Characterization of Acid- and Pepsin-soluble Collagens Extracted from Scales of Carp and Lizardfish Caught in Japan, Bangladesh and Vietnam with a Focus on Thermostability

Sheik Md. Moniruzzaman, Kigen Takahashi, Nur Un Nesa, Sumate Keratimanoch, Emiko Okazaki and Kazufumi Osako

1Department of Food Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan
2Department of Marine Biosciences, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan
3Center of Excellence in Food Processing Pilot Plant, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Wangmai, Pathumwan, Bangkok 10330, Thailand

Received October 5, 2018; Accepted December 29, 2018

Acid- and pepsin-soluble collagens (ASC and PSC) from scales of carp and lizardfish from temperate and sub-tropical countries were studied in regard to thermostability. SDS-PAGE revealed that ASC and PSC were classified as type I collagen, and the molecular weight of ASC was greater than that of PSC. Both types of collagen from sub-tropical fish scales contained greater imino acids compared to those from temperate fish scales. Carp from Japan and Bangladesh showed similar thermal stability, but lizardfish from Vietnam showed higher stability than lizardfish from Japan for both collagen types. Overall, PSC possessed a slightly lower denaturation temperature than the corresponding ASC, suggesting that a relationship between thermostability and the molecular mass of collagen, as observed in SDS-PAGE, might exist. Maximal solubility was noticed at acidic pH (1-4) and solubility obviously declined at a high salt concentration (>2 %). ASC and PSC from these fish scales could be applicable to the food industry in place of mammalian collagens.

Keywords: denaturation temperature, acid- and pepsin-soluble collagen, carp (Cyprinus carpio), lizardfish (Saurida wanieso), temperate and sub-tropical countries

Introduction

Collagen comprises the main structural protein in vertebrates, accounting for about 30 % of total protein, and is found in the connective tissues of animals (i.e., skin, scale, bone, tendon, etc.) (Foegeding et al., 1996). Twenty-nine non-identical types of collagen have been distinguished, with type I being the most abundant collagen in the human body (McCormick, 2009). Recently, the food, pharmaceutical, and cosmetic industries worldwide have been experiencing a growing demand for collagen. To fulfill this demand, collagen has been commonly isolated from terrestrial mammalians, such as cows and pigs, for commercial applications. However, these widely used mammalian collagens have significant limitations due to substantial socio-cultural, religious and health-related concerns. For example, Muslims and Jews cannot consume pork and its derivatives. Also, Hindus and Buddhists cannot consume bovine products to some extent due to religious restrictions. In addition, porcine and bovine collagens have been associated with several disease outbreaks (i.e., bovine spongiform encephalopathy, transmissible mink encephalopathy, feline spongiform encephalopathy, foot-and-mouth disease, vesicular stomatitis, and Japanese encephalitis), resulting in uneasiness among consumers (Jongjareonrak et al., 2005). Moreover, intense competition exists among manufacturers for the
acquisition of mammalian resources, which has led to enhanced demand and excessive prices. This has propelled scientists to find and develop alternatives to mammalian collagen. Fish collagen is not associated with disease outbreaks and is acceptable for all religions and cultures. Furthermore, the sources of fish collagen are by-products (e.g., scales, skin, fins, swim bladders, bones, and heads) of fish processing industries, accounting for approximately 50-70% of the raw waste materials produced, and their disposal can cause severe environmental problems. In this context, the production and application of fish collagen satisfies human consumption needs as well as reduces waste and pollution.

In general, in the Association of Southeast Asian Nations region, lizardfish is considered to be a low-value fish, whereas in Southern Japan, it is regarded as high-grade raw material for surimi and kamaboko gel (Okuda, 2001). The largest catch of this fish was 7,716 tonnes (t) in Japan in 1999. Carp is the national fish of Japan and is a popular food fish in Bangladesh. Approximately 3,015 and 111,966 t of carp were produced in Japan and Bangladesh, respectively, in the 2016-17 fiscal year (DoF, 2017; Statistics Department of MAFF, 2018). By-products of these fish species consist of about 5% scales, which are usually discarded. Utilization of these scales could be advantageous based on availability and price compared to commercial collagens from other marine animals. At present, collagen from the scales of several marine and freshwater fishes has been isolated and characterized. Nevertheless, acid- and pepsin-soluble collagens (ASC and PSC, respectively) from the scales of carp from Japan and Bangladesh and of lizardfish from Japan and Vietnam have not been reported. It is assumed that habitat and body temperature affect the structure, composition (especially imino acids), and properties, such as thermal stability, of collagen. Additionally, we assumed that the thermostability of ASC and PSC is associated with the molecular weight (MW) of collagen. However, no information exists on the spatio-temporal variation in collagen properties from by-products of marine and freshwater fishes of temperate and sub-tropical countries. Therefore, the aim of this study was to clarify the properties of collagen from the scales of lizardfish caught in Japan and Vietnam, and carp caught in Japan and Bangladesh.

