Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 104 (2007) 593–601
Food Chemistry
www.elsevier.com/locate/foodchem

Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*)

Sitthipong Nalinanon [a], Soottawat Benjakul [a,*], Wonnop Visessanguan [b], Hideki Kishimura [c]

[a] *Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90112, Thailand*
[b] *National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Paholyothin Road, Klong1, Klong Luang, Pathumthani 12120, Thailand*
[c] *Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Science, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan*

Received 28 September 2006; received in revised form 5 November 2006; accepted 11 December 2006

Abstract

Extraction and some properties of pepsin-solubilised collagens from the skin of bigeye snapper (*Priacanthus tayenus*) were investigated. Addition of bigeye snapper pepsin (BSP) at a level of 20 kUnits/g of defatted skin resulted in an increased content of collagen extracted from bigeye snapper skin. The yields of collagen from bigeye snapper skin extracted for 48 h with acid and with BSP were 5.31% and 18.74% (dry basis), respectively. With pre-swelling in acid for 24 h, collagen extracted with BSP at a level of 20 kUnits/g of defatted skin for 48 h had a yield of 19.79%, which was greater than that of collagen extracted using porcine pepsin at the same level (13.03%). The skin collagen was characterised to be type I with no disulfide bond. Electrophoretic study revealed slight differences in molecular weight between acid-solubilised collagen and all pepsin-solubilised collagens. The molecular weights of α1 and α2 chains in acid-solubilised collagen were estimated to be 120 and 112 kDa, respectively, whereas α1 and α2 chains of pepsin-solubilised collagens had molecular weights of 118 and 111 kDa, respectively. The result suggested that these pepsin-solubilised collagens might undergo partial cleavage in the telopeptide region by pepsin treatment. The maximum transition temperature (T_{max}) of acid-solubilised collagen was observed at 32.5 °C, which was slightly higher than that of pepsin-solubilised collagens (by about 1 °C). Generally, all collagens were highly solubilised in the pH range of 2–5 and sharply decreased at the neutral pH. No changes in solubility were observed in the presence of NaCl up to 3% (w/v) and the decrease was more pronounced with increasing NaCl concentration.

Keywords: Pepsin; Fish collagen; Extraction; Telopeptide

1. Introduction

Bigeye snapper (*Priacanthus tayenus*) is one of the raw materials commonly used for surimi production in Thailand (Benjakul, Chantarasuwan, & Visessanguan, 2003; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). Surimi and surimi products of Thailand have been exported to several countries, especially the USA, with a volume of 1753 metric tons and a value of 2417 million US dollars in 2004 (Department of export promotion, 2005). During surimi manufacturing, byproducts such as head, viscera, skin and bone are generated in large quantity. These solid wastes constitute 50–70% of the original raw material, depending on the process used (Kim & Park, 2004; Morrissey, Park, & Huang, 2000). Surimi byproducts have mostly been sold for animal feed and fertiliser production with low value. Nevertheless, fish skin contains a large amount of collagen. Nagai and Suzuki (2000) reported that the collagen contents in the fish skin waste of Japanese seabass, chub mackerel and bullhead shark were 51.4%, 49.8% and 50.1% (dry basis), respectively.

Collagen is a major structural protein in the connective tissue of animal skin and bone (Foegeding, Laneir, & Hul-

* Corresponding author. Tel.: +66 7428 6334; fax: +66 7421 2889.
E-mail address: soottawat.b@psu.ac.th (S. Benjakul).

0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.12.035

tin, 1996; Liu, Li, & Guo, 2007; Ogawa et al., 2003). Generally, collagen has a wide range of applications in cosmetic, biomedical, pharmaceutical, leather and film industries (Ogawa et al., 2004; Slade & Levine, 1987). In general, traditional sources of collagen are bovine and porcine skins and bones. Porcine collagen is unacceptable for some religions, for example, Judaism and Islam, while those from bovine sources are at risk of contamination with bovine spongiform encephalopathy (BSE) (Choi & Regenstein, 2000; Fernández-Diaz, Montero, & Gòmez-Guillèn, 2001). Therefore, fish skin, being a byproduct from fish processing, is an important source for collagen production as a replacement for mammalian sources. Normally, a low yield of collagen is obtained with the traditional process. Since pepsin has been reported to cleave peptides in the telopeptide region, the extraction of collagen partially cleaved by pepsin rendered a higher yield (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005; Nagai, Yamashita, Taniguchi, Kanamori, & Suzuki, 2001; Nagai & Suzuki, 2002). Due to a surplus of fish viscera, especially stomach, pepsin of fish origin can be recovered and used to increase the extraction efficiency of collagen. Therefore, the objective of this study was to extract and characterise the collagen from bigeye snapper (*P. tayenus*) skin using a pepsin-aided process.

2. Materials and methods

2.1. Chemical reagents

Bovine hemoglobin, β-mercaptoethanol (βME), L-tyrosine, bovine serum albumin, pepsin from porcine stomach mucosa (EC 3.4.23.1; powderised; 750 U/mg dry matter) and protein marker were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid, Folin-Ciocalteu's phenol reagent, acetic acid and *p*-dimethylamino-benzaldehyde were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), Coomassie Blue R-250 and *N*,*N*,*N*′,*N*′-tetramethyl ethylene diamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). Type I collagen from calf skin was purchased from Elastin products Co. Inc. (Owensville, MO, USA).

2.2. Fish stomach and skin preparation

Internal organs and skin of bigeye snapper (*P. tayenus*), the discards from surimi processing, were obtained from Man A Frozen Foods Co. Ltd., Songkla, Thailand. Those discards were packed in a polyethylene bag, kept in ice with a solid/ice ratio of 1:2 (w/v) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 1 h. Upon arrival, pooled internal organs were excised and only stomach was collected and placed in a polyethylene bag. For the skin, the residual meat was removed manually and cleaned samples were washed with tap water. The skin was then cut into small pieces (0.5 × 0.5 cm) and placed in a polyethylene bag. Both stomach and skin were stored at −20 °C until used.

