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Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes

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Abstract

Acid-soluble collagens (ASCs) were extracted from the skins of several underutilised fishes, namely dusky spinefoot (*Siganus fuscescens*), sea chub (*Kyphosus bigibbus*), eagle ray (*Myliobatis tobijei*), red stingray (*Dasyatis akajei*) and yantai stingray (*Dasyatis laevigata*). The yields of the ASCs from skins of dusky spinefoot and sea chub were about 3.4–3.9%, and from ray species were about 5.3–5.7%, on a dry weight basis. According to the electrophoretic pattern, ASCs consisted of two different α-chains (α1 and α2) and were classified as type I collagen. However, the molecular weights of α2-chain for ray species were lower than those of bony fishes. ASC from ray species contained a higher content of imino acids than those from dusky spinefoot and sea chub. The denaturation temperatures (T_d) of ray species were about 33 °C, which was about 5 °C higher than those of dusky spinefoot and sea chub. The high T_d of ray species suggested the possibility of its utilisation as a substitute for mammalian collagen.

Keywords: Collagen; Underutilised fish; Ray; Skin; Denaturation temperature

1. Introduction

The collagens are a family of fibrous proteins found in all multicellular animals, and they represent about 25% of their total proteins and are the predominant protein of the extracellular matrix. They comprise the major structural element of all connective tissues and are also found in the interstitial tissue of virtually all organs, where they contribute to the stability of tissues and organs, and maintain their structural integrity (Gelse, Poschl & Aigner, 2003). So far, it has been established that even though the identified collagens have common characteristics, they include more than 20 different types, varying considerably in their complexity and diversity of their structure. Due to their unique chemical features, collagens have been utilised in various fields of industry, such as leathers and film, beauty and cosmetics, biomedical and pharmaceutical applications, materials and food (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Morimura et al., 2002; Swatscheka, Schattona, Kellermannd, Muller, & Kreuterc, 2002). In addition, as the denatured collagens, most commonly known as gelatins, they also find important applications in the food and the biomedical industries. Generally, in the collagen industry, skins and bones of mammals, cattle and pig, etc., have been used as raw materials. Nevertheless, after the outbreaks of bovine spongiform encephalopathy and foot-and-mouth disease, it has been of great interest to find collagens from alternative sources, such as those that can be obtained from the aquatic environment (Senaratne, Park, & Kim, 2006).

The use of fish collagen, instead of mammalian collagen, has numerous rewards. During food processing, fish solids

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waste constitute 50–70% of the original raw material, depending on the processes used and types of products (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). Also fish collagen could be used by the Islamic and Hindu nations, which cannot use mammalian collagen, owing to religious constraints.

In Ariake Sound, fishes such as dusky spinefoot, sea chub and ray species have been considered of low economic importance because of their characteristic bad odour and taste, and unpleasant appearance. Furthermore, among those fishes, dusky spinefoot, sea chub and eagle ray, have recently increased their population, and as a result, have been regarded as the cause of whitening of seaweed (a phenomenon called "isoyake" in Japan) and feeding damage to valuable shellfish. For this reason, nowadays, great quantities of those fishes have been caught by Nagasaki prefecture; however, the cost of disposal or management has become a problem, and prompt solutions are required. There are few instances of the industry taking advantage of this important resource of fish collagen. With this and the above considerations in mind, we obtained and studied the acid-soluble collagen materials from underutilised fish, in an effort to evaluate their potential utilisation.

2. Materials and methods

2.1. Fish skin preparation

Dusky spinefoot (*Siganus fuscescens*), sea chub (*Kyphosus bigibbus*), eagle ray (*Myliobatis tobijei*), red stingray (*Dasyatis akajei*) and yantai stingray (*Dasyatis laevigata*) were caught in Ariake Sound, Nagasaki, Japan. The skins were removed, cut into small pieces (1×1 cm^2) with scissors, and kept at −30 °C until used.

