

Extraction of gelatin from bigeye snapper (*Priacanthus tayenus*) skin for gelatin hydrolysate production

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Abstract: Bigeye snapper skin was pretreated with 0.025 N NaOH for 2 h and 0.02 M acetic acid for 2 h. Minced pretreated fish skin extracted at 80, 90, 100°C for 1, 2, 3 h and 110, 120, 130°C for 30 min were compared. The result showed that protein content and hydroxyproline (Hyp) yield of skin gelatin increased with increasing extraction temperature and time ($p < 0.05$). The samples extracted at 120 or 130°C for 30 min and 100°C for 3 h showed the highest Hyp yield (76.36, 68.44 and 68.24%, respectively). Peptide bonds hydrolyzed, expressed as degree of hydrolysis, of samples ranged from 3.54 to 4.92%. SDS-PAGE pattern of gelatin indicated that hydrolysis of gelatin occurred during extraction at 100 to 130°C. The degradation of the fish gelatin resulted in the low bloom strength of gelatin gel. Bloom strength and L^* , a^* , b^* values of gelatin gel (6.67%, pH 7) extracted at 120°C, 30 min were 62.6 g and 32.58, -1.67, 6.14, respectively. The solubility of gelatin was greater than 85% at all pH treated (1-10).

Keywords: Gelatin extraction, fish skin, bigeye snapper, hydroxyproline, gel

Introduction

Gelatin is an ingredient widely used in food, pharmaceutical, cosmetic, and photographic industries because of its unique functional and technological properties (Riaz and Chaudry, 2004; Gimenez *et al.*, 2005; Karim and Bhat, 2009). Commercial gelatins are mostly obtained from pig and cow skins and bones. However, the use of gelatin from those resources is restricted due to the outbreaks of bovine spongiform encephalopathy (BSE) or “mad cow disease” and religion reasons. Therefore, there is an increasing interest in the production of fish gelatin as an alternative for mammalian counterpart (Jamilah and Harvinder, 2002). Due to its low gel strength of fish gelatin with low gelling temperature, it has been used for film preparation (Jongjareonrak *et al.*, 2006). Additionally, the production of hydrolysate from fish gelatin containing bioactive peptides can be an alternative way to exploit fish gelatin (Kim *et al.*, 2001).

Fish skin gelatin hydrolysates have been found to have noticeable antioxidant activities and might be a candidate to be a natural antioxidant (Kim *et al.*, 2001; Wu *et al.*, 2003; Mendis *et al.*, 2005; Yang *et al.*, 2008). It had been reported that skin gelatin hydrolysates from cobia (Yang *et al.*, 2008) and bigeye snapper (Phanturat *et al.*, 2010) contained antioxidant peptides, with noticeable free-radical scavenging activities and reducing power, while those from Alaska pollack (Kim *et al.*, 2001), hoki fish (Mendis *et al.*, 2005) and cobia showed linoleic

acid peroxidation exhibition (Yang *et al.*, 2008).

Bigeye snapper (*Priacanthus* spp.) is an important fish used for surimi production in Thailand (Benjakul *et al.*, 2009). During surimi processing, a large amount of skin generated as low-value by-products. Therefore, production of fish skin gelatin or gelatin hydrolysate can be a promising way to add market value to the product.

Most previous studies of extraction fish skin gelatin mainly focused on mild treatment process to maintain the functional properties of gelatin, such as Górmuez-Guillén (2005) extract gelatin from dover sole skin at 45°C for 16-18 h and Jongjareonrak *et al.* (2006) extracted bigeye snapper skin at 45°C for 12 h. Moreover, the studies of gelatin extraction from cod skin at 45-100°C for 15-120 min (Kołodziejaska *et al.*, 2008), skates skin at 40-70°C for 3-6 h (Cho *et al.*, 2006) and grass carp skin at 40-80°C for 6-24 h (Kasankala *et al.*, 2007) showed that gelatin yield increased with increasing in extraction time and temperature. The aims of this study were to extract bigeye snapper skin gelatin as a substrate for gelatin hydrolysate production using high extraction temperatures and to characterize its properties.

Materials and Methods

Chemicals

Bovine serum albumin and protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250 and N,N,N',N'-tetramethyl ethylene

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diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Type I collagen from calf skin was purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Food grade acetic acid and sodium hydroxide was obtained from local supplier. All the chemicals used were of analytical grade.

