International Food Research Journal 19(1): 251-257 (2012)

Study on some properties of acid-soluble collagens isolated from fish skin and bones of rainbow trout (*Onchorhynchus mykiss*)

[1]Shahiri Tabarestani, H., [2] Maghsoudlou, Y., [3*]Motamedzadegan, A., [2]Sadeghi Mahoonak, A. R. and [4]Rostamzad, H.

[1]*Department of Food Science and Technology, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *Food Engine Department, Natural Resources and Agricultural Sciences University, Gorgan, Iran*
[3]*Department of Food Science, Sari Agricultural Sciences and Natural Resources University, Iran. PoBox 578*
[4]*Fisheries Department, Natural Resources and Agricultural Sciences Universities, Gorgan, Iran*

Abstract: To make more effective use of fish–byproduct resources, acid-soluble collagen (ASC) was isolated from the skin and bones of rainbow trout (*Onchorhynchus mykiss*) with yields of 9.448% and 1.122% on a wet weight basis, respectively. Based on their electrophoretic pattern, both collagens were classified as type I with slightly different amino acid compositions and low imino acid content. From the result, both collagens were rich in inter- and intra-molecular cross-linked components, ß and γ components with bone collagen having more band intensity. Similar changes in viscosity of collagens from the skin and bone of rainbow trout were observed. Collagens from the skin and bones had minimum solubility at pH 9 and 7, respectively. No changes in solubility were observed in the presence of NaCl up to 3% (w/v). However, a sharp decrease in solubility was found above 3% NaCl.

Keywords: Collagen, acid-soluble collagen rainbow trout, *Onchorhynchus mykiss*, fish skins, fish bones

Introduction

Collagens are generally extracellular structural proteins involved in formation of connective tissue structure and are known to occur in genetically distinct forms identified as type I through XIX. They vary considerably in their complexity and the diversity of their structure. Also, the different types show variations in amino acid composition and physical properties that also seems to be correlated with the temperature of the source animal's environment. The main sources of industrial collagen are limited to those from pig and bovine skin and bones.

Due to collagen's unique chemical features, they have been used in various ways, such as leathers and films, beauty aids and cosmetics, biomedical and pharmaceutical applications, and food (Morimura *et al*., 2002; Kittiphattanabawon *et al*., 2005). However, the occurrence of bovine spongiform encephalopathy (BSE), and foot/mouth disease (FMD) along with religious constraints has resulted in an anxiety among users of collagen and collagen-derived products from land-based animals in recent years and thus increasing attention has been paid to alternative collagen sources, especially fish skin and bones which comprise about 30% of the total fish weight available after fish fillet preparation (Shahidi, 1994).

So far, skin and bone collagen from several fish species have been isolated and characterized (Kimura *et al*., 1991; Ciarlo *et al*., 1997; Nagai and Suzuki, 2000a, 2000b; Yata *et al*., 2001; Nagai *et al*., 2002; Sadowska *et al*., 2003). Rainbow trout (*Onchorhynchus mykiss*) is a worldwide favorite fish species produced in large amounts in northern Iran. As there is an increased consumer demand for its fillet, optimal utilization of their wastes after filleting, especially evaluation of its potential use as an important source of collagen could be profitable. Hence, the objective of the present study was to extract collagen from the skin and bone of rainbow trout (*Onchorhynchus mykiss*) and investigate the biochemical properties of prepared collagens.

Material and Methods

Fish skin and bone preparation

Cultured Rainbow trout (*O. mykiss*)- on the same feeding regime, growth temperature of 12-15°C, mean size of 25 cm and age of 3 years- were delivered on ice to process plant, Kian Maahi Khazar Co.,Ltd (Babolsar, Iran) and freezed at -20°C till skinning and deboning. Skins and bones were taken off at –20°C and kept frozen till use.The skins of about 50 Kg of fish were cut into small pieces (1×1 cm^2) with scissors,

*Corresponding author.
Email: *amotgan@yahoo.com*

placed in polyethylene bags and kept at -25°C until used (within a week). Bones mainly backbone were scrapped to remove attached flesh, cut in to small pieces (1–2 cm in length), and powdered by mixing the samples in liquid nitrogen for 20 seconds (Nagai & Suzuki, 2000b) using a blender (National *Model* MK-K77,Tokyo, Japan) for 5 min. The prepared samples were kept at -25°C until used. All chemicals were of analytical grade.