2.3. Preparation of bigeye snapper pepsin (BSP)

Frozen stomach was thawed using running water (26–28 °C) until the core temperature reached −2 to 0 °C. The sample was cut into pieces of a dimension of 1 × 1 cm. Samples were finely ground in liquid nitrogen using a National Model MX-T2GN blender (Taipei, Taiwan) to a powder form according to the method of Klomklao, Benjakul, and Visessanguan (2004). Stomach powder was suspended in 50 mM sodium phosphate buffer at pH 7.2 at ratio of 1:9 (w/v). The mixtures were stirred continuously at 4 °C for 30 min. The suspension was centrifuged for 30 min at 4 °C at 7700*g*, using a Sorvall Model RC-B Plus refrigerated centrifuge (Newtown, CT, USA) to remove the tissue debris. The pH of the supernatant was adjusted to 3 with 1 M HCl and the mixture was allowed to stand at 4 °C for 10 min. The suspension was centrifuged for 30 min at 4 °C at 5000*g*, using a refrigerated centrifuge. The supernatant was then fractionated with ammonium sulfate (0–20% saturation). The pellet was recovered by centrifuging at 5000*g* for 30 min at 4 °C. The pellet was then dissolved and dialysed against 50 mM Na-acetate buffer (pH 3) at ratio of 1:15 (v/v) at 4 °C, three times. Proteolytic activity was assayed using the hemoglobin-TCA-Lowry assay (An, Seymour, Wu, & Morrissey, 1994) at pH 2.5 and 45 °C for 20 min. One unit was defined as the enzyme releasing 1 nmole of tyrosine per min (nmole of Tyr/min).

2.4. Extraction and characterisation of pepsin-solubilised collagen from bigeye snapper skin

2.4.1. Extraction

The collagens were extracted following the method of Jongjareonrak et al. (2005) and Kittiphattanabawon et al. (2005) with a slight modification. All preparation procedures were performed at 4 °C.

2.4.2. Preparation of skin for collagen extraction

To remove non-collagenous proteins, the skin was mixed with 0.1 N NaOH at a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred for 6 h. The alkaline solution was changed every 2 h. Then, the alkali-treated skins were washed with cold water until neutral or faintly basic pHs of wash water were obtained. The treated skins were then defatted with 10% butyl alcohol with a solid/solvent ratio of 1:10 (w/v) for 18 h and the solvent was changed every 6 h. Defatted skins were washed with 10 volumes of cold water, three times.

2.4.3. Effect of pepsin on collagen extraction and composition

2.4.3.1. Effect of pepsin levels and reaction time on extraction and composition of collagen. Defatted skins were

soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) and subjected to limited hydrolysis with bigeye snapper pepsin (BSP) or porcine pepsin (PP) at levels of 10 and 20 kUnits/g of defatted skin. The mixture was stirred at 4 °C for 24 and 48 h. At the designated time, the mixture was immediately submerged and held in an ice-bath. Prior to determination of hydroxyproline content (Bergman & Loxley, 1963) and SDS–PAGE (Laemmli, 1970), the mixture was centrifuged at 3500*g* for 10 min at 4 °C. The extraction yield was calculated, based on hydroxyproline content of the extract in comparison with that of defatted skin.

The concentration of pepsin used for solubilising the collagen, which was able to extract collagen with the highest yield and still retained the major components (β, α1 and α2) of collagen structure, was selected for further steps.

2.4.3.2. Effect of acid swelling process, in combination with pepsin, on extraction and composition of collagen. Defatted skins were soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) for 24 or 48 h, followed by limited hydrolysis with BSP or PP at 20 kUnits/g of defatted skin. After addition of pepsin, the mixture was stirred at 4 °C for 24 h and 48 h. At the designated time, the mixture was submerged and held in an ice-bath to terminate the hydrolysis. The mixtures were centrifuged at 3500*g* for 10 min at 4 °C and the supernatants were subjected to determination of hydroxyproline content (Bergman & Loxley, 1963) and SDS–PAGE (Laemmli, 1970). The yield was then calculated.

The extraction condition rendering the highest yield and retaining the major components (β, α1 and α2) of collagen structure was chosen for further steps.

2.4.4. Comparative studies on different collagen extracting methods

2.4.4.1. Acid solubilisation process. Defatted skins were soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) for 48 h. The mixture was filtered with two layers of cheese cloth. The filtrate was collected and subjected to precipitation.

2.4.4.2. One-step acid/pepsin solubilisation process. Defatted skins were soaked in 0.5 M acetic acid containing BSP or PP at 20 kUnits/g of defatted skin with a solid/solvent ratio of 1:15 (w/v). The mixture was stirred at 4 °C for 48 h. The mixture was then filtered with two layers of cheese cloth. The filtrate was collected for precipitation.

2.4.4.3. Two-step acid/pepsin solubilisation process. Defatted skins were soaked in 0.5 M acetic acid with a solid/solvent ratio of 1:15 (w/v) for 24 h. The mixture was filtered with two layers of cheese cloth. The filtrate was collected. The residue was re-extracted by suspending in 0.5 M acetic acid containing BSP or PP at 20 kUnits/g of defatted skin with a solid/solvent ratio of 1:15 (w/v). The mixture was stirred at 4 °C for 48 h and filtered with two layers of cheese cloth. Both filtrates were combined and subjected to precipitation.

2.4.5. Collagen precipitation

The collagen solutions from Sections 2.4.4.1, 2.4.4.2 and 2.4.4.3 were precipitated according to the method of Kittiphattanabawon et al. (2005). The solutions were precipitated by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M tris(hydroxylmethyl)aminomethane, pH 7.5. The resultant precipitates were collected by centrifuging at 20,000*g* for 60 min. The pellets were dissolved in 0.5 M acetic acid, dialysed against 10 volumes of 0.1 M acetic acid and distilled water, three times, respectively, and freeze-dried.

