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Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of Brownstripe red snapper (*Lutjanus vitta*)

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Abstract

Acid-solubilised collagen (ASC) and pepsin-solubilised collagen (PSC) were successfully extracted from the skin of Brownstripe red snapper (*Lutjanus vitta*) with yields of 9% and 4.7%, respectively, on the basis of wet weight. Both ASC and PSC consisted of two different α chains (α1 and α2), and were characterised to be type I with no disulfide bond. PSC had a lower content of high molecular weight cross-links, than did ASC. Peptide maps of ASC and PSC hydrolysed by V8 protease and lysyl endopeptidase showed some differences in peptide patterns between the two fractions and were totally different from those of calf skin collagen type I, suggesting differences in amino acid sequences and collagen conformation. Maximum solubility in 0.5 M acetic acid was observed at pH 3 and pH 4 for ASC and PSC, respectively. A sharp decrease in solubility was observed in the presence of NaCl, above 2% and 3%, (w/v) for ASC and PSC, respectively. T_{max} values of both collagen fractions were similar and shifted to a lower value in the presence of acetic acid, suggesting some changes in the collagen structure caused by acid induction.

Keywords: Collagen; Brownstripe red snapper; Skin; ASC; PSC; Solubility

1. Introduction

Collagen is an abundant protein in vertebrates and constitutes ≈30% of total animal protein (Muyonga, Cole, & Duodu, 2004). Tendon, skin, bone, vascular system and connective tissue sheaths surrounding muscle are the organs of animals that are mainly structured by collagen (Foegeding, Lanier, & Hultin, 1996). Furthermore, collagen has been found in fish skin, bone and scale (Ikoma, Kobayashi, Tanaka, Walash, & Mann, 2003; Kimura, 1992; Nagai & Suzuki, 2000; Nomura, Sakai, Ishi, & Shirai, 1996). Nineteen variants of collagen have been reported, named type I–XIX (Bailey, Paul, & Knott, 1998). So far, the main sources of collagen are limited to those of land-based animals, such as bovine or porcine skin and bone. However, the outbreaks of bovine spongiform encephalopathy (BSE) and the foot-and-mouth disease (FMD) crisis have resulted in anxiety among users of collagen and collagen-derived products of land animal origin (Helcke, 2000). Additionally, collagen obtained from porcine skin or bone cannot be used as a component of some foods due to aesthetic and religious objections (Sadowska, Kolodziejska, & Niecikowska, 2003). Therefore, alternative sources, such as fish processing waste,

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including skin, bone or scale, have received increasing attention for collagen extraction.

The fish processing industry is an important income generator for Thailand. During processing, a large amount of wastes is generated. Fish solid wastes constitute 50–70% of the original raw material, depending on the processes used and types of products. Those wastes have been of interest as high-protein human foods, instead of employing them as pet foods (Montero, Jimenez-Colmenero, & Borderias, 1991; Shahidi, 1994). Among the value-added products derived from those wastes, collagen from skin, scale and bone has increasingly been of interest owing to its abundance. Collagen contents (acid-solubilised collagen; ASC) in fish skins of young and adult Nile perch were 63.1% and 58.7%, respectively (Muyonga et al., 2004), whereas those of Japanese sea-bass, chub mackerel and bullhead shark were 51.4%, 49.8% and 50.1%, respectively (Nagai & Suzuki, 2000). Collagen contents generally vary with fish species, age and season (Ciarlo, Paredi, & Fraga, 1997; Foegeding et al., 1996; Montero et al., 1991; Nagai, Araki, & Suzuki, 2002). Type I collagen is a fibrous collagen and is the major type in fish waste materials, including skin, bone, scale and fins of various fish species (Ikoma et al., 2003; Kimura, 1983; Kimura, 1992; Nagai & Suzuki, 2000; Nagai et al., 2002; Sato, Yoshinaka, Itoh, & Sato, 1989). Recently, biochemical properties of black drum and sheapshead seabream skin collagen, (subtropical fish) have been characterised (Ogawa et al., 2003). Nevertheless, collagen from different species and habitats might be different in terms of molecular compositions and properties (Foegeding et al., 1996). So far, little information regarding the characteristics of marine fish skin collagen, especially from commercially important species involving those used for surimi production, has been reported. Therefore, this investigation aimed to isolate and characterise acid- and pepsin-solubilised collagens (PSC) from the skin of Brownstripe red snapper (*Lutjanus vitta*) which is one of the main fish species used for surimi production in Thailand.

2. Materials and methods

2.1. Chemicals

β-Mercaptoethanol (βME), pepsin (EC 3.4.23.1) powdered; 750 U/mg dry matter, *Staphylococcus aureus* V8 protease (EC 3.4.21.19) and protein markers were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Sodium dodecyl sulfate (SDS), acetic acid, and Tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt, Germany). *Achromobacter lyticus* Lysyl endopeptidase (EC 3.4.21.50) was from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

2.2. Fish skin preparation

Brownstripe red snapper (*Lutjanus vitta*), with an average length of 22–25 cm, were caught from the Songkhla coast along the Gulf of Thailand, stored in ice and off-loaded 24–36 h after capture. Upon arrival at the dock in Songkhla, fish were stored in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai. Fish were washed using tap water. Skins were then removed, descaled, and cut into small pieces (0.5×0.5 cm). Skins were kept on ice prior to collagen extraction.