Isolation and purification of acid soluble collagens from skin and bones

Collagen isolation by the method of Nagai and Suzuki (2000a) with a slight modification was done at 4°C to reduce chain fragmentation. The skin and bone were treated with 0.1 N NaOH at a sample/alkali solution ratio of 1:10 (w/v) for 6 h to remove non-collagenous proteins and pigments. Then, the skin and bone were rinsed with cold distilled water repeatedly until a neutral pH was reached. Deproteinised skins were defatted by soaking (1:10 w/v) in 10% butyl alcohol for 18 h with the solvent changed every 6 h. Defatted samples were washed with cold water, followed by soaking in 0.5 M acetic acid (1:30 w/v) for 24 h. The mixture was filtered through two layers of cheese cloth. The residue was re-extracted under the same conditions. Both filtrates were combined and salt (to make 2.6 M NaCl solution) was added to the supernatant to precipitate the collagen extract at neutral pH (in 0.05 M Tris-HCl, pH 7.5). The resulting precipitates were separated at 4°C by centrifugation at 20000g for 1 h (Sigma laboratory centrifuge, 3k30, UK). The pellet was dissolved in 0.5M acetic acid (1:5 w/v), dialysed using 12 kDa cut-off dialysis tube against 0.1 M acetic acid and distilled water, sequentially and then freeze-dried at -65°C at 0.016 torr pressure (Operon Co.,Ltd, FDU-7012, Korea).

To extract collagen from bones, deproteinised bones were decalcified with 0.5 M Ethylenediaminetetraacetic acid tetrasodium salt(EDTA 4NA) (pH 7.4, 1:10 w/v) for 40 h. The solution was changed every 10 h. The residue was washed thoroughly with cold water (4°C). Collagen was then extracted from the bone in the same manner as that used for skin. The lyophilised skin and bone were ground to a powder using a laboratory mill (Myson, Tokyo, Japan) and sieved with a 100 mesh (0.15 mm) sieve.

Proximate analysis

Moisture, ash, fat and protein content were determined by AOAC (2000) methods 985.14, 923.03, 920.39 and 990.03, respectively. Collagen content in whole skin and bone tissue was determined based on the hydroxyproline index using the method of Woessner (1961) using L-hydroxyproline (Sigma-Aldrich) as the standard. Standard solutions were prepared at several concentrations between 0 and 2.5 µg/mL based on powder weight. Finally, the absorbances were read at 557 nm using a spectrophotometer (PG Instruments, T80+UV-VIS, UK) and the Hyp concentration of the samples was calculated using the standard curve (Woessner, 1961). The percentage of Hyp in the protein was calculated using the following formula:

$$\text{Hyp\%} = [\text{Hyp conc. (mg/mL)} / \text{protein conc. (mg/mL)}] \times 100$$

The protein concentration of gelatin solutions was determined using Biuret method as described by (Zhou and Regenstein, 2006). Bovine serum albumin (BSA; ZISTCHIMI, Tehran, Iran) was used as the reference protein in the range of 0 to10 mg/mL.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis (SDS–PAGE) was performed by the method of Laemmli (1970) using the discontinuous Tris-glycine buffer (pH 8.3) system, with a home made 4% stacking gel and an 8% separating gel. Samples (2mg/ml) were dissolved in 50 mM acetic acid, mixed with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence of 10% βME, using a sample/sample buffer ratio of 1:1 (v/v), and heated in boiling water for 5 min. The samples (10 µg) were applied to each well and separated at 50V till reaching to stacking gel and 70V from then on. After electrophoresis, protein bands were stained for 30 min with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained for 12 h- according to our pretest - with 30% (v/v) methanol and 10% (v/v) acetic acid. A high molecular weight marker (Fermentous Chemical Co., St. Louis, Mo., USA) was used to estimate the molecular weight of proteins. The gels were densitometred by obtaining volumograms on a photodocumentation system from GENIUS tech and by using the version.6.08 GeneSnap software (Syngene, USA).