Fish skin preparation

Bigeye snapper (*Priacanthus tayenus*) skins were obtained from Songkhla dock, Songkhla, Thailand. Residual meat was removed manually and cleaned fish skin was washed with tap water. The skin was cut into small pieces ($1.0 \times 1.0 \text{ cm}^2$), placed in polyethylene bag and stored at -20°C for not longer than 3 months.

Gelatin extraction

The skin was pretreated by 2 processes according to Kittiphattanabawon (2004). To remove non-collagenous proteins, skin was soaked in 0.025 N NaOH at a skin/alkaline solution ratio of 1:10 (w/v) with the continuous stirring for 2 h. The alkaline solution was changed every 1 h. Then, the deproteinised skin was washed with tap water until neutral or faintly basic pH of wash water was obtained.

For swelling process, deproteinised skin was then soaked in 0.02 M acetic acid at a skin/acid solution ratio of 1:10 (w/v) with the continuous stirring for 2 h. The acid solution was changed every 40 min. Then, the swelled skin was washed with tap water until neutral or faintly basic pH of wash water was obtained.

Pretreated fish skin (PFS) was extracted by distilled water at a skin/water ratio of 1:10 (w/v) at 80 and 90°C in water bath (Model W350, Memmert, Schwabach, Germany), $100 \pm 2^\circ\text{C}$ (manual control) for 1, 2, 3 h with continuous stirring and 1:2 (w/v) at 110, 120, 130°C for 30 min in autoclave (Model SX-500, Tomy, Tokyo, Japan). The extracts were then filtered using two layers of cheesecloth and subjected to analysis.

Determination of protein content

Protein content in the supernatant was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard.

Determination of hydroxyproline (Hyp) yield

Hyp content of skin and extracts were determined according to the method of Bergman and Loxley (1963) with a slight modification (Nalinanon *et al.*, 2007). The samples were hydrolysed with 6 M HCl at 110°C for 24 h in an oil bath (model B-490, BUCHI,

Flawil, Switzerland). The hydrolysate was clarified with activated carbon and filtered through Whatman No. 4 filter paper. The filtrate was neutralized with 10 M and 1 M NaOH to obtain a pH 6.0-6.5. The neutralize sample (0.1 ml) was transferred into a test tube and isopropanol (0.2 ml) was added and mixed well; 0.1 ml of oxidant solution (mixture of 7% (w/v) chlororamine T and acetate/citrate buffer, pH 6, at a ratio of 1:4 (v/v)) was added and mixed thoroughly; 1.3 ml of Ehrlich's reagent solution (mixture of solution A; 2 g of p-dimethyl-amino-benzaldehyde in 3 ml of 60% (v/v) perchloric acid (w/v)) and isopropanol at a ratio of 3:13 (v/v) were added. The mixture was agitated and heat at 60°C for 25 min in the water bath (Model W350, Memmert, Schwabach, Germany) and then cooled for 2-3 min in running water. The solution was diluted to 5 ml with isopropanol. Absorbance was measured against water at 558 nm using a double-beam spectrophotometer (UV-16001, SHIMADZU, N.S.W., Australia). A Hyp standard solution, with concentration ranging from 10-60 ppm, run simultaneously. Hydroxyproline content was calculated and express as $\mu\text{g/g}$ sample. Hyp Yield of sample was calculated based on hydroxyproline content in gelatin in comparison with that of 100 g of pretreated fish skin (PFS).

Determination of degree of hydrolysis

The α -amino acid content was determined according to the method of Benjakul and Morrissey (1997). To properly diluted hydrolysate samples (125 μl), 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.01% TNBS solution were added. The solution was mixed thoroughly and 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 room temperature for 15 min. The absorbance was read at 420 nm and α -amino acid was expressed in terms of L-leucine. DH was calculated as follows:

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

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 gelatin. L_{max} is total α -amino acid in the original gelatin obtained after acid hydrolysis (6 M HCl at 100°C for 24 h).