2.3. Skin collagen preparation

The collagen was extracted according to the method of Nagai & Suzuki (2000) with a slight modification. All processes were carried out at 4 °C. Skin was soaked in 0.1 M NaOH with a sample/solution ratio of 1:30 (w/v) for 24 h with gentle stirring. The solution was changed every 8 h to remove noncollagenous proteins and pigments. Alkali-treated skins were then washed with distilled water until neutral or faintly basic pHs of wash water were obtained. Fat was removed in 10% (v/v) butyl alcohol with a sample/solution ratio of 1:30 (w/v) for 24 h with gentle stirring and a change of solution every 8 h. Defatted skins were thoroughly washed with distilled water. The matter was soaked in 0.5 M acetic acid with a sample/solution ratio of 1:30 (w/v) for 24 h with gentle stirring. The mixture was then centrifuged at 20,000*g* for 1 h at 4 °C. The supernatants were collected and kept at 4 °C. The precipitate was re-extracted in 0.5 M acetic acid with a sample/solution ratio of 1:30 (w/v) for 16 h with gentle stirring, followed by centrifugation at 20,000*g* for 1 h at 4 °C. The supernatants obtained were combined with the first extract. The combined extracts were precipitated by the addition of NaCl to a final concentration of 2.6 M in 0.05 M Tris–HCl (pH 7.5). The resultant precipitate was collected by centrifugation at 20,000*g* for 1 h at 4 °C and then dissolved in 10 volumes of 0.5 M acetic acid. The solution obtained was dialysed with 10 volumes of 0.1 M acetic acid in a dialysis membrane with molecular weight cut-off of 30 kDa for 12 h at 4 °C with change of solution every 4 h. Subsequently, the solution was dialysed with 10 volumes of distilled water with changes of water until neutral pH was obtained. The dialysate was freeze-dried and referred to as ASC. Undissolved residue, obtained after acid extraction, was thoroughly rinsed with distilled water, suspended in 2 volumes of 0.5 M acetic acid and subjected to limit hydrolysis with 10% (w/v) pepsin (EC 3.4.23.1; powderised; 750 U/mg dry matter, Sigma Chemical Co. (St. Louis, Mo, USA)) for 48 h at 4 °C with gentle stirring. The viscous solution was centrifuged at 20,000*g* for 1 h at 4 °C. To terminate the pepsin reaction, the supernatant obtained was dialysed against

10 volumes of 0.02 M sodium phosphate buffer (pH 7.2) in a dialysis membrane with molecular weight cut off of 30 kDa for 24 h at 4 °C with change of solution every 4 h. The dialysate obtained was centrifuged at 20,000*g* for 1 h. The pellet obtained was dissolved in 10 volumes of 0.5 M acetic acid. The solution was further precipitated by the addition of NaCl to a final concentration of 2.6 M in 0.05 M Tris–HCl (pH 7.5). The resultant precipitate was collected by centrifugation at 20,000*g* for 1 h at 4 °C and re-dissolved in 10 volumes of 0.5 M acetic acid. The solution was dialysed with distilled water and freeze-dried in the same manner as for ASC preparation. Dry matter was referred to as PSC.

2.4. Electrophoretic analysis

Protein patterns of collagen samples were analysed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970). Collagen samples were dissolved in 0.02 M sodium phosphate buffer (pH 7.2) containing 1% (w/v) SDS and 3.5 M urea. The sample mixtures were gently stirred at 4 °C for 12 h to dissolve total proteins. Supernatants were collected after centrifuging at 3000*g* for 3 min at 4 °C. Solubilised collagen samples were mixed with the sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol) with and without 10% (v/v) βME, using the sample/sample buffer ratio of 1:1 (v/v). Samples were loaded on to the PAGEL®-Compact precast gel (5% gel) and subjected to electrophoresis at a constant current of 20 mA/gel using a Compact-PAGE apparatus (Atto Co., Tokyo, Japan). 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. High-molecular-weight markers (Sigma Chemical Co., St. Louis, Mo, USA) were used to estimate the molecular weights of proteins. Calf skin acid-soluble type I collagen (Sigma Chemical Co., St. Louis, Mo, USA), porcine cartilage acid-soluble type II collagen, porcine skin acid-soluble type III collagen, and porcine placenta acid-soluble type V collagen (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) were used as standard collagens.

2.5. Amino acid composition

Collagen samples were hydrolysed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2(2-aminoethyl)indole at 115 °C for 24 h, and the hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 ml was applied to an amino acid analyser (MLC-703; Atto Co., Tokyo, Japan).

2.6. Peptide mapping of collagen

Peptide mapping of collagen samples was performed according to the method of Saito, Kunisaki, Urano, & Kimura (2002) with a slight modification. The freeze-dried samples (0.2 mg) were dissolved in 0.1 ml of 0.1 M sodium phosphate, pH 7.2 containing 0.5% (w/v) SDS. After the addition of 10 μl of the same buffer containing 5 μg of *S. aureus* V8 protease (EC 3.4.21.19, 800 unit/mg solid Sigma Chemical Co., St. Louis, Mo., U.S.A.) or 0.05 μg of lysyl endopeptidase from *A. lyticus* (EC 3.4.21.50; 4.5 AU/mg protein; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) to collagen solutions, the reaction mixture was incubated at 37 °C for 25 min or 5 min for V8 protease and lysyl endopeptidase, respectively. The reaction was terminated by subjecting the reaction mixture to boiling water for 3 min. Peptides generated by the protease digestion were separated by SDS–PAGE using 7.5% gel. Peptide mapping of calf skin collagen acid-soluble type I was conducted in the same manner and the peptide maps were compared.

2.7. Collagen solubility test

2.7.1. Solubility determination

The solubility of collagens was determined in 0.5 M acetic acid at various pH levels and NaCl concentrations according to the method of Montero et al. (1991) with a slight modification. Collagen samples were dissolved in 0.5 M acetic acid with gentle stirring at 4 °C for 12 h to obtain the final concentrations of 3 and 6 mg/ml.

2.7.2. Effect of pHs on collagen solubility

Eight ml of collagen solutions (3 mg/ml) were transferred to a centrifuge tube and the pH was adjusted with either 6 M NaOH or 6 M HCl to obtain a final pH ranging from 1 to 10. The volume of sample solutions was made up to 10 ml with distilled water, previously adjusted to the same pH as the collagen sample solutions tested. The solutions were stirred gently for 30 min at 4 °C and centrifuged at 10,000*g* for 30 min at 4 °C. Protein content in the supernatants was determined by the method of Lowry, Rosebrough, Farr, & Randall (1951) using bovine serum albumin as a protein standard. Relative solubility of collagen samples was calculated in comparison with that obtained at the pH rendering the highest solubility.

