Food Chemistry 116 (2009) 318–322

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Characterisation of acid-soluble collagen from skin of silver carp (*Hypophthalmichthys molitrix*) ☆

Junjie Zhang [a,*], Rui Duan [a], Yuanyong Tian [b], Kunihiko Konno [b]

[a] School of Marine Science and Technology, Huaihai Institute of Technology, Lianyungang 222005, China
[b] Faculty of Fisheries Science, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

ARTICLE INFO

Article history:
Received 7 December 2008
Received in revised form 17 January 2009
Accepted 18 February 2009

Keywords:
Silver carp
ASC
Chromatography
Denaturation temperature
Peptide map

ABSTRACT

Acid-soluble collagen (ASC) from the skin of silver carp (*Hypophthalmichthys molitrix*) was isolated and some properties of ASC were investigated. SDS–PAGE patterns showed ASC from silver carp skin was type I collagen. Sulfopropyl-Toyopearl 650(M) column chromatography indicated that ASC from silver carp skin was composed of three kinds of α chains, α_1, α_2 and α_3. Hydroxyproline and proline content of ASC from silver carp skin was 192 residues/1000 residues, which was similar to that of ASC from carp skin. Denaturation temperature (T_d) of ASC from silver carp skin was around 29 °C. The results showed that some properties of ASC from silver carp skin were similar to those of ASC from carp skin. However, the peptide map of ASC from silver carp skin digested by pepsin was distinguished with that of ASC from carp skin.

1. Introduction

Collagen is the most abundant protein in the living body, comprising approximately 30% of the total protein content. Collagen and gelatin have broad applications in many fields, such as food, pharmaceutical, cosmetic, biomedical materials, photographic gelatin and leather industries.

The skin and bone of pig and bovine are generally the main sources of collagen and gelatin. However, the outbreaks of bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE) have resulted in anxieties among users of cattle collagen and collagen-derived products. Besides, the use of collagen from pig skin and bone is not allowed in some regions due to religious reasons. Fish offal, such as skins, scales, as well as bones is tissues that are mainly structured by collagen. Although the physical and chemical properties of aquatic animal collagen are different from those of mammalian collagen, the fish collagen is unlikely to be related to BSE and TSE and will not be forbidden for religious reasons.

China is the largest producer of freshwater fish in the world. According to the statistical data from the Ministry of agriculture (2006), the yield of freshwater fish was 20,093,500 tons in 2005. Silver carp is one of the major species produced in China. The consumption of silver carp was about 3,524,800 tons in 2005. With the rapid development of fishery, some processing companies were established. As the byproducts of fishery processing, around 170,000 tons of silver carp skin was generated annually. For the processors, interest is growing in obtaining higher value from the processing wastes. The production of value-added products is a promising means to increase revenue for the producers and to decrease the environmental pollution.

Many papers focused on the practical utilisation of marine animals to produce collagen (Piez, 1965; Rigby, 1968). Some concerned collagens from freshwater fish such as carp (Duan, Zhang, Du, Yao, & Konno, 2009; Kimura, Miyauchi, & Uchida, 1991) and grass carp (Zhang et al., 2007). However, few concerned the properties of collagen from silver carp skin. Therefore, the objective of this research was to isolate and characterise the acid-soluble collagen (ASC) from the skin of silver carp (*Hypophthalmichthys molitrix*).

2. Materials and methods

2.1. Raw materials

Live cultured carps and silver carps (average body weight of 850 g) were obtained from a free market in Lianyungang, Jiangsu province (February 21st, 2008). The skins were removed manually and washed with chilled tap water to get rid of the adhering tissues. The samples were then placed in polyethylene bags and stored at −25 °C until used.

☆ The paper has not been published previously at any form. It is not under consideration for publication elsewhere, and its publication is approved by all authors.

* Corresponding author. Tel.: +86 13851290162; fax: +86 518 85893890.
E-mail address: zjjdr180@yahoo.com.cn (J.J. Zhang).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.02.053

2.2. Chemical reagents

All reagents were of analytical grade. Type I collagen from calf skin was purchased from Sigma chemical company (St. Louis, MO, USA).

2.3. The approximate analyses of fish skins

The protein, moisture, fat, ash contents of fish skins were determined according to the method of AO AC(1999). The hydroxyproline content was determined using the colourimetric method recommended by the ISO (Anonymous, 1978) after the material was hydrolysed in 6 M hydrochloric acid for 8 h at 110 °C. The hydrolysed samples (50 μl) were mixed with a buffered chloramines-T regent (450 μl, pH 6.5) and the oxidation was allowed to proceed for 25 min at room temperature. Ehrilich's aldehyde reagent (500 μl) was added to each sample, mixed gently, and the chromophore was developed by incubating the samples at 65 °C for 20 min. The absorbance of reddish purple complex was measured at 550 nm using a Model 1601 UV–vis spectrophotometer (Shimdzu, Japan).