Materials and Methods

Fish scales Scales of carp (Cyprinus carpio) were collected from Miyazaki Prefecture, Japan (temperate country), on January 2017 and from Natore, Bangladesh (sub-tropical country), on March 2017. Lizardfish (Saurida waniesso) scales were collected on February 2017 from Nagasaki Prefecture, Japan (temperate country) and from a frozen seafood company in Cần Thơ city, Vietnam (sub-tropical country). The scales acquired from Japan were brought to our laboratory under a chilled condition, and samples from Bangladesh and Vietnam were frozen before air-shipping to the laboratory, where the scales were cleaned with cold deionized water, placed in polyethylene bags and stored immediately at -30°C prior to analysis.

Determination of moisture, protein and ash contents Moisture, crude protein and ash contents were determined following the standard protocol of AOAC (2000).

Extraction of acid-soluble collagen from sampled fish scales Extraction of acid-soluble collagen was carried out according to the method of Nagai and Suzuki (2000) with slight modifications. Fish scale samples were cut into small pieces with scissors and immersed in 0.1 M sodium hydroxide with continuous stirring by an overhead stirrer at a sample/sodium hydroxide solution ratio of 1:8 (w/v) for 6 h. The sodium hydroxide solution was replaced after 3 h. Then the samples were washed in chilled distilled water until a neutral pH was attained. The pH of the wash water was determined using a digital pH meter (LAQUA Model 9680 JF80; HORIBA Scientific, Tokyo, Japan). Demineralization of scales was performed by treating with 0.5 M ethylenediaminetetraacetic acid disodium salt solution (pH 7.5) at a sample/ethylenediaminetetraacetic acid disodium salt solution of 1:10 (w/v) for 24 h and washing with chilled distilled water.

After pretreatment, fish scales were extracted with 0.5 M acetic acid at a sample/acetic acid ratio of 1:3 (w/v) for 3 days under constant stirring by a magnetic stirrer. Next, the extract was centrifuged at 20,000 × g for 1 h using a centrifuge (SUPREMA 21; Tomy Seiko Co., Ltd, Tokyo, Japan). The obtained supernatant was salted out by adding sodium chloride to a final concentration of 2.6 M in the presence of 0.05 M Tris (hydroxymethyl) aminomethane (pH 7.5), while the residue was kept for pepsin-soluble collagen extraction. The resulting precipitate was collected by centrifugation at 20,000 × g for 30 min. The resultant pellet was dissolved with a minimum volume of 0.5 M acetic acid, subsequently dialyzed against 0.1 M acetic acid for 48 h, followed by distilled water for another 48 h, with replacement of the solution every 12 h. Thereafter, the resulting dialysate was freeze-dried by using a freeze dryer (Model DC401; Yamato Scientific Co., Ltd, Tokyo, Japan) and referred to as “acid-soluble collagen, ASC” and stored at -30°C until analysis. All the preparation procedures were conducted below 4°C.

Extraction of pepsin-soluble collagen from sampled fish scales The undissolved scale residue obtained after acid extraction was used for pepsin-soluble collagen extraction following the method of Chuaychan et al. (2015) with a minor modification. The residue was immersed in 0.5 M acetic acid containing 1% pepsin (Pepsin 1:10,000, from Porcine Stomach Mucosa; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at a sample/solvent ratio of 1:10 (w/v) for 48 h with continuous stirring using a magnetic stirrer at 4°C. Next, the mixture was centrifuged, precipitated, dissolved, dialyzed, and freeze-dried as previously described. The obtained collagen was referred to as “pepsin-soluble collagen, PSC” and stored at -30°C until
further analysis.