2.5. Characterisation of collagen

2.5.1. Hydroxyproline content

Collagens were analysed for hydroxyproline content according to the method of Bergman and Loxley (1963) with a slight modification. 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 neutralised with 10 M and 1 M NaOH to obtain a pH of 6.0–6.5. The neutralised 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*-dimethylamino-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 heated at 60 °C for 25 min in a water bath (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. A hydroxyproline standard solution, with concentration ranging from 10 to 60 ppm, was also included. Hydroxyproline content was calculated and expressed as mg/g of sample. The conversion factor for calculating the collagen content from hydroxyproline of bigeye snaper skin was 7.7 (Kittiphattanabawon et al., 2005).

2.5.2. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). The collagen samples were dissolved in 0.02 M sodium phosphate containing 1% SDS and 3.5 M urea (pH 7.2). The mixtures were centrifuged at 8500*g* for 5 min at room temperature to remove undissolved debris. Solubilised samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence or absence of 10% β-ME. Samples were loaded onto a polyacrylamide gel

made of 7.5% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel, using a Mini Protein II unit (Bio-Rad Laboratories Inc., Richmond, CA, USA). After electrophoresis, the 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. High-molecular-weight markers (Sigma Chemical Co., St. Louis, MO, USA) were used to estimate the molecular weights of proteins. Type I collagen from calf skin was used as a standard.

2.5.3. Differential scanning calorimetry (DSC)

Prior to analysis, collagen samples were prepared, following the methods of Rochdi, Foucat, and Renou (2000) and Komsa-Penkova, Koyonava, Kostov, and Tenchov (1999) with a slight modification. The samples were rehydrated by adding 0.05 M acetic acid to dried samples at a solid/solution ratio of 1:40 (w/v). The mixture was allowed to stand for 2 days at 4 °C.

Differential scanning calorimetry (DSC) was performed, using a Model DSC 7 (Norwalk, USA). Temperature calibration was done using the indium thermogram. The collagen solutions (5–10 mg) were accurately weighed into aluminium pans and sealed. The samples were scanned at 1 °C/min over the range of 20–50 °C, using ice water as the cooling medium. An empty pan was used as the reference. Total denaturation enthalpy (ΔH) was estimated by measuring the area in the DSC thermogram. The maximum transition temperature (T_{max}) was estimated from the thermogram.

2.5.4. Collagen solubility

2.5.4.1. Preparation of collagen solution. The collagen solubility was determined by the method of Montero, Jimenez-Colmenero, and Borderias (1991) with a slight modification. The collagens were dissolved in 0.5 M acetic acid to obtain a final concentration of 3 mg/ml and the mixture was stirred at 4 °C until collagen was completely solubilised.

2.5.4.2. Effect of pH on collagen solubility. Collagen solution (8 ml) was added to a 50 ml centrifuge tube (NUNC, Roskilde, Denmark) and the pH was adjusted with either 6 N NaOH or 6 N HCl to obtain the final pHs ranging from 1 to 10. The volume of solutions was made up to 10 ml by distilled water, previously adjusted to the same pH as the collagen solution. The solution was centrifuged at 10,000*g* at 4 °C for 30 min. Protein content in the supernatant was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard. Relative solubility was calculated in comparison with that obtained at the pH rendering highest solubility.

2.5.4.3. Effect of NaCl on collagen solubility. Collagen solutions (5 ml) with a concentration of 6 mg/ml were mixed with 5 ml of NaCl in 0.5 M acetic acid at various concentrations (0%, 2%, 4%, 6%, 8%, 10% and 12% (w/v)). The mixture was stirred continuously at 4 °C for 30 min, followed by centrifuging at 10,000*g* at 4 °C for 30 min. Protein content in the supernatant was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Relative solubility was calculated as mentioned above.

2.6. Statistical analysis

All data were subjected to analysis of variance (ANOVA) and differences between means were evaluated by Duncan's multiple range test (Steel & Torrie, 1980). SPSS Statistic Program (Version 10.0) (SPSS, 1.2, 1998) was used for data analysis.

3. Results and discussion

3.1. Effect of pepsin on collagen extraction and composition

3.1.1. Effect of pepsin levels and reaction time

The yields of collagen extracted from bigeye snapper skin using BSP and PP for 24 and 48 h are shown in Table 1. The highest yield (53.68%) was obtained when the skin was extracted using BSP at 20 kUnits/g of defatted skin for 48 h. At the same enzyme level and reaction time, BSP showed a higher extraction efficiency than did PP ($P < 0.05$), as evidenced by the greater yield. High enzyme level generally led to a greater yield of collagen extracted. Additionally, a longer reaction time rendered a higher yield. Hydrolysis at the telopeptide region was more pronounced with sufficient reaction time, particularly at the higher enzyme levels. From this result, a much lower yield was obtained in the absence of pepsins, both BSP and PP.

Table 1
Total hydroxyproline (Hyp) and yield of collagen extracted from the skin of bigeye snapper using bigeye snapper pepsin (BSP) or porcine pepsin (PP) at different levels for different times

Extraction time (h)	Treatments	Total extracted Hyp (mg/g defatted skin)	Yield[B] (%)
24	Control	1.35	5.67 ± 0.39a[C]
	BSP 10[A]	6.86	28.8 ± 1.37e
	BSP 20	8.08	33.9 ± 0.71f
	PP 10	4.79	20.1 ± 1.05c
	PP 20	5.30	22.2 ± 1.07d
48	Control	1.78	7.49 ± 2.36b
	BSP 10	12.2	51.2 ± 0.40h
	BSP 20	12.8	53.7 ± 0.71i
	PP 10	8.06	33.8 ± 0.40f
	PP 20	10.4	43.8 ± 0.41g

Means ± SD from triplicate determinations.
[A] Numbers denote activity of enzyme in kUnits/g of defatted skin.
[B] Yield was calculated, based on hydroxyproline content in the collagen in comparison with that of the skin.
[C] Different letters in the same column indicate the significant difference ($P < 0.05$).