2.2. Preparation of acid-soluble collagens

Collagens were extracted according to the method of Yoshinaka, Sato, and Ikeda (1976), with a slight modification. All procedures to prepare acid-soluble collagen (ASC) were carried out at 4 °C. The cleaned skins were extracted with 20 volumes (v/w) of 50 mM acetic acid for 3 days with stirring. The extract was centrifuged at 9000*g* for 1 h by high speed refrigerated centrifuge (KUBOTA 7930, KUBOTA Co., Tokyo, Japan), and the supernatant was salted out, by adding NaCl, to give a final concentration of 10%. The resulting precipitates were collected by centrifugation at 9000*g* for 1 h. The precipitate was dissolved in 20 volumes (v/w) of 50 mM acetic acid and then any insoluble material was removed by centrifugation at 9000*g* for 1 h. The supernatant was salted out by adding NaCl to 20%, and the resulting precipitates were separated by centrifugation as described above. The precipitate was dissolved in 5 volumes (v/w) of 50 mM acetic acid. After centrifuging at 9000*g* for 1 h, the supernatant was dialysed against 50 volumes (v/v) of 0.01 M Na_2HPO_4 for 3 days with a change of solution twice per day. The precipitate was obtained by centrifugation at 9000*g* for 1 h, and then lyophilised.

2.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1970), using 6% separating gel and 3% stacking gel. The samples, dissolved in 50 mM acetic acid, were mixed with the sample buffer (20 mM Tris–HCl buffer (pH 8.0) containing 8 M urea and 2% (w/v) SDS), using the sample/sample buffer ratio of 1:1 (v/v), and heated in boiling water for 5 min. The prepared samples (5–10 μg) were applied to each well and separated at 20 mA/gel. Type I collagen from tiger puffer (*Takifugu rubripes*) was used as a standard of α-chains and β-component of collagen mobilities (Mizuta, Fujisawa, Nishimoto, & Yoshinaka, 2005). After electrophoresis, protein bands were stained using the collagen staining method, as described by Fairbanks, Steck, and Wallach (1971). The gel was initially stained by soaking in 10% acetic acid containing 0.05% CBB R-250 and 25% 2-propanol overnight at room temperature. The gel was then stained in 10% acetic acid containing 0.005% CBB R-250 and 10% 2-propanol. After 6–9 h, the gel was soaked in 10% acetic acid containing 0.002% CBB R-250 overnight. Then the background of the gel was extensively destained with 10% acetic acid.

2.4. Amino acid composition

Twenty milligrams of the lyophilised samples of ASC were hydrolysed in 6 N HCl at 110 °C for 22 h under vacuum pressure. The hydrolysates, neutralised with 4 N and 0.1 N NaOH, were applied to an automated amino acid analyser (ALC 1000, Shimadzu Seisakusho Co. Ltd., Kyoto, Japan), and the amino acids were quantified with respect to the known standards. Cysteine (Cys) and methionine (Met) were analysed by the procedure of Moore (1963).

2.5. Determination of denaturation temperature

Collagen samples were prepared by the method of Kittiphattanabawon et al. (2005) with a slight modification. The freeze-dried collagen samples were rehydrated in 50 mM acetic acid solution with a sample and solution ratio of 1:40 (w/v). The mixture was allowed to stand for 2 days at 4 °C. Calorimetric measurements were performed by differential scanning calorimetry (DSC) (DSC 120, Seiko Instruments Inc., Tokyo, Japan). The instrument was calibrated for temperature and enthalpy using indium and tin as standards. The samples (5–10 mg) were weighed accurately into aluminum pans and sealed. The samples were scanned over the range of 20–50 °C with a heating rate of 1 °C/min using ice water as the cooling medium. An empty sealed pan was used as the reference. The denaturation temperature (T_d) was estimated from the thermogram. The change of enthalpy (ΔH) was determined from the peak area and expressed in mJ/mg sample material.

2.6. Collagen solubility test

The collagen solubility was measured in 50 mM acetic acid at various pHs and NaCl concentrations, according to the method of Jongjareonrak et al. (2005). Collagens were dissolved in 50 mM acetic acid to obtain a final concentration of 3 and 6 mg/ml and the mixture were stirred with rotator at 4 °C until collagen was completely solubilised.

2.6.1. Effect of pH on collagen solubility

Eight milliliters of collagen solution (3 mg/ml) in the centrifuge tube were adjusted across the pH range (of 1–10) with 6 N HCl or 6 N NaOH, and then the volume was made up to 10 ml with distilled water. The solutions were stirred gently for 30 min at 4 °C and centrifuged at 20,000*g* for 30 min at 4 °C. Protein concentration in the supernatant was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (BSA) as a standard. Relative solubility of collagen samples was calculated in comparison with that obtained at the pH rendering the highest solubility.