Electrophoretic analysis

Protein patterns of gelatin and gelatin gel samples were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The

samples (1 g) were dissolved in 10 ml of 5% (w/v) SDS solution. The mixture was heated at 85°C for 1 h in a water bath to dissolve total proteins. Supernatants were collected after centrifuging at 3000 g for 3 min. The supernatants were then mixed with sample buffer (0.5M Tris-HCl, pH 6.8 containing 4% (w/v) SDS and 20% (v/v) glycerol at the ratio of 1:1 (v/v)). Samples (20 µg protein) were loaded into the polyacrylamide gel made of 5% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15mA per gel using a Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Calf skin acid-soluble type I collagen (Sigma Chemical Co., St. Louis, Mo., USA) was used as a standard collagen.

The gelatin extracted by the condition giving the highest Hyp yield was dried in hot air oven (Model FD115, BINDER, Tuttlingen, Germany) at $50 \pm 2^\circ\text{C}$. The dried gelatin with moisture content of 1-2% was subjected to further analysis.

Solubility of skin gelatin

The effect of pHs on gelatin solubility was determined by the method of Montero *et al.*, (1991) with a slight modification (Kittiphattabawon, 2004). The dried gelatin was dissolved in distilled water at 60°C to obtain a final concentration of 2% (w/v) and the mixture was stirred at room temperature until the gelatin was completely solubilised. The gelatin solution was adjusted to different pHs (1–10) with either 6 N NaOH or 6 N HCl. The volume of solution was made up to 10 ml with distilled water, previously adjusted to the same pH of gelatin solution. The solution was centrifuged at 8500 g at room temperature for 10 min. Protein content in the supernatant was determined by the Biuret method (Robinson and Hodgen, 1940) using bovine serum albumin as a standard. Relative solubility was calculated in comparison with the 2% (w/v) gelatin solution.

Gelatin gel properties

Bloom strength

Gelatin gel was prepared according to the British Standard 757:1975 method (BSI (British Standards Institution), 1975) with a slight modification. Gelatin (2.0 g) was mixed with 30 ml of distilled water in a 50 ml-beaker (PYREX[®], USA) to obtain a final concentration of 6.67% (w/v). The mixture was stirred thoroughly and left at room temperature for 30 min to allow the gelatin to absorb water and swell.

The mixture was then incubated at 42°C for 30 min in a temperature-controlled water bath with occasional stirring. The beakers were then kept in a temperature-controlled chamber at 10°C and allowed to stand for 16–18 h before determination of bloom gel strength.

Bloom gel strength at 10°C was determined by a Model TA-XT2i Texture analyzer (Stable Micro System, Surrey, UK) using a load cell of 5 kg equipped with a 1.27 cm diameter flatfaced cylindrical Teflon[®] plunger. The dimensions of the sample were 3.8 cm in diameter and 2.7 cm in height. The maximum force (in grams) taken, when the penetration distance of 4 mm was obtained, was recorded. The speed of the plunger was 0.5 mm/s.

Turbidity of gelatin solution

Gelatin solution was prepared by the method of Fernández-Díaz *et al.* (2001) with a slight modification (Kittiphattabawon, 2004). Gelatin was dissolved with distilled water at 60°C to obtain the final concentration of 6.67% (w/v). The solution was stirred until the gelatin was solubilised completely. Turbidity of gelatin solution was measured by reading the percent transmittance at 360 nm using a double-beam spectrophotometer (UV-16001, SHIMADZU, N.S.W., Australia).

Color measurement of gelatin gel

The gelatin gel was prepared as described in gel preparation for bloom strength measurement. The color of gelatin gel was measured by a Hunter lab colour metre (ColorFlex, HunterLab Reston, USA) and reported by the CIE system. L^* , a^* and b^* parameters indicate lightness, redness/greenness and yellowness/ blueness, respectively.

Statistical analysis

All experiments were run in duplicate. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 10.0 for window, SPSS Inc, Chicago, IL).