Amino acid composition

According to the method of Sato *et al*. (1992), the lyophilised samples of ASCs (20 mg) were hydrolysed under reduced pressure (6 N HCl, 115°C, 24 h) in the presence of 2-mercaptoethanol in a Pico Tag system (Waters Corporation, Milford, MA), Using a PicoTag column (3.9 ×150mm;Waters) at a flow rate of 1ml min^{-1} with UV detection. Breez® software was applied to data analysis. The procedure of Moore (1963) was used to determine total Cys and methionine (Met)

as cysteic acid and methionine sulfone, respectively. The analysis was performed using a Beckman 12^0C amino acid analyzer equipped with 15 cm Beckman PA- 35 resin column.

Viscosity measurement

Samples (0.03% collagen in 0.1 M acetic acid) were prepared and subjected to viscosity measurements using a Brookfield viscometer (model DV II+, Brookfield Engineering Labs Inc., Stoughton, MA, USA.) with spindle No.1 and a speed of 90 rpm, heated using water bath of rotary from 5 to 50°C with a heating rate of 5°C/min. 600 ml of the sample solution were incubated for 30 min at each temperature before the measurements. The relative viscosity was calculated in comparison to that obtained at 5°C, which was adjusted using ice flake.

Collagen solubility test

The collagen solubility was determined by the method of Montero *et al*. (1991) with a slight modification. The collagens were dissolved in 0.5 M acetic acid to obtain a final concentration of 3 mg/ml and the mixture was stirred at 4°C until the collagen was somewhat completely solubilised and no pellet observed after 5 min settlement.

Effect of pH on collagen solubility

Eight ml of collagen solution (3 mg/ml) in a centrifuge tube were adjusted across the pH range of (1 to 12) with HCl or NaOH dilution series and then the volume was made up to 10 ml with distilled water. The solutions were centrifuged at 20,000 g for 30 min at 4°C. Protein concentration in the supernatant was determined by Biuret method (Zhou and Regenstein, 2006) using weighed out bovine serum albumin (BSA; ZISTCHIMI, Tehran, Iran) as a standard. Protein solubility was calculated using the following equation:

$$\text{Solubility} = \frac{\text{(Protein content of the supernatant)}}{\text{(Total protein content in the sample)}}$$

$$\text{Relative solubility} = \frac{\text{(solubility at given pH)}}{\text{(highest solubility in the range of pH)}}$$

Effect of NaCl on collagen solubility

Five ml 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 process thereafter was like that for pH solubility.

Statistical analysis

The data were presented as means ± standard deviation of three determinations. Statistical analyses were done using a one-way ANOVA and the Student's t-test Multiple comparisons of means were done using the LSD test. A probability value of <0.05 was considered significant. All computations were made using SAS 9.1 (SAS Institute Inc., Cary, NC).

