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Isolation and characterisation of acid-solubilised collagen from the skin of Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Acid-solubilised collagen (ASC) was extracted from the skin of Nile tilapia (*Oreochromis niloticus*) and characterisation was studied. The results indicated that the yield of ASC was 39.4% on the basis of dry weight. This ASC was rich in glycine (35.6%). The amount of imino acids, proline and hydroxyproline, in ASC was 210 residues per 1000 residues. The ultraviolet (UV) absorption spectrum of ASC showed that the distinct absorption was at 220 nm. ASC showed transition curve at maximum temperature (T_{max}) of 32.0 °C in 0.05 M acetic acid, about 12 °C lower than that of calf skin collagen. Maximum solubility (0.75 mg/ml) in 0.5 M acetic acid was observed at pH 3. Solubility reached the minimum at pH 7. A sharp decrease in solubility was observed in 2% (w/v) NaCl or above. Biochemical studies indicated that ASC was composed of the α1α2α3 heterotrimers.

1. Introduction

Collagen not only plays important roles in the development of muscle tissue but also has been utilised as a material for foods, cosmetics, pharmaceuticals and experimental reagents. So far, the main sources of collagen are limited to those of land-based animals, such as the skin and bone of bovine, and porcine. However, the outbreaks of bovine spongiform encephalopathy (BSE) and the foot-and-mouth disease (FMD) crisis have resulted in anxiety amongst users of collagen and collagen-derived products of land animal origins (Helcke, 2000). Besides, the collagen extracted from pigs cannot be used due to religious barriers. Therefore, many scientists have been focusing their experiments to marine animals to produce collagen (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Nagai, Araki, & Suzuki, 2002; Nagai & Suzuki, 2000; Ogawa et al., 2003). Fish industrial by-products such skins, bones and scales are rich in collagen. Recently, biochemical properties of Brownstripe red snapper (*Lutjanus vitta*) and black drum (*Pogonias cromis*) and sheepshead sea bream skin collagen have been characterised (Jongjareonrak et al., 2005; Ogawa et al., 2003). However, a little information regarding the characteristics of Nile tilapia (*Oreochromis niloticus*) skin collagen has been reported. Tilapia (including all species) is the second most important group of farmed fish after carps. According to the statistics of FAO, by 2005 annual China's production of tilapia had risen to nearly 1 million ton, which account for about 50% of tilapia production in the world. Tilapia is usually processed as whole frozen tilapia and frozen fillet and fresh fillet in factories. During processing of tilapia to fillets, large quantities of the by-products are produced. Collagen content in the skin was about 27.8% (Xiaoyan et al., 2008). If the skin can be re-utilised for the production of collagen, it may increase the economic value of the fish. Therefore, the aim of this study was to isolate and characterise collagen from the skin of *Oreochromis niloticus*.

2. Materials and methods

2.1. Preparation of fish skin

The *Oreochromis niloticus* skin was obtained from a local fish-processing factory. Skin was descaled and cut into small pieces (0.5 × 0.5 cm) and stored at −25 °C until used.

2.2. Chemicals

Type I collagen from calf skin was purchased from Sigma Chemical Co.

2.3. Preparation of collagen from the skin

2.3.1. Removal of noncollagenous proteins and fat from the skin

The collagen was extracted according to the method of Nagai and Suzuki (2000) with a slight modification. All the preparative

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procedures were performed at 4 °C. Skin was soaked in 0.1 M NaOH solution in ratio of 1:30 (w/v) for 48 h with a gentle stirring. To remove noncollagenous proteins and pigments, the solution was changed every 8 h. Then the skin was washed with distiled water until neutral pH of wash water was obtained. Fat was removed in 10% (v/v) butyl alcohol solution in ratio of 1:30 (w/v) for 24 h with a gentle stirring and renewed every 8 h. Defatted skin was thoroughly washed with distiled water.

2.3.2. Isolation of ASC

Defatted skin was soaked in 0.5 M acetic acid with a sample/solution ratio of 1:50 (w/v) for 3 days with a gentle stirring. Then the mixture was centrifuged at 21,200*g* for 30 min 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 2 days with a gentle stirring, followed by centrifugation at 21,200*g* for 30 min at 4 °C. The second supernatant obtained was combined with the first extract. The collagen in the combined extracts were precipitated by the addition of NaCl to a final concentration of 0.9 M and followed by precipitation of the collagen by the addition of NaCl to the final concentration of 2.3 M in 0.05 M Tris-HCl (pH 7.5). The resultant precipitate was collected by centrifugation at 21,200*g* for 30 min at 4 °C and then dissolved in 10 volumes of 0.5 M acetic acid. The solution obtained was dialysed against 10 volumes of 0.1 M acetic acid in a dialysis membrane with molecular weight cut-off of 12 kDa for 24 h at 4 °C with a change of solution every 4 h. Subsequently, the solution was dialysed against 10 volumes of distiled water with changes of water until neutral pH was obtained. The dialysate was freeze-dried and referred to as ASC.