Results and Discussion

Hydroxyproline yield and protein content

The Hyp yield and protein content of gelatin extracted at different conditions were shown in Table 1. The highest yield was obtained from the extraction conditions at 120 or 130°C for 30 min and 100°C for 3 h (76.36, 68.44 and 68.24%, respectively). The Hyp yield was much higher than that of bigeye snapper (*P. tayenus*) skin extracted at 45°C for 12 h (22.2%) and

Table 1. Hydroxyproline (Hyp) yield, protein content and degree of hydrolysis of gelatin extracted from bigeye snapper skin at various temperatures and times

Extraction condition		Hyp Yield (%)	Protein content extracted from 100 g of PFS (g)	Degree of hydrolysis (%)
Temperature (°C)	Time (h)			
80	1	50.79 ± 1.78 ^{de}	14.85 ± 1.11 ^e	4.01 ± 0.10 ^b
	2	57.06 ± 5.06 ^{cde}	18.76 ± 0.34 ^{cd}	4.40 ± 0.52 ^{ab}
	3	53.36 ± 10.73 ^{cde}	18.83 ± 0.62 ^{cd}	4.19 ± 1.16 ^{ab}
90	1	61.22 ± 1.61 ^{bc}	19.36 ± 1.96 ^{cd}	4.37 ± 0.35 ^{ab}
	2	57.55 ± 1.44 ^{cde}	20.85 ± 0.42 ^{bc}	3.60 ± 0.04 ^b
	3	55.01 ± 3.10 ^{cde}	23.24 ± 1.68 ^a	3.75 ± 0.22 ^b
100	1	52.40 ± 5.06 ^{cde}	15.52 ± 1.18 ^e	4.10 ± 0.41 ^{ab}
	2	48.06 ± 7.30 ^e	17.03 ± 1.91 ^{de}	4.15 ± 0.07 ^{ab}
	3	68.24 ± 6.15 ^{ab}	18.94 ± 0.81 ^{cd}	4.09 ± 0.24 ^{ab}
110	0.5	60.77 ± 4.90 ^{bcd}	16.15 ± 0.95 ^e	4.32 ± 0.15 ^{ab}
120	0.5	76.36 ± 1.64 ^a	20.34 ± 0.91 ^c	4.92 ± 0.03 ^a
130	0.5	68.44 ± 1.15 ^{ab}	24.43 ± 0.71 ^{ab}	3.68 ± 0.02 ^b

Means ± SD from triplicate determinations (n=2). Different letters in the same column indicate the significant differences (p<0.05).

pepsin-aided process in combination with a protease inhibitor (pepstatin A) (40.3%) (Nalinanon *et al.*, 2008). The results is in agreement with those of Cho *et al.* (2006), Kasankala *et al.* (2007), Kołodziejaska *et al.* (2008) and Kittiphattanabawon *et al.* (2010), who reported the increasing extraction time and temperature resulted in the increased yield of gelatin from brown headed bamboo shark and blacktip shark (45-75°C, 6-12 h), cod skin (45-100°C, 15-120 min), skates skin (40-70°C, 3-6 h) and grass carp skin (40-80°C, 6-24 h). The acid treatment causes swelling of skin gelatin and removal of non-collagenous protein and hot water extraction cause breaking down the triple helix structure of collagen to produce smaller gelatin molecules. Thus as the heating temperature was increased, the rate of collagen break down increased and more gelatinous proteins were formed (Fonkwe and Singh, 1997, Chew *et al.*, 2007). Complete solubilization of samples was achieved when the extraction temperature higher than 100°C was employed. The retorting treatment resulted in sufficient denaturation of soluble collagen and the strong thermohydrolysis provides an effective method for the production of skin gelatin hydrolysates (Yang *et al.*, 2008).

Degree of hydrolysis

Degree of hydrolysis, defined as the percentage of peptide bonds hydrolyzed, of gelatin extracted at different conditions was in range of 3.60-4.92% as shown in Table 1. Different extraction temperatures and times slightly affected degree of hydrolysis of gelatin. Yang *et al.* (2008) reported that hot water extraction caused thermohydrolysis and subsequent solubilization of swollen gelatin. The retorting treatment resulted in sufficient denaturation of soluble collagen and the strong thermohydrolysis provided an effective method for the production of skin gelatin hydrolysate.