Results and Discussions

Proximate analyses of rainbow trout skin and bone and their collagen

The proximate analyses of rainbow trout skin, bone and their collagen are shown in Table1. Skins and bones were found to contain similar moisture content in the range of (41.6-48%). The amounts of protein in skin was approximately 2-fold greater than that of bone. Conversely, lower ash and fat contents were observed in skin than bone. On the other hand, skin had a higher content of hydroxyproline in comparison with bone. Acid soluble collagens were extracted with the yields of 9.448% and 1.122% on the basis of wet weight, from skin and bone, respectively which were in accordance with the results obtained for their hydroxyproline content. Proximate analyses for rainbow trout skin and bone collagen showed that the ash and fat content could be removed effectively and reduced to (0.21-0.83%) and (0.31-0.51%), respectively with protein content ranging from 89.6% to 96.2%. The acid soluble collagens from skin and bone of rainbow trout (o.mykiss) were extracted and characterized. Collagens derived from fish species living in cold environments have lower contents of hydroxyproline and they exhibit lower thermal stability than those from fish living in warm environments. This is because hydroxyproline is involved in inter-chain hydrogen bonding, which stabilises the triple helical structure of collagen. Sadowska *et al*. (2003) reported that hydroxyproline content of cod skin was 14.6 mg/g sample, while Kittiphattanabawon *et al*. (2005) reported higher hydroxyproline content(19.5 mg/g sample) for bigeye snapper skin which the latter was slightly lower than that of rainbow trout skin in the presented study (20.4 mg/g sample).

SDS - PAGE patterns of collagens from skin and bone

The collagens extracted from the fish skin and bone were analysed by SDS-PAGE (Figure 1). The electrophoretic patterns and migration of collagens from skin and bone were approximately similar, comprised at least two different α chains, α_1 and α_2

Table 1. Proximate analyses and hydroxyproline content of skin and bone of rainbow trout and their ASC

Sample	Proximate composition (% wet wt)				Hydroxyproline (mg/gr sample)
	Moisture	Ash	Fat	Protein	
Skin	41.6± 0.06 ^{b}	5.45±0.10 ^{b}	13.12±0.20 ^{b}	41.12± 0.01 ^{c}	20.4 ±0.41 ^{c}
Bone	48.03± 0.70 ^{a}	13.4±0.20 ^{a}	18.29±0.10 ^{a}	20.27± 0.40 ^{d}	8.76±0.23 ^{d}
Skin collagen	3.49±0.40 ^{d}	0.21±0.10 ^{d}	0.31±0.07 ^{c}	96.2± 0.75 ^{a}	67.2±0.20 ^{a}
Bone collagen	8.67±1.30 ^{c}	0.83±0.49 ^{c}	0.51±0.20 ^{c}	89.6±1.20 ^{b}	56.4±1.10 ^{b}

Average± SD from triplicate determination. Values followed by different letters in a column are significantly different at $p < 0.05$.

with ß-component. From this result, it was suggested that collagens from skin and bone of rainbow trout were type I collagens. Type I collagen in skin and bone of rainbow trout was a heteropolymer of two α_1 chains and one α_2 chain. The molecular masses of the collagen subunits for α_1 and α_2 were about 117 kDa and 111 kDa for skin and about 114 kDa and 108kDa for bone, respectively. However, α chain molar ratio (α_1/ α_2) of ASC in bone (1.45) was slightly higher than that of α chain molar ratio (α_1/ α_2) for collagens extracted from skin (1.13). Moreover, the mobility of the α_2 band in skin collagen was lower than that of bone collagen. From the result, both collagens were rich in inter- and intra-molecular cross-linked components, ß and γ components with bone collagen having more band intensity. From the SDS-PAGE result of ASC in the presented study, it was suggested that collagens from skin and bone of rainbow trout were type I collagens. Type I collagen is the main component in all connective tissue, including bones and skins (Montero *et al*., 1990; Ciarlo *et al*., 1997; Nagai and Suzuki, 2000b) and has functions that include mechanical protection of tissues and organs or physiological regulation of the cell environment. The results of electrophoretic patterns and migration of collagens from skin and bone is in accordance with those of skin and bone from other fish species (Ciarlo *et al*., 1997; Nagai and Suzuki, 2000a, 2000b). Saito *et al*. (2001) reported that Complete primary structure of rainbow trout type I collagen consist of alpha1(I) alpha2(I)alpha3(I) heterotrimers. However, the α_3 chain, if present, could not be separated under the electrophoretic conditions employed in the presented study, since α_3 chain has an indistinguishable molecular mass from α_1 chain and cannot be separated from the corresponding α_1 chain, even if an α_3 chain is present (Nagai and Suzukib, 2000; Jongjareonrak *et al*., 2005; Kittiphattanabawon *et al*., 2005).