**Analysis of extracted ASC and PSC**

**Extraction yield of ASC and PSC** The yield of collagen was calculated based on the dry weight of the scale sample according to the following equation:

\[
\text{Yield (\%)} = \frac{\text{Weight of lyophilized collagen (g)}}{\text{Weight of dry fish scale (g)}} \times 100 \quad \text{Eq. 1}
\]

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)** SDS-PAGE of collagen from sampled fish scales was determined following the method of Laemmli (1970) with minor modifications. Both ASC and PSC were dissolved in 0.1 M acetic acid and centrifuged at 10,000 \( \times g \) for 10 min to remove undissolved matter. Then the supernatant was mixed with sample buffer (0.5 M Tris (hydroxymethyl) aminomethane, pH 6.8, containing 10% (w/v) SDS, 20% (v/v) glycerol, and 0.1% Bromophenol Blue) in the presence of 10% (v/v) \( \beta \)-mercaptoethanol at a collagen/sample buffer ratio of 1:1 (v/v) and boiled for 3 minutes. Each sample (about 10 \( \mu \)g) was loaded onto the polyacrylamide gel (7.5%) and electrophoresed in an electrophoresis instrument (AE-6530; ATTO Corporation, Tokyo, Japan) at a constant voltage of 250 V and current of 20 mA for 75 minutes. After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Blue R-250 in 30% (v/v) methanol and 10% (v/v) acetic acid and then de-stained with 30% (v/v) methanol and 10% (v/v) acetic acid. High-molecular-weight protein markers (PageRuler Unstained Protein Ladder; Thermo Scientific, Vilnius, Lithuania) were used to estimate the molecular weight of proteins.

**Analysis of amino acid composition** Amino acid composition was analyzed following the method of Le et al. (2014) with a slight modification. Twenty milligrams of freeze-dried collagen was hydrolyzed in 6 M hydrochloric acid at 110\( ^\circ \)C for 22 h under vacuum. The hydrolysate was neutralized with 6 M and 0.6 M sodium hydroxide and filtered through a cellulose membrane filter. Then the filtrate was used to determine sulfur-containing amino acids using an amino acid analysis system. Afterwards, the degrees of proline hydroxylation (%) and lysine hydroxylation (%) were calculated as follows:

\[
\text{Degrees of Pro hydroxylation (\%)} = \frac{\text{Hydroxyproline content}}{\text{Hydroxyproline content+Proline content}} \times 100 \quad \text{Eq. 2}
\]

\[
\text{Degrees of Lys hydroxylation (\%)} = \frac{\text{Hydroxylysine content}}{\text{Hydroxylysine content+Lysine content}} \times 100 \quad \text{Eq. 3}
\]

**Differential scanning calorimetry (DSC)** Denaturation temperatures of all the collagen samples were evaluated by using DSC (Pyris 1; PerkinElmer Co., Ltd., Yokohama, Japan) according to Kittiphathanabawon et al. (2005) with a slight modification. The ASC and PSC were rehydrated in deionized water at a sample/solution ratio of 1:40 (w/v) and the mixture was retained for 48 h at 4\( ^\circ \)C with gentle shaking by a sawed shaker prior to analysis. DSC was calibrated by using indium as the standard and an empty sealed aluminum pan was used as the reference. Collagen samples (14-16 mg) were exactly weighed into aluminum pans and sealed. Next, the aluminum pans were scanned over a range of 20-50\( ^\circ \)C, with a heating rate of 1 \( ^\circ \)C/min and using iced water as a cooling medium. The denaturation temperature (\( T_d, ^\circ \)C) was documented from each endothermic peak, and enthalpy change (\( \Delta H, \text{J/g} \)) was estimated by measuring the equivalent area under each endothermic peak of the DSC transition curves.

**Solubility of collagen** The solubility of collagen was determined at various pH values and sodium chloride concentrations according to the method of Montero et al. (1991) as modified by Le et al. (2014). Both ASC and PSC samples were dissolved in 0.1 M acetic acid with gentle shaking by a TAITEC Invitro shaker (Wave-SI; TAITEC Corporation, Saitama, Japan) at 4\( ^\circ \)C overnight to obtain final concentrations of 3 mg/mL (for pH) or 6 mg/mL (for sodium chloride).

To investigate the effect of pH on collagen solubility, 8 mL of collagen solution (3 mg/mL) was transferred to a 50 mL centrifuge tube and adjusted with either 6 M hydrochloric acid or 6 M sodium hydroxide to obtain a final \( \text{pH} \) range of 1-10. Then the final volume of the solution was made up to 10 mL by adding deionized water. The mixture was stirred gently for 30 min at 4\( ^\circ \)C, and then centrifuged at 20,000 \( \times g \) for 30 min at 4\( ^\circ \)C. For both ASC and PSC, the protein content in the supernatants was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the protein standard. Then the relative solubility was calculated in comparison with that obtained at the \( \text{pH} \) giving the highest solubility using the following equation:

\[
\text{Relative solubility (\%)} = \frac{\text{Solubility at pH x}}{\text{Solubility at pH x}} \times 100
\]
Table 1. Moisture, protein and ash contents of fish scales (based on dry weight)

