531-540). New York: Mercel Dekker.
- 4 Simpson, B.K., & Haard, H.F. (1984). Trypsin from Greenland cod (*Gadus ogac*).  
5 Isolation and comparative properties. *Comparative Biochemistry and*  
6 *Physiology Part B*, 79, 613-622.
- 7 Simpson, B.K., Simpson, M.V., & Haard, N.F. (1989). On the mechanism of enzyme  
8 action: digestive proteases from selected marine organisms. *Biotechnological*  
9 *Application Biochemistry*, 11, 226-234.
- 10 Torrissen, K.R. (1984). Characterization of proteases in the digestive tract of Atlantic  
11 salmon (*Salmo salar*) in comparison with rainbow trout (*Salmo gairdneri*).  
12 *Comparative Biochemistry and Physiology Part B*, 77, 669-674.
- 13 Vissessanguan, W., Ogawa, M., Nakai, S., & An, H. (2000). Physicochemical changes  
14 and mechanism of heat-induced gelation of arrowtooth flounder myosin.  
15 *Journal of Agricultural and Food Chemistry*, 48, 1016-1023.
- 16 Yamashita, M., & Konagaya, S. (1991). Hydrolytic action of salmon cathepsin B and  
17 L to muscle structural proteins in respect of muscle softening. *Nippon Suisan*  
18 *Gakkaishi*, 57,1917-1922.
- 19  
20  
21  
22  
23  
24  
25

1 Table 1

2 Purification of trypsin from skipjack tuna spleen

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purity (fold)	Yield (%)
Crude extract	20378	673.7	30.2	1.0	100.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (30-70%)	14459	45.8	131.9	4.4	70.9
Sephacryl S-100	13585	30.8	470.3	15.6	66.7
Benzamidine-Sepharose 4 Fast Flow	10514	8.2	674.9	22.3	51.6