2.4. Amino acid composition

Collagen sample was dissolved in 6 M HCl solution and hydrolyzed at 110 °C for 24 h. The solvent was analysed with an amino acid analyser (HIRACHI L8800).

2.5. UV absorption spectrum

UV absorption spectrum of ASC was measured using a Shimadzu-uv-2501Pc. The ASC (0.04 mg) was dissolved in 100 ml 0.02 M sodium acetate buffer, pH 4.8 containing 2 M urea. The solution was placed into a quartz cell with a path length of 1 mm. UV spectrum was measured at wavelength 190–350 nm at a scan speed of 2 nm/s with an interval of 1 nm.

2.6. Thermal transition measurement

The ASC sample was rehydrated in 0.05 M acetic acid solution in ratio of 1:40 (w/v). The mixture then stood for 2 days at 4 °C. The thermal transition of collagen was measured using Perkin–Elmer differential scanning calorimetry (DSC) (Pyris1 DSC-7, Norwalk, PE, USA). Temperature calibration was performed using the Indium thermogram. The rehydrated sample (5–10 mg) was accurately weighed into aluminium pans, sealed, and scanned over the range of 20–50 °C at a heating rate of 5 °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 maximum transition temperature (T_{max}) was estimated from the maximum peak of DSC transition curve. DSC transition curve of type I collagen of calf skin was conducted in the same manner and was compared.

2.7. Collagen solubility test

2.7.1. Solubility determination

The solubility of ASC from the skin of *Oreochromis niloticus* was determined in 0.5 M acetic acid at various pH levels and NaCl concentrations according to the method of Montero, Jimennez-Colmenero, and Borderias (1991) with a light modification. Collagen sample was dissolved in 0.5 M acetic acid with gentle stirring at 4 °C for 12 h to obtain the final concentration of 3 and 6 mg/ml.

2.7.2. Effect of pHs on collagen solubility

Eight millilitre of collagen solution (3 mg/ml) was 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 solution was made up to 10 ml with distiled water previously adjusted to the same pH as the collagen solution tested. The solution was stirred gently for 30 min at 4 °C and centrifuged at 10,000*g* for 30 min at 4 °C. Protein content in the supernatant was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a protein standard.

2.7.3. Effect of salt concentration on collagen solubility

Five millilitre of collagen solution (6 mg/ml) in 0.5 M acetic acid was 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 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 et al. (1951) using bovine serum albumin as a protein standard.

2.8. Determination of subunit composition

Denatured collagen chains were separated on an ion exchange column according to the established methods for collagen (Piez, Eigner, & Lewis, 1963). Purified collagen was dissolved in 0.02 M sodium acetate buffer, pH 4.8, containing 2 M urea, and denatured by heating to 45 °C and the solution was centrifuged at 12,000 *g* for 20 min. The supernatant was then loaded onto a 1.6 × 20 cm column with CM-cellulose (CM-52) resin equilibrated with the same buffer. The denatured collagen chains were eluted with a linear gradient of 0–0.15 M NaCl over a total volume of 200 ml into a fraction collector and the subunit components were detected by absorbance at 220 nm (UV-9900; Tokyo rikakikai Co., Tokyo, Japan), and the fractions indicated by the numbers were examined by SDS–PAGE at 7.5% gel.

3. Result and discussion

3.1. Isolation of ASC from the skin of Oreochromis niloticus

ASC was isolated from *Oreochromis niloticus* skin, its yield was 39.4% (on the dry basis). The skin was not completely solubilised by 0.5 M acetic acid. This suggested that the yield of ASC from the skin of *Oreochromis niloticus* was much higher than that from Brownstripe red snapper skin (9%) and lower than that from the skins of Japanese sea-bass (51.4%), chub mackerel (49.8%), and bullhead shark (50.1%) (Nagai & Suzuki, 2000).

3.2. Amino acid composition

The amino acid composition of ASC was expressed as residues per 1000 total amino acid residues and was shown in Table 1.

