รายงานวิจัยฉบับสมบูรณ์

โครงการ สารยับยั้งเอนไซม์โปรติเนสจากไข่ปลาทูน่าพันธุ์ครีบเหลือง (*Thunnus albacores*): การทำบริสุทธิ์ การจำแนกคุณลักษณะและการประยุกต์ใช้สำหรับการปรับปรุงคุณภาพซูริมิ

โดย รองศาสตราจารย์ ดร. สรรพสิทธิ์ กล่อมเกล้า

กรกฎาคม 2558

สัญญาเลขที่ RSA5580027

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รองศาสตราจารย์ ดร.สรรพสิทธิ์ กล่อมเกล้า มหาวิทยาลัยทักษิณ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยทักษิณ
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. และมหาวิทยาลัยทักษิณ ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัย และมหาวิทยาลัยทักษิณ สำหรับเงินอุดหนุนโครงการวิจัยเรื่อง “สารยับยั้งเอนไซม์โปรติเนสจากไข่ปลาทูน่าพันธุ์ครีบเหลือง (*Thunnus albacores*): การทำบริสุทธิ์ การจำแนกคุณลักษณะและการประยุกต์ใช้สำหรับปรับปรุงคุณภาพเจลซูริมิ” จำนวน 1,200,000 บาท ขอขอบพระคุณคณะเทคโนโลยีและการพัฒนาชุมชน มหาวิทยาลัยทักษิณ และคณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์ที่ให้การสนับสนุนด้านอุปกรณ์และเครื่องมือวิทยาศาสตร์สำหรับการวิจัยครั้งนี้

รองศาสตราจารย์ ดร.สรรพสิทธิ์ กล่อมเกล้า
หัวหน้าโครงการฯ

บทคัดย่อ

รหัสโครงการ: RSA5580027

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จากการศึกษาการทำบริสุทธิ์สารยับยั้งเอนไซม์ทริปซินจากไข่ปลาทูน่าพันธุ์ครีบเหลืองโดยการให้ความร้อนที่อุณหภูมิ 60 องศาเซลเซียส เป็นเวลา 10 นาทีตามด้วยการใช้โครมาโตกราฟีชนิดต่าง ๆ จนกระทั่งสารยับยั้งเอนไซม์ทริปซินที่ผ่านการทำบริสุทธิ์ปรากฏเป็นแถบโปรตีนเดี่ยวบนเจลของ native-PAGE โดยสารยับยั้งมีความบริสุทธิ์เพิ่มขึ้น 11.29 และมีผลผลิตร้อยละ 46.02 สารยับยั้งเอนไซม์ทริปซินมีน้ำหนักโมเลกุลเท่ากับ 70 กิโลดาลตัน เมื่อตรวจสอบด้วยวิธีเจลฟิวเตรชันและ SDS-PAGE สารยับยั้งเอนไซม์ทริปซินมีกิจกรรมสูงสุดที่พีเอช 7 และ 50 องศาเซลเซียส มีความคงตัวต่อพีเอชในช่วงพีเอช 5-8 และมีความคงตัวในช่วงอุณหภูมิ 20-60 องศาเซลเซียส เป็นเวลา 10 นาที โซเดียมคลอไรด์ที่ระดับความเข้มข้นร้อยละ 0-3 ไม่มีผลต่อกิจกรรมของสารยับยั้งเอนไซม์ทริปซินที่ผ่านการทำบริสุทธิ์ จากการศึกษาผลของสารยับยั้งเอนไซม์ทริปซินที่ผ่านการทำบริสุทธิ์บางส่วนจากไข่ปลาทูน่าพันธุ์ครีบเหลือง (TIYTR) ที่ระดับแตกต่างกัน (ร้อยละ 0-3) ต่อกิจกรรมการย่อยสลายโปรตีนและสมบัติการเกิดเจลซูริมิปลาตาหวานหนังบาง (*Priacanthus macracanthus*) พบว่า TIYTR สามารถยับยั้งกิจกรรมการย่อยสลายโปรตีนในเจลคามาโบโกะ (40/90 องศาเซลเซียส) และเจลโมโดริ (60/90 องศาเซลเซียส) ซึ่งประสิทธิภาพการยับยั้งขึ้นกับระดับความเข้มข้นของ TIYTR ที่ใช้ เมื่อระดับความเข้มข้นของ TIYTR เพิ่มขึ้นไม่เกินร้อยละ 3 ปริมาณโปรตีนไมโอซินเส้นหนักในเจลทั้งสองชนิดมีปริมาณเพิ่มขึ้นซึ่งสอดคล้องกับการเพิ่มขึ้นของค่าแรงเจาะทะลุและระยะทางก่อนเจาะทะลุ รวมถึงการลดลงของการย่อยสลายโปรตีนโดยเห็นได้จากปริมาณเปปไทด์ที่ละลายได้ในกรดไตรคลอโรอะซิติกมีค่าลดลง ค่าความขาวของเจลคามาโบโกะและโมโดริมีค่าลดลงเล็กน้อยเมื่อระดับความเข้มข้นของ TIYTR มีค่าเพิ่มขึ้น (p<0.05) อย่างไรก็ตามความสามารถในการอุ้มน้ำของเจลทั้งสองเพิ่มขึ้นเมื่อความเข้มข้น TIYTR เพิ่มขึ้น (p<0.05) การเติม TIYTR โปรตีนพลาสมาจากเลือดวัว (BPP) และโปรตีนจากไข่ขาว (EW) ที่ระดับความเข้มข้นร้อยละ 3 ในเจลซูริมิปลาตาหวานหนังบางส่งผลให้ค่าแรงเจาะทะลุและระยะทางก่อนเจาะทะลุมีค่าเพิ่มขึ้น อย่างไรก็ตาม TIYTR และโปรตีนจากพลาสมาเลือดวัวมีประสิทธิภาพในการเพิ่มความแข็งแรงของเจลซูริมิได้ดีกว่าโปรตีนจากไข่ขาว ดังนั้น TIYTR สามารถใช้เป็นสารยับยั้งเอนไซม์โปรตีเนสทางเลือกใหม่ที่มีราคาถูกสำหรับปรับปรุงความแข็งแรงของเจลซูริมิ

คำสำคัญ: โปรติเนส สารยับยั้งเอนไซม์ทริปซิน ไข่ปลา ซูริมิ สมบัติการเกิดเจล

Abstract

Project Code: RSA5580027

Project Title: Proteinase inhibitor from yellowfin tuna (*Thunnus albacores*) roe: Purification, characterization and application for improvement of gel quality of surimi

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Project Period: July 16, 2012 – July 15, 2015

Trypsin inhibitor was purified to homogeneity from the roe of yellowfin tuna (*Thunnus albacores*) by heat-treatment at 60°C for 10 min, followed by a series of chromatographic separations to obtain a single band using native-PAGE. It was purified 11.29-fold with a yield of 46.02%. Purified trypsin inhibitor had an apparent molecular weight of 70 kDa when analyzed using SDS-PAGE and size exclusion chromatography. Maximal activity was recorded at pH 7.0 and 50°C. The purified inhibitor was stable in temperature ranges from 20 to 60°C for 10 min and in the pH range of 5 to 8. NaCl concentration up to 3% did not significantly affect the inhibitory activity of purified trypsin inhibitor. Effects of partially purified trypsin inhibitor from the roe of yellowfin tuna (*Thunnus albacores*) (TIYTR) at different levels (0-3.0%) on proteolysis and gelling properties of bigeye snapper (*Priacanthus macracanthus*) surimi were also investigated. TIYTR showed inhibitory activity against proteolysis in kamaboko (40/90°C) and modori (60/90°C) gels in a concentration-dependent manner. Myosin heavy chain (MHC) was more retained in both gels when the level of TIYTR increased up to 3.0%. This was associated with the increased breaking force and deformation as well as lowered protein degradation as evidenced by the decrease in trichloroacetic acid-soluble peptide content (p<0.05). Whiteness of kamaboko and modori gels slightly decreased with increasing TIYTR levels (p<0.05). However, water-holding capacity of both gels was improved as TIYTR level increased (p<0.05). Incorporation of TIYTR, beef plasma protein (BPP) and egg white (EW) at a level of 3.0% resulted in the increased breaking force and deformation of surimi gels. Nevertheless, TIYTR and BPP showed the higher gel strengthening effect than EW. Therefore, TIYTR could be used as an alternative cheap proteinase inhibitor to improve gel strength of surimi.

Keywords: Proteinase, Trypsin inhibitor, Fish roe, Surimi, Gel properties

CONTENTS

CHAPTER 1

OPTIMUM EXTRACTION AND RECOVERY OF TRYPSIN INHIBITOR FROM YELLOWFIN TUNA (*THUNNUS ALBACORES*) ROE AND ITS BIOCHEMICAL PROPERTIES

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Optimum extraction and recovery of trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe and its biochemical properties

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Summary

Effect of lipid removal, extraction medium and extraction time on the isolation and recovery of trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe was investigated. Trypsin inhibitor extracted from defatted tuna roe showed the higher specific inhibitory activity than extracted from origin tuna roe. Optimal extraction medium was attained by shaking the defatted yellowfin tuna roe powder in 10 mM Na-phosphate buffer (pH 7.0) containing 0.5 M NaCl ($P<0.05$). The extraction time affected the inhibitor recovery significantly ($P<0.05$). The extraction time of 30 min was optimum for recovery of trypsin inhibitor from yellowfin tuna roe. The biochemical properties of trypsin inhibitor from yellowfin tuna roes were also determined. The inhibitor was heat stable up to 60°C and over a broad pH range (5-8). Increasing the concentration of salt (up to 3%, w/v) did not significantly decrease the trypsin inhibitory activity. However, the activity decreased when trypsin inhibitor was incubated with metal ions at ambient temperature for 30 min.

Keywords Trypsin inhibitor, proteinase, isolation, tuna, roe

Introduction

Proteinase inhibitors are proteins or peptides capable of inhibiting the catalytic activities of proteolytic enzymes (Choi et al., 2002). Proteinase inhibitors commonly accumulate in high quantities in plant seeds, bird eggs and various body fluids. Proteinase inhibitors are also found in mammalian and marine blood plasma and fish roe and viscera (Choi et al., 2002). Proteinase inhibitors in fish roe can have a major impact on nutritional value as they inhibit pancreatic serine proteinases, thus impairing protein digestion. However, fish roe can be used as a potential source of proteinase inhibitor and it can be of a variety of applications such as medicine, agriculture and food technology. Proteinase inhibitor can be used to improve texture in various foods, for example, sausages, meat balls, and low-salt fish products.

Thailand is the world's largest canned tuna producer and exporter. By the year 2008, tuna products with total amount of 483,894 tons and value of 61,036 million baht were exported (Department of Foreign Trade, 2009). Tuna roe, a by-product generated from fish processing (approximately 1.5-3.0% of total weight), is generally used as animal feed or for pet food preparation in Thailand. Based on our preliminary study, yellowfin tuna roe contained high trypsin inhibitory activity. The recovery of trypsin inhibitor from yellowfin tuna roe can increase its value and reduce waste disposal or treatment, thereby lowering environmental pollution. Nevertheless, no information regarding the biochemical properties of trypsin inhibitor from the roe of yellowfin tuna have been reported. Hence, yellowfin tuna roe that can be a cheap and promising source of proteinase inhibitor, especially trypsin inhibitor can be recovery and isolated for utilizations.

The objectives of this investigation were to extract and characterized the trypsin inhibitor from yellowfin tuna roe.

Material and Methods

Chemicals

*N*α-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), trypsin from porcine pancreas, sodium chloride, tris (hydroxymethyl) aminomethane, dimethylsulfoxide, sodium caseinate and bovine serum albumin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Extraction of trypsin inhibitor from yellowfin tuna roe

Raw materials

Roes of yellowfin tuna (*Thunnus albacores*), with a size of 51.8±2.4 g/roe, were obtained from Tropical Canning (Thailand) Public Co. Ltd., Songkhla, Thailand. The samples (5 kg) were placed in ice using a roe:ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung, within 2 h. The whole roes were cleaned using cold water (4°C). The roes were immediately frozen and stored at -20°C until used.

Preparation of defatted roe powder for trypsin inhibitor extraction

Frozen roes 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 homogenized with three volumes of acetone at -20°C for 30 min according to the method of Klomklao et al. (2010a) with a slight modification. The homogenate was filtrated in vacuo on Whatman No. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20°C for 30 min, and then the residue was air-dried at room temperature. Defatted roe powder obtained was stored at -20°C until used.

Effect of lipid removal on trypsin inhibitor extraction

Samples, non-defatted and defatted roes, were mixed with distilled water at a ratio of 1:9 (w/v) and shaken (BW 201 Shaking bath, Tokyo, Japan) for 30 min at 150 rpm at room temperature. The extract was recovered by centrifuging at 10,000×g for 30 min. The trypsin inhibitory activity and protein content in the extracts were determined and the specific inhibitory activity of the extracts were compared.

Effect of extraction media on trypsin inhibitor extraction

Different extraction media including distilled water, 10 mM Tri-HCl, pH 7.0 and 10 mM Na-phosphate buffer, pH 7.0 were used to extract trypsin inhibitor. The defatted roe powder was added to the extraction medium at a ratio of 1:9 (w/v) and shaken (BW 201 Shaking bath, Tokyo, Japan) for 30 min at 150 rpm at room temperature. The extract was recovered by centrifuging at 10,000×g for 30 min. The trypsin inhibitory activity and protein content in the extract were determined and the specific inhibitory activity of the extracts obtained using

different media were compared. The extraction media, which was able to extract the trypsin inhibitor with the highest specific trypsin inhibitory activity, was selected for further steps.

Effect of NaCl concentration on trypsin inhibitor extraction

Defatted roe powder was suspended in 10 mM Na-phosphate, pH 7.0 containing different NaCl concentration (0, 0.25, 0.5, 0.75 and 1.0 M) at a ratio of 1:9 (w/v) and shaken for 30 min at 150 rpm at room temperature. The mixture was centrifuged at 10,000×g for 30 min and the supernatants were subjected to determination of trypsin inhibitory activity and protein content. The extraction medium rendering the highest specific trypsin inhibitory activity was chosen for further study.

Effect of extraction time on trypsin inhibitor extraction

Defatted roe powder was mixed with 10 mM Na-phosphate buffer (pH 7.0) containing 0.5 M NaCl with a solid/solvent ratio of 1:9 (w/v) and shaken for 0.25, 0.5, 1, 2 and 3 h. At designated time, the mixture was centrifuged at 10,000×g for 30 min and the supernatants were subjected to determination of trypsin inhibitory activity and protein content. The extraction time rendering the highest specific trypsin inhibitory activity was chosen for further study.

Characterization of trypsin inhibitor from yellowfin tuna roe

Thermal and pH stability

Trypsin inhibitor extracts from yellowfin tuna roe were diluted with distilled water to obtain 60-70% inhibition. The solutions were incubated at 30, 40, 50, 60, 70, 80, 90 and 100°C for 10, 20 and 30 min and then cooled in iced-water. The residual trypsin inhibitory activity was determined and reported as the relative activity compared to the original activity.

The effect of pH on trypsin inhibitor stability was evaluated by measuring the residual activity after incubation at various pHs for 30 min at room temperature. Different buffers used included McIlvaine buffers (0.2 M Na phosphate and 0.1 M Na citrate) for pH 2.0-7.0 and 0.1 M glycine-NaOH for pH 8.0-11.0.

Salt stability

Trypsin inhibitor was incubated at room temperature for 30 min in the presence of NaCl ranging from 0 to 3%. The mixture was tested for inhibitory activity against trypsin. The residual inhibitory activity was reported.

Effect of metal ions

The solutions with various metal ions such as KCl, NaCl, $MgCl_2$ and $CaCl_2$ were added in the trypsin inhibitor to a final concentration of 10 mM. The trypsin inhibitory activity was assayed after incubation for 30 min at room temperature. The residual inhibitory activity was reported.

Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured by the method of Benjakul et al. (2000) with a slight modification using BAPNA as substrate. A solution containing 200 µl of inhibitor solution, 200 µl (20µg/ml) porcine pancreas trypsin was preincubated at 37°C for 15 min. Then, 1,000 µl of the mixtures containing 800 µl of 0.5 mM BAPNA and 200 µl of distilled water (prewarmed to 37°C) was added and vortexed immediately to start the reaction. After incubating for 10 min, 900 µl of 30% acetic acid (v/v) was added to terminate the reaction. The reaction mixture was centrifuged at 8,000×g for 5 min (Eppendorf Micro Centrifuge). Residual activity of trypsin was measured by the absorbance at 410 nm due to p-nitroaniline released. One unit of proteolytic activity was defined as an increase of 0.01 absorbance unit/ml.min under the assay condition. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduces the enzyme activity by one unit.

Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical analysis

A completely randomized design was used throughout this study. All data were subjected to analysis of variance (ANOVA) and the differences between means were carried out using Duncan's Multiple Range Test. For effect of lipid

removal on trypsin inhibitor recovery, the independence t-test was used for pair comparison. (Steel & Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows; SPSS Inc.).

Results and discussion

Optimum extraction of trypsin inhibitor from yellowfin tuna roe

The effect of lipid removal on trypsin inhibitor recovery from the roe of yellowfin tuna is displayed in Table 1. Total trypsin inhibitory activity and specific inhibitory activity of defatted tuna roes was generally higher than that of the control (non-defatted roe) ($P<0.05$). The results indicated that the lipid removal facilitated the release of trypsin inhibitor from tuna roes.

The effect of extraction media on trypsin inhibitor extraction from yellowfin tuna roe is shown in Table 2. Defatted roe extract using 10 mM Na-phosphate buffer, pH 7.0 showed higher trypsin inhibitory activity than those extracted with distilled water and 10 mM Tris-HCl buffer (Table 2) ($P<0.05$). Additionally, specific inhibitory activity was greater in the extract using Na-phosphate buffer, compared with other two extracts (Table 2). The results suggested that Na-phosphate buffer had a greater ability to extract trypsin inhibitor with the higher purity than did distilled water and Tris-HCl buffer.

Na-phosphate buffer containing different NaCl concentration were used to extract the trypsin inhibitor from yellowfin tuna roe (Table 3). When the concentration of NaCl was increased from 0 to 0.5 M, trypsin inhibitory activity increased ($P<0.05$). However, there was no further increase in the activity with

NaCl above 0.5 M. This probably due to the denaturation of yellowfin tuna roe trypsin inhibitor at high salt concentration. Factors involved in protein solubility and recovery include particle size of tissue, temperature, length of extraction time, pH, ionic strength, type and concentration of extraction medium as well as the hydration characteristics of proteins (Benjakul et al., 2000 ; Klomklao et al., 2011). From the results, 10 mM Na-phosphate buffer containing 0.5 M NaCl was shown to the best extraction medium for yellowfin tuna roe trypsin inhibitor.

Fig. 1 showed the effect of extraction time on the recovery of trypsin inhibitor from yellowfin tuna roe. A higher extraction efficiency was found when the extraction time increased up to 0.5 h (Fig. 1a) ($P<0.05$). Increased extraction time allowed the protein, especially trypsin inhibitors to be more dissolved. The inhibitory activity was slightly increased for yellowfin tuna roe with the extraction time of 0.5 h (Fig. 1a) ($P<0.05$). However, the decrease in inhibitory activity was observed when the extraction time of 1-3 h was used ($P<0.05$) (Fig. 1a), indicating reduced stability of trypsin inhibitor from yellowfin tuna roe during the extraction. This contributed to the loss of specific activity (Fig. 1b). High mechanical shear generated by shaking and the incorporation of air bubbles and adsorption of protein molecules to the air-liquid interface can cause denaturation of protein (Klomklao et al., 2010b; Damodaran, 1996). Therefore, an extraction time of 0.5 h was selected for extraction of trypsin inhibitor from yellowfin tuna roe.

Table 1. Effect of lipid removal on the recovery of trypsin inhibitor from yellowfin tuna roes

Sample	Total trypsin inhibitory activity (Units)	Total protein (mg)	Specific trypsin inhibitory activity (Units/mg protein)
Non-defatted roe	80.52±0.15a	206.46±0.05b	0.39±0.11a
Defatted roe	97.50±0.19b	199.80±0.03a	0.48±0.01b

*The samples was shaken in distilled water at ambient temperature for 30 min and trypsin inhibitory activity was analyzed using BAPNA as substrate.
**Mean±SD from triplicate determinations
The different letters in the same column denote the significant differences ($P<0.05$).

Table 2. Effect of extractants on the recovery of trypsin inhibitor from yellowfin tuna roes

Extractants	Total trypsin inhibitory activity (Units)	Total protein (mg)	Specific trypsin inhibitory activity (Units/mg protein)
Distilled water	93.76±6.91a	190±1.00a	0.49±0.40a
10 mM Tris-HCl, pH 7.0	113.69±2.76b	209±1.52b	0.54±0.12a
10 mM Na-Phosphate, pH 7.0	154.83±6.32c	196.67±15.28ab	0.78±0.03b

*The samples was shaken in different media at ambient temperature for 30 min and trypsin inhibitory activity was analyzed using BAPNA as substrate.
**Mean±SD from triplicate determinations
The different letters in the same column denote the significant differences ($P<0.05$).

Table 3. Effect of NaCl concentration on the recovery of trypsin inhibitor from yellowfin tuna roes

NaCl Concentration (M)	Total trypsin inhibitory activity (Units)	Total protein (mg)	Specific trypsin inhibitory activity (Units/mg protein)
0	154.83±5.26a	198.83±0.76a	0.77±0.01a
0.25	213.40±14.15c	200±1.00a	1.06±0.07c
0.5	257.41±2.50d	210.3±1.96b	1.22±0.01d
0.75	218.58±3.12c	219±1.00c	0.99±0.12b
1	199.33±5.13b	245±1.00d	0.81±0.03a

*The samples was shaken in 10 mM Na-Phosphate, pH 7.0 in different NaCl concentration at ambient temperature for 30 min and trypsin inhibitory activity was analyzed using BAPNA as substrate.
**Mean±SD from triplicate determinations
The different letters in the same column denote the significant differences ($P<0.05$).