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25

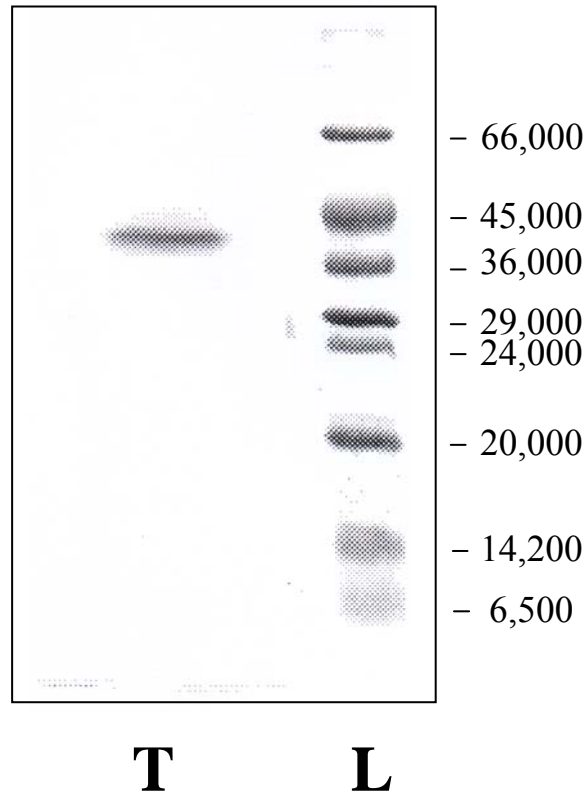


Fig. 1

1

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

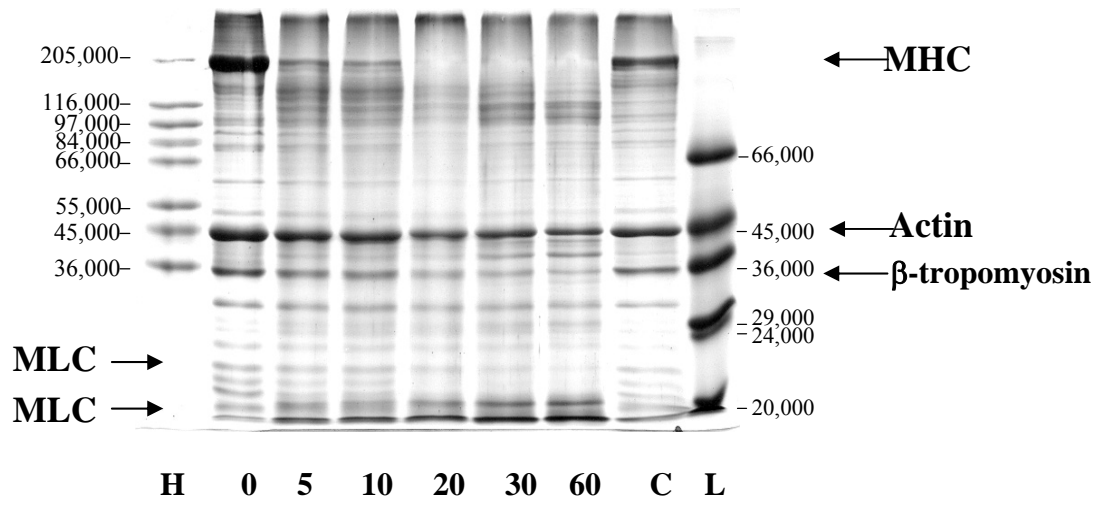


Fig. 2



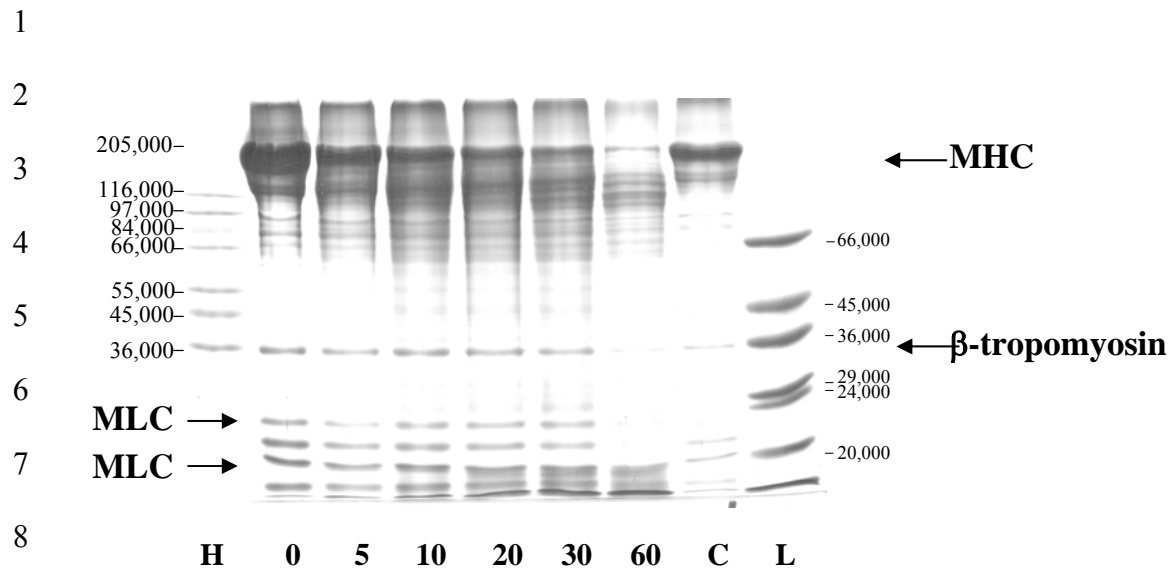


Fig. 3

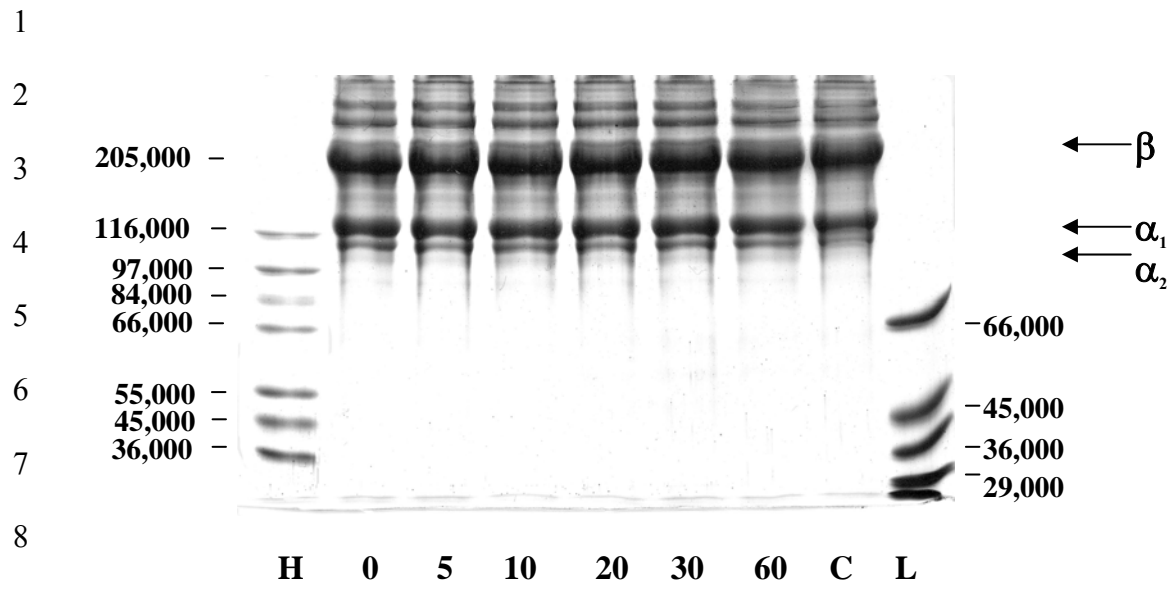


Fig. 4

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25

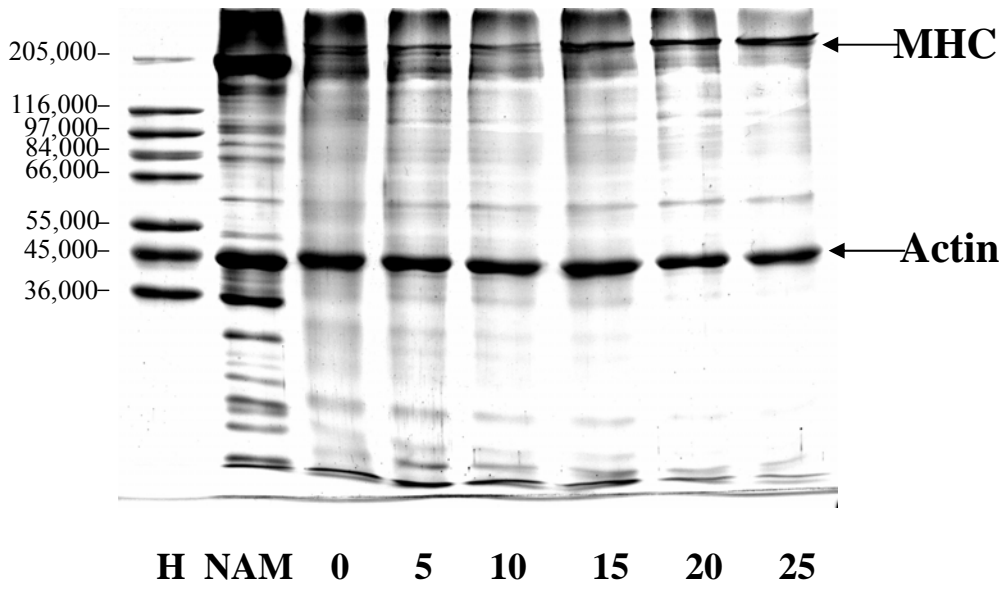


Fig. 5

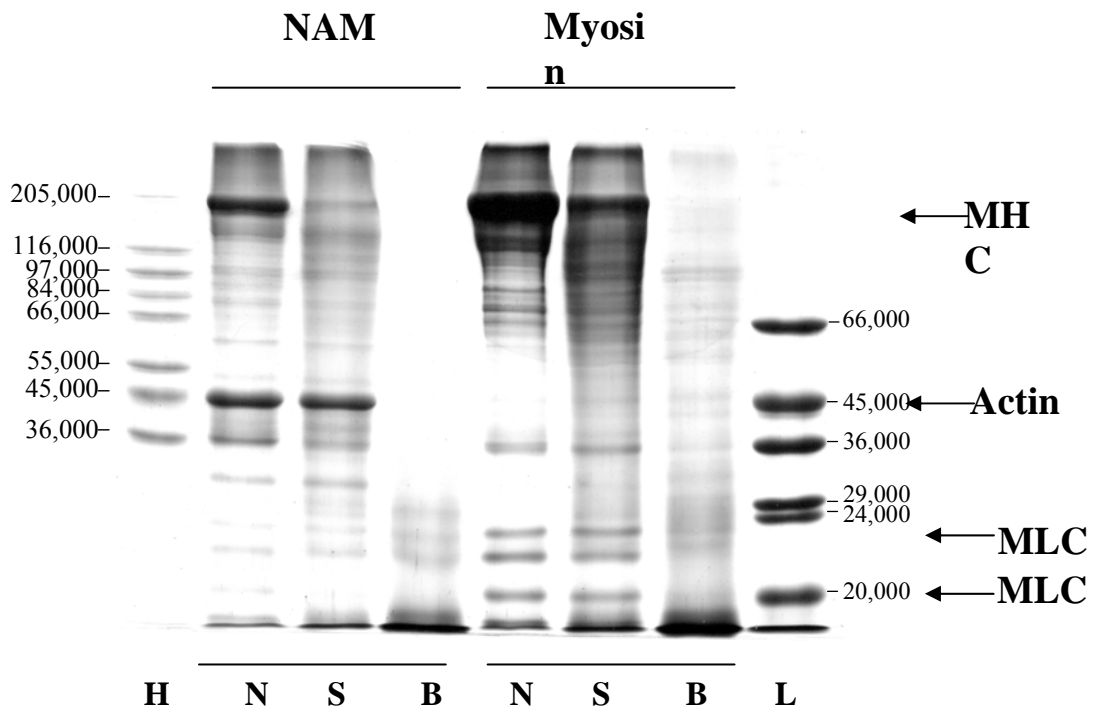


Fig. 6