From the results, amino acid composition of ASC isolated from *Oreochromis niloticus* skin was rich in glycine (35.6%). In general, glycine occours uniformly at every third residue throughout most of collagen molecules. ASC consisted of the unique amino acids such as proline and hydroxyproline. The amount of these acids, proline and hydroxyproline in ASC was 210 residues per 1000 residues. The content of imino acids in ASC from *Oreochromis*

Table 1
Amino acid composition of ASC from the skin of *Oreochromis niloticus* (residues per 1000 total amino acid residues).

Amino acids	
Hydroxyproline	82
Aspartic acid	42
Threonine	22
Serine	32
Glutamic acid	69
Proline	128
Glycine	356
Alanine	119
Valine	17
Methionine	5
Isoleucine	8
Leucine	20
Tyrosine	3
Phenylalanine	13
Lysine	20
Histidine	6
Arginine	58
Total	1000

niloticus was very similar to that in Brownstripe red snapper skin collagen (212) and higher than that in skins collagen from carp (157) and ocellate puffer (170) (Montero, Borderias, Turnay, & Leyzarbe, 1990; Nagai et al., 2002). Proline and hydroxyproline contents are correlated with species and their living habitat (Foegeding, Lanier, & Hultin, 1996; Love, Yamaguchi, Creach, & Lavety, 1976).

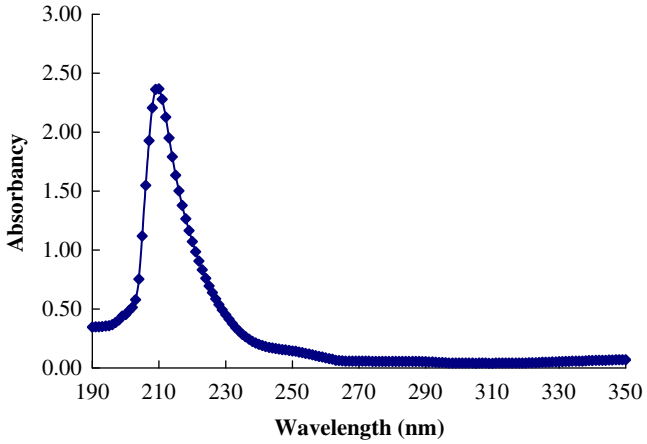

Fig. 1. UV absorption spectrum of ASC from *Oreochromis niloticus* skin.

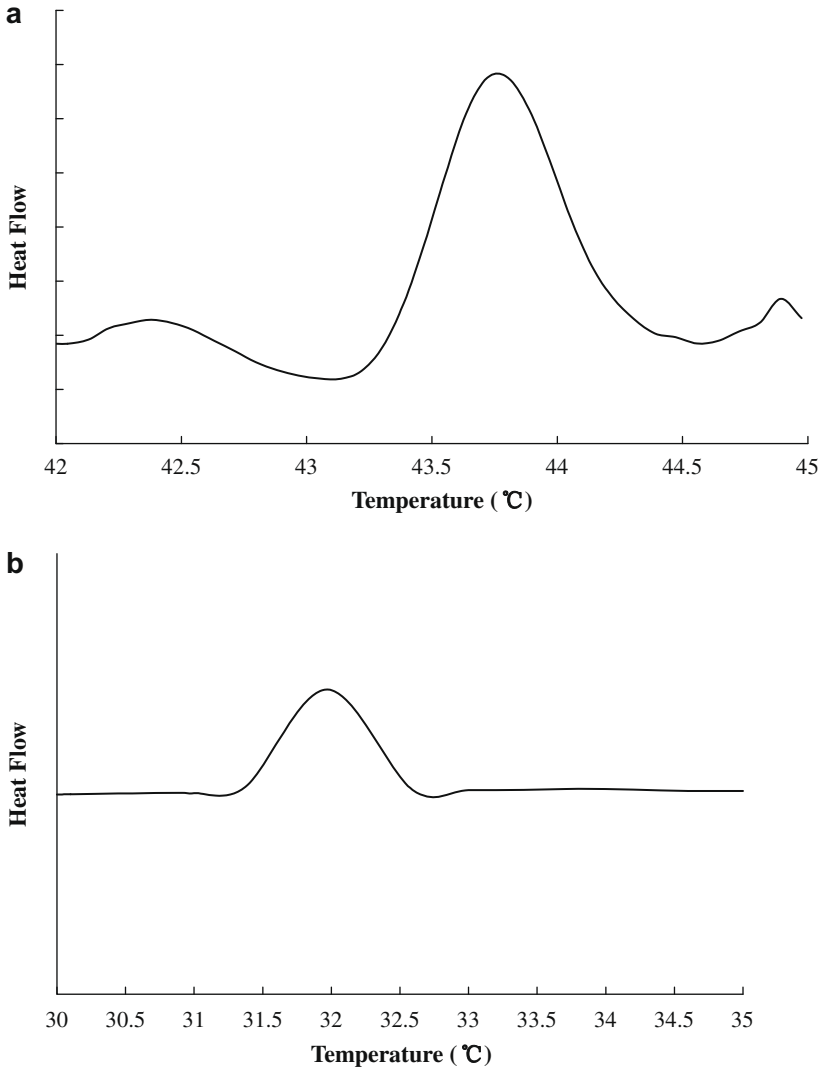

Fig. 2. DSC curves of type I collagen (a) from calf skin and (b) from *Oreochromis niloticus* skin in 0.05 M acetic acid.