2.6.2. Effect of NaCl on collagen solubility

Five milliliters of collagen (6 mg/ml) in 0.05 M acetic acid were mixed with 5 ml of NaCl in 0.05 M acetic acid at various concentrations (0%, 2%, 4%, 6%, 8%, 10% and 12% (w/v)). The mixture was stirred continuously for 30 min at 4 °C, followed by centrifuging at 20,000*g* for 30 min at 4 °C. Protein concentration in the supernatant was measured by the method of Lowry et al. (1951) using BSA as a standard. Relative solubility was calculated as before.

3. Results and discussion

3.1. SDS-PAGE patterns of collagens from the skins of underutilised fishes

The collagens extracted from the fish skins were analysed by SDS-PAGE under reducing conditions (Fig. 1). The patterns of SDS-PAGE were compared with that of ASC from tiger puffer skin (Mizuta et al., 2005), showing the typical patterns of a type I collagen with the bands of α1(I), α2(I) and β-component. The electrophoretic patterns and migration of collagens from skins of dusky spinefoot, sea chub and ray species were basically similar to that of tiger puffer. From this result, it was suggested that all collagens were type I collagen formed as a heterotrimer by two identical α1-chains and one α2-chain. However, the band intensity of α1-chain was not twofold greater than that of α2-chain for all collagens extracted. It was suggested that these collagens might contain inter- and intra-molecular crosslinked components, β (dimer) and γ (trimer) components. Crosslinking of α-chains was observed in other collagens (Kittiphattanabawon et al., 2005; Muyonga, Cole, & Duodu, 2004; Nagai, Araki, & Suzuki, 2002). In addition, Kimura, Kamimura, Takema, and Kubota (1981) described that the α2-chain exists as a β_{12} dimer combined with α1-chain in shark skin collagen. Similar results were reported for the type I collagen of other elasmobranches (Hwang, Mizuta, Yokoyama, & Yoshinaka, 2007; Nomura, Yamano, & Shirai, 1995).

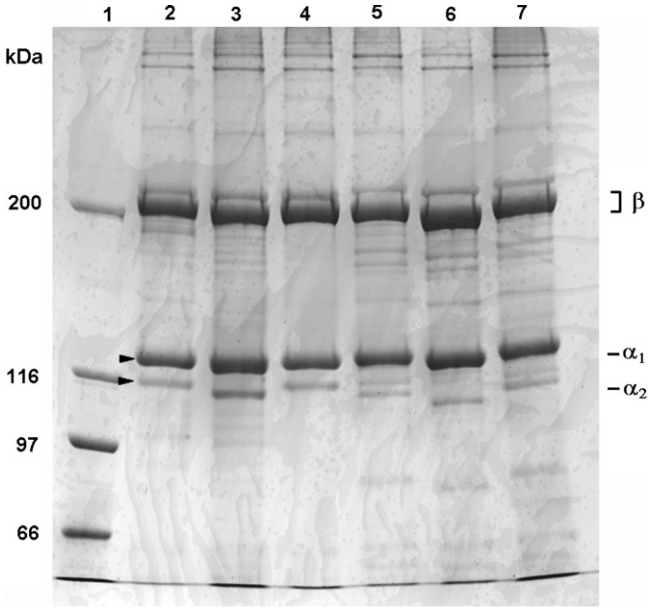

Fig. 1. SDS-PAGE patterns of ASC from the skins of underutilised fishes on 6% gel in the presence of β-mercaptoethanol. Lane 1, high molecular weight protein markers; lane 2, tiger puffer; lane 3, dusky spinefoot; lane 4, sea chub; lane 5, eagle ray; lane 6, red stingray; lane 7, yantai stingray.

The mobility of α-chains and β-chains for ray species were a little different from that of tiger puffer. Their protein bands of ASC subunit were detected at approximately 120 kDa for α1 and 112–114 kDa for α2, respectively. In particular, the mobility of the band, which was thought to be α2, was lower than that of bony fish. Moreover, the β-components, which are dimers of α-chains, for both red stingray and yantai stingray were also lower than those of tiger puffer, dusky spinefoot and sea chub. This pattern was observed in other elasmobranches, skate and shark (Hwang et al., 2007; Nomura et al., 1995). From these facts, we considered that the existence of α2-chains which have lower molecular weight may be a distinctive molecular formation of ray species.