2.7.3. Effect of salt concentration on collagen solubility

Five ml of collagen solutions (6 mg/ml) in 0.5 M acetic acid were mixed with 5 ml of cold NaCl in acetic acid of various concentrations (0%, 2%, 4%, 6%, 8%, 10% and 12% (w/v)), to obtain the final NaCl concentrations of 1%, 2%, 3%, 4%, 5% and 6% (w/v). The mixtures were stirred gently at 4 °C for 30 min and centrifuged at 10,000*g* for 30 min at 4 °C. Protein content in the

supernatants was determined by the method of Lowry et al. (1951) and relative solubility was calculated in comparison with that found at the salt concentration exhibiting the highest solubility.

2.8. Thermal transition measurement

Collagen samples were prepared by the method described by Rochdi, Foucat, & Renou (2000) with a slight modification. The freeze-dried collagen samples were rehydrated in deionised water or 0.05 M acetic acid solution with a sample/solution ratio of 1:40 (w/v). The mixtures were allowed to stand for 2 days at 4 °C.

The thermal transition of collagens was measured using Perkin–Elmer Differential Scanning Calorimetry (DSC) (Model DSC-7, Norwalk, CT, USA). Temperature calibration was performed using the Indium thermogram. The rehydrated samples (5–10 mg) were accurately weighed into aluminium pans, sealed, and scanned over the range of 20–50 °C with a heating rate of 1°C/min. Ice water was used as a cooling medium and the system was equilibrated at 20 °C for 5 min prior to the scan. The empty aluminium pan was used as the reference. The maximum transition temperature (T_{max}) was estimated from the maximum peak of DSC transition curve.

2.9. Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test (Steel & Torrie, 1980). Analysis was performed using a SPSS package (SPSS 8.0 for windows, SPSS Inc, Chicago, IL).

3. Results and discussion

3.1. Isolation of ASC and PSC from Brownstripe red snapper skin

Acid-solubilised collagen and pepsin-solubilised collagen were isolated from Brownstripe red snapper skin with yields of 9.0% and 4.7% (wet weight basis), respectively. The skin was not completely solubilised by 0.5 M acetic acid extraction. This result was in agreement with Jongjareonrak, Benjakul, Visessanguan, & Tanaka (accepted) who reported the incomplete solubilisation of bigeye snapper skin in 0.5 M acetic acid. The result suggested that the collagen molecules in Brownstripe red snapper skin were most likely cross-linked by covalent bonds through the condensation of aldehyde groups at the telopeptide region as well as the inter-molecular cross-linking, leading to a decrease in solubility of collagen (Burghagen, 1999; Foegeding et al., 1996). With further limited pepsin digestion, the cross-linked molecules at the telopeptide region were cleaved without damaging the integrity of the triple helix. Therefore, the collagen with the predominant monomeric molecules could be solubilised in the presence of acid (Hickman et al., 2000). From the result, the major fraction of collagen from Brownstripe red snapper skin was ASC (66% based on extractable collagen weight) and a lower content of PSC was found (34%). The greater content of ASC fraction in Brownstripe red snapper skin was in accordance with those reported in bigeye snapper (*Priacanthus marcracanthus*) skin (85%) (Jongjareonrak et al., accepted) and hake (*Merluccius hubbsi*) skin (85%) (Ciarlo et al., 1997). However, the PSC fraction in Brownstripe red snapper skin was 2-fold higher than those obtained from those two species. It is suggested that collagen with more inter-molecular cross-links is present to a greater extent in the skin of Brownstripe red snapper, than in bigeye snapper and hake skin.

3.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The electrophoretic patterns of ASC and PSC were analysed in the presence and absence of βME (Fig. 1). Generally, no differences in the electrophoretic patterns of ASC or PSC, with or without βME, were observed, indicating that both collagen fractions contained no disulfide bonds. Montero, Borderias, Turnay, & Leyzarbe (1990) reported that similar electrophoretic diagrams of collagen from the skin of both hake and trout were observed in the presence or absence of βME. From this result, α1- and α2-chains were found as the major constituents for both ASC and PSC. High-molecular-weight components (High MW components), including β- and γ-components, as well as their cross-linked molecules, were also observed in both fractions.

High MW cross-linked molecules in collagen increase with animal age (Foegeding et al., 1996) and starving fish has more cross-linked collagen than those that are well fed (Love, Yamaguchi, Creach, & Lavety, 1976; Sikorski, Kolakowska, & Pan, 1990). However, the cross-linking rate of collagen in fish skins is extremely slow and the highly cross-linked molecule is rarely found (Cohen-Solal, Louis, Allian, & Meunier, 1981). High MW cross-linked molecules in ASC were possibly digested, as evidenced by the lower density of β- and γ-component bands with a concomitant increase in band intensity of α-chains in the PSC fraction. Pepsin cleaves the cross-link containing telopeptide, and β-chain is concomitantly converted to two α-chains (Sato et al., 2000). The band intensity of the α1-chain was 2-fold higher than that of the α2-chain for both ASC and PSC. The α1- and α2-chain patterns were similar to that of standard collagen type I from calf skin (Lane 2). It is suggested that type I collagen is the major collagen in