<table>
<thead>
<tr>
<th>Species</th>
<th>Moisture (%)</th>
<th>Before removal of non-collagenous protein and demineralization</th>
<th>Ash (%)</th>
<th>After removal of non-collagenous protein and demineralization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Protein (%)</td>
<td>Protein (%)</td>
<td>Ash (%)</td>
</tr>
<tr>
<td>Carp (Japan)</td>
<td>55.67 ± 0.77</td>
<td>51.03 ± 1.69</td>
<td>23.36 ± 1.59</td>
<td>33.68 ± 1.19</td>
</tr>
<tr>
<td>Carp (Bangladesh)</td>
<td>57.12 ± 1.94</td>
<td>47.25 ± 1.69</td>
<td>25.86 ± 0.84</td>
<td>41.65 ± 0.47</td>
</tr>
<tr>
<td>Lizardfish (Japan)</td>
<td>56.65 ± 2.13</td>
<td>42.61 ± 1.09</td>
<td>43.65 ± 0.73</td>
<td>37.49 ± 1.22</td>
</tr>
<tr>
<td>Lizardfish (Vietnam)</td>
<td>64.85 ± 0.74</td>
<td>34.19 ± 1.10</td>
<td>51.88 ± 0.64</td>
<td>31.64 ± 0.97</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (n = 4). Different superscripts in the same column demonstrate statistical differences (p < 0.05).

Solubility (%) = 

\[
\left( \frac{\text{Protein concentration (mg/mL) of supernatants}}{\text{Protein concentration (mg/mL) of sample (highest solubility)}} \right) \times 100
\]

\[\cdots\cdot\cdot\cdot\text{Eq. 4}\]

To investigate the effect of sodium chloride concentration on collagen solubility, 5 mL of collagen solution (6 mg/mL) was mixed with 5 mL of sodium chloride in 0.1 M acetic acid at various concentrations to obtain the final concentrations of 0%, 1%, 2%, 3%, 4%, 5%, and 6%. The mixture was stirred gently for 30 min at 4 °C and centrifuged at 20,000 × g for 30 min at 4 °C. Protein content in the supernatants was measured as shown above. Relative solubility of the collagen samples was calculated and compared with the control (without sodium chloride) using the following equation:

Solubility (%) = 

\[
\left( \frac{\text{Protein concentration (mg/mL) of supernatants}}{\text{Protein concentration (mg/mL) of the control (without NaCl)}} \right) \times 100
\]

\[\cdots\cdot\cdot\cdot\text{Eq. 5}\]

Statistical analysis All the experiments were carried out in at least triplicate and the data were provided as means ± standard deviation. Differences between variables were examined using Duncan’s multiple range tests (DMRT). Analysis was conducted using SPSS software (SPSS Version 18.0 for Windows).

Results and Discussion

Proximate composition of sampled fish scales The proximate composition of the sampled fish scales is presented in Table 1. The lowest moisture content was approximately 55%. Carp and lizardfish scales from Japan contained higher crude protein than those from Bangladesh and Vietnam, respectively. On the other hand, the ash contents of carp and lizardfish scales from Japan were lower than those from Bangladesh and Vietnam, respectively. The results of protein and ash contents coincide with other freshwater and marine fish scales reported from temperate and sub-tropical countries (Matmaroh et al., 2011; Zhang et al., 2010). Moreover, immersing the sample in 0.1 M sodium hydroxide for 6 h removed the non-collagenous proteins, and treatment with 0.5 M ethylenediaminetetraacetic acid disodium salt solution (pH 7.5) for 24 h efficaciously eliminated the ash in the scales. These might increase the efficacy of collagen extraction by disrupting the matrix of the scales.

Isolation of ASC and PSC from scales The extraction yields (on a dry weight basis) of ASC and PSC from scales of carp caught in Japan were 0.97% and 1.37%, respectively, and those of carp caught in Bangladesh were 1.21% and 1.73%, respectively (data not shown). On the other hand, the yields from lizardfish scales caught in Japan were 0.44% and 0.72%, respectively, and those of lizardfish caught in Vietnam were 0.91% and 1.15%, respectively. The collagen contents in the carp scales from Japan and Bangladesh were 8.61% and 8.95%, respectively, while the recoveries of total collagen were 27.2% and 32.8%, respectively. The collagen contents in lizardfish scales from Japan and Vietnam were 7.56% and 8.78%, respectively, while the recoveries of total collagen were 15.27% and 23.52%, respectively. The results demonstrated that more collagen in the form of PSC could be recovered from the scale residue after pepsin treatment followed by ASC extraction.