Amino acid compositions of collagens from skin and bone

The amino acid compositions of ASC extracted from the skin and bone of rainbow trout are shown

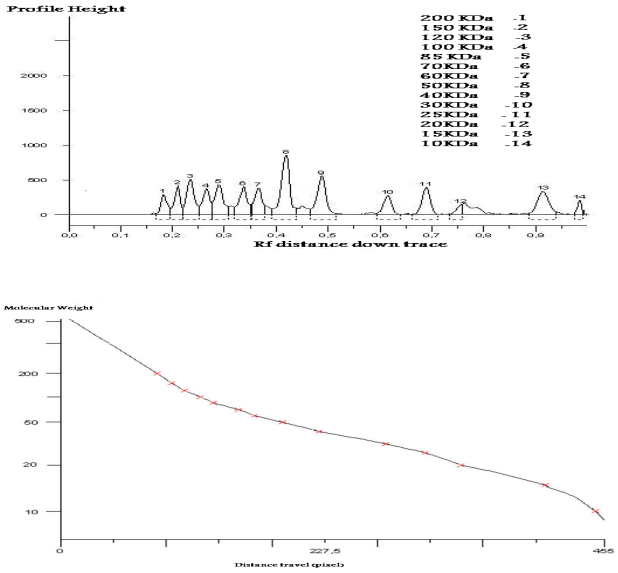

Figure 1. SDS-PAGE pattern of acid soluble collagens from skin and bones of rainbow trout .Classification is based on molecular weight (b-components (~200 kDa), a_1 (120 kDa) and a_2(100-120 kDa)

in Table 2. The amino acid contents were expressed as residues per 1,000 total amino acid residues. Generally, both collagens had slightly different amino acid profiles and glycine as the major amino acid. There were relatively high contents of proline, glutamic acid, hydroxyproline, argenin and alanine, while the contents of tyrosine and cysteine were very low and tryptophan was not detected. The imino acid contents of collagens from the skin and bone were 185 and 156 residues/1000 residues, which were lower than those of collagens from high temperature and tropical fish species, and were similar to those of collagens from cold-water fish (Gilsenan and Ross-Murphy, 2000; Gudmundsson and Hafsteinsson, 1997; Jongjareonrak *et al*., 2005; Muyonga *et al*., 2004). The degrees of hydroxylation of proline in collagens from the skin and bone were 39.13% and 42.3%, respectively. Collagen from the skin and bone had degrees of lysine hydroxylation of 47.78% and 34.68%, respectively. Hydroxylated proline plays a role in stabilising the triple helix (Ramachandran, 1988) and hydroxylated lysine contributes to the formation and stabilisation of cross-links, giving rise to complex, non-hydrolysable bonds (Stimler and Tanzer, 1977; Asghar and Henrickson, 1982). Generally, type I collagen consisted of low amounts of cysteine (0.2%) and methionine (1.24–1.33%), which play an essential role in disulfide bond formation. However, type III and IV collagens contain oxidisable cysteine residues (Foegeding *et al*., 1996). In the presented study, skin collagen contained higher amount of all amino acid except in

the case of glycine which was found in higher level in bone collagen. The degree of hydroxylation of proline and lysine influences the thermal stability of collagen (Kimura *et al*., 1988). It appeared that it was the higher hydroxylated proline content in bone collagen of rainbow trout, rather than the extent of lysine hydroxylation, which seemed to be the reason for the higher denaturation temperature observed for bone collagens.