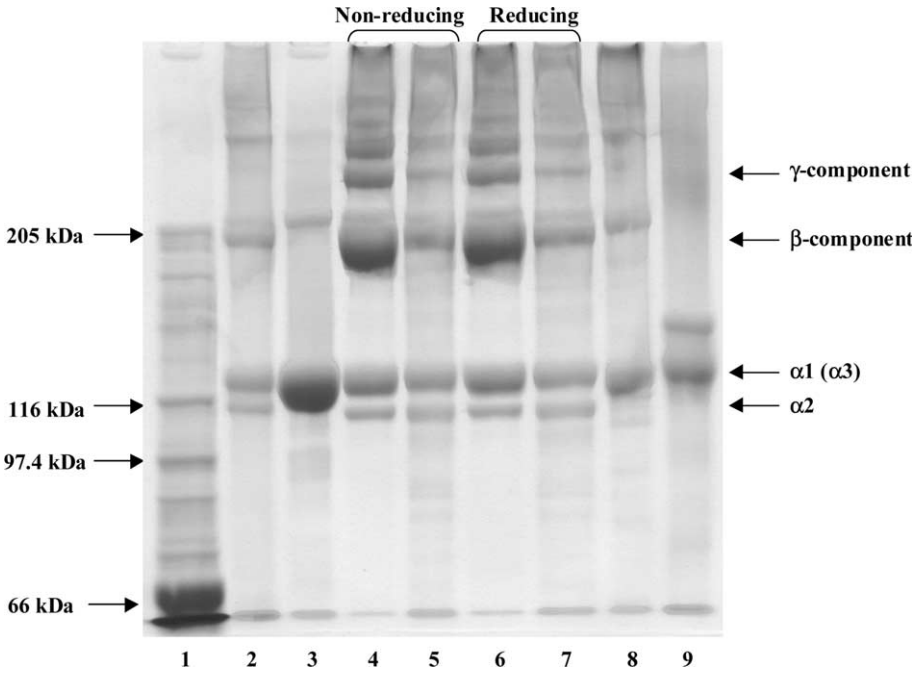

Fig. 1. Protein pattern of ASC and PSC from Brownstripe red snapper skin under reducing and non-reducing conditions. Lane 1: high MW protein markers; lanes 2, 3, 8, and 9: collagen types I, II, III, and V, respectively; lanes 4 and 5: ASC and PSC under non-reducing conditions; lanes 6 and 7, ASC and PSC under reducing conditions.

both ASC and PSC from Brownstripe red snapper skin. This observation is in agreement with the findings reported for skin collagens from hake (Ciarlo et al., 1997; Montero et al., 1990), trout (Montero et al., 1990), Nile perch (Muyonga et al., 2004), blackdrum and sheephead seabream (Ogawa et al., 2003) and bigeye snapper (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). Type I collagen consists of two α1- and one α2-chain as the major component ($[\alpha1]_2\alpha2$). Since the α3-chain has a molecular mass indistinguishable from α1 and as it cannot be separated from α1 under the electrophoretic conditions employed, the co-presence of α3 with α1 might be possible.

3.3. Amino acid composition

The amino acid composition of ASC and PSC is expressed as residues per 1000 total amino acid residues and is shown in Table 1. Generally, ASC and PSC extracted from Brownstripe red snapper skin had similar amino acid profiles. From the results, ASC and PSC were rich in glycine (25.2% and 23.5%), alanine (14.3% and 14.2%), and proline (13.1% and 13.5%). In general, glycine occurs uniformly, at every third residue throughout most of collagen molecules, except for the first 14 amino acids from the N-terminus and the first 10 from the C-terminus (Burghagen, 1999; Foegeding et al., 1996; Wong, 1989). Both the ASC and the PSC fraction consisted of proline, hydroxyproline and hydroxylysine, which are unique amino acids found in collagen. The amounts of imino acids, proline and hydroxyproline, in ASC and PSC were 212 and 221 residues per 1000 residues, respectively. The contents of imino acids in ASC and PSC from Brownstripe red snapper skin were relatively high and were similar to that of ASC from calf skin (215 residues per 1000 residues) (Herbage, Bouillet, & Bernengo, 1977). Imino acids contribute to the denaturation temperature and

Table 1
Amino acid composition of ASC and PSC from Brownstripe red snapper skin (residues per 1000 total amino acid residues)

Amino acids	ASC	PSC
Hydroxyproline	81	86
Aspartic acid	50	49
Threonine	29	30
Serine	37	39
Glutamic acid	81	79
Proline	131	135
Glycine	252	235
Alanine	143	142
Valine	18	17
Methionine	15	14
Isoleucine	7	8
Leucine	24	24
Tyrosine	4	2
Phenylalanine	15	16
Hydroxylysine	9	15
Lysine	33	34
Histidine	7	6
Arginine	65	68
Total	1000	1000

the stability of the helix structure of collagen (Ikoma et al., 2003). ASC and PSC had high levels of hydroxyproline and hydroxylysine and were similar to ASC from calf skin reported by Herbage et al. (1977). This result suggested a high degree of oxidation of hydroxylated residues of proline and lysine in collagens from Brownstripe red snapper skin. The formations of hydroxyproline and hydroxylysine were catalysed by proline hydroxylase and lysine hydroxylase, respectively (Foegeding et al., 1996; Wong, 1989). Imino acid contents of collagen from Brownstripe red snapper skin were relatively high, compared with those reported for collagen from skins of carp, hake, trout, ocellate puffer, black drum, sheephead and bigeye snapper, which contained imino acids ranging from 158 to 211 residues per 1000 residues (Jongjareonrak et al., accepted; Kimura, 1992; Kittiphattanabawon et al., 2005; Montero et al., 1990; Nagai et al., 2002; Ogawa et al., 2003). Proline and hydroxyproline contents vary with species and their living habitat (Foegeding et al., 1996; Love et al., 1976). The pyrolidine rings of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain and help to strengthen the triple helix (Wong, 1989). Thus, collagen from Brownstripe red snapper might have a different molecular conformation from that of other species owing to the high content of imino acids.

3.4. Peptide mapping of collagen

Peptide maps of collagens (ASC and PSC) digested by V8 protease and lysyl endopeptidase, in comparison with calf skin collagen type I, were revealed by SDS–PAGE (7.5% gel) as shown in Fig. 2. Generally, band intensity of α1- and α2-chains, as well as high MW cross-link, γ- and β-components of ASC, PSC and calf skin collagen type I, decreased after limited digestion by V8 protease (Lanes 5–7) and lysyl endopeptidase (Lanes 8–10). A concomitant increase in small peptides was also observed. Calf skin collagen type I digested by V8 protease (Lane 5) underwent a slight decrease in band intensity of the α-, β-components, and high MW cross-linked molecules and there was an appearance of peptide fragments with MW of 154.7, 95.0, 83.6 and 38.6 kDa. For ASC and PSC, the α-component and high MW cross-linked molecules were more hydrolysed after digestion with V8 protease. The results suggest that the α-component and high MW cross-linked molecules from calf skin collagen type I are more tolerant to hydrolysis by V8 protease than are ASC and PSC from Brownstripe red snapper skin. V8 protease shows a high specific preference for glutamic acid and aspartic acid residues of proteins (Vercaigne-Marko, Kosciarz, Nedjar-Arroume, & Guillochon, 2000). Due to the lower contents of glutamic