SDS-PAGE of ASC and PSC SDS-PAGE of ASC and PSC was performed under reducing conditions and the protein patterns are shown in Figure 1. The SDS-PAGE pattern showed that both ASC and PSC isolated from lizardfish and carp consisted of two different α chains, α-1 (123-129 kDa) and α-2 (110-116 kDa), as major components and the band density of α-1 was higher than that of α-2, suggesting that all ASC and PSC were type I collagen. Other than α chains, all the ASC and PSC contained high-MW β-chains (dimers) of approximately 187 kDa. Additionally, ASC from lizardfish from both Japan and Vietnam also contained γ-chains (trimers) to some extent (Duan et al., 2009; Le et al., 2014). However, in the case of carp, bands below 100 kDa were observed, whereas these bands were not observed in lizardfish. These bands could be the product of collagen degradation, which indicates that some of them could be more susceptible to hydrolysis during ASC extraction.

Based on the SDS-PAGE results, it was observed that the
PSC of all sampled fish scales had a lower MW than the corresponding ASC. However, the PSC and ASC from the scales of lizardfish from Vietnam had a similar MW. The results implied that ASC from the sampled fish scales had a higher degree of cross-linking than the corresponding PSC. This might be due to the fact that after pepsin treatment, the high cross-linked structures in ASC were possibly digested, as confirmed by the low density of β- and γ-component bands, with a consequent increase in the band density of α-chains in the corresponding PSC. Pepsin cleaves the cross-links containing telopeptide, and β-chain is concomitantly converted into two α-chains (Sato et al., 2000). As a result, the MW of PSC was lower than that of ASC. These results agreed with those of Nalinanon et al. (2007), who observed a similar trend of decreasing MW of PSC after pepsin treatment. The MWs of ASC and PSC from scales of lizardfish from Japan were lower than those of lizardfish scales from Vietnam. In contrast, the ASC and PSC of freshwater carp from both Japan and Bangladesh showed a similar pattern in terms of MW. Our results suggested that the differences in MW between ASC and PSC might have an effect on collagen properties.

**Amino acid composition of sampled fish scales** The amino acid composition of ASC and PSC isolated from the fish scales sampled varied among the species and regions, as illustrated in Table 2. The results indicated that glycine was the dominant amino acid in all extracted collagen, followed by alanine. Apart from these, both ASC and PSC from the sampled fish scales were abundant in hydroxyproline, proline, and glutamic acid. Furthermore, smaller proportions of cysteine, tyrosine, histidine and hydroxylysine were also detected, revealing a similarity with collagen extracted from other freshwater and marine fish species (Duan et al., 2009; Ogawa et al., 2004). Generally, glycine represents about one-third of the total residues. However, a computational study suggested that replacing the glycine residues of collagen with d-alanine or d-serine would stabilize the triple helix (Tsai et al., 2005), and thus glycine residues in collagen serve as substitutes for non-natural d-amino acids. The electrophoretic pattern correlated with the amino acid composition of ASC and PSC isolated from the sampled fish scales, confirming that these were type I collagen from residual counts of amino acids.

**Thermal stability of ASC and PSC** Variations in total proline and hydroxyproline contents (imino acids) of carp and lizardfish scales might be due to variations in habitat.
Amino acid composition of acid- and pepsin-soluble collagen from fish scales

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues/1000 total residues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASC</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>48 ± 2a</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>88 ± 2b</td>
</tr>
<tr>
<td>Threonine</td>
<td>24 ± 2a</td>
</tr>
<tr>
<td>Serine</td>
<td>39.5 ± 4.7i</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>73 ± 5.8i</td>
</tr>
<tr>
<td>Proline</td>
<td>9.4 ± 8.1i</td>
</tr>
<tr>
<td>Glycine</td>
<td>295.9 ± 11.9ab</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (n = 3). Different superscripts in the same row demonstrate statistical differences (p < 0.05).

