Enzymatic hydrolysis of starry triggerfish (*Abalistes stellaris*) muscle using liver proteinase from albacore tuna (*Thunnus alalunga*)

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Abstract Proteinases from liver extract from albacore tuna (*Thunnus alalunga*) were used to produce protein hydrolysate from starry triggerfish (*Abalistes stellaris*) muscle. Hydrolysis conditions for preparing protein hydrolysate from starry triggerfish muscle were optimized. Enzyme level, reaction time and fish muscle/buffer ratio significantly affected the hydrolysis ($p < 0.05$). Optimum conditions for triggerfish muscle hydrolysis were 5.5 % liver extract, 40 min reaction time and fish muscle/buffer ratio of 1:3 (w/v). The freeze-dried protein hydrolysate was characterized with respect to chemical composition, amino acid composition and color. The product contained 91.73 % protein, 2.04 % lipid and 6.48 % ash. The protein hydrolysate exhibited high amount of essential amino acids (45.62 %). It was light yellow in color ($L^* = 82.94$, $a^* = 0.84$, $b^* = 22.83$). The results indicate that the extract from liver of albacore tuna could be used to produce fish protein hydrolysate and protein hydrolysate from

starry triggerfish muscle may potentially serve as a good source of desirable peptide and amino acids.

Keywords Protein hydrolysate · Proteinases · Tuna · Industrial application

Introduction

Proteins from fish processing byproducts can be modified to improve their quality and functional characteristics by enzymatic hydrolysis (Shahidi 1994). Utilizing proteolytic enzymes, fish protein hydrolysate (FPH) can be prepared with the peptides having new and/or improved properties. Generally, protein can be hydrolyzed by chemical process or protease enzymes. However, FPH obtained from these two methods are different in quality (Wisuthipheat and Kongruang 2015). Enzymatic hydrolysis of food proteins is an efficient way to recover potent bioactive peptides. Fish protein hydrolysates have been shown to have potential for nutritional or pharmaceutical applications (Thiansilakul et al. 2007; Wu et al. 2003). Protein hydrolysis decreases the peptide size, and thereby making hydrolysates the most available amino acid source for various physiological functions of human body. Protein hydrolysates are used as readily available sources of protein for humans and animals due to their good functional properties (Neklyudov et al. 2000).

The starry triggerfish (*Abalistes stellaris*), or flat-tailed triggerfish belongs to order Tetraodontiformes and is a member of the Balistidae family. Generally, it is widely used as fish meal. In Thailand, starry triggerfish has not been used for human consumption due to its tough skin. To increase the value of this fish species, the production of new value-added products such as protein hydrolysates, with nutritive value and bioactivity, can pave the way for its full utilization. Many factors

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affect the bioactivity of protein hydrolysates, e.g. type and concentration of proteinases (Benjakul and Morrissey 1997), steps of hydrolysis (Thiansilakul et al. 2007), etc. Protein hydrolysates have been produced and characterized from several sources, e.g. toothed ponyfish (*Gazza minuta*) (Klomklao et al. 2013), Catla (*Catla catla*) (Bhaskar and Mahendrakar 2008), yellowfin tuna (*Thunnus albacores*) (Guerard et al. 2001) and bigeye snapper (*Priacanthus macracanthus*) (Phanturat et al. 2010).

Fish viscera generated during processing contain a variety of enzymes including proteinases. Proteinases are potential enzymes for industrial applications and could produce the new bioactive molecules (Klomklao et al. 2005). Albacore tuna (*Thunnus alalunga*) is the potential raw materials for canned production in Thailand (Nalinanon et al. 2008). Processing wastes generated during canned tuna processing, especially viscera can be used as the essential source of proteinases. Sripokar et al. (2015) reported that the liver of albacore tuna contained high proteolytic activity and major proteinases were heat-activated alkaline proteinases, most likely trypsin-like serine proteinases.

Recovery and use of proteinases from fish viscera for fish protein hydrolysate are a promising approach to minimize the economics and ecologicals problem of this processing waste. Also, the production of protein hydrolysate from fish muscle using albacore tuna liver extract has not been reported. Hence, the purpose of this work was to study the production and characterization of hydrolysate from starry triggerfish muscle using albacore tuna liver extract.