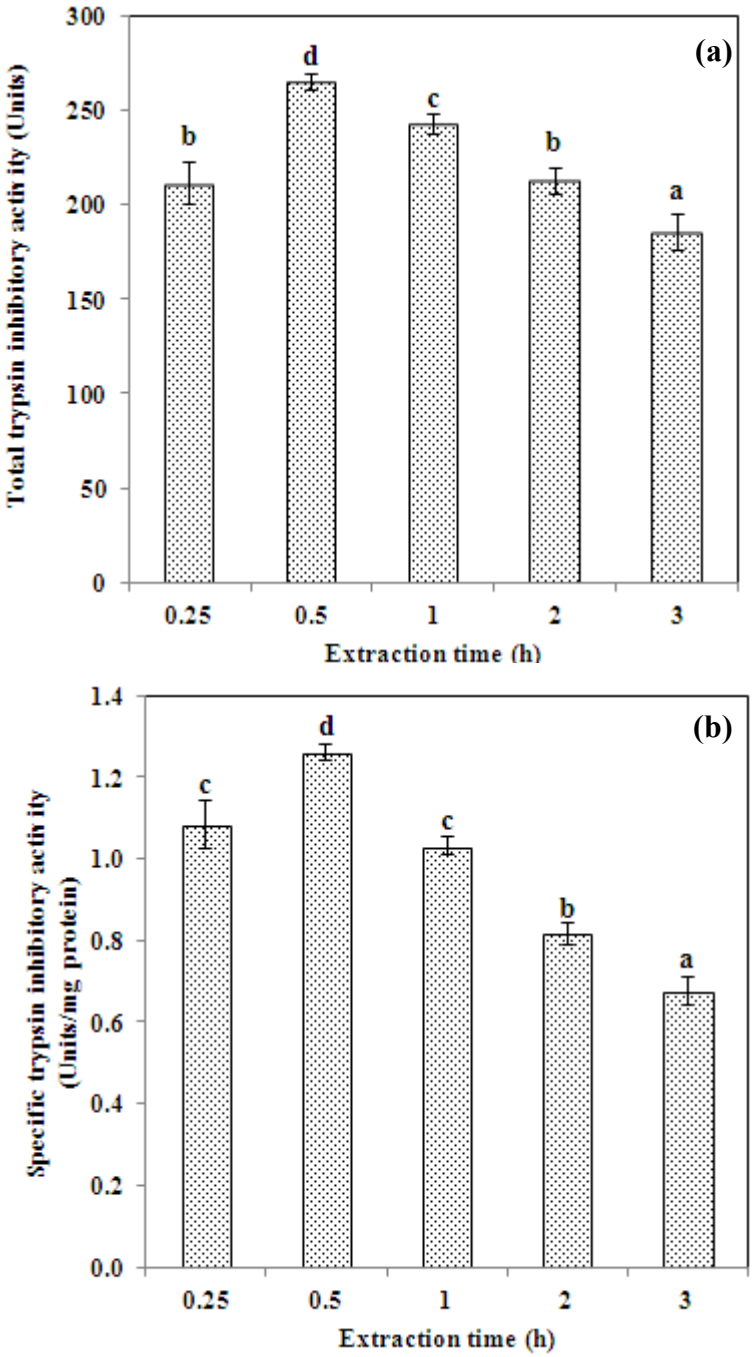

Figure 1. Effect of extraction time on the trypsin inhibitory activity (a) and the specific trypsin inhibitory activity (b) of yellowfin tuna roe extract. Bar indicate standard deviation from triplicate determination. Different letters on the bars indicate the significant differences ($P<0.05$).

Characterization of trypsin inhibitor from yellowfin tuna roe

pH and thermal stability

The pH stability of the trypsin inhibitor from yellowfin tuna roe is depicted in Fig. 2a. The inhibitor was stable over a wide pH range (pH 5-8). However, slight loss of activity was observed at low and high pHs (pH 2-4 and 9-11). Therefore, the inhibitor was generally stable in the neutral pH range. In general, most proteins are stable within a particular pH range, but denaturation (unfolding) of proteins can occur due to the strong electrostatic repulsion of ionized groups inside the molecules at extreme pH value (Damodaran, 1996). Choi et al. (2002) reported that the trypsin inhibitor from the egg of skipjack tuna (*Katsuwonus pelamis*) was stable in the pH range from 4.0 to 10.0. The differences in pH stability indicated the different molecular properties including bonding stabilizing the structure as well as the trypsin inhibitor conformation among the various species. (Benjakul et al., 2000).

For thermal stability, the inhibitor was stable when incubated at temperature up to 60°C for 10-30 min with the residual activity more than 95% (Fig. 2b). Nevertheless, the sharp decrease in trypsin inhibitory activity was noticeable at temperature above 70°C. No activity remained at 90 and 100°C, suggesting complete loss in activity caused by thermal denaturation of trypsin inhibitor. In general, the stability of trypsin inhibitor decreased when the heating time was increased. A heating time of 30 min cause the highest loss of activity at every temperature above 60°C used. It was presumed that trypsin inhibitors from yellowfin tuna roe underwent denaturation during high temperature heating. Choi

et al. (2002) reported that the trypsin inhibitor from the egg of skipjack tuna was stable at temperatures below 40°C.

Salt stability

The effect of NaCl on the inhibitory activity of trypsin inhibitor from yellowfin tuna roe was investigated. No marked changes in relative activity were observed when NaCl was added up to 3.0% (data not shown). From the result, trypsin inhibitor showed high salt stability up to 3%, which might be useful in surimi-base products in which 2-2.5% salt are commonly used.

Effect of metal ions

The activity of trypsin inhibitor in the presence of different monovalent and divalent metal ions was determined. All of the metal ions including K^+, Na^+, Mg^{2+} and Ca^{2+} had a negative effect on the trypsin inhibitor activity with 12.32-32.05% loss in inhibitory activity (Table 4). It was presumed that metal ions bound by inhibitors induces a structural modification that changes their conformational stability for their biological activity (Bijina et al., 2011). However, the activity of the trypsin inhibitor from the egg of skipjack tuna was increased in the presence of metal ions such as K^+, Na^+, Mg^{2+} and Ca^{2+} (Choi et al., 2002). These findings suggest a different in the structure of the metal ion binding site among different marine fish trypsin inhibitors.

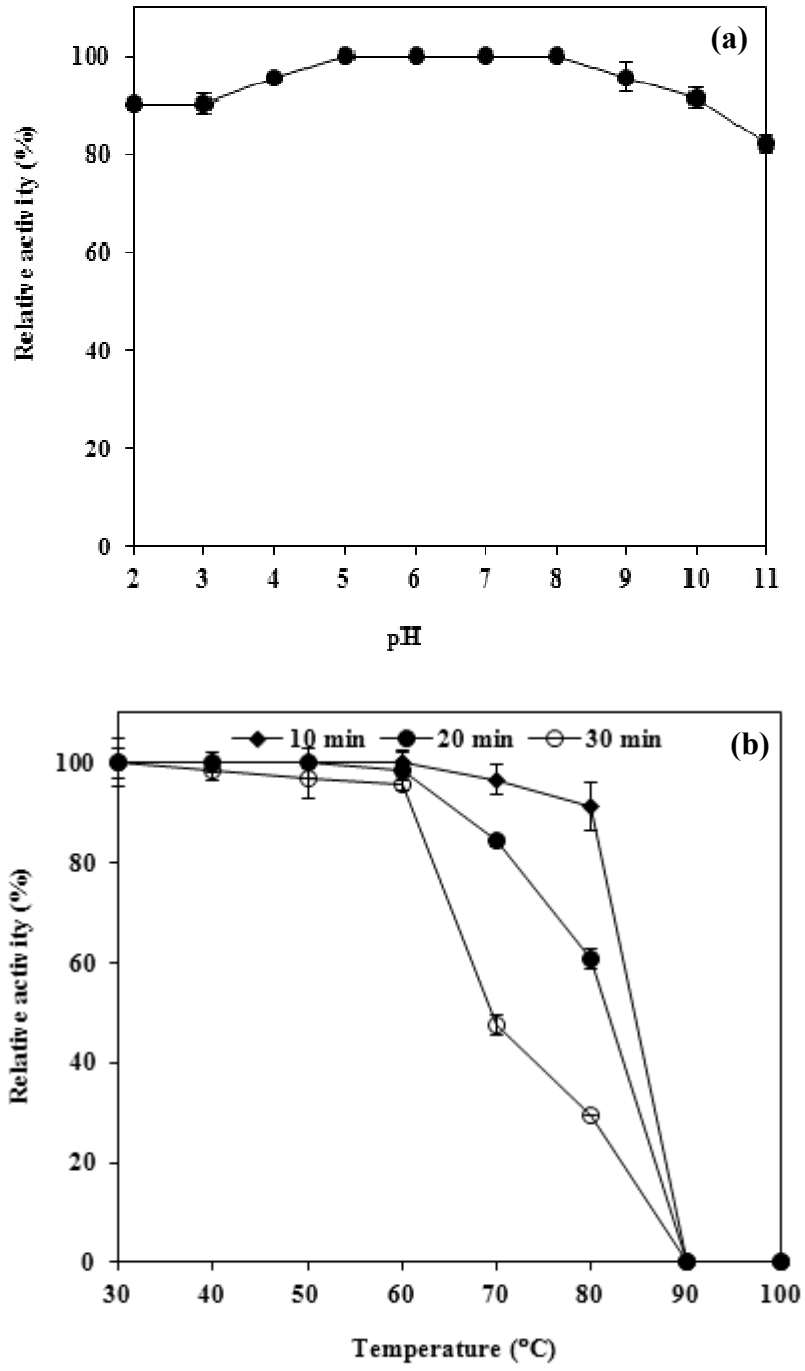

Figure 2. pH (a) and thermal (b) stability of trypsin inhibitor from yellowfin tuna roe. Bar indicate standard deviation from triplicate determination.

Table 4. Effect of metal ions on the recovery of trypsin inhibitor from yellowfin tuna roes

Metal ions	Relative activity (%)
Control	100d
K^+	75.85±0.18b
Na^+	87.68±3.84c
Mg^{2+}	67.95±2.24a
Ca^{2+}	85.99±1.34c

*Mean±SD from triplicate determination
The different letters in the same column denote the significant differences ($P<0.05$).

Conclusion

Trypsin inhibitor from yellowfin tuna roe was successfully extracted by 10 mM Na-phosphate buffer (pH 7.0) containing 0.5 M NaCl. It was stable at various pHs, heat treatment, and was also stable at high salt concentration up to 3%. Based on, the properties, the trypsin inhibitor from yellowfin tuna roe can be used as an alternative additive from improving the quality of surimi gels, especially those suffering, caused by trypsin or trypsin-like serine proteases.

Acknowledgments

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CHAPTER 2

PURIFICATION AND CHARACTERIZATION OF TRYPSIN INHIBITOR FROM YELLOWFIN TUNA (*THUNNUS ALBACORES*) ROE

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(SUBMITTED)

PURIFICATION AND CHARACTERIZATION OF TRYPSIN INHIBITOR FROM YELLOWFIN TUNA (*THUNNUS ALBACORES*) ROE

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ABSTRACT

Trypsin inhibitor was purified to homogeneity from the roe of yellowfin tuna (*Thunnus albacores*) by heat-treatment at 60°C for 10 min, followed by column chromatographies on Sephacryl S-200, Sephadex G-50 and DEAE-cellulose. The trypsin inhibitor was purified 11.29-fold with a yield of 46.02%. Yellowfin tuna trypsin inhibitor migrated as a single band using native-PAGE. Purified trypsin inhibitor had an apparent molecular weight of 70 kDa when analyzed using SDS-PAGE and size exclusion chromatography. No inhibitory activity was obtained under reducing condition (βME). Maximal activity was recorded at pH 7.0 and 50°C. The purified inhibitor was stable in temperature ranges from 20 to 60°C for 10 min and in the pH range of 5 to 8. NaCl concentration up to 3% did not significantly affect the inhibitory activity of purified trypsin inhibitor. However, the activity decreased when trypsin inhibitor was incubated with metal ions (Cu^{+}, Na^{+}, Mg^{2+} and Ca^{2+}) at ambient temperature for 30 min.

KEYWORDS: trypsin inhibitor; proteinase; isolation; tuna; roe

PRACTICAL APPLICATIONS

Trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe, the byproducts of tuna processing, can be purified. The yellowfin tuna trypsin inhibitor is a salt stable peptide and could be useful for food applications, especially surimi.

INTRODUCTION

The tuna processing industry has been economically important for Thailand as the essential income generator. In terms of volume, Thailand is the world's largest canned tuna producer and exporter. In 2014, tuna products with total amount of 240,121 tons and value of 29,372 million baht were exported (Department of Foreign Trade, 2015). Among tuna, yellowfin, skipjack and tongol are important for canning in Thailand. During the butchering process, large amount of roe are collected from females in spawning period and generally used as animal feed with low market value. Fish roe, accounting for 1.5-3.0% of total weight, has biotechnological potential as a source of proteinase inhibitor, especially trypsin inhibitor (Klomklao et al. 2014; Choi et al. 2002). The isolation and recovery of trypsin inhibitor from tuna roe can increase its value and reduce waste disposal or treatment, thereby lowering environmental pollution. Hence, tuna roe can serve as a cheap and promising source of proteinase inhibitor for further applications.

An enzyme inhibitor is any substance that reduces the rate of enzyme-catalyzed reaction (Whitaker, 1994). Proteinase inhibitors are agents that block the activity of proteolytic enzymes that break down proteins. (Klomklao et al., 2014). Proteinase inhibitors are commonly accumulated in high quantities in plant seeds, bird eggs and various body fluids. Proteinase inhibitors are also found in mammalian and marine blood plasma and fish roe and viscera (Choi et al., 2002). Proteinase inhibitors can be versatile tools in the fields of medicine, agriculture and food technology (Choi et al., 2002; Sabotic and Kos, 2012; Klomklao et al., 2014). In medicine, proteinase inhibitors can be used as diagnostic or therapeutic

agents for viral, bacterial, fungal and parasitic diseases as well as for treating cancer and immunological, neurodegenerative and cardiovascular diseases (Sabotic and Kos, 2012). They can be involved in crop protection against plant pathogens and herbivorous pests as well as against abiotic stress such as drought (Sabotic and Kos, 2012). For food technology, proteinase inhibitor can be used to improve texture in various foods, for example, sausages, meat balls, and low-salt fish products (Benjakul et al., 2000; Klomklao et al., 2015). Proteinase inhibitors have been isolated and characterized from the hepatopancreas of squid (*Todarodes pacificus*) (Kishimura et al. 2001) and the skin mucus of pufferfish (*Takifugu pardalis*) (Nagashima et al. 2004). Choi et al. (2002) also purified and characterized trypsin inhibitor from the egg of skipjack tuna *(Katsuwonus pelamis*). Recently, yellowfin tuna roe was reported to possess high trypsin inhibitory activity (Klomklao et al. 2014). However, molecular and biochemical properties of trypsin inhibitor in yellowfin tuna roe, have not been studied. The objectives of this study were to purify and characterize trypsin inhibitor from the roe of yellowfin tuna.

MATERIALS AND METHODS

Chemicals

*N*α-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), porcine pancreas trypsin, β-mercaptoethanol (βME), sodium chloride, tris (hydroxymethyl) aminomethane, dimethylsulfoxide, sodium caseinate, ammonium sulfate, sodium dodecyl sulfate (SDS), Coomassie Blue R-250, *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and bovine serum albumin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Sephacryl S-200 and Sephadex G-50 was obtained from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was obtained from Whatman (Maidstone, England).

Fish sample preparation

Roes of yellowfin tuna (*Thunnus albacores*), with a size of 51.8±2.4 g/roe, were obtained from Tropical Canning (Thailand) Public Co. Ltd., Songkhla, Thailand. These samples (5 kg) were placed in ice using a roe:ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology, Thaksin University, Phatthalung, within 2 h. The whole roes were cleaned using cold water (4°C) and immediately frozen and stored at -20°C until used.

Preparation of roe extract

Frozen roes 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 homogenized with three volumes of acetone at -20°C for 30 min according to the method of Klomklao et al. (2010a). The homogenate was filtered in vacuo on Whatman No. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20°C for 30 min, and the residue was air-dried at room temperature.

To prepare the extract, roe powder was mixed with 10 mM Na-phosphate buffer (pH 7.0) containing 0.5 M NaCl at a ratio of 1:9 (w/v) and shaken (BW 201 Shaking bath, Tokyo, Japan) for 30 min at 150 rpm at room temperature. The roe extract was recovered by centrifuging at 10,000×g for 30 min at 4°C.

Purification of yellowfin tuna roe trypsin inhibitor

Roe extract was heated at different temperatures (50-100°C) for 10 min and then cooled with ice water. To remove the heat coagulated debris, the extracts were centrifuged at 10,000×g for 10 min at 4°C. The activity and specific activity of trypsin inhibitor in the supernatant obtained were measured. The heat treatment which gave a supernatant with highest specific activity was chosen for further study.

The heat-treated extract was subsequently chromatographed on a Sephacryl S-200 column (3.9×64.0 cm), which was equilibrated with approximately two bed volumes of 10 mM Tris-HCl buffer (pH 7.0). The sample was loaded onto the column and eluted with the same buffer at a flow rate of 0.5 ml/min at 4°C. Fractions of 5 ml were collected and the main trypsin inhibitor

fractions were pooled and further purified by Sephadex G-50. Absorbance at 280 nm (A_{280}) of each fraction was also measured.

Pooled fractions with trypsin inhibitory activity from the Sephacryl S-200 column were concentrated by lyophilization and dissolved in distilled water prior to size exclusion chromatography. The sample was applied to a Sephadex G-50 column (3.9×64.0 cm) previously equilibrated with approximately two bed volumes of 10 mM Tris-HCl buffer (pH 7.0). The sample was loaded onto the column at 4°C and then eluted with the same buffer at a flow rate of 0.1 ml/min. Fractions of 5 ml were collected and subjected to A_{280} measurement. The fractions with trypsin inhibitory activity were pooled and dialyzed against 10 mM Tris-HCl buffer (pH 7.0) for 24 h at 4°C.

After Sephadex G-50 chromatography, the sample was then chromatographed on a DEAE-cellulose (Whatman, England) column (2.2×18.0 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0). The sample was loaded onto the column at a flow rate of 0.5 ml/min at 4°C and the column was washed with 10 mM Tris-HCl buffer (pH 7.0) until A_{280} was less than 0.05. Elution was performed with a linear gradient of 0-0.25 M NaCl in 10 mM Tris-HCl buffer (pH 7.0) at a flow rate of 0.5 ml/min. Fractions of 5 ml were collected and A_{280} was measured. The fractions with trypsin inhibitory activity were pooled and used for further studies.

Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured by the method of Benjakul et al. (2000) with a slight modification using BAPNA as substrate. A solution

containing 200 µl of inhibitor solution and 200 µl (40µg/ml) porcine pancreas trypsin was preincubated at 37°C for 15 min. 1.0 ml of the mixtures containing 800 µl of 0.5 mM BAPNA and 200 µl of distilled water (prewarmed to 37°C) were added and vortexed immediately to start the reaction. After incubating for 10 min, 900 µl of 30% acetic acid (v/v) was added to terminate the reaction. The reaction mixture was centrifuged at 8,000×g for 5 min (MIKRO200, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Residual activity of trypsin was measured by the absorbance at 410 nm (UV-16001, SHIMADZU, Japan) due to p-nitroaniline released. One unit of proteolytic activity was defined as an increase of 0.01 absorbance unit/ml.min under the assay condition. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduces the enzyme activity by one unit.

Effect of temperature on trypsin inhibitor activity and stability

Optimum temperature for maximal activity of trypsin inhibitor was determined by assaying at different incubation temperatures (30-100°C) and expressed as the relative inhibitory activity.

For thermal stability, trypsin inhibitor extracts from yellowfin tuna roe were incubated at 30-100°C for 10-30 min and then cooled in iced-water. The residual trypsin inhibitory activity was determined and reported as the relative activity compared to the original activity.

Effect of pH on trypsin inhibitor activity and stability

Optimum pH for activity of the trypsin inhibitor was determined by performing protease inhibitor assay at different pH ranging from 2 to 11. Different buffers were used for different pH conditions: 0.2 MMcIlvaine buffers (0.2 M Na phosphate and 0.1 M Na citrate) for pHs 2.0-7.0 and 0.1 M glycine-NaOH for pHs 8.0-11.0.

For pH stability, purified inhibitor solution was incubated at various pHs (2.0-11.0) for 30 min at room temperature. Residual activity was then determined. Different buffers were used including 0.2 M McIlvaine buffers (0.2 M Na phosphate and 0.1 M Na citrate) for pHs 2.0-7.0 and 0.1 M glycine-NaOH for pHs 8.0-11.0.

Effect of NaCl on trypsin inhibitor activity

Trypsin inhibitor was incubated at room temperature for 30 min in the presence of NaCl ranging from 0 to 3%. The mixture was tested for inhibitory activity against trypsin.

Effect of metal ions on trypsin inhibitor activity

Different metal ions (K^+, Na^+, Mg^{2+} and Ca^{2+}) were added to the trypsin inhibitor to obtain a final concentration of 10 mM. The trypsin inhibitory activity was assayed after incubation for 30 min at room temperature and the residual inhibitory activity reported.

Polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with SDS-PAGE sample buffer in the presence or absence of βME and boiled for 3 min. The sample (20 μg) was loaded on the gel made of 4% stacking and 15% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II cell apparatus (Atto Co., Tokyo, Japan). 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.

Native-PAGE was performed using 15% separating gels in a similar manner, except that the sample was not heated and the addition of SDS and reducing agent were omitted.

Inhibitory activity of trypsin inhibitor by electrophoresis

DEAE-cellulose fraction was separated on SDS-PAGE, followed by inhibitory activity staining using casein as a substrate with the slightly modified method of Garcia-Carreno et al. (1993) and Klomklao et al. (2010a). The gels were washed in 2.5% Triton X-100 for 15 min to remove SDS and renature the proteins. The gel was washed with distilled water before soaking in trypsin solution (0.2 mg/ml) at 0-4°C for 45 min. The gels were then washed again with distilled water and incubated with 1% casein in 0.1 M glycine-NaOH, pH 9.0 for 90 min at 37°C. The gel was washed again with distilled water, fixed and stained with Coomassie blue R-250. After destaining, the bands with inhibitory activity were compared to the control gel and molecular weight markers.

Size exclusion chromatography

The molecular weight of purified trypsin inhibitor was determined using size exclusion chromatography on a Sephacryl S-200 column. The trypsin inhibitor separated on size exclusion chromatography was estimated for its molecular weight by plotting available partition coefficient (K_{av}) against the logarithm of molecular weight of the protein standards. The elution volume (V_e) was measured for each protein standard and the trypsin inhibitor. Void volume (V_o) was estimated by the elution volume of blue dextran (M_r 2,000,000). The standards used included aprotinin (M_r 6,500), trypsinogen (M_r 24,000), bovine serum albumin (M_r 66,000) and catalase (M_r 232,000).

Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical analysis

A completely randomized design was used throughout this study. All data were subjected to analysis of variance (ANOVA) and the differences between means were carried out using Duncan's Multiple Range Test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows; SPSS Inc.).

RESULTS AND DISCUSSION

Purification of trypsin inhibitor from yellowfin tuna roe

The yellowfin tuna roe extracts were subjected to heat treatment at different temperatures. The trypsin inhibitory activities of yellowfin tuna roe extract were quite constant up to 60°C. A marked decrease in activity was obtained at 80°C (Table 1). Specific trypsin inhibitory activity was increased markedly when the heating temperature increased up to 60°C (Table 1) but decreased at 70°C. Therefore, heat treatment at 60°C for 10 min was introduced to the purification process to rapidly remove undesired proteins. The heat treatment of extract from yellowfin tuna roe rendered the purification at 2.62-fold (Table 2). Some heat labile proteins were removed, resulting in higher specific inhibitory activity. The yield was increased to 118.86% from this step. Heat treatment might induce conformational change of trypsin inhibitor by destroying the noncovalent bonds (Benjakul et al., 2000). The modified configuration of trypsin inhibitor might favour interaction between trypsin inhibitor and trypsin (Benjakul et al. 2000). Klomklao et al. (2011) had reported that heat-treatment of mung bean extract at 90°C for 10 min resulted in increased specific trypsin inhibitory activity by 6.46-fold.

Heat-treated extract was further purified by gel filtration on Sephadex S-200 to obtain a single peak. Purification of 5.09-fold with a yield of 86.80% was observed (Table 2). Gel filtration had been used to remove other proteins in adzuki bean seed trypsin inhibitor fraction, leading to higher purity of trypsin inhibitor (Klomklao et al. 2010a).