Without pepsins, the collagen extracted was mainly acid-solubilised collagen, which was more solubilised with increasing extraction times. The electrophoretic patterns of the resultant collagen, under reducing conditions, are shown in Fig. 1. All collagens extracted under different conditions had similar components. The β-chain was found to be the dominant constituent. α-Chains, both α1 and α2, were also observed in all collagens extracted. From the protein pattern, it was revealed that the collagen obtained was type I collagen, which was predominant in fish skin collagen (Hwang, Mizuta, Yogoyama, & Yoshinaka, 2007; Jongjareonrak et al., 2005; Kittiphattanabawon et al., 2005; Ogawa et al., 2003). This result suggested that the covalent cross-linking at the telopeptide regions of collagen molecules through the condensation of aldehyde groups, as well as the inter-molecular cross-linked molecules, were not readily solubilised by acid extraction (Jongjareonrak et al., 2005). These cross-linking molecules generally cause decreased solubility of collagen (Burghagen, 1999; Foegeding et al., 1996). Cross-linked molecules at the telopeptide region were cleaved by pepsins, both BSP and PP, without damaging the integrity of the triple helix of collagen. With increasing extraction time, collagens referred to as 'pepsin-solubilised collagen' localised in the loosened structure caused by partial cleavage at the telopeptide region, could be extracted more completely. From this result, higher band intensities of β- and α-chains was noticeable in pepsin-solubilised collagen using BSP than in collagen extracted using PP as the extraction aid. Furthermore, slightly lower molecular weights were obtained in β- and α-chains of pepsin-solubilised collagen than in acid-solubilised collagen (without pepsin addition). This result suggested that small portions of peptides at the telopeptide region were cleaved and removed, leading to the formation of collagens with slightly lower MW.

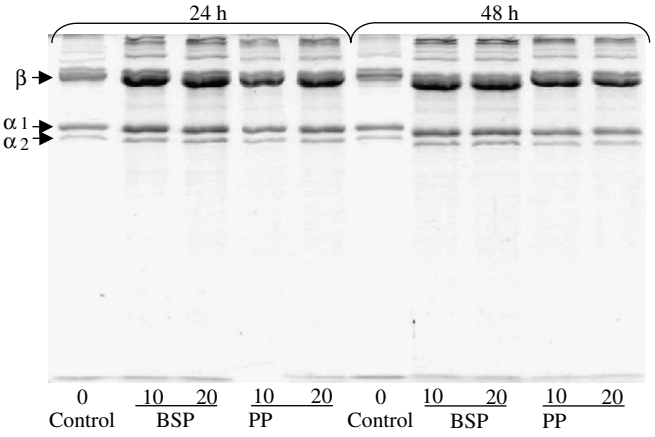

Fig. 1. SDS–PAGE patterns of collagens from the skin of bigeye snapper extracted, using bigeye snapper pepsin (BSP) or porcine pepsin (PP) at different levels, for different times. Numbers denote pepsin activity in kUnits/g of defatted skin.

3.1.2. Effect of pre-swelling process in combination with pepsin

The collagen extraction was generally maximised with the pre-swelling process. The skin was swollen with 0.5 M acetic acid for 24 h (A24) and 48 h (A48) prior to the hydrolysis using BSP or PP for 24 or 48 h. Total hydroxyproline and the yields of all treatments are shown in Table 2. The yield of collagen extracted increased with increasing pre-swelling time and pepsin treatment time. The highest yield (~65%) was found when BSP was added with a reaction time of 48 h, regardless of pre-swelling time. The results suggested that swollen skin, which was treated with acetic acid, possibly had a porous and loose structure caused by charge repulsion. As a result, the penetration of pepsin into the skin matrix could be enhanced. Thus, hydrolytic reaction of pepsin toward collagen was augmented. From this result, BSP had a greater ability to extract or solubilise the collagen from the skin than did PP. With a shorter hydrolysis time (24 h), swelling for a longer time led to increased yield of collagen extracted. At the same pre-swelling time and hydrolysis time, BSP exhibited greater extracting ability than did PP. Therefore, both pre-swelling time and hydrolysis time affected the collagen extraction from bigeye snapper skin. Electrophoretic study of collagens extracted under different conditions revealed that β, α1 and α2 were the major components. No differences in protein pattern were noticeable among samples (Fig. 2). Proteins with molecular weights lower than the α2-chain were negligible in collagens treated with pepsin. This result indicated that pepsin possibly preferred to cleave the non-helical telopeptide regions of collagen structure at low temperature (4 °C). Generally, pepsin activity is enhanced at low pH (Lin & Liu, 2006). At room temperature (26–28 °C), pepsin showed high activity and

Table 2
Total hydroxyproline (Hyp) and the yield of collagen extracted from the skin of bigeye snapper with acid pre-swelling process, followed by the treatment of bigeye snapper pepsin (BSP) or porcine pepsin (PP) at different levels for different times

Swelling time (h)	Pepsin/hydrolysis time (h)	Total extracted Hyp (mg/g defatted skin)	Yield[B] (%)
24	–	1.90	7.29 ± 1.20a[C]
24	BSP/24[A]	10.7	42.80 ± 0.41d
24	BSP/48	16.2	65.03 ± 0.81g
24	PP/24	10.2	41.02 ± 0.40c
24	PP/48	13.7	54.02 ± 1.06e
48	–	2.31	9.29 ± 0.88b
48	BSP/24	14.8	59.41 ± 1.49f
48	BSP/48	16.2	65.14 ± 0.41g
48	PP/24	11.1	44.52 ± 0.82d
48	PP/48	16.4	65.72 ± 1.22g

Means ± SD from triplicate determinations.
[A] Numbers denote extraction time (h).
[B] Yield was calculated, based on hydroxyproline content in the collagen in comparison with that of the skin.
[C] Different letters in the same column indicate the significant difference ($P < 0.05$).