Materials and methods

Chemical

β -Mercaptoethanol (β ME), 2,4,6-trinitrobenzenesulfonic acid (TNBS), *L*-leucine and bovine serum albumin were procured from Sigma Chemical Co. (St. Louis, MO, USA). Tris (hydroxymethyl) aminomethane (Tris-HCl), sodium sulphite and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) and Coomassie Brilliant Blue R-250 were procured from Bio-Rad Laboratories (Hercules, CA, USA). All of chemicals used were of analytical grade.

Fish sample preparation

Albacore tuna (*Thunnus alalunga*) internal organs were obtained from Tropical Canning (Thailand) Public Co. Ltd., Hat Yai, Songkhla. The samples were packed in polyethylene bags, kept in ice with a sample/ice ratio of 1:3 (w/w) and transported to the research laboratory within 2 h. Pooled

internal organs were separated and only the liver was collected, immediately frozen and stored at -20°C until used.

Starry triggerfish (*Abalistes stellaris*) with the length of 30–35 cm were purchased from the dock in Trang, Thailand. The fish, off-loaded approximately 18–24 h after capture, were placed on ice at a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Science and Technology, Thaksin University, Phatthalung, within 2 h. Upon arrival, the fish were filleted and the ordinary muscle was collected and ground to uniformity. A portion of mince (500 g) was placed in a polyethylene bag and stored at -20°C until used.

Preparation of liver extract

Frozen livers were thawed using running water ($26\text{--}28^{\circ}\text{C}$) 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 into powder in three volumes of acetone at -20°C for 30 min according to the method of Klomklao et al. (2007). The homogenate was filtrated in vacuum on Whatman No. 4 filter paper. The residue obtained was then homogenized in two volumes of acetone at -20°C for 30 min. The residue was left at room temperature until dried and free of acetone odor.

To prepare the liver extract, defatted liver powder was suspended in 50 mM Na-phosphate, pH 7.0 at a ratio of 1:9 (w/v) and stirred continuously at 4°C for 30 min. The suspension was centrifuged at $5000\times g$ for 30 min at 4°C using a Sorvall Model RC-B Plus centrifuge (Newtown, CT, USA). The supernatant obtained was collected and referred to as "liver extract".

Study on the optimum condition for production of starry triggerfish protein hydrolysate using tuna liver extract

Effect of extract concentration on DH

Effect of liver extract concentrations (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 10, 15 and 20 % (w/w)) on degree of hydrolysis (DH) was studied. Different amounts of liver extract were added to the suspension of starry triggerfish mince in 0.1 M glycine-NaOH buffer, pH 8.5 (1:2 ratio, w/v) and the reaction was carried out at 55°C , for 30 min (Sripokar et al. 2015). The termination of hydrolytic reaction was done by heating the reaction mixture at 90°C for 15 min in a water bath. The supernatant was obtained by centrifuging at $5000 g$ for 10 min. DH was then determined. The extract concentration, which was able to hydrolyze fish mince with the highest DH, was selected for further study.

Effect of reaction time on DH

To study the effect of reaction time on DH, starry triggerfish mince was added with 0.1 M glycine-NaOH buffer (pH 8.5) at

the ratio of 1:2 (w/v). The reaction was initiated by adding 5.5 % liver extract. The mixtures were shaken at 55 °C for 5, 10, 15, 20, 30, 40, 60, 80 and 100 min. At designated time, the reaction was stopped and the supernatant was prepared in the same manner as previously described. DH was then determined. The reaction time rendering the highest DH was chosen for further steps.

Effect of starry triggerfish muscle and buffer ratio on DH

Starry triggerfish mince was mixed with 0.1 M glycine-NaOH buffer, pH 8.5 at a ratio of 1:0.5, 1:1, 1:2, 1:3, 1:4 and 1:5 (w/v). Liver extract at a level of 5.5 % was added and the reaction was maintained for 40 min at 55 °C. The hydrolysate obtained was determined for DH. The buffer ratio used for mixing the mince showing the highest DH was selected for further study.