To refine the pooled fraction obtained from previous step, pooled active fractions were subjected to a Sephadex G-50 column. Purification of 5.45-fold with a yield of 64.71 was obtained. Choi et al. (2002) also found that the use of Sephadex G-100 for purification of trypsin inhibitor from skipjack tuna egg led to an increase in activity by 10.96-fold.

Pooled active Sephadex G-50 fractions were further purified using a DEAE-cellulose column. After this step, only one activity peak was found and a large amount of contaminated protein was removed, resulting in a substantial increase in purification fold. Purity was increased by 11.29-fold with yields of 46.02%. Prasad et al. (2010) also chromatographed the trypsin inhibitor from black gram seeds on an anion exchanger, DEAE-cellulose, resulting in 3.22- fold purity.

TABLE 1. EFFECT OF HEAT TREATMENT AT DIFFERENT TEMPERATURES ON PURIFICATION OF TRYPSIN INHIBITORS FROM YELLOWFIN TUNA ROE.

Temperature (°C)	Total activity* (Units)	Total protein* (mg)	Specific inhibitory activity* (Units/mg protein)
50	130.02±4.85d	133.52±3.23e	0.97±0.04c
60	131.28±6.29d	110.93±2.51d	1.18±0.06e
70	109.70±4.71c	106.20±20.25cd	1.03±0.04d
80	74.02±7.21b	95.15±3.23bc	0.77±0.06b
90	0a	90.88±2.14b	0a
100	0a	41.96±0.36a	0a

*Mean±SD from triplicate determination
The different letters in the same column denote the significant differences ($P<0.05$).

TABLE 2.PURIFICATION OF TRYPSIN INHIBITORS FROM YELLOWFIN TUNA ROE.

Purification steps	Total activity (Units)	Total protein (mg)	Specific inhibitory activity (Units/mg protein)	Purity (fold)	Yield (%)
Crude Extract	110.45	245.33	0.45	1	100
Heat treatment	131.28	110.93	1.18	2.62	118.86
Sephacryl S-200	95.87	41.95	2.29	5.09	86.80
Sephadex G-50	71.47	29.24	2.45	5.45	64.71
DEAE-cellulose	50.83	10.01	5.08	11.29	46.02

Protein pattern and activity staining of trypsin inhibitors from yellowfin tuna roe

Purity of the purified trypsin inhibitor was examined via native gel electrophoresis. The inhibitor migrated as a single band on native- PAGE (Fig. 1a), suggesting homogeneity of the inhibitor.

Based on the protein patterns under non-reducing condition, purified trypsin inhibitor was shown to be a70 kDa protein(Fig. 1b) similar to that estimated by gel chromatography on a Sephacryl S-200 column (Fig. 2). Under reducing conditions, the protein band with molecular mass of 70 kDa disappeared with the concomitant occurrence of proteins with molecular mass of 40 kDa and 30 kDa (Fig. 1b). The results suggested that purified trypsin inhibitor from yellowfin tuna roe consisted of subunits stabilized by disulfide bond. Choi et al. (2002) reported that the molecular mass of the purified trypsin inhibitor was 78 kDa as estimated by gel filtration and 39 kDa by SDS-PAGE under reducing conditions.

Activity staining of trypsin inhibitor (Fig. 1c) revealed that the protein with apparent molecular size of 70kDa inhibited trypsin under nonreducing conditions. It was concluded that this protein band was trypsin inhibitor. After reduction with β-mercaptoethanol (βME), no activity band was found (Fig 1c). It is postulated that the cleavage of disulfide bond by the action of βME may have led to protein denaturation and loss of functionality. Klomklao et al. (2010a) also reported that trypsin inhibitor purified from adzuki bean seeds was not observed under reducing conditions. Therefore, the intact structure in the native

conformation of inhibitor in yellowfin tuna roe was prerequisite for inhibitory action.

Effect of temperature on the activity and stability of trypsin inhibitor

The effect of various temperatures on trypsin inhibitory activity is depicted in Fig. 3a. The highest inhibitory activity was found at 50°C. Above 50°C, the relative inhibitory activity decreased and reached the lowest point at 100°C. After temperatures above the optimum point, the structure of the enzyme or inhibitor molecules is disrupted resulting in decreased activity (Wati et al., 2009).

The trypsin inhibitor was stable when incubated at temperatures up to 60°C for 10-30 min with residual activity higher than 90% (Fig. 3b). However, a sharp decrease in activity was observed at temperatures above 60°C. No activity remained at 90 and 100°C, suggesting complete loss of activity caused by thermal denaturation. Generally, the stability of trypsin inhibitor decreased with increasing heating time. A heating time of 30 min caused the highest loss of activity at every temperature above 50°C (Fig. 3b). Choi et al. (2002) also found that the trypsin inhibitor from skipjack tuna egg was stable at temperatures below 40°C. From the results, purified trypsin inhibitor from yellowfin tuna roe was stable up to 30 min at 60°C and makes it potentially useful in thermal processes such as gelation of surimi.

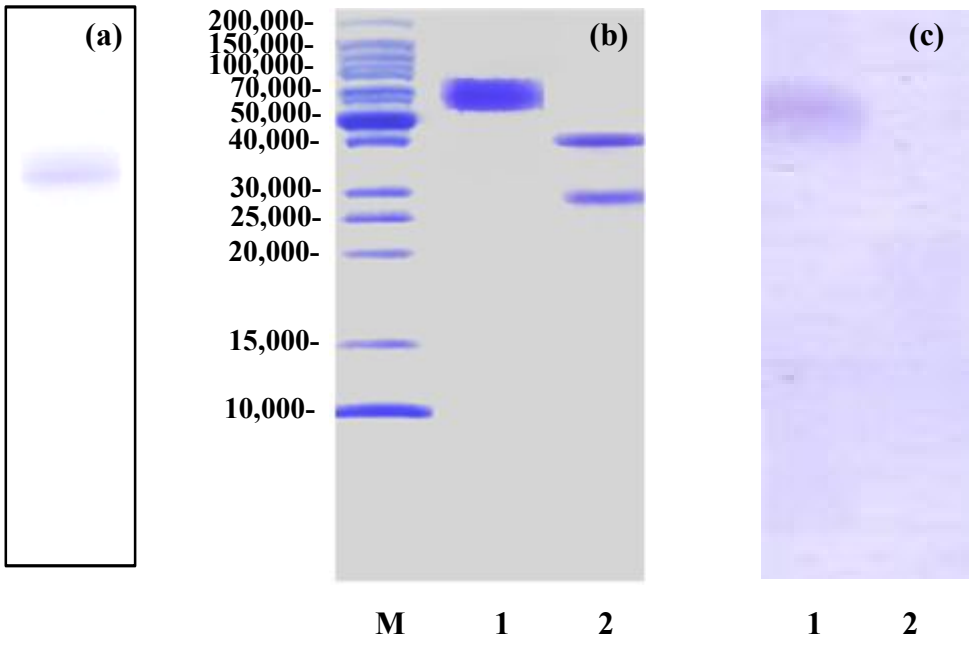

FIG.1. NATIVE-PAGE (A), SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (B) AND INHIBITORY ACTIVITY STAINING FOR TRYPSIN (C) OF PURIFIED TRYPSIN INHIBITOR FROM YELLOWFIN TUNA ROE. M, molecular weight standard; 1: trypsin inhibitor (without reducing agent); 2: trypsin inhibitor (with reducing agent).

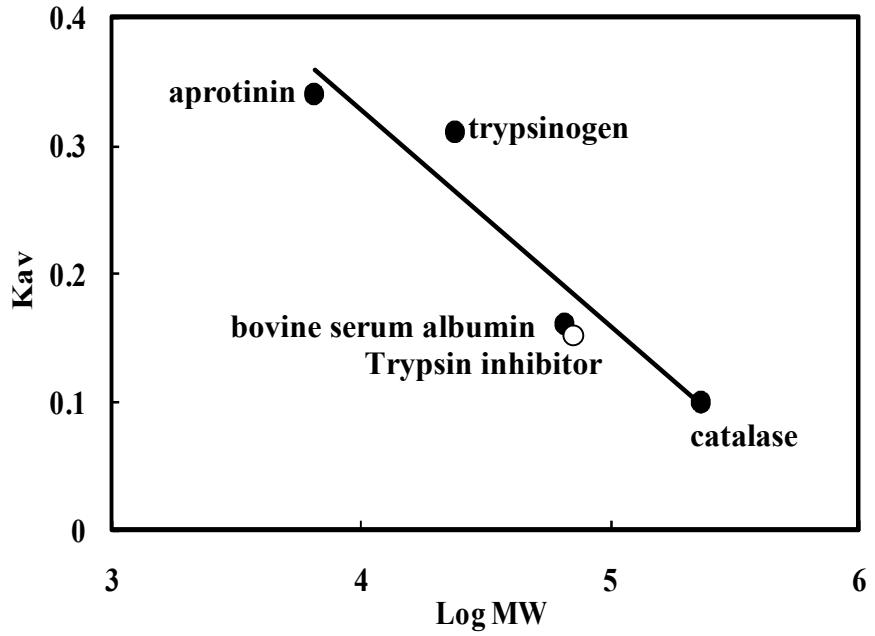

FIG. 2. CALIBRATION CURVE FOR THE MOLECULAR WEIGHT DETERMINATION OF THE PURIFIED TRYPSIN INHIBITOR ON SEPHACRYL S-200 CHROMATOGRAPHY.

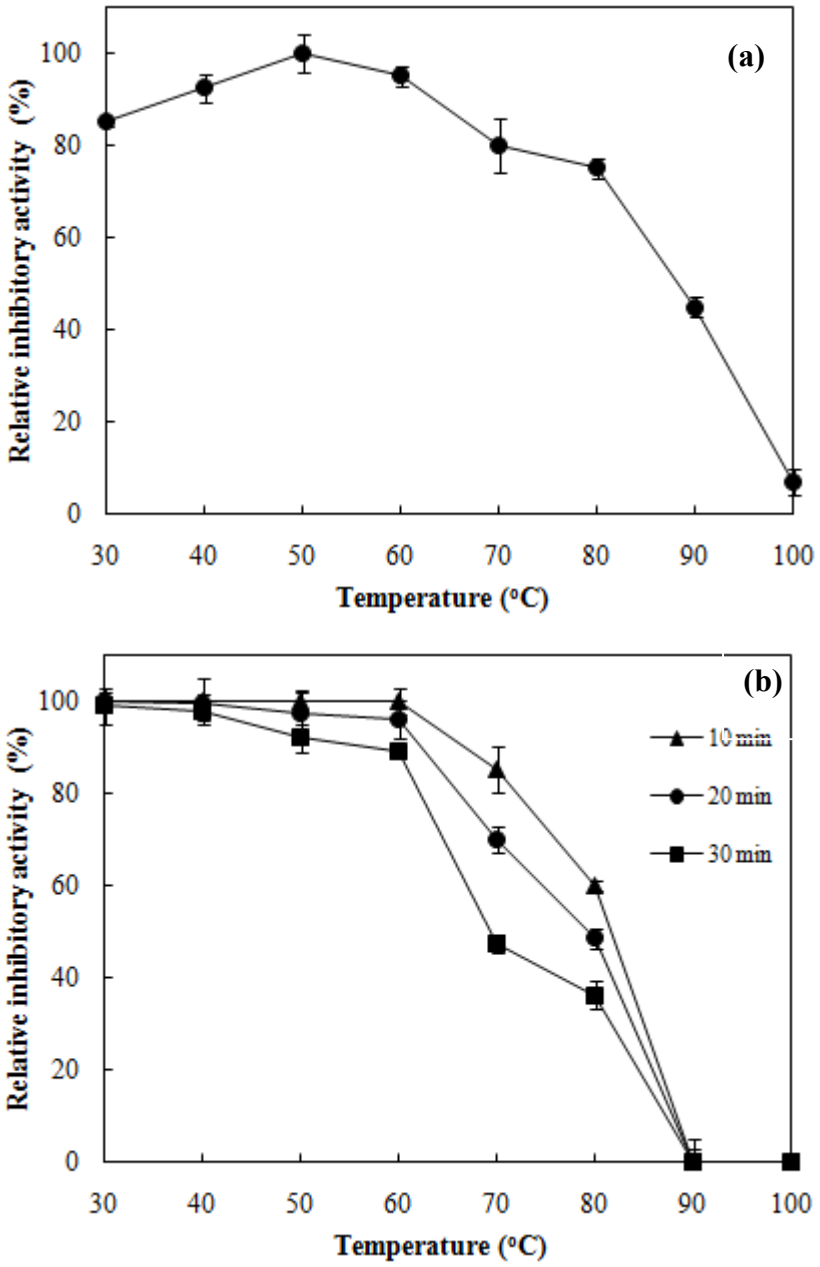

FIG.3. TEMPERATURE PROFILE (A) AND THERMAL STABILITY (B) OF PURIFIED TRYPSIN INHIBITOR FROM YELLOWFIN TUNA ROE. Error bars indicate standard deviations from triplicate determination.

Effect of pH on the activity and stability of trypsin inhibitor

The purified trypsin inhibitor was incubated at pHs 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 and the highest inhibitory activity was observed at pH 7.0. However, decreased relative inhibitory activity of trypsin inhibitor from yellowfin tuna roe was observed at strong acidic and alkaline conditions (Fig. 4a).

pH stability of trypsin inhibitor purified from yellowfin tuna roe was investigated (Fig. 4b). The purified inhibitor was stable over a broad pH range (pH 5-8). However, there was some decrease in activity at low and high pHs. Therefore, the inhibitor was generally stable in the neutral pH ranges. At extreme pH values, however, strong intramolecular electrostatic repulsion caused by high net charge results in swelling and unfolding of the protein molecules which is manifested as loss of inhibitory activity (Damodaran, 1996; Cheftel et al. 1985). Choi et al. (2002) found that the trypsin inhibitor from skipjack tuna egg was stable in the pH range from 4.0 to 10.0.

Effect of salt on the stability of purified trypsin inhibitor

No marked changes in relative inhibitory activity were found when NaCl was added up to 3.0% ($p>0.05$) (Fig. 5) indicating high salt stability. Therefore, purified inhibitor from the roe of yellowfin tuna can be useful in surimi-based products in which 2-2.5% salt is commonly used without severe loss of inhibitory activity.

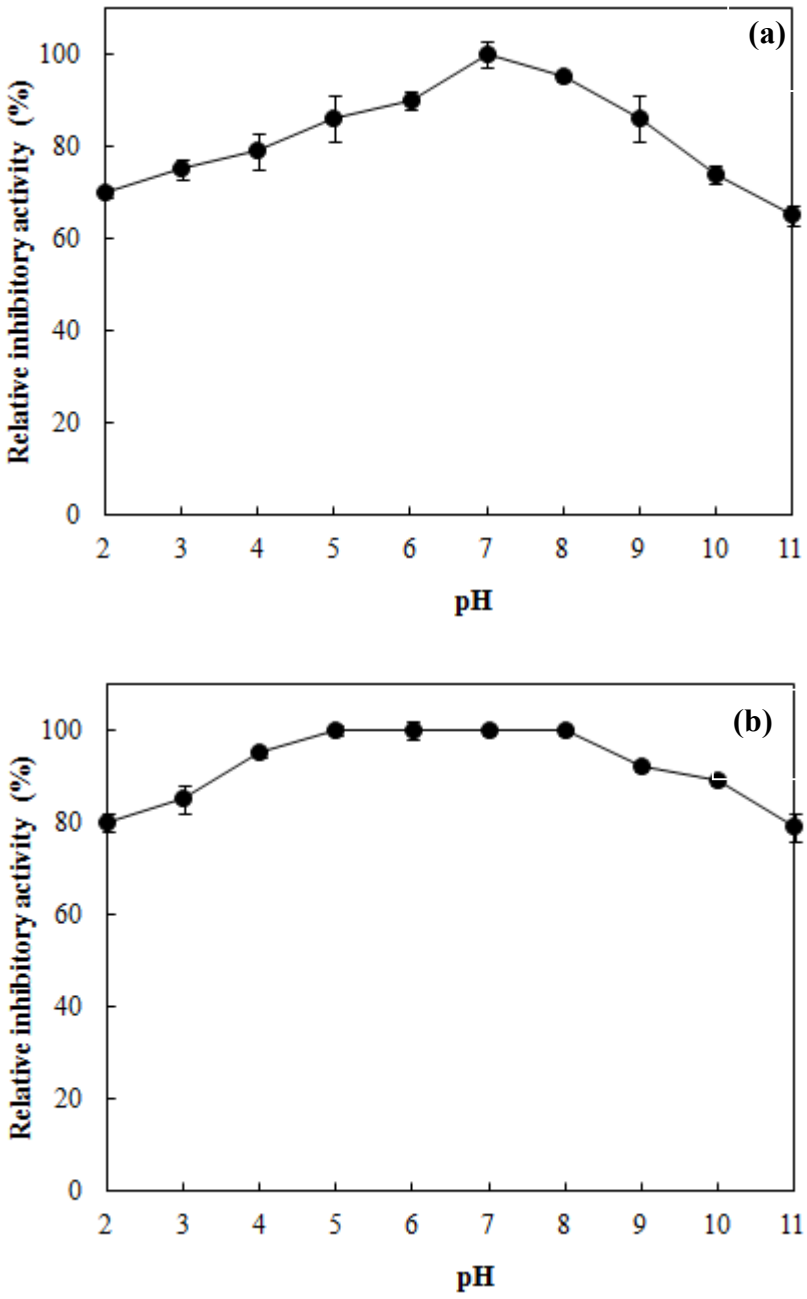

FIG. 4. pH PROFILE (A) AND pH STABILITY (B) OF PURIFIED TRYPSIN INHIBITOR FROM YELLOWFIN TUNA ROE. Error bars indicate standard deviations from triplicate determination.

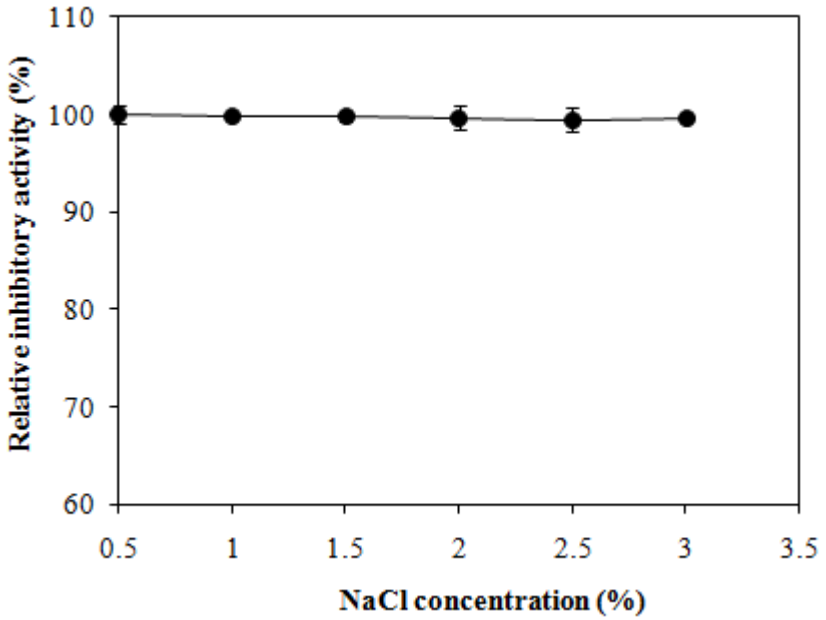

FIG. 5. EFFECT OF SALT CONCENTRATION ON THE STABILITY OF PURIFIED TRYPSIN INHIBITOR FROM YELLOWFIN TUNA ROE. Error bars indicate standard deviations from triplicate determination.

Effect of metal ions on the activity of purified trypsin inhibitor

The metal ions studied, including K^+, Na^+, Mg^{2+} and Ca^{2+} had a negative effect on the trypsin inhibitory activity with 14-32% loss of activity (Table 3). It was presumed that metal ions bound by inhibitors induced structural modification that changed their conformational stability required for biological activity (Bijina et al. 2001). However, Choi et al. (2002) reported that the activity of the trypsin inhibitor from skipjack tuna egg was increased in presence of same metal ions.

TABLE 3. EFFECT OF METAL IONS ON THE ACTIVITY OF TRYPSIN INHIBITOR FROM YELLOWFIN TUNA ROES

Metal ions	Relative inhibitory activity (%)*
Control	100d
K^+	75.85±0.18b
Na^+	87.68±3.84c
Mg^{2+}	67.95±2.24a
Ca^{2+}	85.99±1.34c

*Mean±SD from triplicate determination
The different letters in the same column denote the significant differences ($P<0.05$).

CONCLUSION

Yellowfin tuna roe has been recognized as a source of lipid and protein, but so far it had not been reported as a source of trypsin inhibitor. In this study, the potential of yellowfin tuna roe as a source of trypsin inhibitor is indicated. The purified trypsin inhibitor from yellowfin tuna roe had a molecular mass of 70 kDa and was stable at high salt concentration up to 3%. Thus, the purified trypsin inhibitor from yellowfin tuna roe can be used as an alternative additive for improving the quality of surimi gels, especially the rapid softening caused by trypsin or trypsin-like serine proteases.

ACKNOWLEDGMENTS

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CHAPTER 3

INHIBITION OF BIGEYE SNAPPER (*PRIACANTHUS MACRACANTHUS*) PROTEINASES BY TRYPSIN INHIBITOR FROM YELLOWFIN TUNA (*THUNNUS ALBACORES*) ROE

JOURNAL OF FOOD BIOCHEMISTRY

(ACCEPTED)

INHIBITION OF BIGEYE SNAPPER (*PRIACANTHUS MACRACANTHUS*) PROTEINASES BY TRYPSIN INHIBITOR FROM YELLOWFIN TUNA (*THUNNUS ALBACORES*) ROE

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ABSTRACT

The inhibitory effect of partially purified trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe (TIYTR) on proteolysis by bigeye snapper (*Priacanthus macracanthus*) proteinases was investigated. TIYTR inhibited sarcoplasmic proteinases and autolysis of bigeye snapper mince and its washed mince at 60°C in a concentration dependent manner. Myosin heavy chain (MHC) in the mince and the washed mince of bigeye snapper was better retained when higher concentrations of TIYTR were used. The presence of NaCl (3.0% w/w) enhanced the inhibitory activity of TIYTR slightly (3.5-5.8%). Both TIYTR and beef plasma protein (BPP) (at a level of incorporation of 3%, w/w) showed higher inhibition of bigeye snapper proteinases than egg white (EW) ($P<0.05$). Based on inhibition studies, it is suggested that the trypsin inhibitor from the roe of yellowfin tuna can be a potential aid to suppress the gel weakening of bigeye snapper surimi, elicited by trypsin-like proteinases, during either setting or heating.

KEYWORDS: trypsin inhibitor; proteinase; proteolysis; bigeye snapper; tuna roe

PRACTICAL APPLICATIONS

Yellowfin tuna (*Thunnus albacores*) roe (TIYTR) is an abundant and underutilized resource that can be used as a source of trypsin inhibitors. Trypsin inhibitors from the roe of yellowfin tuna play a role in the inhibition of proteolysis in bigeye snapper (*Priacanthus macracanthus*) muscle. Thus, it can be a potential

alternative for commercial trypsin inhibitor to inhibit proteolysis and enhance surimi gel strength.