3.2. Amino acid compositions

The amino acid compositions of ASC extracted from the skins of tiger puffer and underutilised fish are shown in Table 1. The amino acid contents were expressed as residues per 1000 total amino acid residues. The amino acid profiles were similar in all fishes determined. The collagens were rich in Gly, Ala, Pro and Hyp, as are all collagens. The contents of Cys, Met, Tyr and His were very low, also like other collagens. In particular, Gly accounted for more than 30% of all amino acids in whole samples, except for

Table 1
Amino acid compositions of acid-soluble collagen from the skin of underutilised fishes and tiger puffer

	Amino acid content (residues/1000 residues)					
	Tiger puffer	Dusky spinefoot	Sea chub	Eagle ray	Red stingray	Yantai stingray
Asp	49.7	61.6	50.9	42.7	43.1	43.2
Thr	26.2	35.9	26.7	30.6	27.4	28.4
Ser	44.8	43.4	42.0	40.2	33.0	34.6
Glu	77.6	103	78.0	86.0	80.1	81.8
Pro	109	83.9	110	113	115	117
Hyp	68.1	50.6	74.2	80.2	102	80.0
Gly	333	252	326	326	335	342
Ala	123	123	127	110	106	102
Cys	0.00	0.00	0.00	0.00	0.00	0.00
Val	27.9	36.5	20.6	28.0	26.9	27.3
Met	8.42	7.39	8.49	7.56	8.71	13.8
Ile	7.11	21.1	9.93	15.3	12.3	11.9
Leu	15.8	41.6	23.0	26.8	23.5	23.9
Tyr	2.40	8.38	3.13	2.80	2.24	1.70
Phe	11.5	18.8	13.7	13.6	9.52	10.2
His	5.95	10.4	6.32	10.8	4.48	5.68
Lys	30.1	44.5	29.4	19.2	24.1	24.4
Arg	59.0	58.4	50.6	47.1	47.0	52.3

the dusky spinefoot (25%). Gly, regularly spaced at every third residue throughout the central region of the α-chain, was the most abundant amino acid in collagen. In addition, Gly, which is the smallest amino acid having only a hydrogen atom as a side chain, allows the three helical α-chains to pack tightly together, to form the final collagen superhelix (Alberts et al., 2002). The contents of imino acids (Pro and Hyp), another important factor in the formation of the triple-stranded helix, in ASC from ray species were relatively high, compared with those of collagens from the bony fishes. Particularly, the amount of imino acids of red stingray collagen was about 216 residues per 1000 residues, presenting a significantly higher content than any other samples in this study. It is known that the pyrrolidine rings of Pro and Hyp impose restrictions on the conformation of the polypeptide chain and help to strengthen the triple helix (Riesle, Hollander, Langer, Freed, & Vunjak-Novakovic, 1998). It is suggested that the collagen of ray species is relatively stable in comparison with the collagen of other species. In addition, the amount of imino acids, especially Hyp, depends on the temperature in which the fish lives and it affects the thermal stability of the collagens (Kimura, Zhu, Matsui, Shijoh, & Takamizawa, 1988). The collagens obtained from fish species living in cold environments have lower contents of Hyp and they exhibit lower thermal stability than those from fish living in warm environments. Also, the high imino acid content, especially Hyp content, is significant because it affects the functional properties of gelatin that can be derived from collagen (Gomez-Guillen et al., 2002). For that reason, fish gelatins with low imino acid content rarely have been used in industry, although they have potential for use in several applications (Haug, Draget, & Smidsrod, 2004; Muyonga et al., 2004). Red stingray collagen contains the highest amounts of imino acids and it could possibly be used in the gelatin products having good gelling properties.

3.3. Thermal behaviour

High temperatures induce the structural melting or unfolding of the molecule. For collagen, thermal denaturation means unfolding of the triple helix, that is, loss of collagen's unique characteristics. Therefore, the denaturation temperature (T_d) is an important measure of the thermal stability of proteins. Differential scanning calorimetry (DSC) is particularly suited for the study of the thermal denaturation of protein. It measures heat flow between sample and reference zone and provides information about the thermal transitions of protein. The denaturation temperature values of collagen samples, which were calculated from the maximum transition point (the endothermic peak) of the thermal denaturation curves, were summarised in Fig. 2.

Tiger puffer collagen had a T_d of 28.4 °C, which was similar to that of ocellate puffer fish (*T. rubripes*), reported by Nagai et al. (2002). That was about 9 °C lower than that of porcine skin collagen. T_d values of dusky spinefoot and sea chub collagen were about 29 °C. These values were similar to those obtained from other marine collagen: Japanese sea bass fin (29.1 °C) (Kimura et al., 1988), skin of bigeye snapper (28.7 °C) (Kittiphattanabawon et al., 2005), muscle of eel (30.2 °C), common mackerel (26.9 °C), saury (24.0 °C), chum salmon (20.6 °C) and skins of eel (29.3 °C), common mackerel (26.1 °C), saury (23.0 °C), chum salmon (19.4 °C) (Kimura et al., 1988), and cuttlefish outer skin (27.0 °C) (Nagai, Yamashita, Taniguchi, Konamori, & Suzuki, 2001).