Determination of α -amino acids and DH

The α -amino acid content was determined according to the method of Klomklao et al. (2013). Properly diluted hydrolysate samples (125 μ l) were mixed thoroughly with 2.0 ml of 0.2125 M phosphate buffer, pH 8.2, followed by the addition of 1.0 ml of 0.01 % TNBS solution. The mixtures were then placed in a temperature controlled water bath (Model W350, Memmert, Schwabach, Germany) at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled at ambient temperature for 15 min. The absorbance was measured at 420 nm and α -amino acid was expressed in term of *L*-leucine. DH was calculated as follows:

$$DH = [(L_t - L_0) / (L_{max} - L_0)] \times 100$$

where L_t is the amount of α -amino acid released at time t . L_0 is the amount of α -amino acid in the original starry triggerfish muscle homogenate. L_{max} is total α -amino acid in the original starry triggerfish muscle homogenate obtained after acid hydrolysis with 6 N HCl at 100 °C for 24 h.

Production of starry triggerfish protein hydrolysate

Starry triggerfish mince (20 g) was mixed with 0.1 M glycine-NaOH buffer, pH 8.5 at a ratio of 1:3 (w/v) and pre-incubated at 55 °C for 10 min (Klomklao et al. 2013; Sripokar et al. 2015). The enzyme hydrolysis was started by adding albacore tuna liver extract at a level of 5.5 % (w/w). The reaction was conducted at pH 8.5 and 55 °C for 40 min. After 40 min of hydrolysis, the enzyme was inactivated by heating at 90 °C for 15 min in a water bath. The mixture was then centrifuged at 5000 g at 4 °C for 10 min and the

supernatant was collected. Starry triggerfish protein hydrolysate was freeze-dried using a Dura-Top™ μ p freeze-dryer (FTS systems Inc., Stone Ridge, NY, USA). The freeze-dried starry triggerfish protein hydrolysate obtained was subjected to analyses.

Proximate analysis

Moisture, protein, fat and ash contents were determined according to the method of AOAC (2005).

Amino acid analysis

Amino acid compositions of freeze-dried hydrolysate were determined according to the method of Cohen and Michaud (1993). Hydrolysate (10 μ g) was dissolved in 10 mM HCl (10 μ l) and treated with 0.2 M borate buffer pH 9.3 (30 μ l). The sample solution was reacted with 10 mM 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (10 μ l) to form stable unsymmetric urea derivatives. Amino acids were separated by reverse phase HPLC (AccQ Tag column, Waters, Milford, MA, USA) at 37 °C, using gradient mobile phase: deionized water, acetonitrile and eluent A (sodium acetate, phosphoric acid, triethylamine) and detected by a UV detector (Waters 486, Milford, MA, USA) at 254 nm and a fluorescence detector (Jasco FP-920, Great Dunmow, Essex, UK) with excitation wavelength at 250 nm and emission wavelength at 395 nm. The amount of amino acids was calculated, based on the peak area in comparison with that of standard. The amino acid content was expressed as a percentage of total amino acids in the sample.

Color measurement

The color of freeze-dried hydrolysate was measured by the Hunter lab colorimeter and reported by the CIE system. L^* , a^* and b^* parameters, indicating lightness, redness and yellowness, respectively.

Statistical analysis

Experiments were run in triplicate. All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test. For proximate composition, the independence t-test was used for pair comparison (Steel and Torrie 1980). Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows; SPSS Inc., Chicago, IL, USA).