^a Imino acids: hydroxyproline + proline

Temperature and might affect the properties and application of collagen, since imino acids play a pivotal role in determining the thermal stability of collagen (Ikoma et al., 2003; Regenstein and Zhou, 2007). The average recorded minimum and maximum temperatures of the sampling sites during the sampling period were as follows: Miyazaki Prefecture, Japan (18.2-20.6°C); Natore, Bangladesh (23-33°C); Nagasaki Prefecture, Japan (14.3-16.3°C); and Cần Thơ, Vietnam (25.1-27.7°C) (World Sea Temperatures, 2017)). Generally, collagen from fish species of temperate environments has a lower imino acid content than that of fish from tropical or sub-tropical environments (Bae et al., 2008; Regenstein and Zhou, 2007). Our findings supported this because the imino acid contents of ASC and PSC from scales of lizardfish caught in Vietnam were higher than those of lizardfish caught in Japan. However, the imino acid contents of ASC and PSC from carp scales from Japan and Bangladesh might have a higher thermal stability than collagen from the scales of lizardfish, whilst lizardfish from Vietnam might have a higher thermal stability than those from Japan.

The degrees of proline and lysine hydroxylation were calculated as 47.5-53.2% and 25.9-36.6%, respectively, as shown in Table 3. The degree of proline and lysine hydroxylation contributes to the thermal stability of collagen (Kimura et al., 1988). Both the large quantity of hydroxyproline and hydroxylysine present in collagen and the high amount of imino acids, responsible for conferring rigidity on the collagen structure by initiating large numbers of twists into the chain, presumably promote the thermal stability of the molecule (Leach, 1967). Based on these results, a higher degree of proline and lysine hydroxylation in the ASC and PSC of carp from Japan and Bangladesh and lizardfish from Japan and Vietnam might be associated with the higher thermal stability of collagen.

Table 2. Amino acid composition of acid- and pepsin-soluble collagen from fish scales

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>ASC (Japan)</th>
<th>ASC (Bangladesh)</th>
<th>ASC (Lizardfish, Japan)</th>
<th>ASC (Lizardfish, Vietnam)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>48 ± 2a</td>
<td>44 ± 1abc</td>
<td>47 ± 2ab</td>
<td>44 ± 1b</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>88 ± 2b</td>
<td>96 ± 2c</td>
<td>87 ± 1b</td>
<td>96 ± 3c</td>
</tr>
<tr>
<td>Threonine</td>
<td>24 ± 2a</td>
<td>25.3 ± 3.1i</td>
<td>26.5 ± 2.3f</td>
<td>25.6 ± 1.5g</td>
</tr>
<tr>
<td>Serine</td>
<td>39.5 ± 4.7i</td>
<td>37.7 ± 3.5f</td>
<td>39 ± 4.6i</td>
<td>38.3 ± 4.5f</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>73 ± 5.8i</td>
<td>76 ± 6.9a</td>
<td>76.3 ± 4.9g</td>
<td>76 ± 6.1e</td>
</tr>
<tr>
<td>Proline</td>
<td>9.4 ± 8.1i</td>
<td>89.6 ± 7.3a</td>
<td>91.9 ± 8.7f</td>
<td>93.5 ± 12.6d</td>
</tr>
<tr>
<td>Glycine</td>
<td>295.9 ± 11.9ab</td>
<td>309.7 ± 7.8a</td>
<td>291.6 ± 12.9ab</td>
<td>293 ± 11ab</td>
</tr>
</tbody>
</table>

The thermal properties of ASC and PSC from sampled fish scales The Td and AH of ASC and PSC isolated from the sampled fish scales are shown in Table 4. The Td of ASC and PSC were recorded as follows: carp from Japan, 33.0°C (AH = 1.55 J/g) and 32.8°C (AH = 1.0 J/g), respectively; carp from Bangladesh, 32.8°C (AH = 1.25 J/g) and 32.6°C (AH = 1.23 J/g), respectively; lizardfish from Japan, 29.5°C (AH = 0.62 J/g) and 28.1°C (AH = 0.88 J/g), respectively; and lizardfish from Vietnam, 31.3°C (AH = 1.83 J/g) and 31.4°C (AH = 1.29 J/g), respectively. The thermal properties of ASC and PSC from sampled fish scales were shown in Table 4. The thermal properties of ASC and PSC isolated from the sampled fish scales are shown in Table 4. The thermal properties of ASC and PSC were recorded as follows: carp from Japan, 33.0°C (AH = 1.55 J/g) and 32.8°C (AH = 1.0 J/g), respectively; carp from Bangladesh, 32.8°C (AH = 1.25 J/g) and 32.6°C (AH = 1.23 J/g), respectively; lizardfish from Japan, 29.5°C (AH = 0.62 J/g) and 28.1°C (AH = 0.88 J/g), respectively; and lizardfish from Vietnam, 31.3°C (AH = 1.83 J/g) and 31.4°C (AH = 1.29 J/g).
Table 3. Degrees of proline and lysine hydroxylation from fish scale collagen