INTRODUCTION

Protein hydrolysis of fish muscle during post mortem storage and processing has been attributed to endogenous muscle proteinases, and can result in undesirable flavor and texture changes. Muscle proteinases are found in the sarcoplasmic fraction of cellular organelles of muscle tissues, connective tissues and myofibrils (Benjakul and Visessanguan 2000). A number of endogenous proteases have been studied for their relative contributions to post mortem softening of fish flesh and have been found to contribute to myofibrillar proteins degradation to varying degrees (An *et al.* 1996). Among the endogenous proteinases, lysosomal cathepsins are found to play the most influential role in tissue softening of most fish during post mortem storage. However, only cathepsin L, and alkaline proteinases (i.e., trypsin-like and cysteine protease-like; Busconi *et al.* 1984) have been reported as active proteinases involved in the softening phenomenon observed at the elevated temperatures normally employed during surimi processing (Yongsawatdigul and Piyadhammaviboon 2004).

Bigeye snapper is one of the most important species for surimi production in the Southeast Asian region due to its white color and large availability. Bigeye snapper caught in Thailand normally includes two species, *Priacanthus tayenus* and *P. macracanthus*. *P. macracanthus* has a much poorer gel quality compared to *P. tayenus*. Benjakul *et al.* (2003a) reported that mince and washed mince from *P. macracanthus* were degraded at 50°C and 60°C to a higher extent, compared to

their counterparts from *P. tayenus*. Heat-stable alkaline proteinases, especially trypsin-like serine proteinase, have been implicated in gel weakening of bigeye snapper (*P. macracanthus*) surimi (Benjakul *et al.* 2003b). Therefore, various food-grade enzyme inhibitors such as egg white powder, beef plasma powder and whey protein concentrate have been used to prevent texture degradation in surimi gels (Rawdkuen and Benjakul 2008; Benjakul *et al.* 2004). However, these inhibitors are expensive, thus, discovering alternative and cheaper sources of food grade inhibitors to control the endogenous proteinases that adversely impact fish texture would be very useful for the formulation of these surimi-type products.

Tuna roe is an abundant by-product from the commercial processing of canned tuna. Thus far, a large amount of roe are commonly collected and used as animal feed which has low market value. Fish roe, accounting for 1.5-3.0% of total weight, have potential for a range of biotechnological applications such as a source of proteinase inhibitor, especially trypsin inhibitor (Klomklao *et al.* 2014a). Recently, yellowfin tuna roe was shown to contain trypsin inhibitors which can reduce proteinase activity in fish extracts (Klomklao *et al.* 2014a; Klomklao and Benjakul, 2014b). However, the effect of the trypsin inhibitor from yellowfin tuna roe on proteolysis of bigeye snapper has not been studied. Thus, the objectives of the present study were to evaluate the inhibitory effects of trypsin inhibitor from yellowfin tuna roe on proteolysis in bigeye snapper.

MATERIALS AND METHODS

Chemicals

*N*α-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), porcine pancreas trypsin, β-mercaptoethanol (βME), sodium chloride, trichloroacetic acid, tris (hydroxymethyl) aminomethane, dimethylsulfoxide, sodium caseinate, ammonium sulfate, sodium dodecyl sulfate (SDS), Coomassie Blue R-250, *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and bovine serum albumin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Beef plasma protein (BPP) and egg white (EW) powder were obtained from Food EQ Co., Ltd (Bangkok, Thailand).

Preparation of partially purified trypsin inhibitor from yellowfin tuna roe (TIYTR)

The roe of yellowfin tuna (*Thunnus albacores*), with a size of 51.8±2.4 g/roe, were obtained from Tropical Canning (Thailand) Public Co. Ltd., Songkhla, Thailand. The samples (5 kg) were placed in ice using a roe:ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology at the Thaksin University (Phatthalung, Thailand) within 2 h of roe removal. The whole roe were cleaned using cold water (4°C), vacuum-packed and immediately frozen at -20°C until needed for use.

Frozen roe were thawed using running tap water (26-28°C) until the core temperature reaches -2 to 0°C. The samples were cut into pieces with thickness ranging from 1-1.5 cm and homogenized with three volumes of cold acetone at -

20°C for 30 min according to the method of Klomklao *et al.* (2010). The homogenate was filtered under vacuum through Whatman No. 4 filter paper. The residue obtained was then homogenized with two volumes of cold acetone (-20°C) for 30 min, and then the residue was collected by filtering under vacuum through Whatman No. 4 filter paper. The defatted roe was air-dried at room temperature (28-30°C) until dry and free of acetone odor.

To prepare the extract, roe powder was mixed with 10 mM Na-phosphate buffer (pH 7.0) containing 0.5 M NaCl at a ratio of 1:9 (w/v) and shaken (BW 201 Shaking bath, Tokyo, Japan) for 30 min at 150 rpm at room temperature (28-30°C). The roe extract was recovered by centrifuging at 10,000×g for 30 min at 4°C.

Next, the roe extract was partially purified by heat-treatment at 60°C for 10 min (Klomklao and Benjakul, 2014b) and then cooled with ice water. This treatment produced coagulated debris which was subsequently removed by centrifugation at 10,000×g for 10 min at 4°C. The supernatant was freeze-dried and kept at 4°C until needed.

Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured as per the method of Klomklao *et al.* (2011) with a slight modification using BAPNA as substrate. A solution containing 200 µl of inhibitor solution and 200 µl of porcine pancreas trypsin (20µg/ml) was pre-incubated at 37°C for 15 min. To initiate the reaction, 1,000 µl of the mixtures containing 800 µl of 0.5 mM BAPNA and 200 µl of distilled water

(pre-warmed to 37°C) were added and vortexed immediately to start the reaction. After incubating for 10 min, 900 µl of 30% acetic acid (v/v) was added to terminate the reaction, and the reaction mixture was centrifuged at 8,000×g for 5 min at room temperature (28-30°C) (MIKRO200, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Residual activity of trypsin was measured by the absorbance at 410 nm due to p-nitroaniline released (Klomklao *et al.* 2011). One unit of proteolytic activity was defined as an increase of 0.01 absorbance unit/ml.min under the assay conditions. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduces the enzyme activity by one unit.

Bigeye snapper preparation

Bigeye snapper (*P. macracanthus*) were caught from the Songkhla Coast along the Gulf of Thailand, stored in ice and off-loaded approximately 24-36 h after capture. Fish were transported in ice to the Department of Food Science and Technology, Thaksin University, Phatthalung within 2 h. Fish were then filleted, vacuum-pakced and frozen at -20°C until used for sarcoplasmic fluid preparation. To prepare fish mince, the fish were filleted and then ground through a 4 mm plate.

Preparation of Sarcroplasmic proteinases (Sp)

Sarcoplasmic proteinases (Sp) were prepared by centrifuging 50 g of finely chopped fillets of bigeye snapper at 5,000xg for 30 min at 4°C using a Sorvall

Model RC-B Plus centrifuge (Newtown, CT, USA). The supernatant was used as Sp.

Washed mince preparation

Washed mince was prepared according to the method of Benjakul *et al.* (2001) with a slight modification. The comminuted flesh was homogenized with 5 volumes of 50 mM NaCl for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was centrifuged at 10,000×g for 10 min at 4°C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). The washing process was repeated twice. The final precipitate was designated as "washed mince".

TIYTR inhibition of bigeye snapper sarcoplasmic proteinases

Sp was mixed with the partially purified trypsin inhibitor to obtain the final concentration of 0.5, 1, 2 and 3% (w/v). Sp (100 µl) was incubated with the solution of trypsin inhibitor (100 µl) for 20 min at room temperature. The residual activity was determined using the casein-TCA-Lowry method as described by Klomklao *et al.* (2010) at 60°C for 30 min. The activity determined in the absence of the trypsin inhibitor was used as the control.

The commercially available protease inhibitors, namely, beef plasma protein (BPP) and dry egg white (EW) powder, were used in comparison with TIYTR. For this, the Sp was mixed with TIYTR and commercial inhibitors to obtain the final concentration of 3% (w/v). Residual activity was determined as

before using the casein-TCA-Lowry method at 60°C for 30 min (Klomklao *et al.* 2010). The percent inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(Ac-As)}{Ac} \times 100$$

Where As and Ac are the proteolytic activity of sample treated with protease inhibitor and that of the control, respectively. The control was conducted in the same manner except that deionized water was used instead of protease inhibitor.

Effect of TIYTR on autolytic activity

The inhibitory activity of TIYTR against autolysis of the mince and washed mince samples was measured according to the method of Morrissey *et al.* (1993). TIYTR at levels of 0, 0.5, 1, 2 and 3% (w/w) was added to 3 g of mince and washed mince. The mixture was then incubated in a water bath at 60°C for 1 h and then the reaction was stopped by adding 27 ml of 5% cold TCA solution. TCA-soluble peptides released were analyzed using the Lowry assay (Lowry *et al.* 1951). Inhibition of autolysis was expressed as percent of autolytic activity inhibited, compared to that in the control (without trypsin inhibitor addition). % Inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{tyrosine released (without TIYTR)} - \text{tyrosine released (with TIYTR)}}{\text{tyrosine released (without TIYTR)}} \times 100$$

To monitor the protein pattern, another lot of sample was added with hot 5% SDS solution (85°C) to terminate the reaction and solubilize total protein. All samples were subjected to SDS-PAGE (Laemmli 1970).

BPP and EW powder were used in comparison with TIYTR. The mince or washed mince (3 g) samples were mixed with BPP or EW or TIYTR at level of 3% (w/w). Mince and washed mince added with and without protein additives were incubated for 60 min at 60°C. The reaction was terminated by addition of 27 ml of cold 5% trichloroacetic acid (w/v). The soluble peptide released were measured using the Lowry method (Lowry *et al.* 1951).

Effect of NaCl on inhibition of autolysis in bigeye snapper by TIYTR

TIYTR at levels of 3% (w/w) was added to 3 g of the mince and washed mince samples in the presence and absence of 0.5-3% NaCl. The mixture was then incubated in a water bath at 60°C for 1 h and then the reaction was stopped by adding 27 ml of 5% cold TCA solution. TCA-soluble peptides in the supernatant were analyzed using the Lowry assay (Lowry *et al.* 1951).

Sodium dodecyl sulfate-gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970). The samples subjected to different autolytic condition were mixed with 27 ml of 5% SDS solution (85°C). After incubating in an 85°C water bath for 1 h, the samples were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer and boiled for 3 min. The sample (20 µg) were loaded on the gel made of 4% stacking and 10% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel at room temperature (280-30°C) for 1.5 h using a Mini-Protean II cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were removed and stained with 0.2% Coomassie Brilliant Blue R-250 in a 45%

methanol, 10% acetic acid and 45% H_2O staining solution, and destained with a destaining solution comprised of 30% methanol and 10% acetic acid and 60% H_2O.

Protein determination

Protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Statistical analysis

A completely randomized design was used throughout this study. Analysis of variance (ANOVA) was performed and mean comparisons were run by Duncan's Multiple Range Test (Steel and Torrie 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows; SPSS Inc.).

RESULTS AND DISCUSSION

TIYTR inhibition of bigeye snapper sarcoplasmic proteinases

The inhibition of proteolytic activity of Sp from bigeye snapper muscle by different concentrations of TIYTR is depicted in Fig. 1. Inhibitory activity of TIYTR increased with increasing concentrations from 0.5 up to 3%. At 3% (w/v) concentration, TIYTR inhibited bigeye snapper muscle Sp activity by more than 60% (Fig. 1). Thus, TIYTR was able to inhibit proteolytic activity by bigeye snapper muscle sarcoplasmic fluid appreciably. The proteolytic activity is attributed to trypsin-like enzymes because of the capacity to hydrolyze the synthetic substrate, DL-BAPNA. It is suggested that serine proteinases, mainly trypsin-like proteinases, major components of the sarcoplasmic proteinases in bigeye snapper muscle, that cause the softening of muscle during extended iced storage. Also, they have been reported to play an important role in degradation of bigeye snapper myofibrillar proteins during heat-induced gelation, which results in gel weakening (Benjakul *et al.* 2003a). Klomklao and Benjakul (2014b) previously reported on the presence of a 70 kDa trypsin inhibitor in yellowfin tuna roe which could play an important role in preventing the proteolysis.

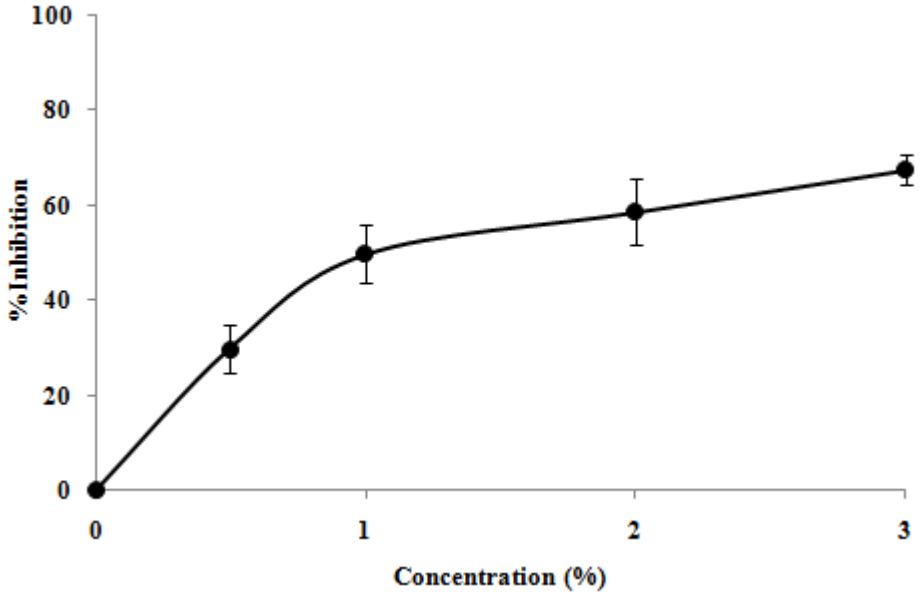

FIG. 1. EFFECT OF TIYTR AT DIFFERENT CONCENTRATIONS ON INHIBITION OF BIGEYE SNAPPER SARCOPLASMIC PROTEINASES. Error bars indicate standard deviations from triplicate determination.

Inhibition of autolysis of bigeye snapper by TIYTR

Inhibitory of autolysis of both mince (unwashed) and washed mince from bigeye snapper by TIYTR is presented in Fig. 2. In general, higher autolytic inhibition was achieved with increasing TIYTR concentration ($P<0.05$) within the inhibitor concentration range investigated. At the same concentration, TIYTR was slightly more effective in inhibiting autolysis of the washed mince samples compared to unwashed mince samples. TIYTR at the level of 3% showed the autolysis inhibition of 79.61 and 74.13% for washed mince and unwashed mince, respectively. This was possibly due to the fact that washed mince contained a lower amount of proteinases. Sarcoplasmic proteinases were leached out during the washing process (Benjakul *et al.* 2001). Nevertheless, myofibril-associated proteinases remained in the muscle and were directly associated with gel softening (Benjakul *et al.* 2003b). From the result, it is suggested that TIYTR functioned as an effective proteinase inhibitor to prevent the degradation of muscle proteins.

The autolytic patterns of mince and washed mince of bigeye snapper incubated at 60°C for 60 min in the absence and presence of TIYTR at levels of 0.5, 1, 2 and 3% are shown in Fig. 3. For both the mince and washed mince samples, a marked degradation of MHC was observed in the sample with no TIYTR as indicated by the lowest band intensity. The result indicated that MHC is the primary target of proteolytic activity. As indicated by An *et al*. (1994), MHC and β-tropomyosin and troponin-T were more susceptible to degradation than actin. A structural disintegration of fish muscle generally occurs within the temperature range from 50 to 70°C, called 'modori'. This phenomenon is induced by thermal stable endogenous proteinases (Benjakul *et al.* 2004). Degradation of

muscle proteins, especially MHC in bigeye snapper mince and washed mince was found at 60°C (Benjakul *et al*. 2003a). Though washing could remove some proteinases, particularly sarcoplasmic proteinases, those associated with myofibrils still remained and probably caused the degradation of MHC. MHC of mince or washed mince was more retained as the concentration of TIYTR increased. The presence and concentration of myosin determine the gel strength of surimi (Benjakul *et al.* 2004). The presence of TIYTR may protect myofibrillar proteins of bigeye snapper muscle by acting as a true inhibitor, which effectively decreased the proteolytic activity on myosin. From the results, the degradation caused by endogenous proteinases, especially trypsin or trypsin-like serine proteinases could be impeded to some extent by the addition of TIYTR.

Effect of NaCl on inhibition of autolysis in bigeye snapper by TIYTR

Figure 4 illustrates the inhibition of autolysis of mince and washed mince of bigeye snapper at 60°C for 60 min by TIYTR at 3% (w/w) in absence and presence of NaCl at levels of 0.5, 1, 2 and 3% (w/w). In general, the presence of NaCl generally enhanced the inhibitory activity of TIYTR at the concentration levels investigated. It was inferred that NaCl solubilize muscle proteins, leading the exposure of proteinase associated with myofibrils. As a consequence, proteinase inhibitors could bind more proteinases, resulting in higher inhibition.

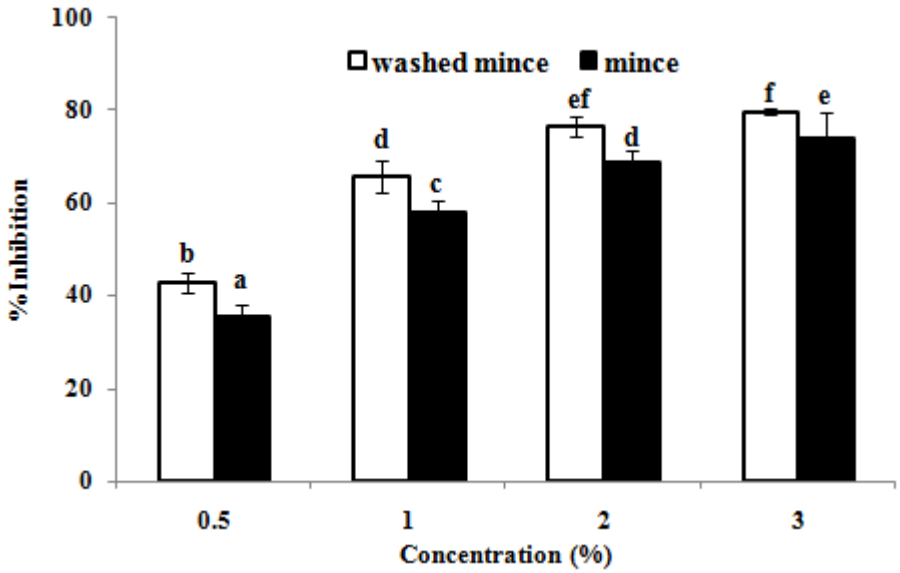

FIG. 2. EFFECT OF TIYTR AT DIFFERENT CONCENTRATIONS ON AUTOLYTIC INHIBITION OF BIGEYE SNANPPER MINCE AND WASHED MINCE. Error bars indicate standard deviations from triplicate determination. Different letters indicate significant differences ($P<0.05$)

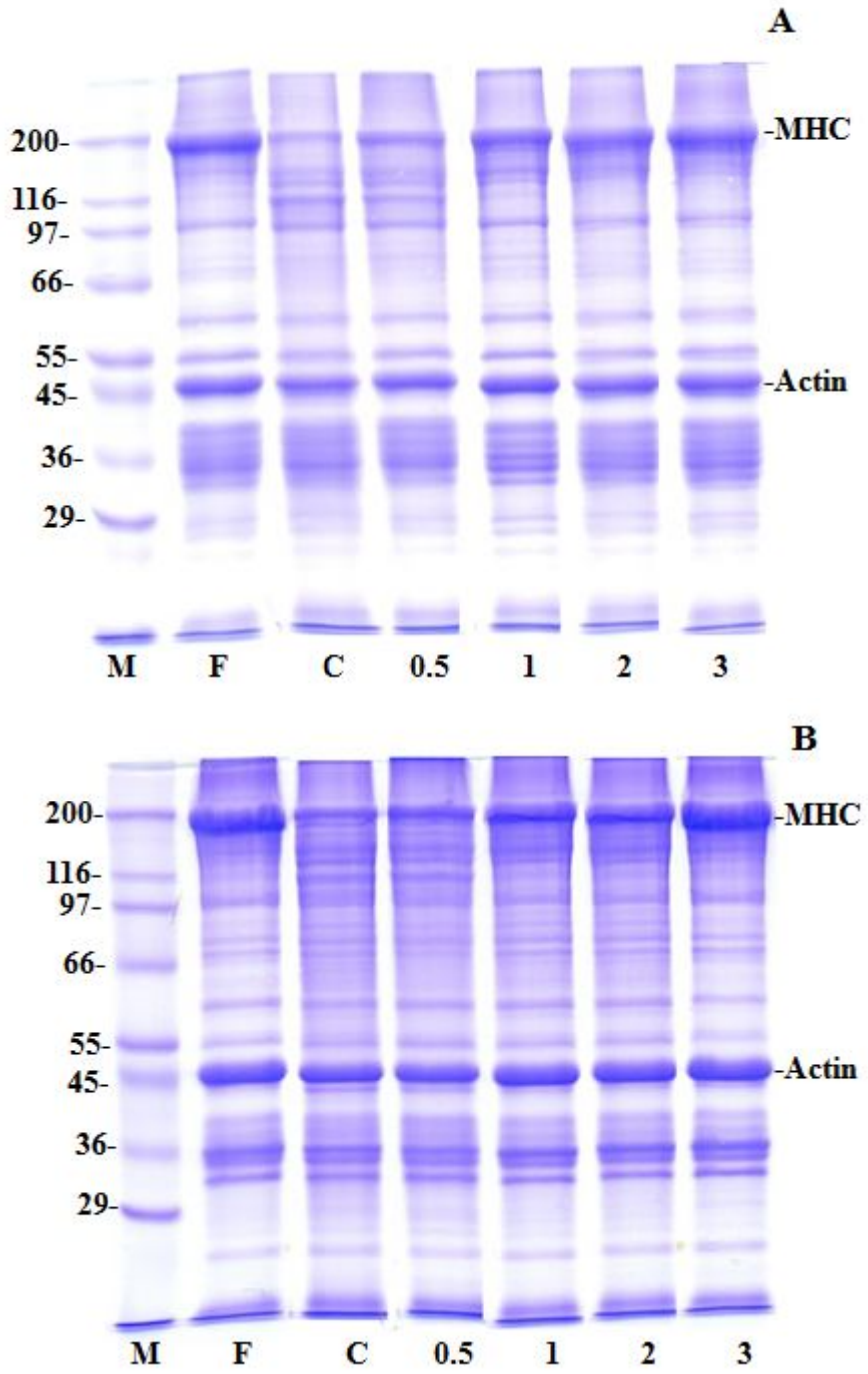

FIG. 3. AUTOLYSIS PATTERN OF BIGEYE SNAPPER MINCE (A) AND WASHED MINCE (B) IN THE ABSENCE AND PRESENCE OF TIYTR AT DIFFERENT CONCENTRATIONS AT 60°C FOR 1 H. M, molecular weight standards; F, mince or washed mince without incubation (control); C, mince or washed mince without TIYTR. Numbers designate the concentration of TIYTR. MHC, myosin heavy chain.