Results and discussion

Optimum condition for starry triggerfish protein hydrolysate production using albacore tuna liver extract

Enzymatic hydrolysis is influenced by several factors like temperature, pH, enzyme to substrate level and time that cooperatively influence the enzyme activity thereby making the process more controllable (Liaset et al. 2000). Based on my previous study, liver proteinase from albacore tuna had optimum pH and temperature at 8.5 and 55 °C, respectively. An appreciable decrease in enzyme activity was observed at temperature above 55 °C, due to thermal denaturation (Sripokar et al. 2015). The optimal pH and temperature were 9.0 and 50 °C, respectively, when toothed ponyfish (*Gazza minuta*) muscle was used as a substrate (Klomklao et al. 2013). Alcalase had maximal activity at pH 8.5 and 55 °C for hydrolysis of Persian sturgeon viscera (Ovissipour et al. 2009). Benjakul and Morrissey (1997) found that Alcalase and Neutrase showed optimum activity against Pacific whiting solid wastes at pH 9.5, 60 °C and pH 7.0, 55 °C, respectively. Therefore, pH 8.5 and temperature 55 °C was chosen as the optimal condition for protein hydrolysate production from starry triggerfish using liver proteinase from albacore tuna.

Effect of liver extract concentration on DH

Degree of hydrolysis (DH), which indicates the percentage of peptide bonds cleaved (Adler-Nissen 1986), is essential because several properties of protein hydrolysates are closely related to DH (Nielsen 1997). The DH of starry triggerfish muscle treated with tuna liver extract increased when the liver extract level was increased (Fig. 1). Significant changes in DH occurred with the liver extract treatment at levels ranging from 0.5 to 5.5 % ($p < 0.05$). However, no increase for DH was found with treatment of liver extract at level above 5.5% (Fig. 1). The value of DH in this study was similar to that obtained in toothed ponyfish hydrolysate treated with catfish viscera extract (Klomklao et al. 2013). Shahidi et al. (1995) reported that considerable soluble protein was released during initial phase and no increase in soluble hydrolysate was observed when additional enzyme was added during the stationary phase of hydrolysis. Morr et al. (1985) suggested that, the overall reaction involved at least two steps. In the first step, the enzyme molecules become associated with and bound to the fish particles. Subsequently, hydrolysis took place, resulting in the release of soluble peptides and amino acids.

When $\log_{10}$ (liver extract concentration) was plotted against DH, a linear relationship and the regressive equations were obtained (Fig. 2). Klomklao et al. (2013) found a similar linear relationship between %DH and log of enzyme

concentration, when hydrolyzing toothed ponyfish muscle with catfish viscera extract. The relationship is further supported by Benjakul and Morrissey (1997) for Pacific white shrimp protein hydrolysate derived with Alcalase and Neutrase and Thiansilakul et al. (2007) for round scad derived with Flavourzyme. From the regression, amount of liver extract required to prepare starry triggerfish muscle protein hydrolysate with required DH could be calculated.

Effect of reaction time on DH

Figure 3 shows the effect of hydrolysis time on DH of starry triggerfish muscle using albacore tuna liver extract. A high rate of hydrolysis was obtained during the initial stage (10–40 min), owing to a large number of peptide bonds available. After 40 min of hydrolysis, the enzymatic reaction more likely reached the steady-state phase. The stability in the hydrolysis rate, observed in the later stage, was mainly due to a decrease in available substrate, enzyme auto-digestion and product inhibition (Khantaphant et al. 2011). Generally, the enzyme absorbs rapidly onto the insoluble protein particles and the polypeptide chains that are loosely bound to the surface are then cleaved. The more compacted core proteins are hydrolyzed more slowly. The rate of enzymic cleavage of peptide bond controls the overall rate of hydrolysis (Benjakul and Morrissey 1997). Fish gelatin was hydrolyzed using proteases from hepatopancreas of Pacific white shrimp, a rapid hydrolysis was found within the first 30–40 min, followed by a slower hydrolysis rate up to 120 min (Senphan and Benjakul 2014). Ovissipour et al. (2009) produced protein hydrolysates from Persian sturgeon (*Acipenser persicus*) viscera by optimizing the hydrolysis time. The highest DH%, 46.13 was achieved at 55 °C after 205 min. Klomklao et al. (2013) investigated the enzymatic hydrolysis of toothed ponyfish (*Gazza minuta*) muscle with viscera extract from hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). A rapid reaction rate of both DH and NR was obtained in the first 15 min. From the results, the reaction time of 40 min was found to be optimal for starry triggerfish protein hydrolysate production.