Electrophoretic analysis

SDS-PAGE pattern gelatin from bigeye snapper

skin under non-reducing condition was shown in Fig.1. Gelatin extracted from both *P. macracanthus* and *P. tayenus* contained α_1 and α_2 -chains as the major components and were characterized to be type I (Benjakul *et al.*, 2009). The intensity of β -chain (α -chain dimmers), α_1 and α_2 -chains and the proteins or peptides with a molecular weight lower than the α -chain were decreased with increasing extraction temperature and time. Complete degradation of gelatin was observed at 120 and 130°C of extraction. The conversion of collagen to gelatin might provide the molecules with varying chain length, mainly due to the cleavage of inter-chain covalent cross-links and unfavorable breakage of some intra-chain peptide linkage (Zhao *et al.*, 2007). Muyonga *et al.* (2004) reported that Nile perch skin gelatin extracted at high temperature was found to contain more peptides (molecular weight less than α -chain) and lower proportion of high molecular weight (greater than β -chain) fractions than low temperature extraction. Apart from β -chain and α -chain, other protein bands might represent the contaminating proteins in gelatin suggesting incomplete removal of non-collagenous protein prior to extraction. Degradation of major components into lower molecular weight fragment or shorter chains might result in lowering properties of gelatin such as bloom strength and viscoelastic properties. Lower setting point was also presumed.

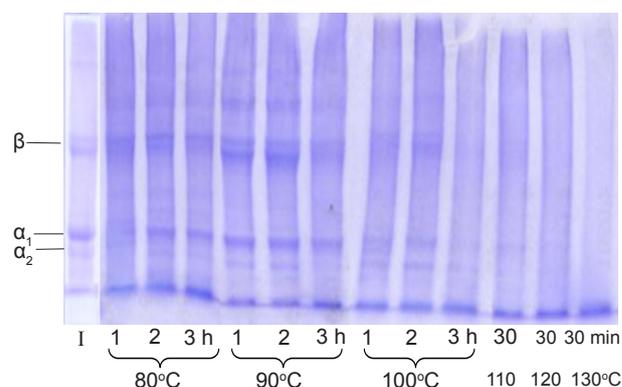


Figure 1. SDS-PAGE pattern of gelatin from bigeye snapper skin extracted at different temperature and time under non reducing condition. I: type I collagen.

Solubility

Gelatin extracted at 120°C for 30 min was observed a high solubility in the wild pH range (1-10). The solubility of the gelatin ranged from 87.37 to 94.28% (data not shown). Similar results of gelatins extracted from both *P. tayenus* and *P. macracanthus* showed relative solubility was greater than 90% at all pH tested (1-10) (Benjakul *et al.*, 2009). However, Bovine serum had the lowest solubility at pH 5. Difference in solubility of different gelatins might result from the difference in molecular weight and the content of polar and non-polar groups in amino acids (Zayas, 1997).

Gelatin properties

The turbidity of 6.67% (w/v) gelatin solution, which extracted at 120°C for 30 min, was 0.842±0.017 % transmittance. Color value of the gelatin gel reported as L*, a*, b* values were 32.58±1.99, -1.67±0.28, and 6.14±1.76, respectively. Benjakul *et al.* (2009) showed that L*, a*, b* values of gelatin extracted from *P. tayenus* and *P. macracanthus* was 29.71, -1.61, 3.22 and 28.55, -1.33, -3.90, respectively. The difference color might be due to the different extraction conditions. In this study the condition used was 120°C for 30 min, while that reported by Benjakul *et al.* (2009) was 45°C for 12 h.

Bloom strength of gelatin gel was 62.6±2.1 g and was much lower than those from bigeye snapper skin extracted at 45°C for 12 h (*P. tayenus*: 227.7±6.20 g, *P. macracanthus*: 254.10±11.13 g). Muyonga *et al.* (2004) and Cho *et al.* (2006) reported that Nile perch and yellow fin tuna skin gelatin extracted at higher temperatures exhibited a lower gel strength. In addition, Kittiphattanabawon *et al.* (2010) revealed bloom strength of gelatin gels from brownbanded bamboo shark and blacktip shark decreased as the extraction temperature (45-75°C) and time (6-12 h) increased. The higher extraction temperature led to the degradation of peptides or proteins as showed in SDS-PAGE pattern (Fig.1) and the structure chain fragments of gelatin could not form the junction zone, in which the strong network could be developed (Kittiphattanabawon *et al.*, 2010). Ledward (1986) reported that degraded α -chains of gelatin were not able to anneal correctly during stabilization overnight by hindering the growth of the existing nucleation sites.

Conclusion

The increasing of extraction temperature and time led to the increasing of Hyp yield of gelatin extracted from bigeye snapper skin. However, the degradation

of gelatin extracted at high temperature contributed to the low bloom strength and other properties.

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