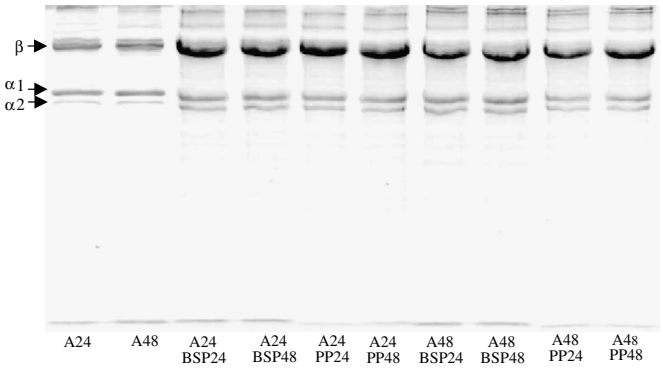

Fig. 2. SDS–PAGE patterns of collagen extracted from the skin of bigeye snapper with acid pre-swelling process, followed by the treatment with bigeye snapper pepsin (BSP) or porcine pepsin (PP) at 20 kUnits/g defatted skin for different times.

randomly cleaved the swollen collagen molecules of bigeye snapper skin. The integrity of collagen was destroyed under severe conditions (data not shown).

3.2. Composition of collagen extracted from bigeye snapper skin under different conditions

The yield, hydroxyproline and collagen contents of different collagens are shown in Table 3. Hydroxyproline and collagen contents of bigeye snapper skin were 57.3 and 440 mg/g of defatted skin, respectively. Approximately 1.5-fold increases of hydroxyproline content were obtained in the resultant collagens. This result suggested that higher content of collagen was found in the extract. Centrifugation after extraction could lead to the removal of non-collagenous substances. As a result, higher hydroxyproline and collagen contents were observed in the extracted collagen than in the skin. From this result, the yield of resultant collagens was obviously increased when pepsins were used in the extraction process. Greater yield was obtained when BSP was added together with extracting acid medium or after an acid pre-swelling process for 24 h. PP showed a lower capacity for extracting collagen from bigeye snapper skin, as evidenced by the lower yield. Among all collagens obtained, that extracted using acid without pepsin addition had the lowest yield (5.31%). It appears that the swollen skins were easily cleaved with pepsin at the telopeptide regions. Therefore, the collagen could be solubilised into the extracting solution in the presence of pepsin to a greater extent than in the absence of pepsin. Different yields (dry weight basis) of pepsin-solubilised collagen from fish skin were reported in different sources, including channel catfish (38.4%) (Liu et al., 2007), ocellate puffer fish (44.7%) (Nagai, Araki, & Suzuki, 2002), black drum (15.8%) and sheephead seabream (29.3%) (Ogawa et al., 2003). Therefore, pepsin improved efficiency of collagen extraction and an increased yield could be obtained.

Table 3
Yield, hydroxyproline and collagen contents of different collagens from the skin of bigeye snapper

Sample[C]	Hydroxyproline (mg/g sample)	Collagen[A] (mg/g sample)	Yield (% dry wt.)
Skin	57.3 ± 0.39a	440 ± 1.31a	–
A48	82.7 ± 0.60b	637 ± 4.66b	5.31 ± 0.11a[B]
BSP48	84.0 ± 3.04b	647 ± 23.43b	18.7 ± 0.09c
A24/BSP48	84.1 ± 5.03b	647 ± 38.77b	19.8 ± 0.12d
A24/PP48	85.1 ± 3.55b	656 ± 27.32b	13.0 ± 0.15b

Means ± SD from triplicate determinations.
[A] The conversion factor for calculating the collagen content from hydroxyproline was 7.7 (Kittiphattanabawon et al., 2005).
[B] Different letters in the same column indicate the significant difference ($P < 0.05$).
[C] A48: collagen extracted with acid for 48 h; BSP48: collagen extracted with acid containing BSP (20 kUnits/g of defatted skin) for 48 h; A24/BSP48: collagen extracted with acid for 24 h, followed by extracting using BSP (20 kUnits/g of defatted skin) for 48 h; A24/PP48: collagen extracted with acid for 24 h, followed by extracting using PP (20 kUnits/g of defatted skin) for 48 h.

3.3. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) patterns of collagens from bigeye snapper skin

SDS–PAGE patterns of collagens, under reducing and non-reducing conditions, are shown in Fig. 3. Generally, no differences in protein patterns of collagens (under both conditions) were observed. This result indicated that no collagens contained any disulfide bonds. All collagens comprised β, α1 and α2 chains as the major constituents. This result was accordance with those found in skin collagen of hake and trout (Montero, Bonderias, Turnay, & Leyzarbe, 1990), Japanese sea-bass and bullhead shark (Nagai & Suzuki, 2000), black drum and sheephead seabream (Ogawa et al., 2004), Pacific whiting (Kim & Park, 2004), young and adult Nile perch (Muyonga, Cole, & Duodu,

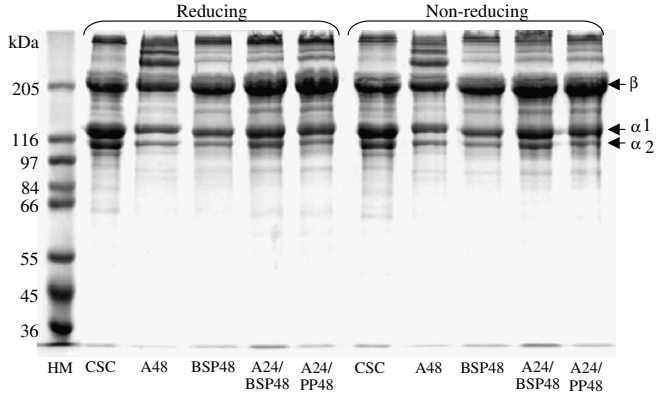

Fig. 3. SDS–PAGE patterns of collagens from the skin of bigeye snapper. A48: collagen extracted with acid for 48 h; BSP48: collagen extracted with acid containing BSP (20 kUnits/g of defatted skin) for 48 h; A24/BSP48: collagen extracted with acid for 24 h, followed by extracting using BSP (20 kUnits/g of defatted skin) for 48 h; A24/PP48: collagen extracted with acid for 24 h, followed by extracting using PP (20 kUnits/g of defatted skin) for 48 h. HM and CSC denote high MW protein markers and collagen type I, respectively.