Effect of fish muscle/buffer ratio on DH

The effect of substrate/buffer ratio on hydrolysis of starry triggerfish muscle using albacore tuna liver extract is shown in Fig. 4. In general, an increase in starry triggerfish mince/buffer resulted in an increase in DH. The ratio above 1:3 did not cause a significant increase in DH ($p > 0.05$). Sufficient buffer gave buffering capacity for the reaction, worked as media for enzyme dispersion and was considered as an important factor for starry triggerfish muscle hydrolysis. From the results, fish muscle/buffer ratio of 1:3 (w/v) was sufficient for enzymatic reaction. Increased water or buffer added to

Fig. 1 Effect of albacore tuna liver extract concentration on DH of starry triggerfish muscle. Bars represent the standard deviation from triplicate determinations

substrate enhanced enzyme homogeneity, promoted tissue swelling, and reduced the localized concentration of hydrolysis products (Surowka and Fik 1994). Klomklao et al. (2013) used catfish viscera extract to recover the proteinases substances from toothed ponyfish muscle and found that an increase in the ratio of added buffer resulted in an increase in DH. Benjakul and Morrissey (1997) reported that an increase in Pacific whiting solid waste/buffer ratio resulted in an increase in α -amino acid concentration as well as NR.

Proximate composition of protein hydrolysate

The proximate composition of starry triggerfish muscle and freeze-dried starry triggerfish protein hydrolysate are depicted in Table 1. The freeze-dried hydrolysate and starry triggerfish muscle contained 10.86 % and 78.27 % moisture, respectively. On dry weight basis, freeze-dried hydrolysate contained higher protein content but lower lipid content than starry triggerfish muscle. The high protein content reported for fish protein hydrolysates is due to the solubilization of proteins

Fig. 2 Relation between $\log_{10}$ (extract concentration) and DH for starry triggerfish muscle treated with albacore tuna liver extract

Fig. 3 Effect of hydrolysis time on DH for starry triggerfish muscle with albacore tuna liver extract. Bars represent the standard deviation from triplicate determinations

during hydrolysis and the removal of insoluble solid matter by centrifugation (Chalamaiah et al. 2010). High protein content of fish protein hydrolysates demonstrates its potential use as protein supplements for human nutrition (Chalamaiah et al. 2012). The low fat content of fish protein hydrolysates is because of removal of lipids with insoluble protein fractions by centrifugation (Chalamaiah et al. 2012). Decreasing lipid content in the protein hydrolysates might significantly increase stability of the material towards lipid oxidation, which may also enhance the product stability (Kristinsson and Rasco 2000). During the hydrolysis process, the muscle cell membranes tend to round up and form insoluble vesicles, leading to the removal of membrane structured lipid (Shahidi et al.

1995). The freeze-dried protein hydrolysate had a higher ash content (6.48 %) than did starry triggerfish mince (3.71 %). The relatively high ash content of fish protein hydrolysates is due to the use of glycine-NaOH buffer during the enzymatic reaction and usage of added acid or base for adjustment of pH of medium (Choi et al. 2009).

Amino acid composition

Table 2 shows the amino acid compositions of the freeze-dried starry triggerfish protein hydrolysate. Protein hydrolysate was rich in glycine, lysine and leucine, which accounted for 15.33, 11.67 and 10.27 % of the total amino acids, respectively. The

Fig. 4 Effect of fish muscle/buffer ratio on DH for starry triggerfish muscle with albacore tuna liver extract. Different letters indicate significant differences ($p < 0.05$). Bars represent the standard deviation from triplicate determinations

Table 1 Proximate composition of freeze-dried hydrolysate produced from starry triggerfish muscle and starry triggerfish muscle

Compositions	Freeze-dried hydrolysate	Starry triggerfish muscle
Protein ^a	91.73 ± 1.16b ^{***}	84.66 ± 2.04a
Lipid ^a	2.04 ± 1.07a	11.89 ± 4.79b
Ash ^a	6.48 ± 1.55b	2.69 ± 1.52a
Moisture	10.86 ± 2.65a	78.27 ± 0.32b