<table>
<thead>
<tr>
<th>Degrees of hydroxylation (%)</th>
<th>Carp (Japan)</th>
<th>Carp (Bangladesh)</th>
<th>Lizardfish (Japan)</th>
<th>Lizardfish (Vietnam)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASC</td>
<td>31.3 ± 0.3^aA</td>
<td>26.8 ± 0.2^abC</td>
<td>30.7 ± 0.3^cC</td>
<td>31.4 ± 0.3^cA</td>
</tr>
<tr>
<td>PSC</td>
<td>38.0 ± 0.2^aA</td>
<td>40.2 ± 0.2^abC</td>
<td>43.4 ± 0.3^A</td>
<td>41.4 ± 0.3^A</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (n = 3). Different superscripts in the same row demonstrate statistical differences (p < 0.05).

Table 4. Denaturation temperature (T_d) and enthalpy change (ΔH) of acid- and pepsin-soluble collagen

<table>
<thead>
<tr>
<th>Fish scales</th>
<th>ASC</th>
<th>PSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carp (Japan)</td>
<td>33.0 ± 0.3^A</td>
<td>28.1 ± 0.1^C</td>
</tr>
<tr>
<td>Carp (Bangladesh)</td>
<td>32.8 ± 0.2^A</td>
<td>36.2 ± 0.3^A</td>
</tr>
<tr>
<td>Lizardfish (Japan)</td>
<td>29.5 ± 0.2^A</td>
<td>31.4 ± 0.3^cD</td>
</tr>
<tr>
<td>Lizardfish (Vietnam)</td>
<td>31.3 ± 0.03^bA</td>
<td>31.4 ± 0.3^cC</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (n = 4). Different superscripts (a, b, c) in the same column demonstrate statistical differences (p < 0.05) and different superscripts for each parameter T_d (A, B) and ΔH (C, D) in the same row demonstrate statistical differences (p < 0.05).

respectively. Individually, ASC and PSC showed a similar pattern in terms of T_d among the fishes, and a higher T_d was noticed in both the ASC and PSC of carp from Japan and Bangladesh, followed by lizardfish from Vietnam and lizardfish from Japan. Nevertheless, in a comparison of the ASC and PSC of the fish, PSC had slightly lower T_d and ΔH compared to the corresponding ASC, except for carp from Bangladesh and lizardfish from Vietnam. This might be attributed to partial cleavage of the telopeptide region as well as lowering of MW caused by pepsin treatment, as evidenced from the SDS-PAGE pattern. This suggested that collagen with a high molecular mass might have a higher thermal stability. These results were in accordance with the variation in imino acid contents as well as the degree of proline hydroxylation and environmental temperature. Shoulders and Raines (2009) found that hydroxylation of proline residues in the Yaa position dramatically increases the thermal stability of the collagen triple helix. Furthermore, the results of T_d from temperate, sub-tropical, and aquacultured species varied slightly from the findings of other studies, e.g., sardine scales (27.0°C; Nomura et al., 1996), carp scales (about 28.0°C; Duan et al., 2009), and silver carp scales (around 29.0°C; Zhang et al., 2010). The comparatively high T_d of ASC and PSC isolated from carp and lizardfish scales demonstrated that the waste products of these fish have structural as well as thermal stability and can be readily employed in food industries.

Effect of pH on the solubility of ASC and PSC The effects of pH on the solubility of ASC and PSC from fish scale in 0.1 M acetic acid are shown in Figure 2. In general, ASC exhibited high solubility (≥83.45-100%) at an acidic pH (1-4) (p < 0.05) and PSC showed high solubility (≥85.18-100%) in the pH range of 1 to 5 (p < 0.05). As the pH increased above 6, a remarkable decrease in ASC solubility (17.5-31.6%) was observed. In contrast, at pH 6, the PSC solubility (48.3-63.3%) remained higher than that of the corresponding ASC. Further, the solubility became constantly low at neutral and slightly alkaline pH ranges (7-9). However, a minor increase in solubility was also observed at pH 10 in both ASC (20.02-29.11%) and PSC (20.32-46.17%). This indicates that ASC and PSC reached the isoelectric point at neutral and slightly alkaline pH ranges.