3.3. UV absorption spectrum

Fig. 1 showed the UV absorption spectrum of ASC at the wavelengths 190–350 nm. Most proteins have a maximum ultraviolet absorption at 280 nm. The numbers of tyrosine and tryptophane residues contribute to the ultraviolet absorption at 280 nm. But the amount of tyrosine in ASC was 3 residues per 1000 residues. ASC isolated from *Oreochromis niloticus* skin showed a maximum absorption at 220 nm. This may be related to the groups C=O, —COOH, $CONH_2$ in polypeptides chains of collagen (Edwards, Farwell, Holder, & Lawson, 1997).

3.4. Denaturation temperature

The T_{max} of ASC was calculated from the thermal denaturation curve. For comparison, T_{max} of calf skin collagen was measured in the same way. Thermal transition of ASC and standard collagen of calf skin rehydrated in 0.05 M acetic acid were shown in Fig. 2.

ASC showed transition curve at maximum temperature (T_{max}) of 32.0 °C, which was about 12 °C lower than that of calf skin collagen and similar to those obtained from other fish skins such as skins of carp (31.7 °C) (Kimura, Zhu, Matsui, Shijoh, & Takamizawa, 1988), and Brownstripe red snapper (31.52 °C) (Jongjareonrak et al., 2005). According to the study of Sikorski, Scott, and Buisson (1984), the thermal stability of protein showed a direct positive correlation with imino acid contents (proline and hydroxyproline). Calf skin collagen has higher hydroxyproline content (94 residues per 1000 residues) (Giraud-Guille, Besseau, Chopin, Durand, & Herbage, 2000) than ASC isolated from *Oreochromis niloticus* skin (82 residues per 1000 residues) and denatures at higher temperature.

3.5. Effect of pHs on collagen solubility

The effect of pH on the solubility of ASC from *Oreochromis niloticus* skin was shown in Fig. 3.

The solubility of ASC reached maximum at pH 3. When the pH was increased to 7, marked decrease in solubility was observed and reached the minimum. On the contrary, when pH increased to 10, caused a slight increase in solubility. According to Vojdani (1996), when 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. In contrast, total net charges of protein molecules are zero and hydrophobic–hydrophobic interaction increases, thereby leading to the precipitation and aggregation at pI. Foegeding et al. (1996) reported that collagen has isoelectric points at pH 6–9. Thus the lowest solubility of ASC from *Oreochromis niloticus* skin observed at pH around 7 was in agreement with the former value.

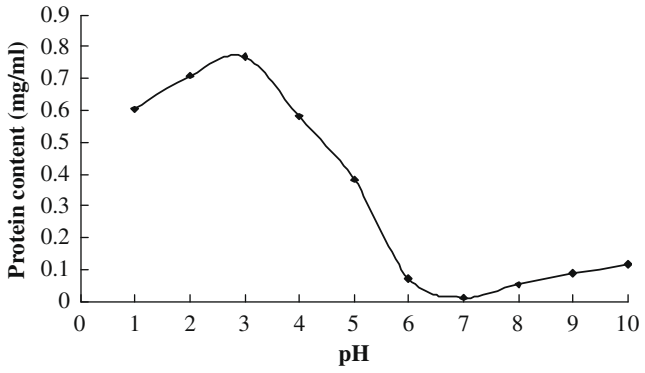

Fig. 3. Protein concentrations of ASC from *Oreochromis niloticus* skin in 0.5 M acetic acid at different pHs.

3.6. Effect of salt concentration on collagen solubility

The effect of NaCl on the solubility of ASC from *Oreochromis niloticus* skin was shown in Fig. 4.

Solubility of ASC in 0.5 M acetic acid was high at NaCl concentrations below 2% (w/v). Solubility of ASC decreased sharply when NaCl content reached 2% and remained at constant low level at 4% NaCl or above. The result suggested that the increasing of ionic strength causing hydrophobic–hydrophobic interaction between protein molecules, and then its solubility reduced.