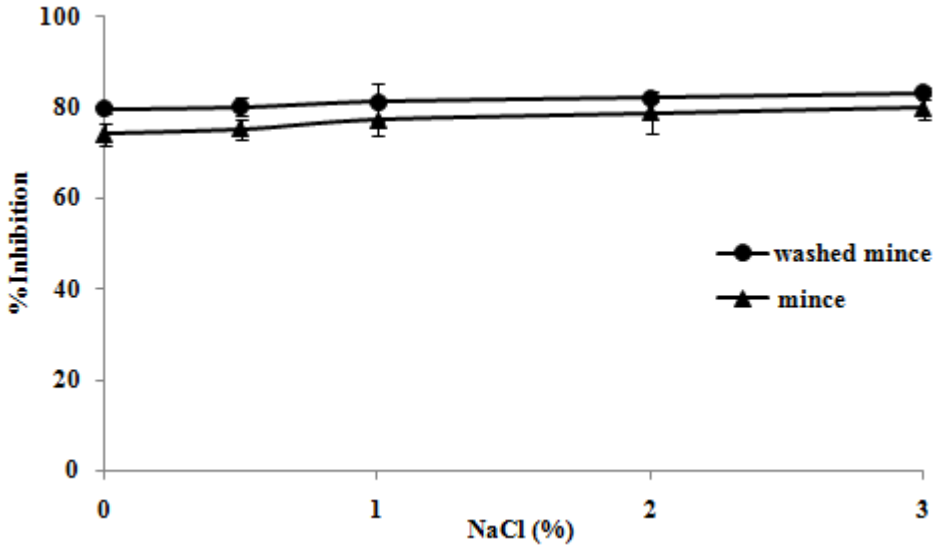

FIG. 4. EFFECT OF NACL AT DIFFERENT CONCENTRATIONS ON BIGEYE SNAPPER AUTOLYTIC INHIBITION BY TIYTR. Error bars indicate standard deviations from triplicate determination.

Comparison of different protease inhibitors on bigeye snapper proteinases activity

The effects of different commercially available inhibitors on the activity of Sp and autolysis of bigeye snapper muscle mince and washed mince samples were compared. All the commercially available protease inhibitors showed inhibitory activity toward Sp and autolysis of bigeye snapper muscle mince and washed mince samples (Fig. 5). There was no difference in inhibitory activity against Sp and autolysis of bigeye snapper mince and washed mince between TIYTR and BPP ($P>0.05$) (Fig. 5). The effect of EW on inhibitory activity of Sp and autolysis was much lower than that of TIYTR and BPP ($P<0.05$). The results indicated that TIYTR, BPP and EW all contained inhibitors, which were able to inhibit proteinase activities in bigeye snapper muscle. BPP was proven to be most effective in both inhibiting proteolytic activity and enhancing the gel strength of surimi (Benjakul *et al.* 2004). The proteinase inhibitory activity of BPP was reported to be due to alpha-2-macroglobulin (α_2-M) and kininogen (Morrissey *et al.* 1993). α_2-M acts as a nonspecific inhibitor for all classes of proteinases (Starkey and Barrett 1977), while kininogen is a specific cysteine proteinase inhibitor (Rawlings and Barret 1990). EW showed inhibitory activity due to the presence of some proteinase inhibitors, such as ovoinhibitor and ovomacroglobulin, which can inhibit cysteine proteinases, serine proteinases and aspartic proteinases (Benjakul *et al.* 2004). Benjakul *et al.* (2004) further reported that BPP showed higher inhibition of lizardfish autolysis, compared to EW. For yellowfin tuna roe, the major inhibitory component was identified as proteins with apparent MW of 70 kDa as shown on inhibitory activity-stained gels (Klomklao

and Benjakul, 2014b). The differences in inhibition efficiency of different inhibitors were presumed to be due to the differences in specificities toward the proteinases tested (Benjakul *et al.* 2004). From the results, TIYTR showed a high capacity to inhibit autolysis of bigeye snapper muscle that TIYTR could be used as a cost-effective proteinase inhibitor in the formulation of surimi-type products.

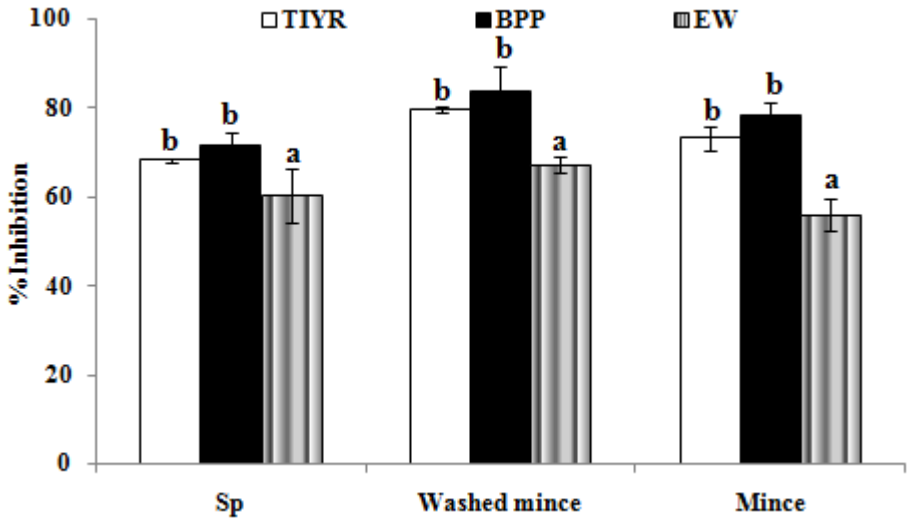

FIG. 5. EFFECT OF TIYTR, BPP AND EW ON INHIBITION OF BIGEYE SNAPPER PROTEINASES. SP, SARCOPLASMIC PROTEINASES. Error bars indicate standard deviations from triplicate determination. Different letters within the same sample indicate significant differences ($P<0.05$)

CONCLUSION

TIYTR showed inhibition against both Sp and autolysis of bigeye snapper muscle. The inhibition toward proteinases in bigeye snapper muscle depended on the concentration of TIYTR used. It displayed effective control of proteolysis in bigeye snapper muscle, comparable to BPP and EW. Thus, TIYTR can potentially be used to inhibit proteolysis and enhance surimi gel strength.

ACKNOWLEDGMENTS

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CHAPTER 4

TRYPSIN INHIBITOR FROM YELLOWFIN TUNA (*THUNNUS ALBACORES*) ROE: EFFECTS ON GEL PROPERTIES OF SURIMI FROM BIGEYE SNAPPER (*PRIACANTHUS MACRACANTHUS*)

LWT-FOOD SCIENCE AND TECHNOLOGY

SUBMITTED

Trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe: Effects on gel properties of surimi from bigeye snapper (*Priacanthus macracanthus*)

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Abstract

Effects of partially purified trypsin inhibitor from the roe of yellowfin tuna (*Thunnus albacores*) (TIYTR) at different levels (0 - 3.0 g/100g) on gelling properties of bigeye snapper (*Priacanthus macracanthus*) surimi were investigated. TIYTR showed inhibitory activity against proteolysis in kamaboko (40/90°C) and modori (60/90°C) gels in a concentration-dependent manner. Myosin heavy chain (MHC) was more retained in both gels when the level of TIYTR increased up to 3.0 g/100g. This was associated with the increased breaking force and deformation as well as lowered protein degradation as evidenced by the decrease in trichloroacetic acid-soluble peptide content ($p<0.05$). Whiteness of kamaboko and modori gels slightly decreased with increasing TIYTR levels ($p<0.05$). However, water-holding capacity of both gels was improved as TIYTR level increased ($p<0.05$). Incorporation of TIYTR, beef plasma protein (BPP) and egg white (EW) at a level of 3.0g/100g resulted in the increased breaking force and deformation of surimi gels. Nevertheless, TIYTR and BPP showed the higher gel strengthening effect than EW. Therefore, TIYTR could be used as an alternative cheap proteinase inhibitor to improve gel strength of surimi.

Keywords: Tuna; Surimi; Trypsin inhibitor; Gel properties; Proteinases

1. Introduction

Gel-forming ability of myofibrillar proteins is the most important functional requirement to provide the superior quality of surimi-based products, which can be affected by both intrinsic and extrinsic factors. The rapid and severe breakdown of myofibrillar proteins, particularly myosin, at higher temperature inhibits the development of three dimensional gel network. This leads to gel weakening of surimi-based products (Kudre, & Benjakul, 2013). Gel weakening phenomenon or "modori" is a major concern in surimi gel manufacture and is induced by endogenous heat-activated proteinases, which are able to degrade myofibrillar proteins (Benjakul, Visessanguan, & Thummaratwasik, 2000). Gel softening varies with species but is generally caused by serine and cysteine proteinases (Rawdkuen, & Benjakul, 2008).

Thailand is one of the most important surimi producing countries in Southeast Asia. Bigeye snapper has become more economically important as a raw material for surimi production due to its white color and large availability. Also, it is not consumed directly due to its appearance and thick skin. Bigeye snapper caught in Thailand normally includes two species, *Priacanthus tayeuns* and *Priacanthus macracanthus*. *P. macracanthus* has a much poorer gel quality compared to *P. tayeuns* (Benjakul, Visessanguan, & Leelapongwattana, 2003). Thus, *P. macracanthus* surimi is considered to be of low value because of its poor gel-forming ability. *P. macracanthus* surimi typically undergoes textural degradation by endogenous proteinases, especially trypsin-like serine proteinases (Benjakul et al., 2003). To improve gel quality of bigeye snapper surimi, some means of inhibiting proteolytic activity must be sought. Food-grade proteinase

inhibitors, commonly used in surimi, include beef plasma (BPP), egg white, potato powder and whey protein concentrate (Morrissey, Wu, Lin, & An, 1993; Yongsawatdigul, & Piyadhammaviboon, 2004). Food-greade proteinases inhibitors have been used to protect myofibrillar proteins from proteolysis caused by indigenous proteinases. However, the application of BPP in surimi has been prohibited by the outbreak of bovine spongiform encephalopathy or mad cow disease. Additionally, some BPP preparations result in off-flavors and off-color. Egg white is high cost and has an undesirable egg-like odour, white off-color problems may be encountered when potato powder is used (Rawdkuen, & Benjakul, 2008). Hence, alternative food-grade proteinase inhibitors for surimi production are still needed.

The roe of tuna is an abundant by-product from the commercial processing of canned tuna and a large amount of roe is produced each year. The roe is normally sold and used as animal feed. Based on our previous study, yellowfin tuna roe contained high trypsin inhibitory activity (Klomklao, Benjakul, & Kishimura, 2014). Recently, Klomklao, Benjakul, and Simpson (2015) reported that partially purified trypsin inhibitor from yellowfin tuna roe (TIYTR) inhibited sarcoplasmic proteinases and autolysis of bigeye snapper mince and its washed mince at 60°C in a concentration-dependent manner. Therefore, the addition of TIYTR possessing the trypsin inhibitory activity should pave the way for gel improvement of bigeye snapper surimi. The objective of this study was to investigate the preventive effects of TIYTR on gelling properties of bigeye snapper (*P. macracanthus*) surimi.

2. Materials and methods

2.1 Chemicals

*N*α-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), trypsin from porcine pancreas, β-mercaptoethanol (βME), sodium chloride, trichloroacetic acid, tris (hydroxymethyl) aminomethane, dimethylsulfoxide, sodium caseinate, ammonium sulfate, sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N',N'*-tetramethyl ethylene diamine (TEMED) and bovine serum albumin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Beef plasma protein (BPP) and EW powder were obtained from Food EQ Co., Ltd. (Bangkok, Thailand).

2.2 Preparation of partially purified trypsin inhibitor from yellowfin tuna roe (TIYTR)

The roes of yellowfin tuna (*Thunnus albacores*), with a size of 51.8±2.4 g/roe, were obtained from Tropical Canning (Thailand) Public Co. Ltd., Songkhla, Thailand. The samples (5 kg) were placed in ice using a roe:ice ratio of 1:2 (g:g) and transported to the Department of Food Science and Technology at the Thaksin University (Phatthalung, Thailand) within 2 h of roe removal. The whole roes were cleaned using cold water (4°C), vacuum-packed and immediately frozen at -20°C until needed for use.

Frozen roes were thawed using running tap water (26-28°C) until the core temperature reaches -2 to 0°C. The samples were cut into pieces with thickness ranging from 1-1.5 cm and homogenized with three volumes of cold acetone at

-20°C for 30 min according to the method of Klomklao, Benjakul, and Kishimura (2010). The homogenate was filtered under vacuum through Whatman No. 4 filter paper. The residue obtained was then homogenized with two volumes of cold acetone (-20°C) for 30 min, and then the residue was collected by filtering under vacuum through Whatman No. 4 filter paper. The defatted roe was air-dried at room temperature (28-30°C) until dry and free of acetone odor.

To prepare the extract, roe powder was mixed with 10 mmol/L Na-phosphate buffer (pH 7.0) containing 0.5 mol/L NaCl at a ratio of 1:9 (w/v) and shaken (BW 201 Shaking bath, Tokyo, Japan) for 30 min at 150 rpm at room temperature (28-30°C). The roe extract was recovered by centrifuging at 10,000×g for 30 min at 4°C.

Next, the roe extract was partially purified by heat-treatment at 60°C for 10 min (Klomklao, Benjakul, & Simpson, 2015) and then cooled with ice water. This treatment produced coagulated debris which was subsequently removed by centrifugation at 10,000×g for 10 min at 4°C. The supernatant was freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The dried powder obtained (TIYTR) was placed in polyethylene bag and kept at 4°C until used.

2.3 Trypsin inhibitory activity assay

Trypsin inhibitory activity was measured by the method of Klomklao, Benjakul, Kishimura, and Chaijan (2011) using BAPNA as substrate. A solution containing 200 µL of inhibitor solution and 200 µL (2 g/L) porcine pancreas trypsin was preincubated at 37°C for 15 min. Then, 1,000 µL of the mixtures containing 800 µL of 0.5 mmol/L BAPNA and 200 µL of distilled water (prewarmed to 37°C) was added and vortexed immediately to start the reaction. After incubating for 10 min, 900 µL of 300 mL/L acetic acid was added to terminate the reaction. The reaction mixture was centrifuged at 8,000×g for 5 min (Eppendorf Micro Centrifuge). Residual activity of trypsin was measured by the absorbance at 410 nm due to *p*-nitroaniline released. One unit of trypsin activity was defined as an increase of 0.01 absorbance unit/ml.min under the assay condition. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduces the enzyme activity by one unit.

2.4 Bigeye snapper preparation

Bigeye snapper (*P. macracanthus*) were caught from the Songkhla Coast along the Gulf of Thailand, stored in ice and off-loaded approximately 24-36 h after capture. Fish were transported in ice to the Department of Food Science and Technology, Thaksin University, Phatthalung within 2 h. Fish were then filleted and kept in ice until used for surimi gel preparation.

2.5 Effect of TIYTR on gel properties of bigeye snapper surimi

2.5.1 Surimi gel preparation

Surimi was prepared according to the method of Benjakul, Visessanguan, Tueksuban, and Tanaka (2004). Fresh bigeye snapper were washed with tap water. The flesh was removed manually and minced into the uniformity. The mince was then washed with cold water (5°C) at a mince/water ratio of 1:2 (g:g). The mixture was stirred gently for 3 min and washed process was repeated twice. Finally the washed mince was subjected to centrifugation using a Model CE 21 K basket centrifuge (Gradniumpiant, Belluno, Italy) with a speed of 700×g for 15 min. The washed mince referred to as 'surimi' was kept in ice until used.

2.5.2 Effect of TIYTR on gel-forming ability of bigeye snapper surimi

Surimi prepared was added with 2.5 g/100g salt and TIYTR was added at level of 0, 0.5, 1, 2 and 3 g/100g. The moisture content was then adjusted to 80 g/100g. The mixture was chopped for 5 min at 4°C to obtain the homogenous sol. The sol was then stuffed into polyvinylidine casing with a diameter of 2.5 cm and both ends of casing were sealed tightly. Kamaboko and modori gels were prepared by incubating the sol at 40 and 60°C for 30 min, respectively, followed by heating at 90°C for 20 min in a water bath (Memmert, Schwabach, Germany). After heating, all gels were immediately cooled in iced water for 30 min and stored at 4°C overnight prior to analysis.

BPP and EW powder were used to comparison with TIYTR. BPP or EW or TIYTR at level of 3 g/100g was added into surimi sol. Kamaboko gels were then prepared as previously described.

2.5.3 Texture analysis

Texture analysis of surimi gels was carried out using a Model TA-XT2 texture analyzer (Stable Micro System, Surrey, UK). Gels were equilibrated at room temperature (25-30°C) before analysis. Ten cylindrical samples (2.5 cm in diameter; 2.5 cm length) were prepared and tested. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the texture analyzer equipped with a spherical plunger (5 mm diameter), with a depression speed of 60 mm/min. (Benjakul et al., 2004).

2.5.4 Determination of whiteness

Gel samples from each treatment were subjected to whiteness measurement using a HunterLab (ColorFlex, Hunter Associates Laboratory, VA, USA). Illuminant C was used as the light source of measurement. L^* (lightness), a^* (redness/greenness) and b^* (yellowness/blueness) were measured. Whiteness was calculated using the following equation (Park, 1994):

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

2.5.5 Determination of expressible moisture content

Expressible moisture content was measured according to the method of Rawdkuen, and Benjakul (2008). Gel samples were cut to a thickness of 5 mm, weight (X) and placed between three pieces of filter papers (Whatman No. 1, Whatman International, Ltd., Maidstone, England) at the bottom and two pieces of paper on the top. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weight again (Y). Expressible moisture content was calculated with the following equation and expressed as the percentage of sample weight:

$$\text{Expressible moisture content (\%)} = [(X-Y)/X]\times 100$$

2.5.6 Determination of TCA-soluble peptide content

TCA-soluble peptide content was determined according to the method of Benjakul et al. (2000). Finely chopped gel sample (3 g) was mixed with 27 ml of 50 g/L TCA. The mixture was homogenized for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was incubated at 4°C for 1 h and centrifuged at 8,000×g for 5 min, using a Mikro 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). TCA-soluble peptides in the supernatant were measured according to the Lowry method (Lowery et al., 1951) and expressed as micromole tyrosine/g sample.

2.5.7 Sodium dodecyl sulfate-gel electrophoresis

Protein patterns of surimi gels were analyzed by SDS-PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 mL of 50 g/L hot SDS (85°C) solution was added to the sample (3 g). The mixture was then homogenized for 2 min at speed of 12,000 rpm. The homogenate was incubated at 85°C for 1 h to dissolve total proteins. The samples were then centrifuged at 8,000×g for 20 min to remove undissolved debris. Protein concentration in the supernatants was determined as per the method of Lowry, Rosebrough, Fan, and Randall (1951). Solubilized samples were mixed at 1:1 ratio with the SDS-PAGE sample buffer (0.5 mol/L Tris-HCl, pH 6.8, containing 40 g/L SDS, 200 mL/L glycerol and 100 mL/L β-ME) and boiled for 3 min. The sample (15 μg) were loaded on the gel made of 40 g/L stacking and 100 g/L separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were stained with 0.2 g/L Coomassie Brilliant Blue R-250 in 500 mL/L methanol and 75 mL/L acetic acid and destained with 500 mL/L methanol and 75 mL/L acetic acid, followed by 50 mL/L methanol and 75 mL/L acetic acid.

2.6 Protein determination

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.7 Statistical analysis

A completely randomized design was used throughout this study. All data were subjected to analysis of variance (ANOVA) and the differences between means were carried out using Duncan's Multiple Range Test (Steel & Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows; SPSS Inc. Chicago, IL, USA).

3. Results and discussion

3.1 Effect of TIYTR on textural properties of bigeye snapper surimi gel

Breaking force and deformation of both kamaboko (40/90°C) and modori (60/90°C) gels containing TIYTR at different levels are depicted in Fig. 1. Breaking force of both kamoboko and modori gels containing TIYTR increased as the amount of TIYTR added increased ($p<0.05$). For the deformation, the increases were noticeable with the addition of TIYTR up to 2 g/100g ($p<0.05$). The lowest breaking force and deformation were observed in modori gel containing no TIYTR ($p<0.05$). Comparatively, breaking force and deformation of kamaboko gels were higher than those of modori gels. This was possibly due to the greater cross-linking of proteins induced by endogenous transglutaminase during setting at 40°C of kamaboko gel. As consequence, high covalent bondings, especially ε-(γ-glutamyl) lysine linkages, were formed. These cross-links were plausibly resistant to hydrolysis induced by proteinases. Although these bondings might be formed to lower extent in modori gel, the degradation still took place in this gel. In the presence of TIYTR with sufficient proteolytic inhibitory activity, the degradation of surimi muscle proteins could be impeded. Klomklao et al. (2015) reported that TIYTR at a level of incorporation of 3 g/100g showed high inhibition of bigeye snapper proteinases. The results suggested that the addition of TIYTR in surimi could strengthen gel via proteinase inhibition.

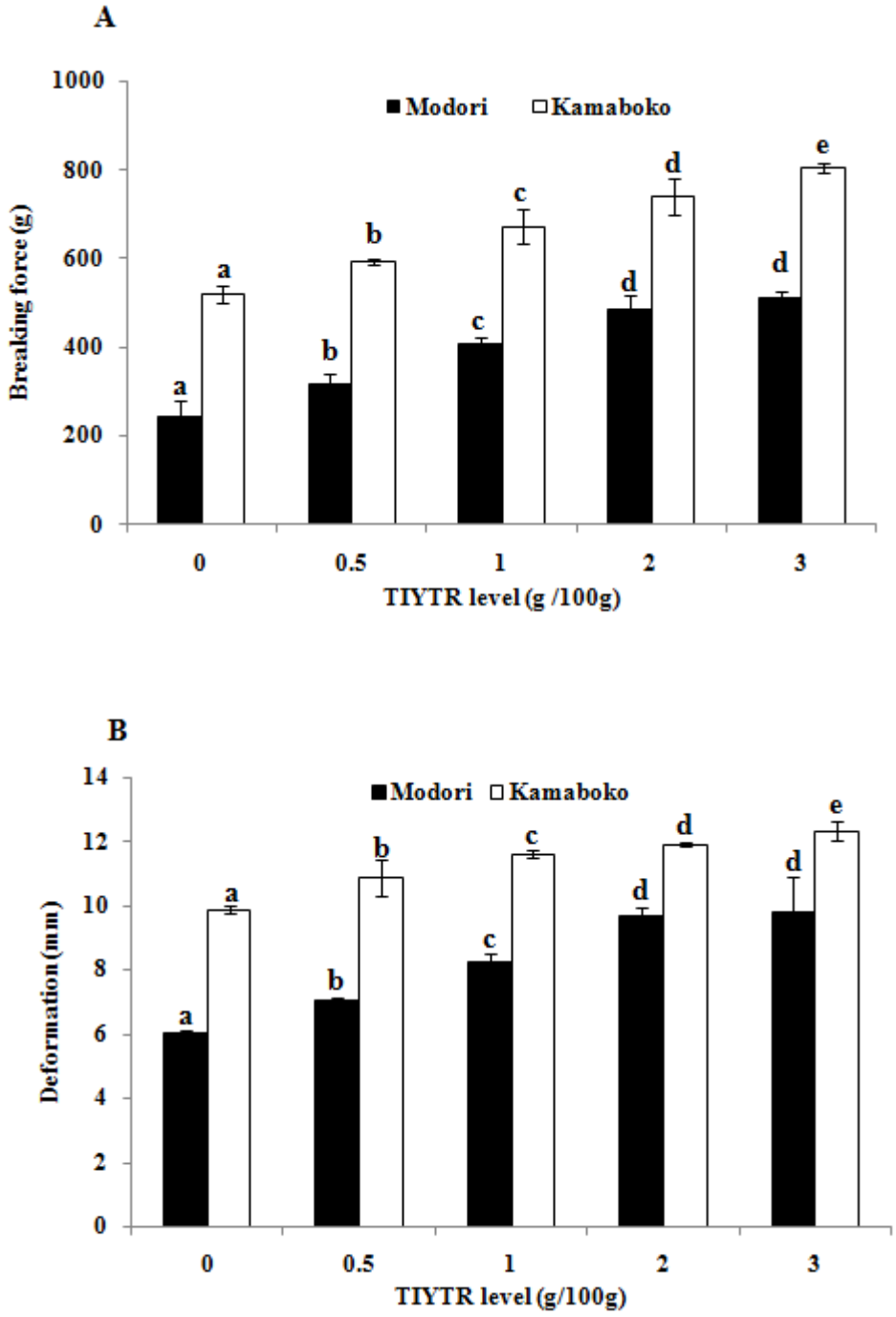

Fig. 1. Breaking force (A) and deformation (B) of modori and kamaboko gels from bigeye snapper surimi added with TIYTR at different levels. Bars indicate standard deviation (n=10). Different letters on the bars within the same gel indicate significant differences (p<0.05).