2004) and channel catfish (Liu et al., 2007). Type I collagen consists of two identical α1 chain and one α2 chain types (Rochdi et al., 2000). Based on electrophoretic patterns and subunit composition, it appears that collagens obtained from different extraction methods are type I collagen. There were no differences in the relative mobilities of α1 and α2 chains between acid-solubilised collagen (A48) and calf skin type I collagen (CSC). However, slight difference in relative mobilities between acid-solubilised collagen (A48) and all pepsin-solubilised collagens with different treatments were observed. The molecular weights of α1 and α2 chains in calf skin type I collagen and acid-solubilised collagen were 120 and 112 kDa, respectively. For pepsin-solubilised collagen, including BSP48, A24/BSP48 and A24/PP48, the molecular weights of the α1 and α2 chains were estimated to be 118 and 111 kDa, respectively. These results suggested that these pepsin-solubilised collagens might undergo partial cleavage at the telopeptide regions by pepsin treatment. As a consequence, a slight decrease in molecular weight of pepsin solubilised collagens was noticeable, in comparison with that of acid-solubilised collagen. Miller (1972) proposed that the mechanism whereby the proteolytic activity of pepsin alters the solubility properties of cartilage collagen involves the degradation of the non-helical region, thus effectively eliminating a site of intermolecular cross-linking. Drake, Davison, Rump, and Schmitt (1966) reported that most of intra- and inter-molecular cross-links found in collagen occur through the telopeptide region. Some of the telopeptides of calf skin tropocollagen are vulnerable to pepsin action since intramolecular cross-links are broken on pepsin digestion and fragments comprising a small fraction of the molecule (approximately 1%) become dialysable (Drake et al., 1966). From this result, higher-molecular-weight components, including γ-chain, were found to a greater extent in acid-solubilised collagen (A48), than in pepsin-solubilised collagens (BSP48, A24/BSP48, A24PP48). These high-molecular-weight components might be degraded to smaller components, such as β, α1 or α2, by pepsin action. Consequently, greater band intensities of β, α1 and α2 chains were noticeable in all pepsin-solubilised collagens than in acid-solubilised collagen. Similar changes were also found in acid-solubilised calf skin tropocollagen when treated with pepsin. Native calf skin tropocollagen consisted of α:β:γ at a ratio of 32:65:3, whereas the components of pepsin treated tropocollagen were changed to 72:23:3 (Drake et al., 1966).

3.4. Thermal stability of collagens from bigeye snapper skin

Thermal transitions of extracted collagens rehydrated in 0.05 M acetic acid are shown in Table 4. The endothermic peak of acid-solubilised collagen (A48) was observed with a T_{max} of 32.5 °C, which was slightly higher than that of pepsin-solubilised collagens (BSP48, A24BSP48 and A24PP48) (by about 1 °C). The decrease in denaturation temperature of pepsin-solubilised collagens might be caused by the molecular weight lowering caused by pepsin digestion, especially in the telopeptide region. T_{max} values of extracted collagens from bigeye snapper skin were higher than those previously reported for collagens from several fish species. Different thermal transition temperatures of skin collagen have been reported for Pacific whiting (27 °C) (Kim & Park, 2004), Japanese sea-bass (26.5 °C), chub mackerel (25.6 °C), bullhead shark (25 °C) (Nagai & Suzuki, 2000) and ocellate puffer fish (28 °C) (Nagai et al., 2002). However, T_{max} values of all collagens from bigeye snapper skin were much lower than those of skin collagen from Nile perch (36 °C) (Muyonga et al., 2004) and calf (40.8 °C) (Komsa-Penkova et al., 1999). Collagen stability is correlated with environmental and body temperature (Rigby, 1968). Low denaturation temperature may be due to a low degree of proline hydroxylation in fish collagens (Hwang et al., 2007). A high content of imino acid is needed for stabilisation of collagen (Xu, Bhate, & Brodsky, 2002). The thermal stability of the collagen triple helix is attributed to the hydrogen-bonded networks, mediated by water molecules, which connect the hydroxyl group of hydroxyproline in one strand to the main chain amide carboxyl of another chain (Babu & Ganesh, 2001). Therefore, differences in hydroxyproline content might determine the denaturation temperatures of collagens from different fish species.

Table 4
The maximum transition temperature (T_{max}) and total denaturation enthalpy (ΔH) of different collagens from the skin of bigeye snapper rehydrated in 0.05 M acetic acid

Samples	T_{max} (°C)[a]	ΔH (J/g)[a]
A48	32.5	0.106
BSP48	31.5	0.800
A24/BSP48	31.3	0.407
A24/PP48	31.8	1.233

A48: collagen extracted with acid for 48 h; BSP48: collagen extracted with acid containing BSP (20 kUnits/g of defatted skin) for 48 h; A24/BSP48: collagen extracted with acid for 24 h, followed by extracting using BSP (20 kUnits/g of defatted skin) for 48 h; A24/PP48: collagen extracted with acid for 24 h, followed by extracting using PP (20 kUnits/g of defatted skin) for 48 h.

[a] Values are means from duplicate determinations.

3.5. Solubility of skin collagens from bigeye snapper skin

The effect of pH on the solubility of different collagens in 0.5 M acetic acid is depicted in Fig. 4. The highest solubility of all collagens was observed at pH 5. Generally, all collagens were highly solubilised in the pH range of 2–5, with relative solubility greater than 80%. A sharp decrease in solubility was observed at the neutral pH. However, different collagens had varying solubilities at pHs ranging from 6 to 10. Pepsin-solubilised collagen (A24/BSP48) showed a higher solubility than did other collagens at pH above 7. This result suggested that A24/BSP48 might possess a lower degree of cross-linking or consisted of weaker

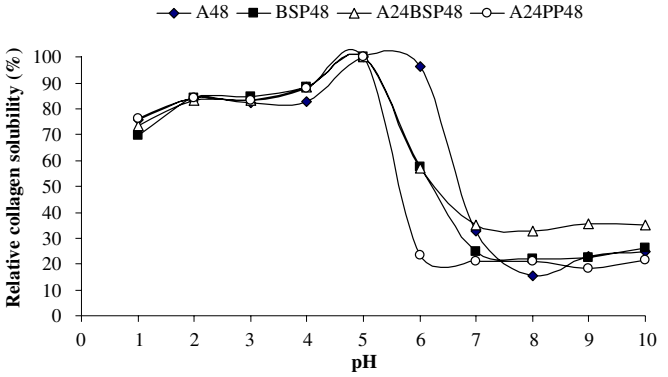

Fig. 4. Relative solubility (%) of collagens extracted from the skin of bigeye snapper by different methods at different pHs. A48: collagen extracted with acid for 48 h; BSP48: collagen extracted with acid containing BSP (20 kUnits/g of defatted skin) for 48 h; A24/BSP48: collagen extracted with acid for 24 h, followed by extracting using BSP (20 kUnits/g of defatted skin) for 48 h; A24/PP48: collagen extracted with acid for 24 h, followed by extracting using PP (20 kUnits/g of defatted skin) for 48 h.