Individually, ASC and PSC from different species showed maximum and minimum solubilities at different pHs (Le et al., 2014). However, the ASC of carp scales from both Japan and Bangladesh and of lizardfish scales from both Japan and Vietnam showed a similar trend in terms of maximum and minimum solubilities. On the other hand, PSCs were found to have their maximum solubilities at different pH values, but their minimum solubilities at the same pH values. The ASC of carp scales from both Japan and Bangladesh showed their maximum and minimum solubilities at pH 1 and pH 7, respectively. However, the PSC of carp scales from Japan and Bangladesh showed their maximum solubilities at pH 2 and pH 1, respectively, and minimum solubilities at pH 8. In contrast, the ASC from lizardfish scales from both Japan and Vietnam showed their maximum and minimum solubilities at pH 2 and pH 6, respectively. On the other hand, PSC from scales of lizardfish from Japan and Vietnam showed maximum solubilities at pH 3 and pH 4, respectively, and minimum solubilities at pH 8. The higher solubilities of PSC might be caused by the breakdown of highly cross-linked structures following pepsin treatment, especially at the telopeptide region.
resulting in a lower molecular mass, as observed in SDS-PAGE. Therefore, a relationship might exist between solubility and thermal stability. The higher solubility is associated with lower stability of collagen; however, there are no reports on this point and further research is required to confirm this hypothesis.

Effect of sodium chloride concentration on the solubility of ASC and PSC

Fig. 2. Effect of pH on the solubility of ASC and PSC from carp scales caught in Japan (A) and Bangladesh (B) and from lizardfish scales caught in Japan (C) and Vietnam (D). CJ = carp caught in Japan; CB = carp caught in Bangladesh; LJ = lizardfish caught in Japan; LV = lizardfish caught in Vietnam.

Fig. 3. Effect of NaCl concentration (%) on the solubility of ASC and PSC from carp scales caught in Japan (A) and Bangladesh (B) and from lizardfish scales caught in Japan (C) and Vietnam (D). CJ = carp caught in Japan; CB = carp caught in Bangladesh; LJ = lizardfish caught in Japan; LV = lizardfish caught in Vietnam.
Characterization of Acid- and Pepsin-soluble Collagens from Fish Scales

(%) on the solubility of ASC and PSC from fish scale in 0.1 M acetic acid are shown in Figure 3. The solubilities of all ASC and PSC from carp and lizardfish scales remained constant (≥86.8-100%) in the presence of sodium chloride up to 2% (w/v) and were pointedly reduced (31.9-57.6%) at a sodium chloride concentration of 3% (w/v). Subsequently, they exhibited a constant low level of solubility (p < 0.05). However, the solubility of PSC was slightly higher than that of ASC. The solubility decreased with increasing sodium chloride concentrations up to 6%. The breakdown of cross-links at the telopeptide region by pepsin digestion as well as the low MW might have resulted in lower PSC stability, and therefore higher solubilities were observed. The reduction in solubility of ASC and PSC might be due to a “salting out” effect, which occurred at comparatively high sodium chloride concentrations (Asghar and Henrickson, 1982). The increase in ionic intensity results in a devaluation in protein solubility by enhancing “hydrophobic-hydrophobic” effects between protein chains and increasing competition for water with ionic salts, followed by accelerated protein precipitation (Damodaran, 1996; Komsa-Penkova et al., 1996). The differences observed in solubility patterns with varying pH and sodium chloride concentrations might be useful information for the practical application of collagen from fish scales.

Conclusion

The ASC and PSC from the scales of carp caught in Japan and Bangladesh and lizardfish caught in Japan and Vietnam were recognized as type I collagen. Pepsin treatment considerably increased the recovery of collagen from fish scales. The SDS-PAGE pattern revealed molecular mass differences among the samples. Amino acid contents were compared side by side with the growth habitat; by-products from fish of a temperate country had lower amino acid contents as well as Tg and enthalpy change values than those of a subtropical country. The Tg of PSCs was somewhat lower than that of the corresponding ASCs and was correlated with the SDS-PAGE pattern. Therefore, MW might be associated with the thermal stability of collagen. The collagen of carp scales showed superior characteristics, i.e., imino acid content, MW, as well as Tg, compared to their counterpart lizardfish. ASC and PSC were soluble in acidic pH and the solubility decreased with increasing sodium chloride concentration. Elucidating the differences in ASC and PSC properties might be useful for industrial applications. Finally, these collagens could be utilized as surrogates for bovine, porcine and other commercial collagen in the context of environmental amelioration, safety aspects, religious acceptance, and comparable properties.

References


*N. Md. Moniruzzaman et al.*


**URL cited**