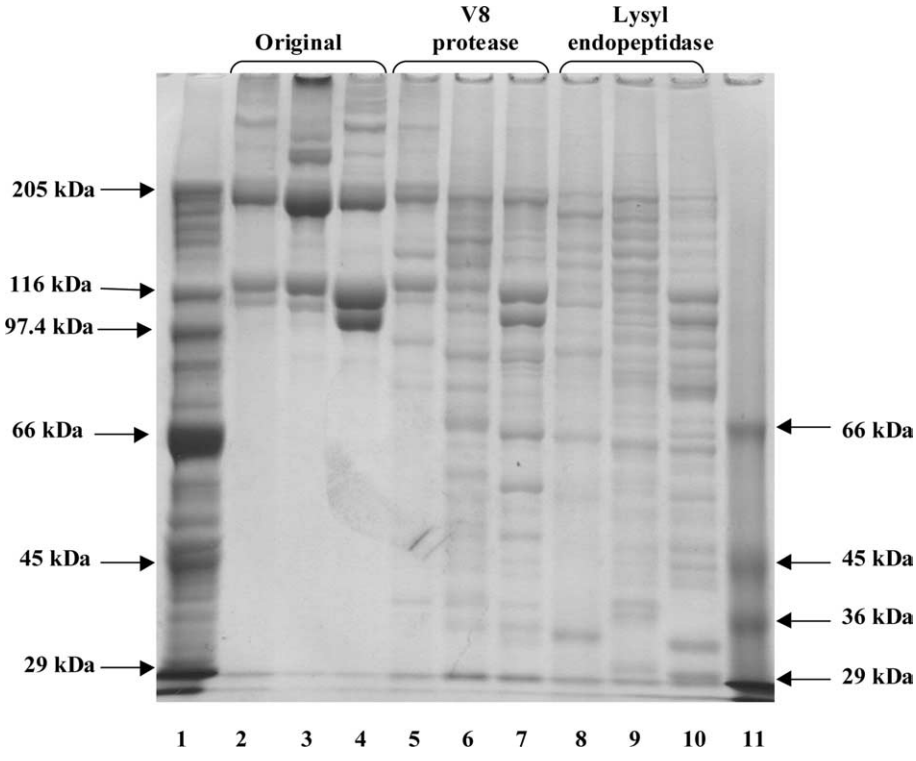

Fig. 2. Peptide maps of ASC and PSC from Brownstripe red snapper skin digested by V8 protease and lysyl endopeptidase. Lanes 1 and 11: high and low MW protein markers, respectively; lanes 2, 3, and 4: collagen type I, ASC, and PSC; lanes 5, 6, and 7: peptide fragments of collagen type I, ASC, and PSC with V8 protease digestion, respectively; lanes 8, 9, and 10: peptide fragments of collagen type I, ASC, and PSC with lysyl endopeptidase digestion, respectively.

acid and aspartic acid residues (75 and 45 residues per 1000 residues) in calf skin collagen (Herbage et al., 1977), ASC (81 and 50 residues per 1000 residues) and PSC (79 and 49 residues per 1000 residues), which had the greater glutamic acid and aspartic acid contents, might be more susceptible to hydrolysis by V8 protease. From this result, ASC was more prone to hydrolysis than PSC. However, similar patterns of peptide fragments were observed. After hydrolysis, the α-component and high MW cross-linked molecules of ASC from the skin of Brownstripe red snapper were degraded into small MW peptides, ranging from 103.6 to 34.0 kDa. In addition, peptide fragments of high MW, ranging from 205 to 116 kDa, were also observed. For PSC, the α-component was hydrolysed to some extent, while high MW cross-linked molecules were totally digested and small MW peptides were also observed with molecular weights ranging from 103.6 to 32.3 kDa.

For the peptide map of collagens digested by lysyl endopeptidase (Lanes 8–10), all collagens were more susceptible to hydrolysis by lysyl endopeptidase than by V8 protease, as evidenced by the disappearance of the β-component (205 kDa) and the high MW cross-linked molecules, with a concomitant increase in small MW fragments. This result was in accordance with the peptide mapping of collagens from skin and bone of bigeye snapper, as reported by Kittiphattanabawon et al. (2005). The α-component and the high MW cross-linked molecules of calf skin collagen type I and ASC from Brownstripe red snapper skin mostly disappeared after hydrolysis, while the α-component of PSC still remained to some extent. The generated peptides from ASC and PSC, after digestion, had MWs ranging from 205 to 30 kDa, while those of calf skin collagen were lesser in number of bands. The differences in peptide map between the different collagens generated by lysyl endopeptidase and V8 protease digestion suggest that there might be some differences in their primary structure, especially the α-helix strand (Nagai et al., 2002; Omura, Urano, & Kimura, 1996; Yoshinaka, Mizuta, Suzuki, & Sato, 1991). Accessibility of susceptible bonds in collagen structure to proteinase might be different, causing varying degrees of hydrolysis between ASC and PSC. Peptide maps of collagens were reported to differ among sources and species (Mizuta, Yamasa, Miyagi, & Yoshinaka, 1999). Thus, ASC and PSC from Brownstripe red snapper skin might be different in terms of domain or cross-links and totally different from calf skin collagen type I in term of sequence and composition of amino acids.

3.5. Effect of pHs on collagen solubility

The effect of pH on the solubility of ASC and PSC from Brownstripe red snapper skin is shown in Fig. 3.