3.7. Subunit composition of collagen of types

The subunit composition of ASC was determined by applying denatured collagen to CM-cellulose chromatography. Resolved

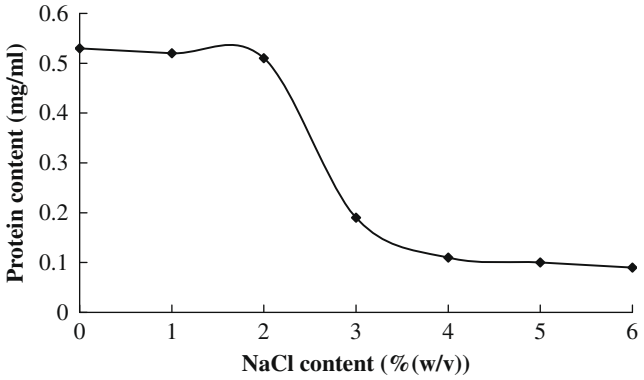

Fig. 4. Solubility of ASC from *Oreochromis niloticus* skin in 0.5 M acetic acid with different NaCl concentrations.

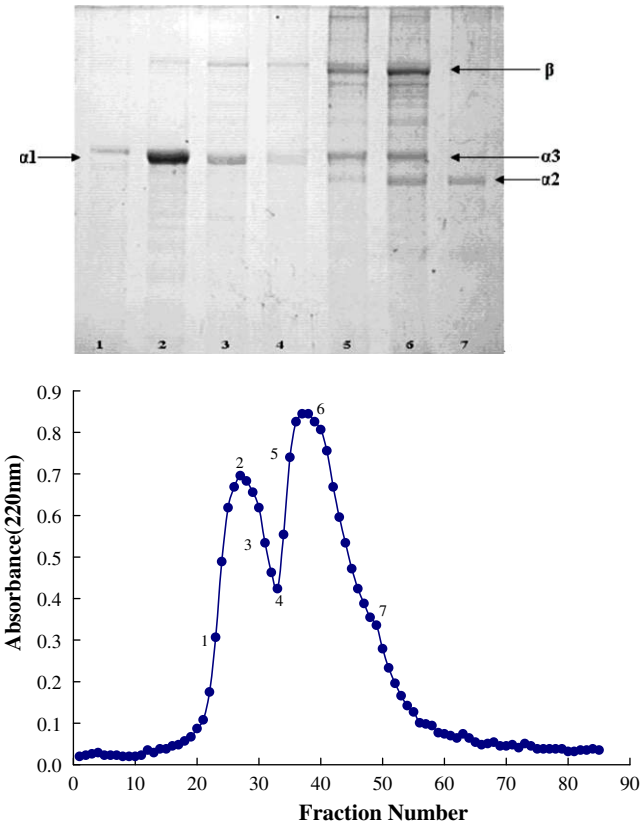

Fig. 5. CM-52 column chromatography of denatured skin collagen from *Oreochromis niloticus* skin. The fractions indicated by the numbers were examined by SDS–PAGE.

fractions were collected and selected fractions were further analysed to recognise each α chain using SDS–PAGE at 7.5% gel. Electrophoretic pattern showed that tilapia skin collagen was composed of three different α chains (Fig. 5). Fractions number 1, 2 and 3 contained α1 chain. Fractions number 6 and 7 had α2, and α3 chain was found in fractions number 5 and 6. By electrophoresis, the α3 was found to migrate at the same position as the α1. The relative amounts of α1, α2 and α3 could be calculated by measuring the area under each peak. The stoichiometry of α1, α2 and α3 was found to be about 1:1:1. It revealed that *Oreochromis niloticus* skin collagen was a heterotrimer containing a chain composition of α1α2α3. The results of the present study were in agreement with the work of Kimura, Ohno, Miyauchi, & Uchida (1987). Kimura et al. suggested that two different heterotrimers of $(\alpha 1)_2$, α2 and α1α2α3 are present in the skin collagen of tilapia. The present study provided evidence for occurrence of the skin α3 chain in tilapia.

4. Conclusion

The yield of ASC from the skin of *Oreochromis niloticus* was 39.4% on the basis of dry weight. It was characterised as type I collagen. Its denaturation temperature (T_{max}) was 32.0 °C, lower than that of calf skin collagen and similar to Brownstripe red snapper skin collagen. ASC was soluble at acidic pH and lost the solubility when salt concentrations increased. Three distinct α chains in the skin collagen of *Oreochromis niloticus* were isolated by CM-cellulose chromatography at pH 4.8, followed by SDS–PAGE at 7.5% gel, and were found to exist as α1α2α3 heterotrimers.

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