3.2 Effect of TIYTR on whiteness of bigeye snapper surimi gel

Figure 2 shows the whiteness of kamaboko and modori gels added with TIYTR at different levels. The slight decrease (p<0.05) in whiteness was noticeable in all gels, as TIYTR levels increased. When TIYTR at a level of 3 g/100g was incorporated to modori and kamaboko gels, the whiteness decreased by 2.88 and 3.15%, respectively, compared with that found in the control gel (without TIYTR). Color properties of surimi gels were largely dependent on the types and amounts of porcine plasma, protein and the legume seed proteinase inhibitors (Benjakul et al., 2004; Klomklao, & Benjakul, 2015). However, no differences in whiteness were observed in both modori and kamaboko gels when TIYTR was added up to 1 and 0.5 g/100g, respectively. Pigments in TIYTR more likely contributed to lowering of gel whiteness. When comparing the whiteness of modori and kamaboko gels, modori gels had a slightly higher whiteness, compared with kamaboko gel, regardless of TIYTR levels used. From the results, TIYTR showed detrimental effect on whiteness of surimi gel, even though it increased the gel strength. Therefore, appropriate amount of TIYTR should be used to maintain the overall quality, both gel strength and whiteness.

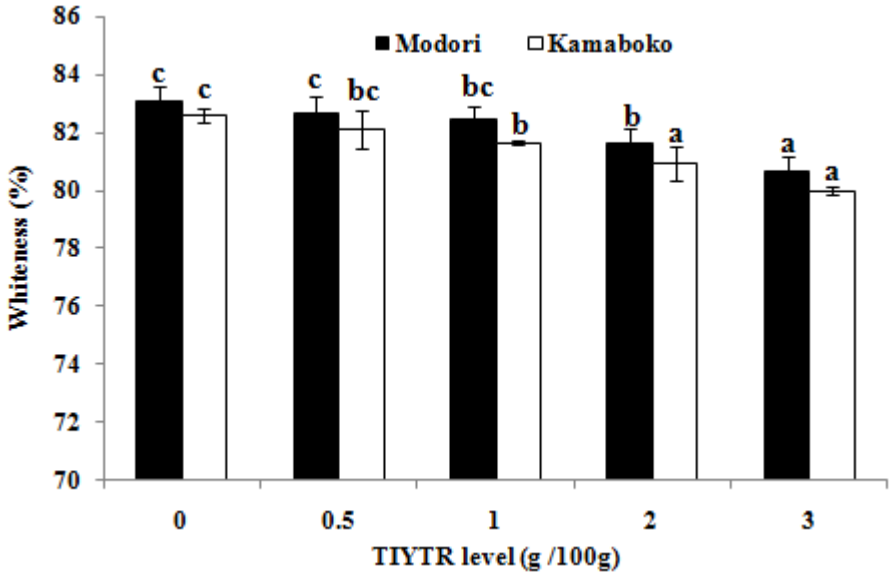

Fig. 2. Whiteness of modori and kamaboko gels from bigeye snapper surimi added with TIYTR at different levels. Bars indicate standard deviation (n=5). Different letters on the bars within the same gel indicate significant differences (p<0.05).

3.3 Effect of TIYTR on expressible moisture content of bigeye snapper surimi gel

Table 1 shows the expressible moisture content of modori and kamaboko gels added with TIYTR at different concentrations. The expressible moisture content of all gels significantly decreased as the levels of TIYTR added increased (p<0.05). This indicated that water-holding capacity of both gels was improved with increasing level of TIYTR resulting in a greater amount of water retained in the gel matrix. Lower expressible moisture contents of kamaboko gels from sardine and threadfin bream surimi were observed when protein isolates from black bean, mung bean and bambara groundnut were added (Oujifard, Benjakul, Ahmad, & Seyfabadi, 2012; Kudre, Benjakul, & Kishimura, 2013; Kudre, & Benjakul, 2013). Protein additives with high water-holding capacity might absorb water, especially when they underwent gelation. The lowest expressible moisture contents were found in either kamaboko or modori gels containing 3g/100g added TIYTR in which the decreases by 27.65 and 46.08%, respectively, were observed. The decrease in expressible moisture content was in accordance with the increased breaking force and deformation of kamaboko and modori gels (Fig. 1). The expressible moisture content of the control kamaboko gel was lower than that of the control modori gel. The higher expressible moisture content of modori gel attributed to the light-scattering effect of released water in modori gel. This result suggested that kamaboko gel had higher water-holding capacity than the modori gel. This might be associated by less degradation of muscle protein in surimi in kamaboko gels, especially when TIYTR was added. Hence, the addition of TIYTR increased water-holding capacity of gel matrix.

Table 1 Expressible moisture content of modori and kamaboko gels from bigeye snapper surimi added with TIYTR at different levels.

Sample	TIYTR level (g/100g)	Expressible moisture content (g/100g)
Modori gel	0	10.96±0.50d
	0.5	8.13±0.76c
	1	6.92±0.49b
	2	6.22±0.25ab
	3	5.91±0.07a
Kamaboko gel	0	4.63±0.54c
	0.5	4.38±0.43bc
	1	4.1±0.11bc
	2	3.90±0.08ab
	3	3.35±0.29a

Values are given as mean±SD from triplicate determinations.
Different letters within the same gel indicate significant differences ($p<0.05$)

3.4 Effect of TIYTR on the degradation of bigeye snapper surimi gel

3.4.1 TCA-soluble peptide content in surimi gel

Degradation of proteins in kamaboko and modori gels with and without TIYTR addition at different concentrations expressed as TCA-soluble peptide content is depicted in Fig. 3. The highest TCA-soluble peptide content was found in both gels without TIYTR addition, indicating the pronounced degradation caused by endogenous proteinases at both heating conditions (40/90 and 60/90°C). This result was in accordance with the lowest breaking force and deformation in the control gels (without TIYTR) (Fig. 1). TCA-soluble peptide content of modori and kamaboko gel decreased as the concentration of TIYTR increased ($p<0.05$). This result suggested that TIYTR showed the inhibitory activity towards degradation of muscle proteins in a dose dependent manner. Generally, TCA-soluble peptide contents in the modori gels were higher than those of the kamaboko gels, indicating the greater degradation of surimi proteins occurred in modori gels compared with kamaboko gels. Degradation occurred during heat-induced gelation is considered to result from the action of heat-activated endogenous proteinases (Benjakul et al., 2003). Accordingly, the decrease in TCA-soluble peptide content in gels, especially modori gel by addition of TIYTR, reflected the ability of TIYTR in inhibiting proteolysis mediated by endogenous proteinases. Inhibition of proteolysis should be an effective means for controlling the undesirable gel weakening of surimi from bigeye snapper during setting at high temperatures. Therefore, protein degradation could be retarded by addition of TIYTR.

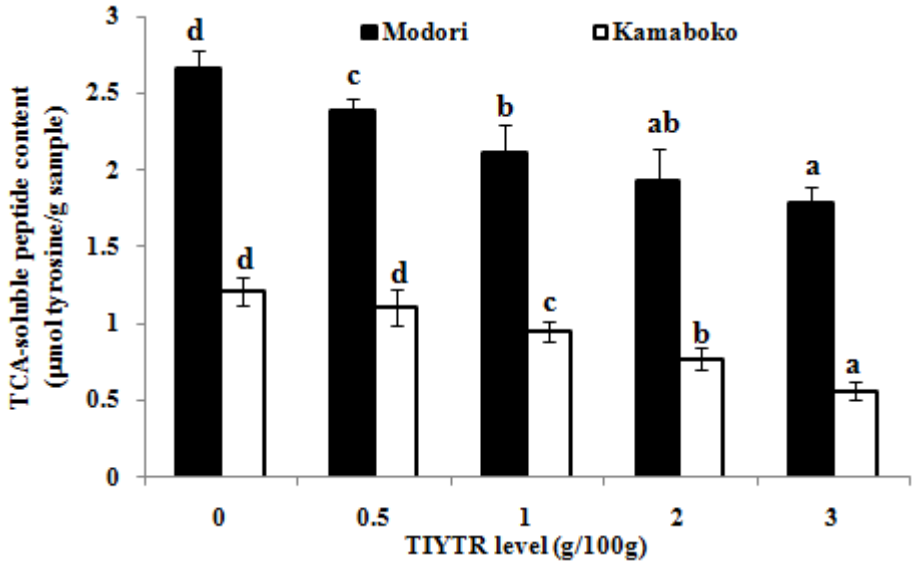

Fig. 3. TCA-soluble peptide content of modori and kamaboko gels from bigeye snapper surimi added with TIYTR at different levels. Bars indicate standard deviation (n=3). Different letters on the bars within the same gel indicate significant differences (p<0.05).

3.4.2 Protein patterns of surimi gel

SDS-PAGE patterns of modori and kamaboko gels added with TIYTR at different concentrations are shown in Fig. 4. The lowest band intensity of MHC was found in modori gel containing no TIYTR, indicating that the highest degradation of MHC caused by endogenous proteinases. The intensity of MHC band increased with increasing the TIYTR concentrations. This result was coincidental with the decrease in TCA-soluble peptide content (Fig. 3) and the increases in breaking force and deformation in modori gel (Fig. 1). The result reconfirmed that TIYTR was able to prevent degradation of MHC in modori gels, thereby lowering the loss in gel strength. For kamaboko gels, slightly higher band intensity of MHC was found when TIYTR at higher levels were added. The addition of TIYTR at higher level might impede cross-linking of MHC during setting mediated by indigenous transglutaminase. Benjakul and Visessanguan (2003) suggested that the decreases in MHC band intensity in bigeye snapper surimi gels were most likely caused by polymerization of MHC, especially during setting. Kudre et al. (2013) reported that MHC was more retained in kamaboko and modori gels when the concentration of protein isolates from black bean and mungbean increased up to 1g/100g.

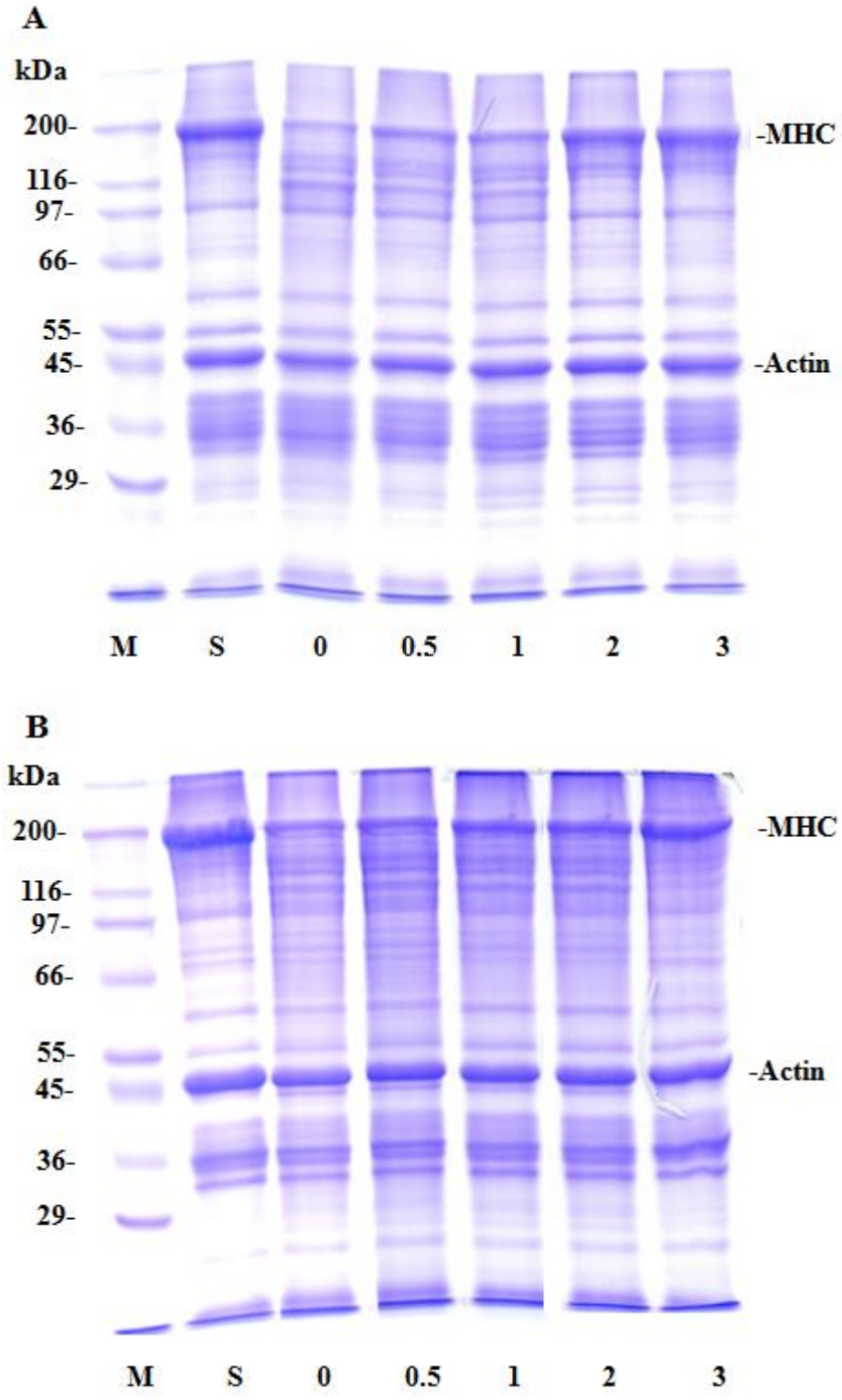

Fig. 4. SDS-PAGE pattern of modori (A) and kamaboko (B) gels from bigeye snapper surimi added with TIYTR at different levels. M, marker; S, surimi sol. Numbers designate the level of TIYTR added (g/100g). MHC, myosin heavy chain.

3.5 Comparison of different proteinase inhibitors on textural properties of bigeye snapper surimi gel

The effects of BPP, EW and TIYTR at the level of 3g/100g on textural properties of bigeye snapper surimi gel were compared (Table 2). Breaking force and deformation of surimi gels increased with addition of BPP, EW or TIYTR. Breaking force of kamaboko gel added with BPP and TIYTR increased by 57.94 and 54.42%, respectively, and deformation increased by 23.99 and 23.48%, respectively, compared with the control. For surimi gels added with EW, a similar gel enhancing effect was observed. However, EW caused the lower increase in breaking force and deformation, compared to BPP and TIYTR. Breaking force increased by 23.26% in kamaboko gel added with EW compared to the control and deformation increased by 21.26%. The results revealed that BPP and TIYTR were more effective in increasing gel strength of bigeye snapper surimi than EW. Apart from proteinase inhibitory activity of α_2-M and kininogen, protein cross-linking activity from both plama transglutaminase and α_2-M was reported (Rawdkuen, & Benjakul, 2008). This could strengthen the gel by myosin and fibrinogen cross-linking and could reduce the availability of myosin as substrate for proteinase action (Rawdkuen, & Benjakul, 2008). As a result, the addition of BPP resulted in the inhibition of proteolysis. Also, the combination effect between cross-linking activity and proteolysis inhibition was found in kamaboko gel as shown by the increase in breaking force and deformation. For EW, the presence of some proteinase inhibitors, such as ovoinhibitor and ovomacroglobulin, can inhibit cysteine proteinases, serine proteinases and aspartic proteinases (Benjakul et al., 2004). Benjakul et al. (2004) reported that EW showed inhibitory effect on

prevention of myosin degradation in lizardfish surimi. EW also functioned as binder in meat (Lu & Chen, 1999). Hence, EW addition presumably contributed to gel strengthening via proteinase inhibition and its binding effect. For TIYTR, protein with apparent molecular weight of 70 kDa, as shown on inhibitory activity-stained gel, was a major inhibitory component (Klomklao et al., 2014). From the results, TIYTR could be an alternative food grade proteinase inhibitor to improve the gel properties of surimi, especially those facing gel weakening.

Table 2 Effect of trypsin inhibitor from yellowfin tuna roe (TIYTR), beef plasma protein (BPP) and egg white (EW) on breaking force and deformation of kamaboko gels from bigeye snapper surimi.

Sample	Breaking force (g)	Deformation (mm)
Control	520.88±20.19a	9.88±0.13a
TIYTR	803.00±10.82c	12.20±0.26b
BPP	822.67±26.10c	12.25±0.26b
EW	657.67±32.02b	11.98±0.38b

Values are given as mean±SD from ten determinations.
Different letters in the same column indicate significant differences (p<0.05)

4. Conclusion

Partially purified trypsin inhibitor from yellowfin tuna roe can be used effectively as trypsin inhibitor in bigeye snapper surimi. Gel strength of surimi containing trypsin inhibitor was improved by the combination of proteolysis inhibition and setting.

Acknowledgments

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Output

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1. **Klomklao, S**., Benjakul, S. and Kishimura, H. 2014. Optimum extraction and recovery of trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe and its biochemical properties. Int. J. Food Sci. Tech. 49: 168-173.
2. **Klomklao, S.**, Benajkul, S. and Simpson, B.K. 2015.Inhibition of bigeye snapper (*Priacanthus macracanthus)* proteinases by trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe. J. Food Biochem. Accepted.
3. **Klomklao, S.,** Benjakul, S., Kishimura, H., Osako, K. and Simpson, B.K. 2015. Purification and characterization of trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe. J. Food Biochem. Submitted.
4. **Klomklao, S.** and Benajkul, S. 2015. Trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe: Effects on gel properties of surimi from bigeye snapper (*Priacanthus macracanthus)*. LWT-Food Science and Technology. Submitted.

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงพาณิชย์

ผลงานวิจัยเรื่องนี้สามารถผลิตสารยับยั้งเอนไซม์ทริปซินจากไข่ปลาทูน่าพันธุ์ครีบเหลืองซึ่งเป็นวัสดุเศษเหลือทิ้งจากการแปรรูปปลาทูน่าบรรจุกระป๋ อง และเป็นปัญหาการกำจัดและการจัดการของโรงงานแปรรูปปลาทูน่า นอกจากนี้ศึกษาการประยุกต์ใช้สารยับยั้งเอนไซม์ทริปซินเพื่อปรับปรุงคุณภาพเจลซูริมิ โดยจากผลงานวิจัยสามารถทำให้เจลซูริมิที่ได้มีคุณภาพสูงขึ้น ดังนั้นผู้ประกอบการซูริมิสามารถลดการนำเข้าสารยับยั้งเอนไซม์ทริปซินทางการค้าซึ่งมีราคาสูง ดังนั้นจึงสามารถเป็นการเพิ่มมูลค่าวัสดุเศษเหลือทิ้งจากโรงงาน และได้มาซึ่งผลิตภัณฑ์ใหม่ในเชิงพาณิชย์และเป็นการเพิ่มมูลค่าผลิตภัณฑ์ซูริมิอีกด้วย ซึ่งผลงานวิจัยนี้ได้ส่งให้ทางโรงงานเพื่อใช้เป็นข้อมูลในการเพิ่มมูลค่าต่อไป

- เชิงวิชาการ

ผลงานวิจัยถูกนำไปใช้ประกอบการเรียนการสอนในหลายวิชาทั้งระดับปริญญาตรีและบัณฑิตศึกษา ได้แก่ วิชาเคมีอาหาร หลักการวิเคราะห์อาหาร เทคโนโลยีภายหลังการจับและการแปรรูปสัตว์น้ำ และเทคโนโลยีและวิศวกรรมของโปรตีน

3. อื่น ๆ

- การเสนอผลงานในที่ประชุมวิชาการ

1. **Klomklao, S**. 2014. Optimum extraction and recovery of trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe and its biochemical characteristics. The 16th Food Innovation ASIA Conference 2014, June 12-13, 2014. BITEC, Bangkok, Thailand.
2. **Klomklao, S.** 2015. Trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe: Autolysis inhibition and inhibitory effect on modori inducing proteinases of bigeye snapper (*Priacanthus macracanthus*) muscle. The Third Higher Education Research Promotion Congress (HERP CONGRESS III), March 9-11, 2015. Nakhon Si Thammarat Rajabhat University, Nakhon Si Thammarat, Thailand.

APPENDIX

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Original article

Optimum extraction and recovery of trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe and its biochemical properties

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Summary Effect of lipid removal, extraction medium and extraction time on the isolation and recovery of trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe was investigated. Trypsin inhibitor extracted from defatted tuna roe showed the higher specific inhibitory activity than extracted from origin tuna roe. Optimal extraction medium was attained by shaking the defatted yellowfin tuna roe powder in 10 mM Na phosphate buffer (pH 7.0) containing 0.5 M NaCl ($P < 0.05$). The extraction time affected the inhibitor recovery significantly ($P < 0.05$). The extraction time of 30 min was optimum for recovery of trypsin inhibitor from yellowfin tuna roe. The biochemical properties of trypsin inhibitor from yellowfin tuna roes were also determined. The inhibitor was stable to heat treatment up to 60C and over a broad pH range (5-8). Increasing the concentration of salt (up to 3%, w/v) did not significantly decrease the trypsin inhibitory activity. However, the activity decreased when trypsin inhibitor was incubated with metal ions at ambient temperature for 30 min.

Keywords Isolation, proteinase, roe, trypsin inhibitor, tuna.

Introduction

Proteinase inhibitors are proteins or peptides capable of inhibiting the catalytic activities of proteolytic enzymes (Choi *et al*., 2002). Proteinase inhibitors commonly accumulate in high quantities in plant seeds, bird eggs and various body fluids. Proteinase inhibitors are also found in mammalian and marine blood plasma and fish roe and viscera (Choi *et al*., 2002). Proteinase inhibitors in fish roe can have a major impact on nutritional value as they inhibit pancreatic serine proteinases, thus impairing protein digestion. However, fish roe can be used as a potential source of proteinase inhibitor and can be of a variety of applications such as medicine, agriculture and food technology. Proteinase inhibitor can be used to improve texture in various foods, for example, sausages, meat balls, low-salt fish products.

