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Food Chemistry 98 (2006) 14–22
Food Chemistry
www.elsevier.com/locate/foodchem

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

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Received 14 March 2005; received in revised form 17 May 2005; accepted 17 May 2005

**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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0308-8146/$ - see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2005.05.047

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/