Mean ± SD from triplicate determination

^a Dry weight basis^{***} The different letters in the same row denote the significant differences ($p < 0.05$)

nutritive value of any ingredient depends on the proteins capacity to fulfill the needs of organisms with respect to essential amino acids. From the result, the protein hydrolysate had an essential amino acid/non-essential amino acid ratio of 0.84. Fish and shellfish have been reported to contain the high essential amino acid/non-essential amino acid ratio (Twasaki and Harada 1985). Protein hydrolysate from toothed ponyfish (*Gazza minuta*) muscle had an essential amino acid/non-essential amino acid ratio of 0.93 (Klomklao et al. 2013). Thiansilakul et al. (2007) reported that the round scad protein hydrolysate had an essential amino acid/non-essential amino acid ratio of 0.92. The ratio of essential amino acid/non-essential amino acid of protein hydrolysate from yellowfin tuna (*Thunnus albacores*) head using Alcalase and Protamax was

Table 2 Amino acid composition of freeze-dried hydrolysate produced from starry triggerfish muscle

Amino acids	Content (%)
Essential amino acids	
Isoleucine	3.72
Leucine	10.27
Lysine	11.67
Methionine	4.85
Phenylalanine	3.47
Threonine	5.77
Valine	5.87
Non-essential amino acids	
Alanine	9.11
Arginine	3.77
Aspartic acid/Asparagine	2.97
Cysteine	0.6
Glutamic acid/Glutamine	8.2
Glycine	15.33
Histidine	2.89
Proline	3.07
Serine	4.52
Tyrosine	3.93

1.04 and 1.43, respectively (Ovissipour et al. 2010). Starry triggerfish protein hydrolysate contained a low level of proline (3.07 %). The presence of proline residues in the centre of the peptides generally contributes to the bitterness. Therefore the peptidase, which can cleave the hydrophobic amino acids and proline, is capable of debittering protein hydrolysate (Capiralla et al. 2002). Hevia and Olcott (1977) reported that bitter peptide from ficin-treated fish concentrate contained glycine, isoleucine, phenylalanine and valine. Thiansilakul et al. (2007) reported that bitter peptide from round scad protein hydrolysate contained leucine, valine, phenylalanine and isoleucine. Since starry triggerfish protein hydrolysate had a high protein content and high essential amino acid/non-essential amino acid ratio, it could be used as a source of protein ingredient for industrial applications.

Color

Color influences the overall acceptability of food products. The freeze-dried protein hydrolysate was light yellow in color ($L^* = 82.94$, $a^* = 0.84$, $b^* = 22.83$) (Table 3). The result was in agreement with Sathivel et al. (2003) who reported that protein hydrolysate from whole herring (*Clupea harengus*) was light yellow in color ($L^* = 89.4$, $a^* = 3.3$, $b^* = 8.0$). However, Thiansilakul et al. (2007) found that round scad protein hydrolysate was brownish yellow in color ($L^* = 58.00$, $a^* = 8.38$, $b^* = 28.32$). The dark color of fish protein hydrolysate was probably from the oxidation of myoglobin and the melanin pigment of the raw materials (Benjakul and Morrissey 1997). Therefore, the varying color of fish protein hydrolysate depended on the composition of the raw material and the hydrolysis condition.

Conclusion

The results of this research clearly revealed that low valued marine fish had a potential to be utilized as protein source for producing protein hydrolysates. Optimum conditions for triggerfish muscle hydrolysis were, 5.5 % liver extract from albacore tuna, 40 min reaction time and fish muscle/buffer ratio of 1:3 (w/v). Hydrolysate from triggerfish muscle may

Table 3 L^* , a^* and b^* -values of freeze-dried hydrolysate produced from starry triggerfish muscle

Color characteristics	Freeze-dried hydrolysate
L^*	82.94 ± 2.45
a^*	0.84 ± 1.87
b^*	22.83 ± 0.64

Values are mean ± SD from triplicate determination

potentially serve as a good source of desirable peptides and amino acids.

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