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Thailand is the world's largest canned tuna producer and exporter. By the year 2008, tuna products with total amount of 483 894 tons and value of 61 036 million baht were exported (Department of Foreign Trade, 2009). Tuna roe, a by-product generated from fish processing (approximately 1.5–3.0% of total weight), is generally used as animal feed or for pet food preparation in Thailand. Based on our preliminary study, yellowfin tuna roe contained high trypsin inhibitory activity. The recovery of trypsin inhibitor from yellowfin tuna roe can increase its value and reduce waste disposal or treatment, thereby lowering environmental pollution. Nevertheless, no information regarding the biochemical properties of trypsin inhibitor from the roe of yellowfin tuna has been reported. Hence, yellowfin tuna roe that can be a cheap and promising source of proteinase inhibitor, especially trypsin inhibitor, can be recoverd and isolated for utilisations. The objectives of this investigation were to extract and characterise the trypsin inhibitor from yellowfin tuna roe.

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Material and methods

Chemicals

*N*α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), trypsin from porcine pancreas, sodium chloride, tris (hydroxymethyl) aminomethane, dimethylsulphoxide, sodium caseinate and bovine serum albumin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Extraction of trypsin inhibitor from yellowfin tuna roe

Raw materials

Roes of yellowfin tuna (*Thunnus albacores*), with a size of 51.8 ± 2.4 g/roe, were obtained from Tropical Canning (Thailand) Public Co. Ltd., Songkhla, Thailand. The samples (5 kg) were placed in ice using a roe/ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology, Faculty of Technology and Community Development, Thaksin University, Phatthalung, within 2 h. The whole roes were cleaned using cold water (4 °C). The roes were immediately frozen and stored at −20 °C until used.

Preparation of defatted roe powder for trypsin inhibitor extraction

Frozen roes 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 homogenised with three volumes of acetone at −20 °C for 30 min according to the method of Klomklao *et al.* (2010a) with a slight modification. The homogenate was filtrated in vacuo on Whatman No. 4 filter paper. The residue obtained was then homogenised in two volumes of acetone at −20 °C for 30 min, and then the residue was air-dried at room temperature. Defatted roe powder obtained was stored at −20 °C until used.

Effect of lipid removal on trypsin inhibitor extraction

Samples, nondefatted and defatted roes, were mixed with distilled water at a ratio of 1:9 (w/v) and shaken (BW 201 Shaking bath, Tokyo, Japan) for 30 min at 150 r.p.m. at room temperature. The extract was recovered by centrifuging at 10 000 × ***g*** for 30 min. The trypsin inhibitory activity and protein content in the extracts were determined, and the specific inhibitory activity of the extracts was compared.

Effect of extraction media on trypsin inhibitor extraction

Different extraction media including distilled water, 10 mM Tris–HCl, pH 7.0 and 10 mM Na phosphate buffer, pH 7.0 were used to extract trypsin inhibitor. The defatted roe powder was added to the extraction medium at a ratio of 1:9 (w/v) and shaken (BW 201 Shaking bath) for 30 min at 150 r.p.m. at room temperature. The extract was recovered by centrifuging at 10 000 × ***g*** for 30 min. The trypsin inhibitory activity and protein content in the extract were determined, and the specific inhibitory activity of the extracts obtained using different media was compared. The extraction media, which was able to extract the trypsin inhibitor with the highest specific trypsin inhibitory activity, were selected for further steps.

Effect of NaCl concentration on trypsin inhibitor extraction

Defatted roe powder was suspended in 10 mM Na phosphate, pH 7.0 containing different NaCl concentration (0, 0.25, 0.5, 0.75 and 1.0 M) at a ratio of 1:9 (w/v) and shaken for 30 min at 150 r.p.m. at room temperature. The mixture was centrifuged at 10 000 × ***g*** for 30 min, and the supernatants were subjected to determination of trypsin inhibitory activity and protein content. The extraction medium rendering the highest specific trypsin inhibitory activity was chosen for further study.

Effect of extraction time on trypsin inhibitor extraction

Defatted roe powder was mixed with 10 mM Na phosphate buffer (pH 7.0) containing 0.5 M NaCl with a solid/solvent ratio of 1:9 (w/v) and shaken for 0.25, 0.5, 1, 2 and 3 h. At designated time, the mixture was centrifuged at 10 000 × ***g*** for 30 min, and the supernatants were subjected to determination of trypsin inhibitory activity and protein content. The extraction time rendering the highest specific trypsin inhibitory activity was chosen for further study.

Characterisation of trypsin inhibitor from yellowfin tuna roe

Thermal and pH stability

Trypsin inhibitor extracts from yellowfin tuna roe were diluted with distilled water to obtain 60–70% inhibition. The solutions were incubated at 30, 40, 50, 60, 70, 80, 90 and 100 °C for 10, 20 and 30 min and then cooled in iced water. The residual trypsin inhibitory activity was determined and reported as the relative activity compared with the original activity.

The effect of pH on trypsin inhibitor stability was evaluated by measuring the residual activity after incubation at various pHs for 30 min at room temperature. Different buffers used included McIlvaine buffers (0.2 M Na phosphate and 0.1 M Na citrate) for pH 2.0–7.0 and 0.1 M glycine-NaOH for pH 8.0–11.0.

Salt stability

Trypsin inhibitor was incubated at room temperature for 30 min in the presence of NaCl ranging from 0% to 3%. The mixture was tested for inhibitory activity against trypsin. The residual inhibitory activity was reported.

Effect of metal ions
The solutions with various metal ions such as KCl, NaCl, $MgCl_2$ and $CaCl_2$ were added in the trypsin inhibitor to a final concentration of 10 mM. The trypsin inhibitory activity was assayed after incubation for 30 min at room temperature. The residual inhibitory activity was reported.

Trypsin inhibitory activity assay
Trypsin inhibitory activity was measured by the method of Benjakul *et al.* (2000) with a slight modification using BAPNA as substrate. A solution containing 200 μL of inhibitor solution, 200 μL (20 μg mL^{-1}) porcine pancreas trypsin was preincubated at 37 °C for 15 min. Then, 1000 μL of the mixtures containing 800 μL of 0.5 mM BAPNA and 200 μL of distilled water (prewarmed to 37 °C) was added and vortexed immediately to start the reaction. After incubating for 10 min, 900 μL of 30% acetic acid (v/v) was added to terminate the reaction. The reaction mixture was centrifuged at 8000 × ***g*** for 5 min (MIKRO200, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). Residual activity of trypsin was measured by the absorbance at 410 nm due to p-nitroaniline released. One unit of proteolytic activity was defined as an increase of 0.01 absorbance unit mL^{-1}.min under the assay condition. One unit of trypsin inhibitory activity was defined as the amount of inhibitor, which reduces the enzyme activity by one unit.

Protein determination
Protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Statistical analysis
A completely randomised design was used throughout this study. All data were subjected to analysis of variance (ANOVA), and the differences between means were carried out using Duncan's multiple range test. For effect of lipid removal on trypsin inhibitor recovery, the independence *t*-test was used for pair comparison. (Steel & Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 10.0 for Windows; SPSS Inc., Chicago, IL, USA).

Results and discussion

Optimum extraction of trypsin inhibitor from yellowfin tuna roe

The effect of lipid removal on trypsin inhibitor recovery from the roe of yellowfin tuna is displayed in Table 1. Total trypsin inhibitory activity and specific inhibitory activity of defatted tuna roes were generally higher than those of the control (nondefatted roe; $P < 0.05$). The results indicated that the lipid removal facilitated the release of trypsin inhibitor from tuna roes.

Table 1 Effect of lipid removal on the recovery of trypsin inhibitor from yellowfin tuna roes*

Sample	Total trypsin inhibitory activity (Units)**	Total protein (mg)	Specific trypsin inhibitory activity (Units mg^{-1} protein)
Nondefatted roe	80.52 ± 0.15[a]	206.46 ± 0.05[b]	0.39 ± 0.11[a]
Defatted roe	97.50 ± 0.19[b]	199.80 ± 0.03[a]	0.48 ± 0.01[b]

The different letters in the same column denote the significant differences ($P < 0.05$).
*The samples were shaken in distilled water at ambient temperature for 30 min, and trypsin inhibitory activity was analysed using *N*α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride as substrate.
**Mean ± SD from triplicate determinations.

Table 2 Effect of extractants on the recovery of trypsin inhibitor from yellowfin tuna roes*

Extractants	Total trypsin inhibitory activity (Units)**	Total protein (mg)	Specific trypsin inhibitory activity (Units mg^{-1} protein)
Distilled water	93.76 ± 6.91[a]	190 ± 1.00[a]	0.49 ± 0.40[a]
10 mM Tris–HCl, pH 7.0	113.69 ± 2.76[b]	209 ± 1.52[b]	0.54 ± 0.12[a]
10 mM Na Phosphate, pH 7.0	154.83 ± 6.32[c]	196.67 ± 15.28[ab]	0.78 ± 0.03[b]

The different letters in the same column denote the significant differences ($P < 0.05$).
*The samples were shaken in different media at ambient temperature for 30 min, and trypsin inhibitory activity was analysed using *N*α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride as substrate.
**Mean ± SD from triplicate determinations.

The effect of extraction media on trypsin inhibitor extraction from yellowfin tuna roe is shown in Table 2. Defatted roe extract using 10 mM Na phosphate buffer, pH 7.0 showed higher trypsin inhibitory activity than that extracted with distilled water and 10 mM Tris–HCl buffer (Table 2; $P < 0.05$). Additionally, specific inhibitory activity was greater in the extract using Na phosphate buffer compared with other two extracts (Table 2). The results suggested that Na phosphate buffer had a greater ability to extract trypsin inhibitor with the higher purity than did distilled water and Tris–HCl buffer.

Na phosphate buffer containing different NaCl concentrations was used to extract the trypsin inhibitor from yellowfin tuna roe (Table 3). When the

Table 3 Effect of NaCl concentrations on the recovery of trypsin inhibitor from yellowfin tuna roes*

NaCl concentration (M)	Total trypsin inhibitory activity (Units)**	Total protein (mg)	Specific trypsin inhibitory activity (Units mg^{-1} protein)
0	154.83 ± 5.26[a]	198.83 ± 0.76[a]	0.77 ± 0.01[a]
0.25	213.40 ± 14.15[c]	200 ± 1.00[a]	1.06 ± 0.07[c]
0.5	257.41 ± 2.50[d]	210.3 ± 1.96[b]	1.22 ± 0.01[d]
0.75	218.58 ± 3.12[c]	219 ± 1.00[c]	0.99 ± 0.12[b]
1	199.33 ± 5.13[b]	245 ± 1.00[d]	0.81 ± 0.03[a]

The different letters in the same column denote the significant differences ($P < 0.05$).

*The samples were shaken in 10 mM Na Phosphate, pH 7.0 in different NaCl concentrations at ambient temperature for 30 min, and trypsin inhibitory activity was analysed using *N*α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride as substrate.

**Mean ± SD from triplicate determinations.

concentration of NaCl was increased from 0 to 0.5 M, trypsin inhibitory activity increased ($P < 0.05$). However, there was no further increase in the activity with NaCl above 0.5 M. This probably was due to the denaturation of yellowfin tuna roe trypsin inhibitor at high salt concentration. Factors involved in protein solubility and recovery include particle size of tissue, temperature, length of extraction time, pH, ionic strength, type and concentration of extraction medium as well as the hydration characteristics of proteins (Benjakul *et al.*, 2000; Klomklao *et al.*, 2011). From the results, 10 mM Na phosphate buffer containing 0.5 M NaCl was shown to be the best extraction medium for yellowfin tuna roe trypsin inhibitor.

Figure 1 showed the effect of extraction time on the recovery of trypsin inhibitor from yellowfin tuna roe. A higher extraction efficiency was found when the extraction time increased up to 0.5 h (Fig. 1a; $P < 0.05$). Increased extraction time allowed the protein, especially trypsin inhibitors, to be more dissolved. The inhibitory activity was slightly increased for yellowfin tuna roe with the extraction time of 0.5 h (Fig. 1a; $P < 0.05$). However, the decrease in inhibitory activity was observed when the extraction time of 1–3 h was used ($P < 0.05$; Fig. 1a), indicating the less stability of trypsin inhibitor from yellowfin tuna roe during the extraction. This contributed to the loss of specific activity (Fig. 1b). High mechanical shear generated by shaking and the incorporation of air bubbles and adsorption of protein molecules to the air–liquid interface can cause denaturation of protein (Damodaran, 1996; Klomklao *et al.*, 2010b). Therefore, an extraction time of 0.5 h was selected for extraction of trypsin inhibitor from yellowfin tuna roe.

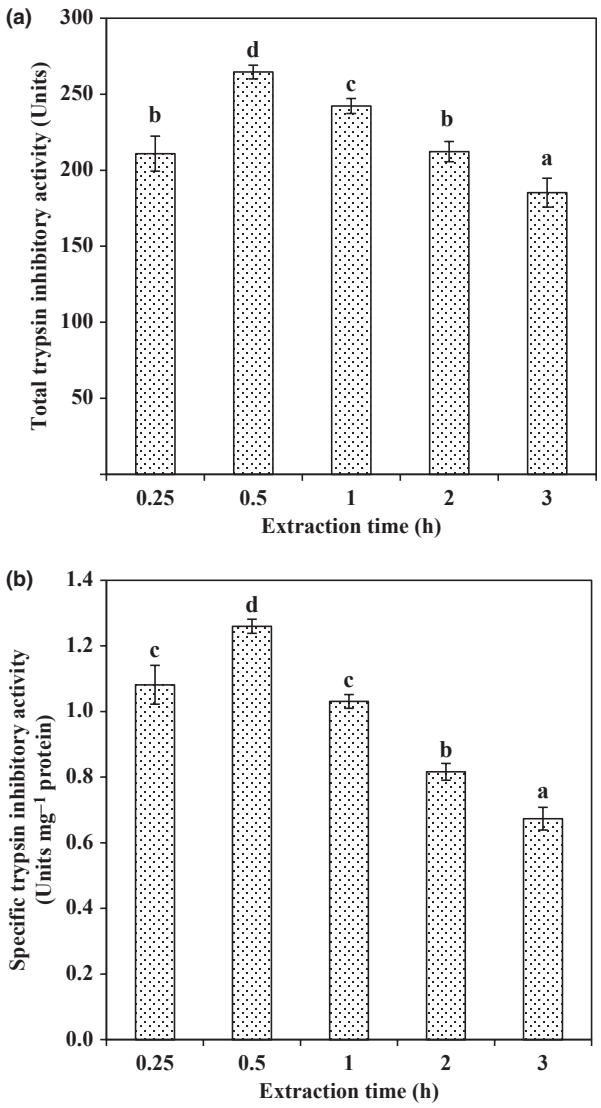

Figure 1 Effect of extraction time on the trypsin inhibitory activity (a) and the specific trypsin inhibitory activity (b) of yellowfin tuna roe extract. Bars indicate standard deviation from triplicate determination. Different letters on the bars indicate the significant differences ($P < 0.05$).

Characterisation of trypsin inhibitor from yellowfin tuna roe

pH and thermal stability

The pH stability of the trypsin inhibitor from yellowfin tuna roe is depicted in Fig. 2a. The inhibitor was stable over a wide pH range (pH 5–8). However, slight loss of activity was observed at low and high pHs (pH 2–4 and 9–11). Therefore, the inhibitor was generally stable in the neutral pH range. In general, most proteins are stable within a particular pH range,

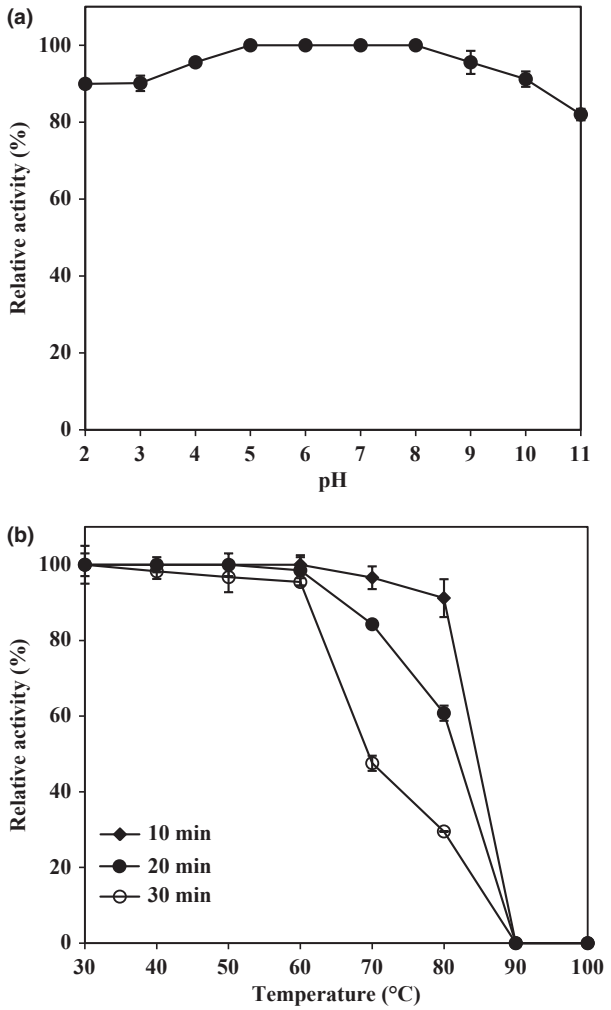

Figure 2 pH (a) and thermal (b) stability of trypsin inhibitor from yellowfin tuna roe. Bars indicate standard deviation from triplicate determination.

but denaturation (unfolding) of proteins can occur due to the strong electrostatic repulsion of ionised groups inside the molecules at extreme pH value (Damodaran, 1996). Choi *et al.* (2002) reported that the trypsin inhibitor from the egg of skipjack tuna (*Katsuwonus pelamis*) was stable in the pH range from 4.0 to 10.0. The differences in pH stability indicated the different molecular properties including bonding stabilising the structure as well as the trypsin inhibitor conformation among the various species. (Benjakul *et al.*, 2000).

For thermal stability, the inhibitor was stable when incubated at temperature up to 60 °C for 10–30 min with the residual activity more than 95% (Fig. 2b). Nevertheless, the sharp decrease in trypsin inhibitory activity was noticeable at temperature above 70 °C. No activity remained at 90 and 100 °C, suggesting complete loss in activity caused by thermal denaturation of trypsin inhibitor. In general, the stability of trypsin inhibitor decreased when the heating time was increased. A heating time of 30 min causes the highest loss of activity at every temperature above 60 °C used. It was presumed that trypsin inhibitors from yellowfin tuna roe underwent denaturation during high-temperature heating. Choi *et al.* (2002) reported that the trypsin inhibitor from the egg of skipjack tuna was stable at temperatures below 40 °C.

Salt stability

The effect of NaCl on the inhibitory activity of trypsin inhibitor from yellowfin tuna roe was investigated. No marked changes in relative activity were observed when NaCl was added up to 3.0% (data not shown). From the result, trypsin inhibitor showed high salt stability up to 3%, which might be useful in surimibase products in which 2–2.5% salt is commonly used.

Effect of metal ions

The activity of trypsin inhibitor in the presence of different monovalent and divalent metal ions was determined. All of the metal ions including K^+, Na^+, Mg^{2+} and Ca^{2+} had a negative effect on the trypsin inhibitor activity with 12.32–32.05% loss in inhibitory activity (data not shown). It was presumed that metal ions bound by inhibitors induce a structural modification that changes their conformational stability for their biological activity (Bijina *et al.*, 2011). However, the activity of the trypsin inhibitor from the egg of skipjack tuna was increased in the presence of metal ions such as K^+, Na^+, Mg^{2+} and Ca^{2+} (Choi *et al.*, 2002). These findings suggest a difference in the structure of the metal ion binding site among different marine fish trypsin inhibitors.

Conclusion

Trypsin inhibitor from yellowfin tuna roe was successfully extracted by 10 mM Na phosphate buffer (pH 7.0) containing 0.5 M NaCl. It was stable at various pHs, heat treatment and also stable at high salt concentration up to 3%. Based on the properties, the trypsin inhibitor from yellowfin tuna roe can be used as an alternative additive from improving the quality of surimi gels, especially those suffering, caused by trypsin or trypsin-like serine proteases.

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INHIBITION OF BIGEYE SNAPPER (*PRIACANTHUS MACRACANTHUS*) PROTEINASES BY TRYPSIN INHIBITOR FROM YELLOWFIN TUNA (*THUNNUS ALBACORES*) ROE

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ABSTRACT

The inhibitory effect of partially purified trypsin inhibitor from yellowfin tuna (*Thunnus albacores*) roe (TIYTR) on proteolysis of bigeye snapper (*Priacanthus macracanthus*) proteinases was investigated. TIYTR inhibited sarcoplasmic proteinases and autolysis of bigeye snapper mince and its washed mince at 60C in a concentration-dependent manner. Myosin heavy chain (MHC) in the mince and the washed mince of bigeye snapper was better retained when higher concentrations of TIYTR were used. The presence of NaCl (3.0% w/w) slightly enhanced the inhibitory activity of TIYTR (3.5–5.8%). Both TIYTR and beef plasma protein (at a level of incorporation of 3% w/w) showed higher inhibition of bigeye snapper proteinases than egg white ($P < 0.05$). Based on inhibition studies, it is suggested that the trypsin inhibitor from the roe of yellowfin tuna can be a potential aid to suppress the gel weakening of bigeye snapper surimi, elicited by trypsin-like proteinases, during either setting or heating.

PRACTICAL APPLICATIONS

Yellowfin tuna (*Thunnus albacores*) roe is an abundant and underutilized resource that can be used as a source of trypsin inhibitors. Trypsin inhibitors from the roe of yellowfin tuna play a role in the inhibition of proteolysis in bigeye snapper (*Priacanthus macracanthus*) muscle. Thus, it can be a potential alternative for commercial trypsin inhibitor to inhibit proteolysis and enhance surimi gel strength.

INTRODUCTION

Protein hydrolysis of fish muscle during post-mortem storage and processing has been attributed to endogenous muscle proteinases and can result in undesirable flavor and texture changes. Muscle proteinases are found in the sarcoplasmic fraction of cellular organelles of muscle tissues, connective tissues and myofibrils (Benjakul and Visessanguan 2000). A number of endogenous proteinases have been studied for their relative contributions to post-mortem softening of fish flesh and have been found to contribute to myofibrillar protein degradation to varying degrees (An *et al.* 1996). Among the endogenous proteinases, lysosomal cathepsins are found to play the most influential role in the tissue softening of most fish during post-mortem storage. However, only cathepsin L and alkaline proteinases (i.e., trypsin-like and cysteine protease-like; Busconi *et al.* 1984) have been reported as active proteinases involved in the softening phenomenon observed at the elevated temperatures normally employed during surimi processing (Yongsawatdigul and Piyahammaviboon 2004).