Viscosity of skin and bone collagen solutions

Changes in the viscosity upon heating are shown in Table 3. Relative viscosity decreased continuously when bone collagen heated up, but in the case of skin collagen there was some fracture in relative viscosity values and then a sudden fall around 30°C and rate of decrease was held up in the temperature range of 30–40 °C. From the result, similar changes in viscosity of collagens from the skin and bone of rainbow trout were observed. Masahiro *et al*. (2004) reported approximately similar results in their studies that the viscosity started declining at 30°C, it decreased completely at 40°C for black drum and 39°C for sheepshead and remained low above 40°C. High viscosity is one of the physicochemical characteristics of collagen and can be accounted for by the high proportion of ß and γ chains, resulting in a higher average molecular weight. Heat-treatment at high temperature can break down the hydrogen bonds, which stabilise collagen structure (Wong, 1989). With increasing temperature, the hydrogen bonds of collagen were gradually broken. Consequently, the triple helix structure of collagen organized by hydrogen bonds was converted into the random coil configuration of gelatin by the process of thermal depolymerisation, which was accompanied by a change in physical properties, such as viscosity.

Effects of pH and NaCl on collagen solubility

Table 4 shows the effect of pH on the solubility of ASC from skin and bone. Highest solubility of collagens from the skin and bone were found at pH 1 and 4, respectively and there was a sudden fall in solubility at neutral pH. Generally, both collagens could be more soluble in the acidic pH ranges (Foegeding *et al*., 1996). At very acidic pH skin collagen had a higher solubility than bone collagen. ASC from the skin and bone of rainbow trout had the minimum solubility at pH 9 and 7, respectively known as pI.

Table 2. Amino acid composition of skin and bone collagens of rainbow trout (residues/1000 residues) based on three separate hydrolysates

Amino Acid	Skin	Bone	P-value
Threonine	17.8±0.06 [a]	16.2±0.03 [b]	0.0034
Serine	22.6±0.06 [a]	20.9±0.04 [b]	0.0012
Glutamic acid	91.2±0.08 [a]	80.9±0.03 [b]	<0.0001
Proline	112±10 [a]	90±0.20 [b]	0.0031
Hydroxy proline	72±10 [a]	66±0.20 [b]	0.0208
Glycine	340±0.73 [b]	352±2.95 [a]	0.032
Alanine	64.7±0.10 [a]	59.8±0.10 [b]	0.0015
Cysteine	0.05±0.001[a]	0.02±0.006 [b]	0.0235
Valine	12.1±0.18 [a]	11.9±0.06 [a]	0.1669
Methionine	12±0.14 [a]	6±0.16 [b]	0.0014
Isoleucine	8.1±0.03 [a]	8±0.15 [b]	0.5271
Leucine	15.3±0.01 [a]	13.5±0.06 [b]	0.0009
Tyrosine	3.5±0.03 [a]	3.3±0.06 [b]	0.0386
Phenylalanine	19.9±0.10 [a]	17.8±0.12 [b]	0.0003
Histidine	15.2±0.03 [a]	15.2±0.10 [a]	0.781
Tryptophan	0 [a]	0 [a]	-
Lysine	25.3±0.03 [a]	22.6±0.06 [b]	0.0001
Hydroxy lysine	14±0.97 [a]	12±0.25 [a]	0.2439

Table 3. Changes in the viscosity of ASC from skin and bone of rainbow trout

Viscosity / Temperature (°C)	Skin Collagen	Bone Collagen
5	98.1±0.15 [a]	97.03±0.84 [a]
10	96.3±0.24 [a]	91.07±0.14 [b]
15	89.2±0.21 [a]	87.3±0.11 [b]
20	78.9±0.08 [b]	79.5±0.11 [a]
25	74.5±0.03 [a]	72. 4±0.26 [b]
30	63. 4±0.25 [b]	66.7±0.11 [a]
35	62.2±0.09 [a]	61.2±0.13 [b]
40	58.7±0.08 [a]	56.8±0.15 [b]
45	52.6±0.19 [a]	50.7±0.12 [b]
50	51.06±0.02 [a]	48.8±0.10 [b]

Average± SD from triplicate determination

The effect of NaCl on the solubility of ASC extracted from skin and bone is shown in Table 5. Similar solubility pattern have been observed for both skin and bone collagen. Solubility of ASC in 0.05 M acetic acid remained constant at NaCl concentrations up to 3%. A dramatic decrease in the solubility of ASCs was observed above 3%. From the result, collagen from bone was more tolerant to salt than collagen from skin, as shown by the greater solubility in the presence of NaCl at 4–6% with a constant change in the mentioned concentrations. When pH values are above and below pI(pH isoelectric), a protein has a net negative or positive charge, respectively (Vojdani, 1996).