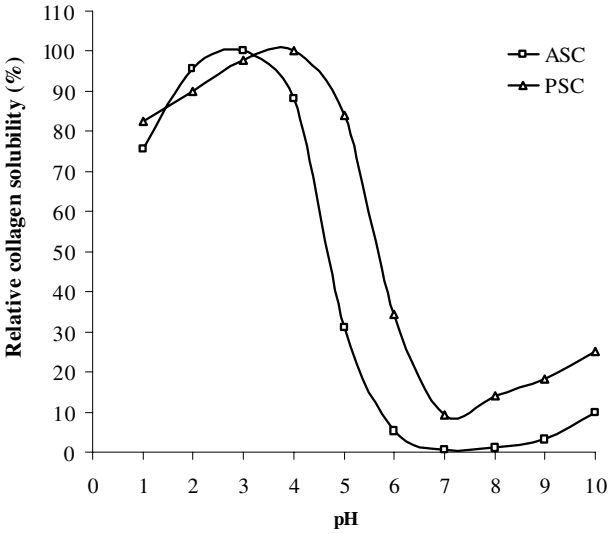

Fig. 3. Solubility of ASC and PSC from Brownstripe red snapper skin in 0.5 M acetic acid at different pHs.

The solubilities of ASC and PSC reached maxima at pH 3 and 4, respectively ($P < 0.05$). In general, both collagens were solubilised to a greater extent in acidic pH ranging from 1 to 4. Marked decreases in solubility were observed and reached the minimum when the pH was increased to 7 ($P < 0.05$). The further increases in pH up to 10 caused a slight increase in solubility. As the pH is lower or higher than pI, the net charge residues of protein molecules are greater and the solubility is increased by the repulsion forces between chains (Vojdani, 1996). In contrast, total net charges of protein molecules are zero and hydrophobic–hydrophobic interaction increases, thereby leading to the precipitation and aggregation at pI. It has been reported that collagen has isoelectric points at pH 6–9 (Foegeding et al., 1996). Thus the lowest solubility of ASC and PSC was obtained at pH around 7. This result was in accordance with the solubility of collagen from trout muscle and skin, which was lowest at pH 7 (Montero et al., 1991). From the result, ASC was solubilised more than PSC at all pHs tested except at pH 2 and 3, suggesting a higher degree of molecular crosslinking of the ASC fraction, or the predominance of stronger bonds than PSC (Montero, Gomez-Guillen, & Borderias, 1999). This was evidenced by the higher contents of high MW cross-link of ASC than of PSC (Fig. 1). The variation in solubility of collagens with pH has been reported for bigeye snapper skin and bone over the pH ranges of 1–10 (Kittiphattanabawon et al., 2005).

3.6. Effect of salt concentration on collagen solubility

The effect of NaCl on the solubility of ASC and PSC extracted from Brownstripe red snapper skin is depicted in Fig. 4. Solubility of ASC and PSC in 0.5 M acetic acid

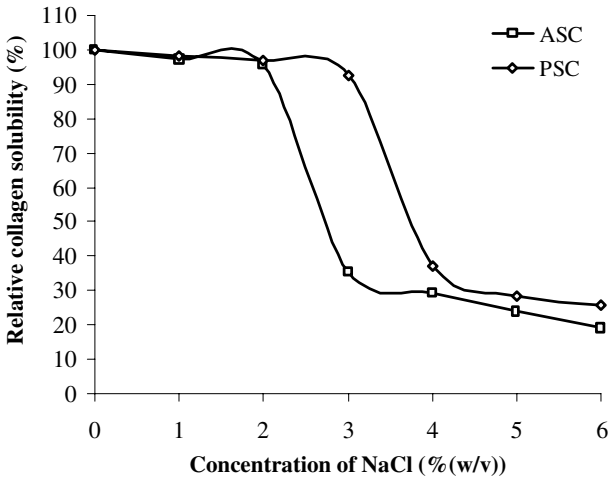

Fig. 4. Solubility of ASC and PSC from Brownstripe red snapper skin in 0.5 M acetic acid with different NaCl concentrations.

remained constant at NaCl concentrations up to 2% ($P > 0.05$). A drastic decrease in the solubility of ASC was observed at 3% NaCl or above ($P < 0.05$). For PSC, a slight decrease in solubility was obtained in the presence of 3% NaCl. A sharp decrease in solubility was observed with 4% NaCl or above. The solubility of collagens from the skin of trout, hake, bigeye snapper (*Priacanthus tayenus*), and bigeye snapper (*P. marcracanthus*) in acetic acid solution generally decreased with increasing NaCl concentration (Kittiphattanabawon et al., 2005; Jongjareonrak et al., accepted; Montero et al., 1999, 1991). The decrease in solubility of collagens could be described by the salting out phenomenon which occurred at relatively low NaCl concentrations (Asghar & Henrickson, 1982). An increase in ionic strength causes a reduction in protein solubility by an enhanced hydrophobic–hydrophobic interaction between protein