In comparison, the collagen of eagle ray, red stingray and yantai stingray had T_d values of 34.1 °C, 33.2 °C and 32.2 °C, respectively. These values were higher than those of other fish, although lower than 36 °C for collagen from skin of Nile perch (*Lates niloticus*) (Muyonga et al., 2004). Fish species with T_d of collagens higher than 30 °C are very limited. Most fish collagens denature at temperatures below 30 °C, indicating that fish collagen is generally less stable than mammalian collagen. The comparatively high T_d of ray species collagen (above 33 °C) indicated their high heat resistances.

At the same time, the ΔH values were indirectly in accordance with the T_d because the enthalpy change associated with collagen denaturation depends on the positional preferences of ionised residues in Gly-X-Y (Holmgren, Taylor, Bretscher, & Raines, 1998). Factors influencing the change of collagen, not only include the content of imino acid and the degree of hydroxylation of Pro, but also the high content of the Gly-Pro-Hyp sequence and collagen thermostability (Burjanadze, 1999). In this study, eagle ray has the highest value of change of enthalpy (ΔH), although both the content of imino acid and hydroxylation of Pro are lower than red stingray. Ikoma, Kobayashi, Tanaka, Walsh, and Mann (2003) reported that the difference in the enthalpy can be attributed to the specific sequences of amino acids and their

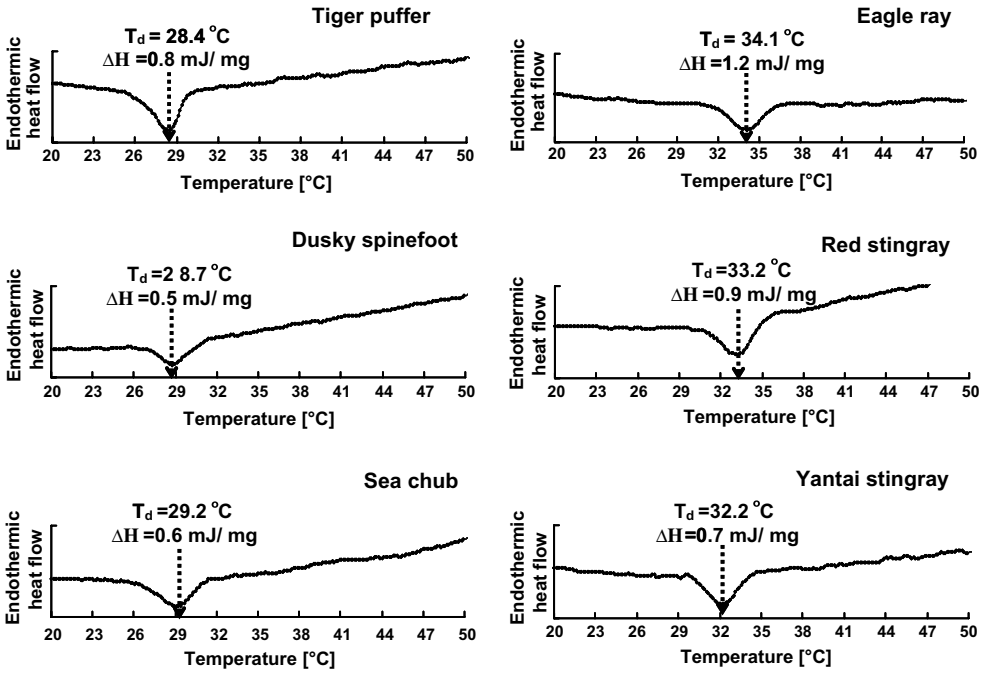

Fig. 2. DSC thermograms of ASC from the skins of tiger puffer and underutilised fishes in 0.05 M acetic acid.

influence on the stability of the triple helix. This is dependent on the formation of hydrogen bonds in the coiled α-chains, and the Gly-Pro-Hyp tripeptide sequence is known to be the most stable in collagen. Therefore, it is suggested that the ASCs from ray species could be easily utilised as a marine collagen resource by treatment with a crosslinking reagent, such as transglutaminase or 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Nomura, Toki, Ishii, & Shirai, 2001; Yunoki, Nagai, Suzuki, & Munekata, 2004), to improve thermal stability.