Bigeye snapper is one of the most important species for surimi production in the Southeast Asian region because of its white color and large availability. Bigeye snapper caught in Thailand normally includes two species: *Priacanthus tayenus* and *Priacanthus macracanthus*. *P. macracanthus* has a much poorer gel quality compared with *P. tayenus*. Benjakul *et al.* (2003a) reported that mince and washed

mince from *P. macracanthus* were degraded at 50 and 60C to a higher extent compared with their counterparts from *P. tayenus*. Heat-stable alkaline proteinases, especially trypsin-like serine proteinase, have been implicated in the gel weakening of bigeye snapper (*P. macracanthus*) surimi (Benjakul *et al*. 2003b). Therefore, various food-grade enzyme inhibitors, such as egg white (EW) powder, beef plasma powder and whey protein concentrate, have been used to prevent texture degradation in surimi gels (Benjakul *et al*. 2004; Rawdkuen and Benjakul 2008). However, these inhibitors are expensive; thus, discovering alternative and cheaper sources of food-grade inhibitors to control the endogenous proteinases that adversely impact fish texture would be very useful for the formulation of these surimi-type products.

Tuna roe is an abundant by-product from the commercial processing of canned tuna. Thus far, a large amount of roe is commonly collected and used as animal feed, which has low market value. Fish roes, accounting for 1.5–3.0% of the total weight, have potential for a range of biotechnological applications such as a source of proteinase inhibitor, especially trypsin inhibitor (Klomklao *et al*. 2014). Recently, yellowfin tuna roe was shown to contain trypsin inhibitors, which can reduce proteinase activity in fish extracts (Klomklao and Benjakul 2015; Klomklao *et al*. 2014). However, the effect of the trypsin inhibitor from yellowfin tuna roe on proteolysis of bigeye snapper has not been studied. Thus, the objective of the present study was to evaluate the inhibitory effects of trypsin inhibitor from yellowfin tuna roe on proteolysis of the bigeye snapper.

MATERIALS AND METHODS

Chemicals

*N*α-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA), porcine pancreas trypsin, β-mercaptoethanol, sodium chloride, trichloroacetic acid (TCA), tris(hydroxymethyl)-aminomethane, dimethyl sulfoxide, sodium caseinate, ammonium sulfate, sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250, *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine and bovine serum albumin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Beef plasma protein (BPP) and EW powder were obtained from Food EQ Co., Ltd. (Bangkok, Thailand).

Preparation of Partially Purified Trypsin Inhibitor from Yellowfin Tuna Roe (TIYTR)

The roes of yellowfin tuna (*Thunnus albacores*), with a size of 51.8 ± 2.4 g/roe, were obtained from Tropical Canning (Thailand) Public Co., Ltd., Songkhla, Thailand. The samples (5 kg) were placed in ice using a roe : ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology at the Thaksin University (Phatthalung, Thailand) within 2 h of roe removal. The whole roes were cleaned using cold water (4C), vacuum-packed and immediately frozen at −20C until needed for use.

Frozen roes were thawed using running tap water (26–28C) until the core temperature reaches −2–0C. The samples were cut into pieces with thickness ranging from 1 to 1.5 cm and homogenized with 3 volumes of cold acetone at −20C for 30 min according to the method of Klomklao *et al.* (2010a,b). The homogenate was filtered under vacuum through Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The residue obtained was then homogenized with 2 volumes of cold acetone (−20C) for 30 min, and then the residue was collected by filtering under vacuum through Whatman No. 4 filter paper. The defatted roe was air-dried at room temperature (28–30C) until dry and free of acetone odor.

To prepare the extract, roe powder was mixed with 10 mM Na-phosphate buffer (pH 7.0) containing 0.5 M NaCl at a ratio of 1:9 (w/v) and shaken (BW 201 Shaking bath, Tokyo, Japan) for 30 min at 150 rpm at room temperature (28–30C). The roe extract was recovered by centrifuging at 10,000 × g for 30 min at 4C.

Next, the roe extract was partially purified by heat treatment at 60C for 10 min (Klomklao and Benjakul 2015) and then cooled with ice water. This treatment produced coagulated debris, which was subsequently removed by centrifugation at 10,000 × g for 10 min at 4C. The supernatant was freeze-dried and kept at 4C until needed.

Trypsin Inhibitory Activity Assay

Trypsin inhibitory activity was measured as per the method of Klomklao *et al*. (2011), with a slight modification using BAPNA as substrate. A solution containing 200 μL of inhibitor solution and 200 μL of porcine pancreas trypsin (20 μg/mL) was pre-incubated at 37C for 15 min. To initiate the reaction, 1,000 μL of the mixtures containing 800 μL of 0.5 mM BAPNA and 200 μL of distilled water (pre-warmed to 37C) was added and vortexed immediately to start the reaction. After incubating for 10 min, 900 μL of 30% (v/v) acetic acid was added to terminate the reaction, and the reaction mixture was centrifuged at 8,000 × g for 5 min at room temperature (28–30C) (MIKRO200, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Residual activity of trypsin was measured by the absorbance at 410 nm due to *p*-nitroaniline released (Klomklao *et al*. 2011). One unit of proteolytic activity was defined as an increase of 0.01 absorbance unit/mL·min under the assay conditions. One unit of

trypsin inhibitory activity was defined as the amount of inhibitor, which reduces the enzyme activity by one unit.

Bigeye Snapper Preparation

Bigeye snapper (*P. macracanthus*) were caught from the Songkhla Coast along the Gulf of Thailand, stored in ice and off-loaded approximately 24–36 h after capture. Fish were transported in ice to the Department of Food Science and Technology, Thaksin University, Phatthalung, Thailand, within 2 h. Fish were then filleted, vacuum-packed and frozen at –20C until used for sarcoplasmic fluid preparation. To prepare fish mince, the fish were filleted and then ground through a 4-mm plate.

Preparation of Sarcoplasmic Proteinases (Sp)

Sp was prepared by centrifuging 50 g of finely chopped fillets of bigeye snapper at 5,000 × g for 30 min at 4C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT). The supernatant was used as Sp.

Washed Mince Preparation

Washed mince was prepared according to the method of Benjakul *et al.* (2001), with a slight modification. The comminuted flesh was homogenized with 5 volumes of 50 mM NaCl for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was centrifuged at 10,000 × g for 10 min at 4C using a Sorvall Model RC-B Plus centrifuge. The washing process was repeated twice. The final precipitate was designated as "washed mince."

TIYTR Inhibition of Bigeye Snapper Sarcoplasmic Proteinases

Sp was mixed with the partially purified trypsin inhibitor to obtain the final concentrations of 0.5, 1, 2 and 3% (w/v). Sp (100 μL) was incubated with the solution of trypsin inhibitor (100 μL) for 20 min at room temperature. The residual activity was determined using the casein–TCA–Lowry method as described by Klomklao *et al.* (2010a,b) at 60C for 30 min. The activity determined in the absence of the trypsin inhibitor was used as the control.

The commercially available protease inhibitors, namely BPP and dry EW powder, were used in comparison with TIYTR. For this, the Sp was mixed with TIYTR and commercial inhibitors to obtain the final concentration of 3% (w/v). Residual activity was determined as before using the casein–TCA–Lowry method at 60C for 30 min (Klomklao *et al.* 2010a,b). The percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(A_c - A_s)}{A_c} \times 100$$

where A_s and A_c are the proteolytic activity of sample treated with protease inhibitor and that of the control, respectively. The control was conducted in the same manner, except that deionized water was used instead of protease inhibitor.

Effect of TIYTR on Autolytic Activity

The inhibitory activity of TIYTR against autolysis of the mince and washed mince samples was measured according to the method of Morrissey *et al.* (1993). TIYTR at levels of 0, 0.5, 1, 2 and 3% (w/w) was added to 3 g of mince and washed mince. The mixture was then incubated in a water bath at 60C for 1 h and then the reaction was stopped by adding 27 mL of 5% cold TCA solution. The released TCA-soluble peptides were analyzed using the Lowry assay (Lowry *et al.* 1951). Inhibition of autolysis was expressed as percentage of autolytic activity inhibited, compared with that in the control (without trypsin inhibitor addition). % Inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Tyrosine released (without TIYTR)} - \text{Tyrosine released (with TIYTR)}}{\text{Tyrosine released (without TIYTR)}} \times 100$$

To monitor the protein pattern, another lot of sample was added with hot 5% SDS solution (85C) to terminate the reaction and solubilize total protein. All samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970).

BPP and EW powder were used in comparison with TIYTR. The mince or washed mince (3 g) samples were mixed with BPP or EW or TIYTR at level of 3% (w/w). Mince and washed mince added with and without protein additives were incubated for 60 min at 60C. The reaction was terminated by addition of 27 mL of cold 5% (w/v) TCA. The released soluble peptides were measured using the Lowry method (Lowry *et al.* 1951).

Effect of NaCl on Inhibition of Autolysis of Bigeye Snapper by TIYTR

TIYTR at level of 3% (w/w) was added to 3 g of the mince and washed mince samples in the presence and absence of 0.5–3% NaCl. The mixture was then incubated in a water bath at 60C for 1 h and then the reaction was stopped by adding 27 mL of 5% cold TCA solution. TCA-soluble peptides in the supernatant were analyzed using the Lowry assay (Lowry *et al.* 1951).

Sodium Dodecyl Sulfate–Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970). The samples subjected to different autolytic conditions were mixed with 27 mL of 5% SDS solution (85C). After incubating in an 85C water bath for 1 h, the samples were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer and boiled for 3 min. The sample (15 μg) was loaded on the gel made of 4% stacking and 10% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel at room temperature (28–30C) for 1.5 h using a Mini-Protean II cell apparatus (Atto Co., Tokyo, Japan). After electrophoresis, the gels were removed and stained with 0.2% Coomassie Brilliant Blue R-250 in a 45% methanol, 10% acetic acid and 45% H_2O staining solution, and destained with a destaining solution composed of 30% methanol and 10% acetic acid and 60% H_2O.

Protein Determination

Protein concentration was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Statistical Analysis

A completely randomized design was used throughout this study. Analysis of variance (ANOVA) was performed and mean comparisons were run by Duncan's multiple range test (Steel and Torrie 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

TIYTR Inhibition of Bigeye Snapper Sarcoplasmic Proteinases

The inhibition of proteolytic activity of Sp from bigeye snapper muscle by different concentrations of TIYTR is depicted in Fig. 1. The inhibitory activity of TIYTR increased with increasing concentrations from 0.5 up to 3%. At 3% (w/v) concentration, TIYTR inhibited bigeye snapper muscle's Sp activity by more than 60% (Fig. 1). Thus, TIYTR was able to inhibit the proteolytic activity of bigeye snapper muscle sarcoplasmic fluid appreciably. The proteolytic activity is attributed to trypsin-like enzymes because of the capacity to hydrolyze the synthetic substrate, DL-BAPNA. It is suggested that serine proteinases, mainly trypsin-like proteinases, major components of the sarcoplasmic proteinases in bigeye snapper muscle, caused the softening of muscle during extended iced storage. Also, they have been reported to play an important role in the degradation of bigeye snapper myofibrillar proteins during heat-induced gelation, which results in gel weakening (Benjakul *et al.* 2003a). Klomklao and Benjakul (2015) previously reported on the presence of a 70-kDa trypsin inhibitor in yellowfin tuna roe, which could play an important role in preventing proteolysis.

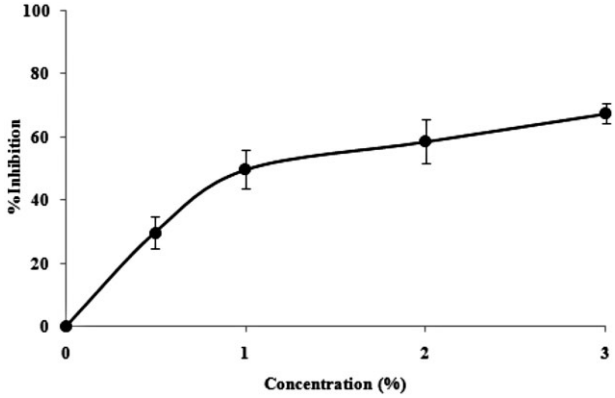

FIG. 1. EFFECT OF TRYPSIN INHIBITOR FROM YELLOWFIN TUNA ROE (TIYTR) AT DIFFERENT CONCENTRATIONS ON INHIBITION OF BIGEYE SNAPPER SARCOPLASMIC PROTEINASES
Error bars indicate standard deviations from triplicate determination.

Inhibition of Autolysis of Bigeye Snapper by TIYTR

Inhibitory of autolysis of both mince (unwashed) and washed mince from bigeye snapper by TIYTR is presented in Fig. 2. In general, higher autolytic inhibition was achieved with increasing TIYTR concentration ($P < 0.05$) within the inhibitor concentration range investigated. At the

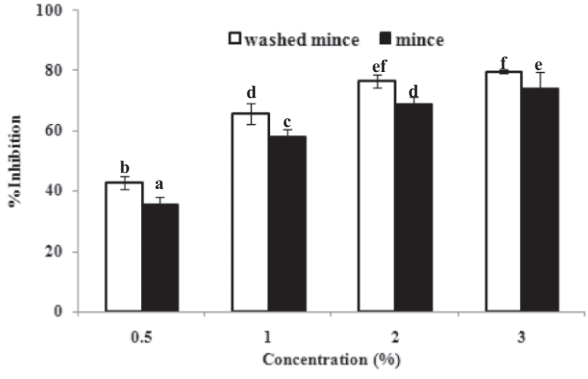

FIG. 2. EFFECT OF TRYPSIN INHIBITOR FROM YELLOWFIN TUNA ROE (TIYTR) AT DIFFERENT CONCENTRATIONS ON AUTOLYTIC INHIBITION OF BIGEYE SNAPPER MINCE AND WASHED MINCE
Error bars indicate standard deviations from triplicate determination. Different letters indicate significant differences ($P < 0.05$).

same concentration, TIYTR was slightly more effective in inhibiting autolysis of the washed mince samples compared with unwashed mince samples. TIYTR at the level of 3% showed the autolysis inhibition of 79.61 and 74.13% for washed mince and unwashed mince, respectively. This was possibly due to the fact that washed mince contained a lower amount of proteinases. Sarcoplasmic proteinases were leached out during the washing process (Benjakul *et al.* 2001). Nevertheless, myofibril-associated proteinases remained in the muscle and were directly associated with gel softening (Benjakul *et al.* 2003b). From the result, it is suggested that TIYTR functioned as an effective proteinase inhibitor to prevent the degradation of muscle proteins.

The autolytic patterns of mince and washed mince of bigeye snapper incubated at 60C for 60 min in the absence and presence of TIYTR at levels of 0.5, 1, 2 and 3% are shown in Fig. 3. For both the mince and the washed mince samples, a marked degradation of MHC was observed in the sample with no TIYTR, as indicated by the lowest band intensity. The result indicated that MHC is the primary target of proteolytic activity. As indicated by An *et al.* (1994), MHC and β-tropomyosin and troponin-T were more susceptible to degradation than actin. A structural disintegration of fish muscle generally occurs within the temperature range from 50 to 70C, called "modori." This phenomenon is induced by thermal-stable endogenous proteinases (Benjakul *et al.* 2004). Degradation of muscle proteins, especially MHC, in bigeye snapper mince and washed mince was found at 60C (Benjakul *et al.* 2003a). Although washing could remove some proteinases, particularly sarcoplasmic proteinases, those associated with myofibrils still remained and probably caused the degradation of MHC. MHC of mince or washed mince was more retained as the concentration of TIYTR increased. The presence and concentration of myosin determine the gel strength of surimi (Benjakul *et al.* 2004). The presence of TIYTR may protect myofibrillar proteins of bigeye snapper muscle by acting as a true inhibitor, which effectively decreased the proteolytic activity on myosin. From the results, the degradation caused by endogenous proteinases, especially trypsin or trypsin-like serine proteinases, could be impeded to some extent by the addition of TIYTR.

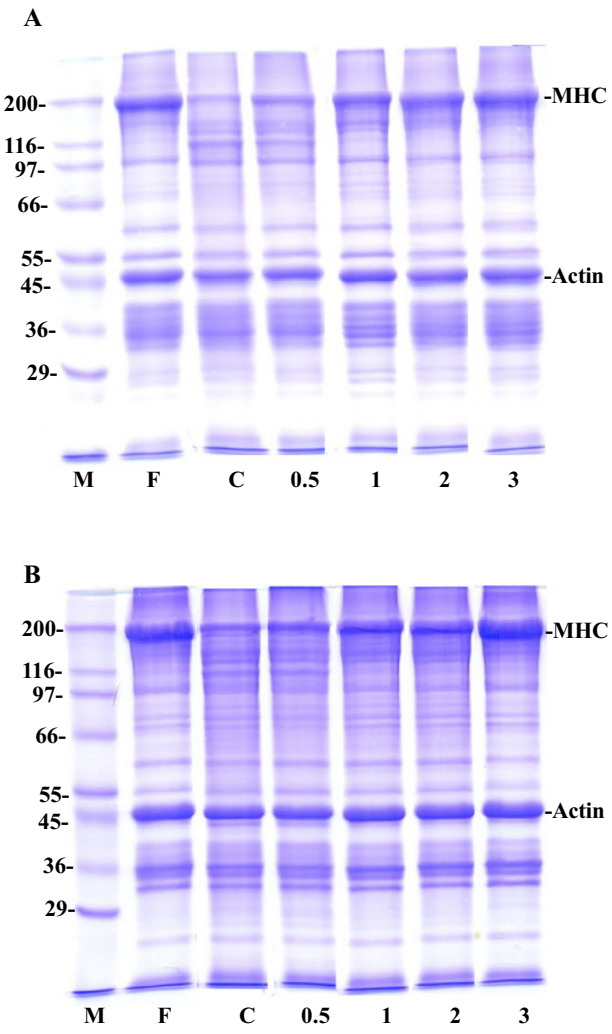

FIG. 3. AUTOLYSIS PATTERN OF BIGEYE SNAPPER MINCE (A) AND WASHED MINCE (B) IN THE ABSENCE AND PRESENCE OF TRYPSIN INHIBITOR FROM YELLOWFIN TUNA ROE (TIYTR) AT DIFFERENT CONCENTRATIONS AT 60C FOR 1 H
M, molecular weight standards; F, mince or washed mince without incubation (control); C, mince or washed mince without TIYTR. Numbers designate the concentration of TIYTR.
MHC, myosin heavy chain.

Effect of NaCl on Inhibition of Autolysis in Bigeye Snapper by TIYTR

Figure 4 illustrates the inhibition of autolysis of mince and washed mince of bigeye snapper at 60C for 60 min by TIYTR at 3% (w/w) in the absence and presence of NaCl at levels of 0.5, 1, 2 and 3% (w/w). In general, the presence of NaCl generally enhanced the inhibitory activity of TIYTR at the concentration levels investigated. It was inferred that NaCl solubilize muscle proteins, leading the exposure of proteinase associated with myofibrils. As a consequence, proteinase inhibitors could bind more proteinases, resulting in higher inhibition.

Comparison of Different Protease Inhibitors on Bigeye Snapper Proteinase Activity

The effects of different commercially available inhibitors on the activity of Sp and autolysis of bigeye snapper muscle mince and washed mince samples were compared. All the commercially available protease inhibitors showed

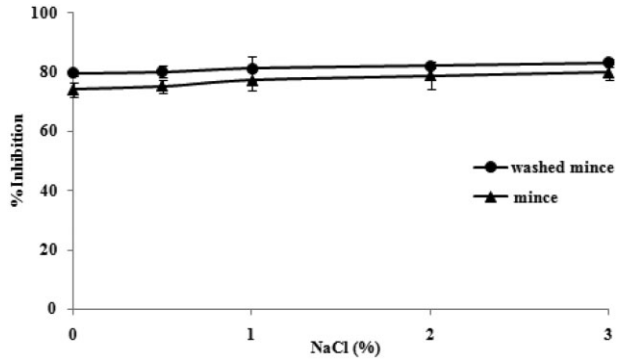

FIG. 4. EFFECT OF NaCl AT DIFFERENT CONCENTRATIONS ON BIGEYE SNAPPER AUTOLYTIC INHIBITION BY TRYPSIN INHIBITOR FROM YELLOWFIN TUNA ROE (TIYTR)
Error bars indicate standard deviations from triplicate determination.

inhibitory activity toward Sp and autolysis of bigeye snapper muscle mince and washed mince samples (Fig. 5). There was no difference in inhibitory activity against Sp and autolysis of bigeye snapper mince and washed mince between TIYTR and BPP ($P > 0.05$) (Fig. 5). The effect of EW on then inhibitory activity of Sp and autolysis was much lower than that of TIYTR and BPP ($P < 0.05$). The results indicated that TIYTR, BPP and EW all contained inhibitors, which were able to inhibit proteinase activities in bigeye snapper muscle. BPP was proven to be most effective in both inhibiting proteolytic activity and enhancing the gel strength of surimi (Benjakul *et al.* 2004). The proteinase inhibitory activity of BPP was reported to be due to alpha-2-macroglobulin (α_2-M) and kininogen (Morrissey *et al.* 1993). α_2-M acts as a nonspecific inhibitor for all classes of proteinases (Starkey and Barrett 1977), while kininogen is a specific cysteine proteinase inhibitor (Rawlings and Barrett 1990). EW showed inhibitory activity due to the presence of some proteinase inhibitors, such as ovoinhibitor and ovomacroglobulin, which can inhibit cysteine proteinases, serine proteinases and aspartic proteinases (Benjakul *et al.* 2004). Benjakul *et al.* (2004) further reported that BPP showed higher inhibition of lizardfish autolysis compared with EW. For yellowfin tuna roe, the major inhibitory component was identified as proteins with apparent molecular weight of 70 kDa, as shown on inhibitory activity-stained gels (Klomklao and Benjakul 2015). The differences in inhibition efficiency of different inhibitors were presumed to be due to the differences in specificities toward the proteinases tested (Benjakul *et al.* 2004). From the results, TIYTR showed a high capacity to inhibit autolysis of bigeye snapper muscle that TIYTR could be used as a cost-effective proteinase inhibitor in the formulation of surimi-type products.

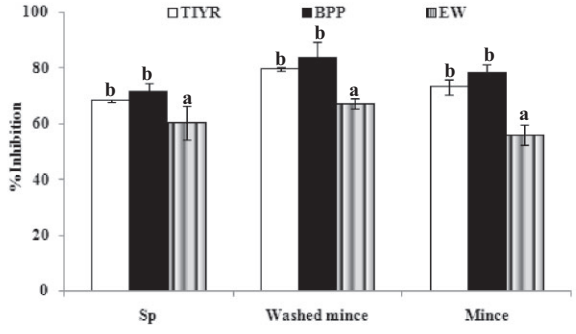

FIG. 5. EFFECT OF TRYPSIN INHIBITOR FROM YELLOWFIN TUNA ROE (TIYTR), BEEF PLASMA PROTEIN (BPP) AND EGG WHITE (EW) ON INHIBITION OF BIGEYE SNAPPER PROTEINASES
Error bars indicate standard deviations from triplicate determination. Different letters within the same sample indicate significant differences ($P < 0.05$).
Sp, sarcoplasmic proteinases.

CONCLUSION

TIYTR showed inhibition against both Sp and autolysis of bigeye snapper muscle. The inhibition toward proteinases in bigeye snapper muscle depended on the concentration of TIYTR used. It displayed effective control of proteolysis in bigeye snapper muscle, comparable to BPP and EW. Thus, TIYTR can potentially be used to inhibit proteolysis and enhance surimi gel strength.

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