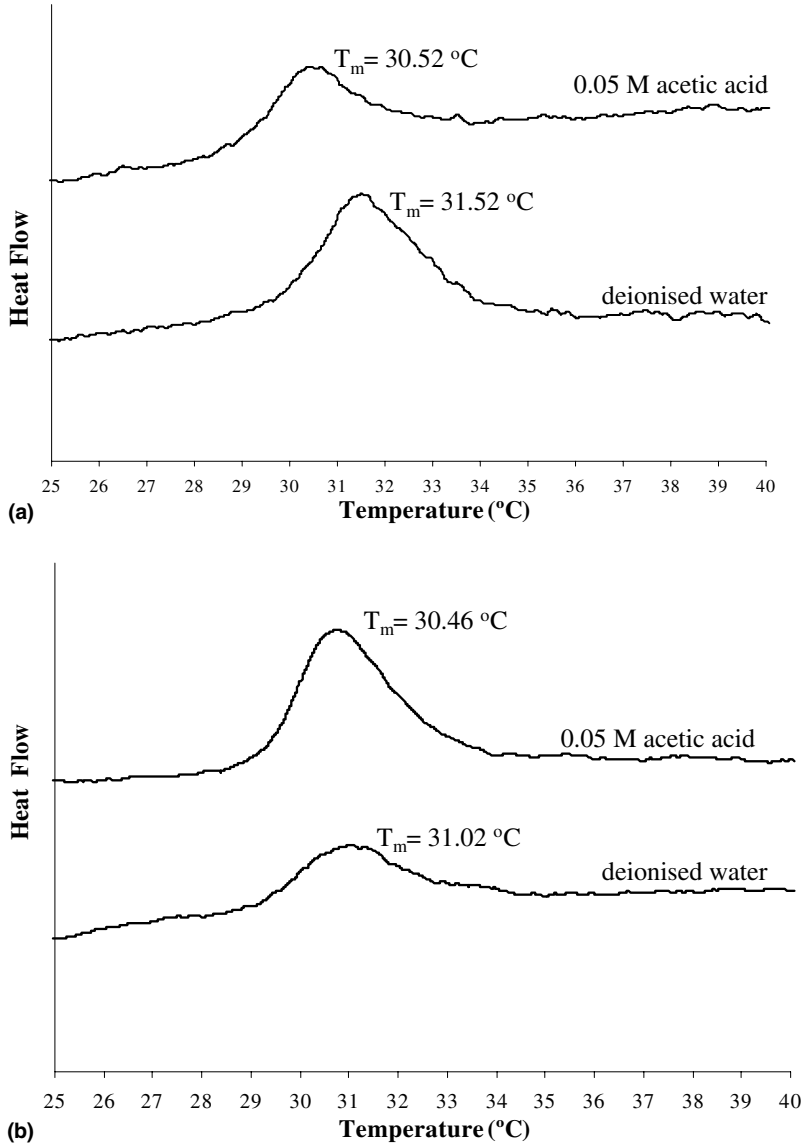

Fig. 5. Thermograms of ASC (a) and PSC (b) from Brownstripe red snapper skin rehydrated in 0.05 M acetic acid and deionised water.

chains, and the competing for water of ionic salts, leading to the induced protein precipitation (Damodaran, 1996; Vojdani, 1996). From the result, similar behaviour was found for both collagen fractions. However, PSC exhibited a greater solubility than ASC at NaCl concentrations greater than 2%. A greater solubility of PSC could be due to the partial hydrolysis of high MW cross-linked molecules by pepsin. In addition, the differences in compositions and molecular species between ASC and PSC fractions might result in such different characteristics.

3.7. Thermal stability of collagen

Thermal transitions of ASC and PSC from Brownstripe red snapper skin rehydrated in deionised water and 0.05 M acetic acid are depicted in Fig. 5. ASC and PSC showed transition curves with maximum temperatures (T_{max}) of 31.52 and 31.02 °C, respectively, in deionised water and at 30.52 and 30.46 °C, respectively, in 0.05 M acetic acid. T_{max} of ASC was similar to that of PSC in both media, suggesting no differences in the denaturation temperature between the two fractions. The triple helix structure was still predominant in the PSC fraction when the skin material was limited to digestion by pepsin (Hickman et al., 2000). T_{max} of ASC and PSC from Brownstripe red snapper skin was much lower than that of mammalian collagen, such as calf skin collagen (37 °C) (Ogawa et al., 2003). Imino acid contents (proline and hydroxyproline) show a direct positive correlation with the thermal stability of protein via hydrogen bonds (Sikorski, Scott, & Buisson, 1984). Therefore, calf skin collagen which has a higher hydroxyproline content (94 residues per 1000 residues) than ASC and PSC (81 and 86 residues per 1000 residues, respectively), denatures at higher temperature. Denaturation temperatures of collagens from Brownstripe red snapper skin were higher than those of collagen from other species, such as hake (10 °C), Baltic cod (15 °C), Alaska pollack (16.8 °C), bullhead shark (25 °C), Japanese seabass (26.5 °C), and ocellate puffer (28 °C) (Ciarlo et al., 1997; Kimura & Ohno, 1987; Nagai & Suzuki, 2000; Nagai et al., 2002; Sadowska et al., 2003). The higher denaturation temperature of ASC and PSC from the skin of Brownstripe red snapper, which is a tropical fish, possibly resulted from the higher content of imino acids (Table 1) than those of temperate fish.

The denaturation temperature of both collagens shifted to a lower temperature in the presence of 0.05 M acetic acid, when compared with those rehydrated in distilled water. Intramolecular hydrogen bonds which play an important role in stabilising the triple helix structure (Ramachandran, 1988) of collagen were cleaved by acetic acid (Gustavson, 1956). Therefore, the helix-coil structure was destroyed, resulting in a decrease in the transition temperature of collagens rehydrated in 0.05 M acetic acid, as observed by the lower T_{max} of both ASC and PSC.

4. Conclusion

ASC and PSC from the skin of Brownstripe red snapper were isolated with yields of 9% and 4.7%, respectively. The collagens were characterised as type I without disulfide bond. ASC exhibited some differences in amino acid profiles, protein patterns, and sequence of primary structure, compared to PSC and was totally different from calf skin collagen. Denaturation temperatures of collagens were similar between ASC and PSC and relatively higher than that of temperate fish. ASC and PSC were soluble at acidic pH and lost the solubility with increasing salt concentrations.

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References

Asghar, A., & Henrickson, R. L. (1982). Chemical, biochemical, functional and nutritional characteristics of collagen in food systems. In C. O. Chichester, E. M. Mrata, & B. S. Schweigert (Eds.). *Advances in food research* (vol. 28, pp. 237–273). London: Academic Press.

Bailey, A., Paul, R. G., & Knott, L. (1998). Mechanisms of maturation and ageing of collagen. *Mechanisms of Aging and Development, 106*, 1–56.

Burghagen, M. (1999). Meat. In H. D. Belitz & W. Grosch (Eds.), *Food chemistry* (2nd ed., pp. 527–580). Berlin, Germany: Springer-Verlag.

Ciarlo, A. S., Paredi, M. E., & Fraga, A. N. (1997). Isolation of soluble collagen from hake skin (*Merluccius hubbsi*). *Journal of Aquatic Food Product Technology, 6*(1), 65–77.

Cohen-Solal, L., Louis, M. L., Allian, J. C., & Meunier, F. (1981). Absence of maturation of collagen crosslinks in fish skin. *FEBS Letters, 123*, 282–284.