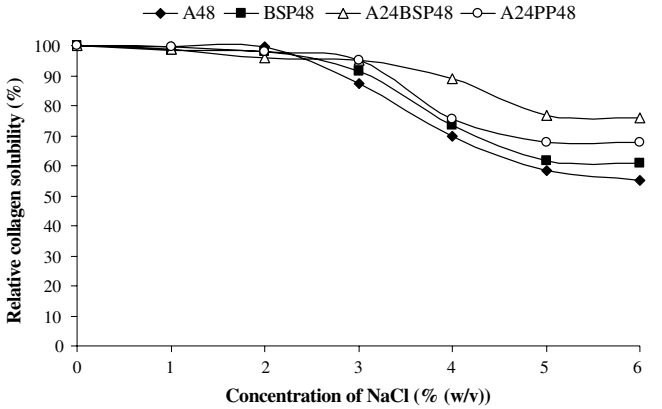

Fig. 5. Relative solubility (%) of collagens extracted from the skin of bigeye snapper by different methods in the presence of NaCl at different concentrations. A48: collagen extracted with acid for 48 h; BSP48: collagen extracted with acid containing BSP (20 kUnits/g of defatted skin) for 48 h; A24/BSP48: collagen extracted with acid for 24 h, followed by extracting using BSP (20 kUnits/g of defatted skin) for 48 h; A24/PP48: collagen extracted with acid for 24 h, followed by extracting using PP (20 kUnits/g of defatted skin) for 48 h.

bonds than did other collagens. The variation in solubility of collagens might be associated with a different isoelectric point (pI), which was altered by pepsin treatment. From this result, it appears that pIs of all collagens were more likely to be in the neutral and alkaline ranges, as evidenced by the lowered solubility in these ranges. At very acidic pH (pH 1), collagen might undergo denaturation to some extent, leading to impaired solubility. When the pH was close to the pI, the molecular charges of collagen became diminished and a decrease in solubility occurred (Montero et al., 1991; Vojdani, 1996). Jongjareonrak et al. (2005) found that acid-solubilsed and pepsin-solubilised collagens from bigeye snapper (*Priacanthus macracanthus*) skin exhibited the lowest solubility at pHs 6 and 7, respectively. Kittiphattanabawon et al. (2005) observed the lowest solubility of acid-solubilsed collagen from bigeye snapper (*P. tayenus*) skin at pH 7.

The effect of NaCl on collagen solubility is shown in Fig. 5. The solubility in 0.5 M acetic acid of all collagens was maintained in the presence of NaCl up to 3%. Solubility of all collagens decreased gradually with increasing NaCl concentration. NaCl, at higher concentration, might result in decreased protein solubility via a 'salting out' effect by increasing hydrophobic interaction and aggregation, and competing with the protein for water. As a result, protein precipitation can be induced (Vojdani, 1996). From this result, pepsin-solubilised collagens (BSP48, A24BSP48 and A24PP48) showed greater solubility than acid-solubilised collagen (A48), especially at high salt concentration. The descending order of collagen solubility at NaCl above 3% was: A24/BSP48 > A24/PP48 > BSP48 and A48. Greater solubility of pepsin-solubilised collagens might be due to the proteolytic action of pepsin in altering collagen structure and reducing the chain length of resultant collagens. Thus, collagens extracted by different methods might have different molecular properties, leading to the varying characteristics of those resultant collagens. However, all collagens were still more than 50% soluble in the presence of NaCl (up to 6%).

4. Conclusion

Efficiency in collagen extraction from the skin of bigeye snapper could be enhanced by incorporating BSP at 20 kUnits/g of defatted skin during a 48 h extraction, after an acid pre-swelling process for 24 h. However, pepsin-solubilised collagens showed slightly lower molecular weights of $\alpha 1$ and $\alpha 2$ than did acid-solubilised collagen. Pepsin might alter collagen structure, which somehow affects thermal stability and solubility of the resultant collagens.

Acknowledgement

The authors would like to express their sincere thanks to the Graduate School of Prince of Songkla University for the financial support.

References

An, H., Seymour, T. A., Wu, J., & Morrissey, M. T. (1994). Assay systems and characterization of Pacific whiting (*Merluccius productus*) protease. *Journal of Food Science, 59*, 277–281.

Babu, I. R., & Ganesh, K. N. (2001). Enhanced triple helix stability of collagen peptides with 4R-Aminoprolyl (Amp) residues: relative roles of electrostatic and hydrogen bonding effects. *Journal of the American Chemical Society, 123*, 2079–2080.

Benjakul, S., Chantarasuwan, C., & Visessanguan, W. (2003). Effect of medium temperature setting on gelling characteristics of surimi from some tropical fish. *Food Chemistry, 82*, 567–574.

Bergman, I., & Loxley, R. (1963). Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Analytical Chemistry, 35*, 1961–1965.

Burghagen, M. (1999). Collagen. In H. D. Belitz & W. Grosch (Eds.), *Food chemistry* (2nd ed., pp. 540–547). Berlin: Springer.

Choi, S. S., & Regenstein, J. M. (2000). Physicochemical and sensory characteristics of fish gelatin. *Journal of Food Science, 65*, 194–199.