Table 4. Effect of pH on the solubility of ASC from skin and bone of rainbow trout

pH / Solubility	Skin Collagen	Bone Collagen
1	95.08±0.16 [a]	79.01±0.04 [b]
2	81.00±0.19 [a]	72.00±0.05 [b]
3	60.08±0.28 [a]	60.07±0.15 [a]
4	74.00±0.13 [b]	91.00±0.12 [a]
5	19.80±0.05 [b]	38.37±0.38 [a]
6	14.40±0.04 [a]	14.28±0.25 [a]
7	23.14±0.16 [a]	0.15±0.03 [b]
8	55.03±0.04 [a]	29.30±0.87 [b]
9	14.26±0.05 [b]	53.48±0.17 [a]
10	25.10±0.30 [b]	40.81±0.19 [a]
11	24.67±0.41 [b]	54.03±0.22 [a]
12	60.02±0.27 [b]	63.04±0.09 [a]

Average± SD from triplicate determination.

As a consequence, more water interacts with the charged proteins (Vojdani, 1996). Works done by Inwoo *et al*. (2007), indicate that the solubility of the skin collagen of fishes varies widely and 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, On the other hand low solubility was observed at pH 7-9, which was similar to pI value obtained in this study. 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).

Table 5. Effect of NaCl on the solubility of ASC extracted from skin and bone of rainbow trout

pH / %NaCl	Skin Collagen	Bone Collagen
0	99.99±0.01[a]	100.00±0 [a]
1	88.17±0.10 [a]	85.23±0.10 [b]
2	77.94±0.04 [a]	75.23±0.14 [b]
3	74.80±0.20 [a]	74.50±0.10 [a]
4	46.71±0.16 [b]	48.95±0.11 [a]
5	44.57±0.21 [b]	47.13±0.10 [a]
6	42.05±0.2 [a]	42.41±0.1 [a]

Average± SD from triplicate determination.

Similar solubility pattern have been observed for both skin and bone collagen 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 (salting out effect) (Jongjareonrak et al., 2004).

Acknowledgement

The authors thank Mr. Gashtasb, manager of Kian Mahi Khazar Fisheries Co, Ltd processing plant in Babolsar for providing the fish bone and skin used as experimental materials.

Reference

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

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

Foegeding, E. A., Lanier, T. C. and Hultin, H. O. 1996. Collagen In : O. R. Fennema (Ed.), Food chemistry,3rd ed.: Pp 902–906. New York: Marcel Dekker, Inc.

Gilsenan, P. M. and Ross-Murphy, S. B. 2000. Rheological characterisation of gelatins from mammalian and marine sources. Journal of Food Hydrocolloids 14(3): 191–195.

Gudmundsson, M. and Hafsteinsson, H. 1997. Gelatin from cod skins as affected by chemical treatments. Journal of Food Science 62(1):c37–39.

Horwitz, W., 2000. Official methods of the Association of Official Agricultural Chemists International 17th ed. AOAC International. Gaitherburg, MD.

Inwoo, B., Kiyoshi, O., Asami, Y., Kazufumi,O., Atsuko,Y. and Kenji, H. 2008. Biochemical properties of acid-soluble collagens extracted from the skins of underutilised fishes. Journal of Food Chemistry 108: 49-54.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., Nagai, T. and Tanaka, M. 2005. Isolation and characterization of acid and pepsin–solubilised collagens from the skin of Brownstripe red snapper (*Lutjanus vitta*). Journal of Food Chemistry 93: 475- 484.