Damodaran, S. (1996). Amino acids, peptides, and proteins. In O. R. Fennema (Ed.), *Food chemistry* (3rd ed., pp. 321–429). New York: Marcel Dekker.

Foegeding, E., Lanier, T. C., & Hultin, H. O. (1996). Characteristics of edible muscle tissue. In O. R. Fennema (Ed.), *Food chemistry* (3rd ed., pp. 879–942). New York: Marcel Dekker.

Gustavson, K. H. (1956). *The chemistry and reactivity of collagen*. New York: Academic Press.

Helcke, T. (2000). Gelatin, the food technologist's friend or foe?. *International Food Ingredients, 1*, 6–8.

Herbage, D., Bouillet, J., & Bernengo, J.-C. (1977). Biochemical and physiological characterization of pepsin-solubilized type-II

collagen from bovine articular cartilage. *Biochemical Journal, 161*, 303–312.

Hickman, D., Sims, T. J., Miles, C. A., Bailey, A. J., Mari, M., & Koopmans, M. (2000). Isinglass/collagen: denaturation and functionality. *Journal of Biotechnology, 79*, 245–257.

Ikoma, T., Kobayashi, H., Tanaka, J., Walash, D., & Mann, S. (2003). Physical properties of type I collagen extracted from fish scales of *Pagrus major* and *Oreochromis niloticas*. *International Journal of Biological Macromolecules, 32*, 199–204.

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

Kimura, S. (1983). Vertebrate skin type I collagen: comparison of bony fishes with lamprey and calf. *Comparative Biochemistry and Physiology Part B, 74*, 525–528.

Kimura, S. (1992). Wide distribution of the skin type I collagen α3 chain in bony fish. *Comparative Biochemistry and Physiology Part B, 102*, 255–260.

Kimura, S., & Ohno, Y. (1987). Fish type I collagen: tissue-specific existence of two molecular forms, α1α2α3, in Alaska pollack. *Comparative Biochemistry and Physiology Part B, 88*, 409–413.

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*, 355–362.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature, 227*, 680–685.

Love, R. M., Yamaguchi, K., Creach, Y., & Lavety, J. (1976). The connective tissue and collagen of cod during starvation. *Comparative Biochemistry and Physiology Part B, 55*, 487–492.

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

Mizuta, S., Yamasa, Y., Miyagi, T., & Yoshinaka, R. (1999). Histological changes in collagen related to textural development of prawn meat during heat processing. *Journal of Food Science, 64*, 991–995.

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

Montero, P., Gomez-Guillen, M. C., & Borderias, A. J. (1999). Functional characterisation of muscle and skin collagenous material from hake (*Merluccius merluccius* L.). *Food Chemistry, 65*, 55–59.

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.

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

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

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

Nomura, Y., Sakai, H., Ishi, Y., & Shirai, D. (1996). Preparation and some properties of type I collagen from fish scales. *Bioscience Biotechnology Biochemistry, 60*, 2092–2094.

Ogawa, M., Moody, M. W., Portier, R. J., Bell, J., Schexnayder, 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.

Omura, Y., Urano, N., & Kimura, S. (1996). Occurrence of fibrillar collagen with structure of $(\alpha_1)_2\ \alpha_2$ in the test of sea urchin *Asthenosoma ijimai*. *Comparative Biochemistry and Physiology Part B, 115*, 63–68.

Ramachandran, G. N. (1988). Stereochemistry of collagen. *Journal of Peptide Protein Research, 31*, 1–16.

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

Sadowska, M., Kolodziejska, I., & Niecikowska, C. (2003). Isolation of collagen from the skin of Baltic cod (*Gadus morhua*). *Food Chemisrty, 81*, 257–262.

Saito, M., Kunisaki, N., Urano, N., & Kimura, S. (2002). Collagen as the major edible component of sea cucumber (*Stichopus japonicus*). *Journal of Food Science, 67*, 1319–1322.

Sato, K., Ebihara, T., Adachi, E., Kawashima, S., Hattori, S., & Irie, S. (2000). Possible involvement of aminotelopeptide in self-assembly and thermal stability of collagen I as revealed by its removal with protease. *Journal of Biological Chemistry, 275*, 25870–25875.

Sato, K., Yoshinaka, R., Itoh, Y., & Sato, M. (1989). Molecular species of collagen in the intramuscular connective tissue of fish. *Comparative Biochemistry and Physiology Part B, 92*, 87–91.

Shahidi, F. (1994). Seafood proteins and preparation of protein concentrates. In F. Shahidi & J. R. Botta (Eds.), *Seafood chemistry, processing technology and quality* (pp. 3–9). Great Britain: Chapman & Hall.

Sikorski, Z. E., Kolakowska, A., & Pan, B. S. (1990). The nutritive composition of the major groups of marine food organisms. In Z. E. Sikorski (Ed.), *Seafood: Resources, nutritional composition, and preservation* (pp. 29–54). Florida: CRC Press.

Sikorski, Z. E., Scott, D. N., & Buisson, D. H. (1984). The role of collagen in the quality and processing of fish. *Critical Reviews in Food Science and Nutrition, 20*, 301–343.

Steel, R. G. D., & Torrie, J. (1980). *Principles and procedures of statistics, a biomaterial approach*. New York: McGraw-Hill.

Vercaigne-Marko, D., Kosciarz, E., Nedjar-Arroume, N., & Guillochon, D. (2000). Improvement of *Staphylococcus aureus*-V8-protease hydrosis of bovine haemoglobin by its adsorption on to a solid phase in the presence of SDS: peptide mapping and obtention of two haemopoietic peptides. *Biotechnological Application Biochemistry, 31*, 127–134.

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

Wong, D. W. S. (1989). *Mechanism and theory in food chemistry*. New York: Van Nostrand Reinhold.

Yoshinaka, R., Mizuta, S., Suzuki, T., & Sato, M. (1991). The immunological study on the effect of pepsin digestion on genetically distinct types of collagen in muscle of kuruma prawn *Peneaus japonicus*. *Comparative Biochemistry and Physiology Part B, 98*, 59–65.