Department of export promotion. (2005). Summary of USA seafood trade in 2004. Ministry of commerce, Thailand (http://www.depthai.go.th).

Drake, M. P., Davison, P. F., Rump, S., & Schmitt, F. O. (1966). Action of proteolytic enzyme on tropocollagen and insoluble collagen. *Biochemistry, 5*, 301–312.

Fernández-Diaz, M. D., Montero, P., & Gòmez-Guillèn, M. C. (2001). Gel properties of collagens from skins of cod (*Gadus morhua*) and hake (*Merluccius merluccius*) and their modification by coenhancers magnesium sulphate, glycerol and transglutaminase. *Food Chemistry, 74*, 161–167.

Foegeding, E. A., Laneir, T. C., & Hultin, H. O. (1996). Characteristics of edible muscle tissues. In O. R. Fennema (Ed.), *Food chemistry* (pp. 902–906). New York, USA: Marcel Dekker Inc.

Hwang, J. H., Mizuta, S., Yogoyama, Y., & Yoshinaka, R. (2007). Purification and characterization of molecular species of collagen in the skin of skate (*Raja kenojei*). *Food Chemistry, 100*, 921–925.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., & Tanaka, M. (2005). Isolation and characterization of collagen from bigeye snapper (*Priacanthus marcracanthus*) skin. *Journal of the Science of Food and Agriculture, 85*, 1203–1210.

Kim, J.-S., & Park, J. W. (2004). Characterization of acid-soluble collagen from Pacific whiting surimi processing byproducts. *Journal of food science, 69*, C637–C642.

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T., & Tanaka, M. (2005). Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). *Food Chemistry, 89*, 363–372.

Klomklao, S., Benjakul, S., & Visessanguan, W. (2004). Comparative studies on proteolytic activity of splenic extract from three tuna species commonly used in Thailand. *Journal of Food Biochemistry, 28*, 355–372.

Komsa-Penkova, R., Koyonava, R., Kostov, G., & Tenchov, B. (1999). Discrete reduction of type I collagen thermal stability upon oxidation. *Biophysical Chemistry, 83*, 185–195.

Laemmli, U. K. (1970). Cleavage of structure protein during the assembly of head bacteriophage T4. *Nature, 277*, 680–685.

Lin, Y. K., & Liu, D. C. (2006). Effects of pepsin digestion at different temperatures and times on properties of telopeptide-poor collagen from bird feet. *Food Chemistry, 94*, 621–625.

Liu, H., Li, D., & Guo, S. (2007). Studies on collagen from the skin of channel catfish (*Ictaurus punctaus*). *Food Chemistry, 101*, 621–625.

Lowry, Q. H., Rosebrough, N. J., Farr, L. A., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 256–275.

Miller, E. J. (1972). Structural studies on cartilage collagen employing limited cleavage and solubilization with pepsin. *Biochemistry, 11*, 4903–4909.

Montero, P., Bonderias, J., Turnay, J., & Leyzarbe, M. A. (1990). Characterization of hake (*Merluccius* L.) and trout (*Salmo irideus* Gibb) collagen. *Journal of Agricultural and Food Chemistry, 38*, 604–609.

Montero, P., Jimennez-Colmenero, F., & Borderias, J. (1991). Effect of pH and the presence of NaCl on some hydration properties of collagenous material from trout (*Salmo irideus* Gibb) muscle and skin. *Journal of the Science of Food and Agriculture, 54*, 137–146.

Morrissey, M. T., Park, J. W., & Huang, L. (2000). Surimi processing waste Its control and utilization. In J. W. Park (Ed.), *Surimi and surimi seafood* (pp. 127–166). New York: Marcel Dekker Inc..

Muyonga, J. H., Cole, C. G. B., & Duodu, K. G. (2004). Characterization of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). *Food Chemistry, 85*, 81–89.

Nagai, T., Araki, Y., & Suzuki, N. (2002). Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chemistry, 78*, 173–177.

Nagai, T., & Suzuki, N. (2000). Isolation of collagen from fish waste material-skin, bone and fins. *Food Chemistry, 68*, 277–281.

Nagai, T., & Suzuki, N. (2002). Preparation and partial characterization of collagen from paper nautilus (*Argo nauta argo*, Linnaeus) outer skin. *Food Chemistry, 76*, 149–153.

Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N., & Suzuki, N. (2001). Isolation and characterization of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). *Food Chemistry, 72*, 425–429.

Ogawa, M., Moody, M. W., Portier, R. J., Bell, J., Schenxnayder, M. A., & Losso, J. N. (2003). Biochemical properties of black drum and sheephead seabream skin collagen. *Journal of Agricultural and Food Chemistry, 51*, 8088–8092.

Ogawa, M., Portier, R. J., Moody, M. W., Bell, J., Schexnayder, M. A., & Losso, J. N. (2004). Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheephead seabream (*Archosargus probatocephalus*). *Food Chemistry, 88*, 495–501.

Rigby, B. J. (1968). Amino-acid composition and thermal stability of the skin collagen of the Antarctic ice-fish. *Nature, 219*, 166–167.

Rochdi, A., Foucat, L., & Renou, J. (2000). NMR and DSC studies during thermal denaturation of collagen. *Food Chemistry, 69*, 295–299.

Slade, L., & Levine, H. (1987). Polymer-chemical properties of gelatin in foods. In A. M. Pearson, T. R. Dutson, & A. J. Bailey (Eds.). *Collagen as food: Advances in meat research* (Vol. 4, pp. 251–266). New York: Nostrand Reinhold.

Steel, R. G. D., & Torrie, J. H. (1980). *Principle and procedure of statistics* (2nd ed.). New York: McGraw-Hill.

Vojdani, F. (1996). Solubility. In G. M. Hall (Ed.), *Methods of testing protein functionality* (1st ed., pp. 11–60). Great Britain: St. Edmundsbury Press.

Xu, Y., Bhate, M., & Brodsky, B. (2002). Characterization of the nucleation step and folding of a collagen triple-helix peptide. *Biochemistry, 41*, 8143–8151.