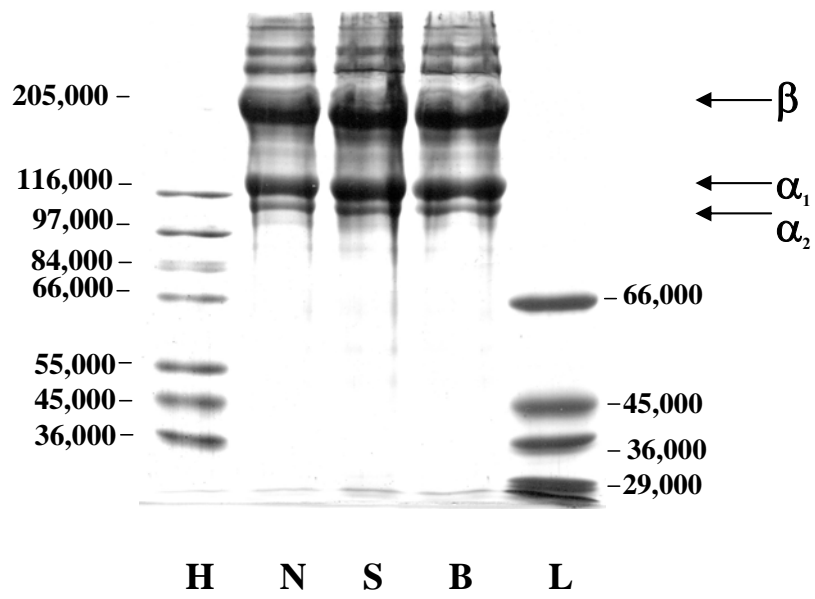


Fig. 7

1 **Figure Legends**

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

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

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

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

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

20 **Fig. 6.** Peptide mapping of sardine NAM and myosin hydrolysed by purified trypsin  
21 from skipjack tuna spleen and trypsin from bovine pancreas. H, high- molecular-  
22 weight standard; L, low-molecular-weight standard; MHC, myosin heavy chain; MLC,  
23 myosin light chain.

24 N, without enzyme addition; S, added with skipjack tuna spleen trypsin (0.25 unit/ 4  
25 mg protein); B, added with bovine pancreas trypsin (0.25 unit/ 4 mg protein).

1 Reaction was conducted at 55°C for 10 min and 20 min for NAM and myosin,  
2 respectively.

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