3.4. Effects of pH and NaCl on collagen solubility

Fig. 3 shows the effect of pH on the solubility of ASC from all samples. All collagens were solubilised in the pH range from 1 to 4, although the pH for the highest solubility differed with the species: dusky spinefoot, eagle ray, red stingray, and yantai stingray at pH 2, tiger puffer at pH 3 and sea chub at pH 4. Low solubility was observed at pH 7–9; the dissolved protein was found to precipitate in this pH range and its relative viscosity was markedly increased. The observation of precipitation and aggregation at a particular pH can be explained, in that hydrophobic interaction among collagen molecules increases at pI, and the total net charge of protein molecules become zero (Jongjareonrak et al., 2005). The differences in maximum and minimum pH for solubility among studied collagens have been attributed to differences in the molecular properties and conformations among the collagens (Kittiphattanabawon et al., 2005).

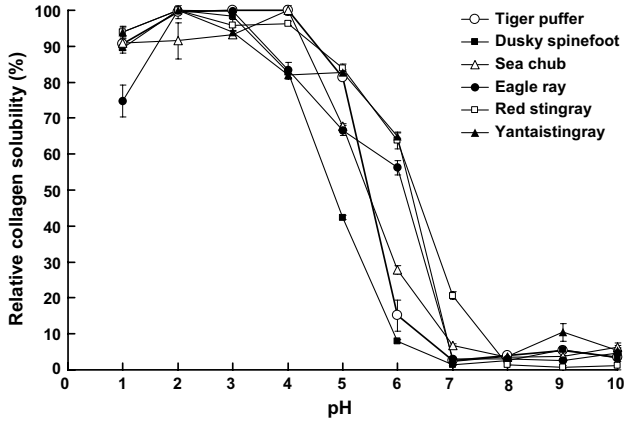

Fig. 3. Relative solubility of ASC from the skins of tiger puffer and underutilised fishes at various pHs. Values are means ± standard deviation ($n = 3$).

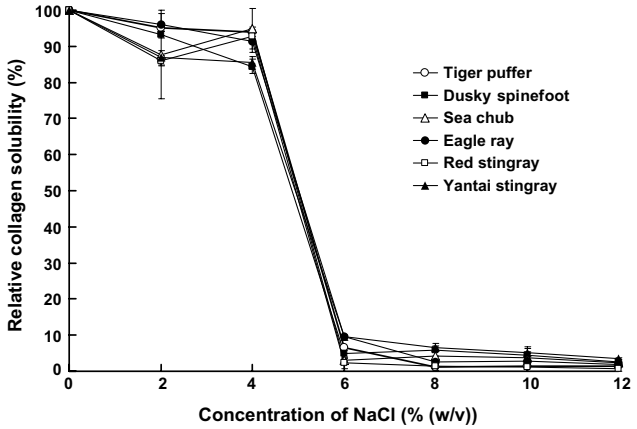

Fig. 4. Effect of NaCl concentration on collagen solubility. Values are means ± standard deviation ($n = 3$).

The effect of NaCl on the solubility of ASC extracted from all samples is shown in Fig. 4. All samples had a similar solubility pattern. Solubility of ASC in 0.05 M acetic acid remained constant at NaCl concentrations up to 4%. A dramatic decrease in the solubility of all ASC was observed above 4%. An increase in NaCl concentration produces a decline in solubility by enhancing hydrophobic–hydrophobic interactions between protein chains, and the competition for water from ionic salts, leads to protein precipitation (Jongjareonrak et al., 2005).

4. Conclusions

The collagens from skins of underutilised fish were extracted and characterised. The yields of the ASCs from skins of dusky spinefoot, sea chub, eagle ray, red stingray and yantai stingray were about 3.9%, 3.4%, 5.3%, 5.7% and 5.5%, on a dry weight basis, respectively. They were classified as type I collagen, consisting of two identical α1-chains and one α2-chain. The denaturation temperatures of ray species were about 33 °C, higher than those of bony fishes, dusky spinefoot and sea chub. Maximum solubility of ASC extracted in this study was observed over the range of pH 2–4, and a sharp decrease in solubility of all samples was observed at and above 4% NaCl. Among these results, the higher denaturation temperature of ASCs from ray species indicates that they have the potential for use as substitutes for mammalian collagens.

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