Kimura, S., Zhu, X.-P., Matsui, R., Shijoh, M. and Takamizawa, S. 1988. Characterization of fish muscle type I collagen. Journal of Food Science 53: 1315–1318.

Kimura, S., Miyauchi, Y. and Uchida, N. 1991. Scale and bone type-I collagens of carp (*Cyprinus carpio*). Comparative Biochemistry and Biophysiology 99B: 473–476.

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

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of head of bacteriophage T4. Nature 277: 680–685.

Masahiro, O., Ralph, J.P., Michael, W., Moody, J.B., Mark, A., Schexnayder, J. and Losso, N. 2004. Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromi*s) and sheepshead seabream (*Archosargus probatocephalus*).

Journal of Food Chemistry 88(4): 495-501.

Montero, P., Borderı´as, J., Turnay, J. and 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.

Moore, S. 1963. On the determination of cystein as cysteic acid. Journal of Biological Chemistry 238: 235-237.

Morimura, S., Nagata, H., Uemura, Y., Fahmi, A., Shigematsu, T. and Kida, K. 2002. Development of an effective process for utilization of collagen from livestock and fish waste. Journal of Process Biochemistry 37: 1403–1412.

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

Nagai, T. and Suzuki, N. 2000a. Isolation of collagen from fish waste material-skin, bone and fins. Journal of Food Chemistry 68: 277–281.

Nagai, T. and Suzuki, N. 2000b. Preparation and characterization of several fish bone collagens. Journal of Food Biochemistry 24: 427–436.

Nagai, T., Yamashita, E., Taniguchi, K., Konamori, N. and Suzuki, N. 2001. Isolation and characterization of collagen from the outer skin waste material cuttlefish (*Sepia Lycidas*). Journal of Food Chemistry 72(4): 425-429.

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

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

Rigby, B. J. 1968. Amino-acid composition and thermal stability of the skin collagen of the antarctic ice-fish. Nature 219(1): 166–167.

Sadowska, M., Kolodziejska, I. and Niecikowska, C. 2003. Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). Journal of Food Chemistry 81: 257–262.

Saito, M., Takenouchi, Y., Kunisaki, Y. and Kimura, S. 2001. Complete primary structure of rainbow trout type I collagen consisting of alpha1(I)alpha2(I)alpha3(I) heterotrimers. European Journal of Biochemistry 268: 2817–2827.

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

Shahidi, F. 1994. Seafood processing by-products In: F. Shahidi and J. R. Botta (Eds.), Seafoods chemistry, processing, technology and quality: pp. 320–334. Glasgow: Blackie Academic and Professional.

Stimler, N. P. and Tanzer, M. L. 1977. Location of the intermolecular crosslinking sites in collagen In: M. Friedman (Ed.), Protein crosslinking nutritional and medical consequences : pp. 133–157. New York: Plenum Press.

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

Woessner, J. F. Jr. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Archives of Biochemistry and Biophysics 93: 440-447.

Wong, D. W. S. 1989. Mechanism and theory in food chemistry. New York: Van Nostrand Reinhold. 428p

Yang, H., Wang, Y., Jiang, M., Oh, J.H., Herring, J. and Zhou, P. 2007. Optimization of Extraction and physical properties of channel catfish (*Ictalurus punctatus*) skin gelatin. Journal of Food Science 72(4): C188-C195.

Yata, M., Yoshida, C., Fujisawa, S., Mizuta, S. and Yoshinaka, R. 2001. Identification and characterization of molecular species of collagen in fish skin. Journal of Food Science 66(2): 247–251.

Zhou, P. and Regenstein, J.M. 2006. Determination of total protein content in gelatin solutions with the Lowry or Biuret assay. Journal of Food Science 71(8): 474-479.and adjunct cultures on the production of exopolysaccharides by *Streptococcus thermophilus* 1275. Journal of Dairy Science 86(11): 3405-3415.