2.4. Amino acid analysis

Acid-soluble collagen samples (10–20 mg) from skins of silver carp, carp and cod were hydrolysed respectively in 6 M HCl at 110 °C for 24 h in the absence of oxygen (Morimura et al., 2002). The hydrolysates were analysed on a Hitachi 835-50 amino acid analyzer.

2.5. Preparation of collagens

The method used for the isolation and purification of collagen is according to Nagai et al. (2000) with some modifications. The skins were mixed with 0.1 M NaOH at a sample/alkali solution ratio of 1:8 (w/v) to remove non-collagenous proteins for 6 h with continuous stirring. The alkali solution was changed every 3 h. Then the samples were washed with cold distilled water, until a neutral pH of the washing water was obtained.

The deproteinised skins were soaked in 10% butyl alcohol with a solid/solvent ration of 1:10 (w/v) overnight to remove the fat, and was then washed with cold distilled water repeatedly. The treated skins were cut into small pieces by scissors at the size of 1 × 1 cm and extracted with 0.5 M acetic acid for 3 days with stirring. The extract was centrifuged at 20,000*g* for 1 h. The supernatant was salted-out by adding NaCl to a final concentration of 2.5 M in the presence of 0.05 M tris(hydroxymethyl) aminomethane, pH 7.0. The resultant precipitate collected by centrifugation at 20,000*g* for 30 min was dissolved in 0.5 M acetic acid. The collagen solution was dialyzed against 50 volumes of 0.1 M acetic acid and distilled water for 2 days, respectively. The dialysis solution was changed three times a day. Then the collagen was lyophilized.

Carp (*Cyprinus carpio*) skin and Atlantic cod (*Gadus morhua*) skin (Taiyuan Food Ltd., Qingdao, china) were also used as raw materials to extracted acid-soluble collagens (ASC from carp skin and cod skin ASC) in the same manner as previously described.

All the operation procedures were carried out at 4 °C.

2.6. SDS-polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970) using 7.5% separating gel and 4% stacking gel. The collagen samples were dissolved in 0.1 M acetic acid. The protein content of each sample was determined in triplicate by the Lowery's method with modification (Regina, Rositza, & Blagovest, 1996). Solubilized samples were mixed with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 5% SDS, 20% glycerol) at 1:2 ratio in the presence of 10% β-ME. Samples were subjected to electrophoresis at a constant current of 15 mA using the vertical cell (Atto Co., Tokyo, Japan). After electrophoresis, the gel was stained with Commassie brilliant blue R250 dissolved in water, methanol and trichloroacetic acid (5:4:1) and de-stained using a solution containing methanol, distilled water and acetic acid at a ratio of 5:4:1. 10 μg of collagen was loaded in each well.

2.7. SP (Sulfopropyl)-Toyopearl column chromatography

The subunit components of ASC from silver carp skin were separated by a SP-Toyopearl 650 M (Tosoh Co. Tokyo, Japan) column chromatography. According to the methods of Kimura and Ohno (1987a), 10 mg ASC from silver carp skin sample was dissolved in 5 ml of 20 mM sodium acetate buffer, pH 4.8, at 4 °C overnight, then denatured at 45 °C for 30 min. After centrifugation at 20 °C for 30 min, the denatured collagen was fractionated on a column of SP-Toyopearl 650 M (1.5 × 10 cm). Elution was achieved by the starting buffer with a linear gradient of 100–250 mM NaCl over a total volume of 300 ml at a flow rate of 90 ml/h. Fractions of 3 ml were collected and the absorbance was monitored at 220 nm. The fractions indicated by the numbers were examined by SDS–PAGE.

2.8. Determination of denaturation temperature

The denaturation temperature (T_d) was measured by the method of Nagai et al. (2000) with a modification. Ten milliliters of 0.75% collagen solution in 0.1 M acetic acid were used for viscosity measurements in circumvolving viscometer (model NDJ-79, Tongji university Labs Inc., Shanghai, China). The collagen solution was heated from 14 to 40 °C with a heating rate of 2 °C/min. At the designated temperature, the solution was held for 20 min prior to viscosity determination. The thermal denaturation curve of the collagen solution was determined by plotting the fractional change of viscosity against temperature. The thermal denaturation temperature, T_d, was expressed as a mid-point temperature between the extrapolated line for native collagen and that for fully denatured collagen on the plot.

2.9. Peptide mapping

The peptide mappings of fish skin collagens were achieved by pepsin (1:10,000, Sigma Chemical Co., St. Louis, Mo., USA) digestion. Lyophilized collagen was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml at 4 °C. The collagens were heated at 50 °C for 30 min and cooled in the ice bath immediately. The pepsin digestion was carried out at an enzyme/substrate ratio of 1/100 (w/w) at 30 °C for 5 h. The digestion was stopped by adding 10 μl 5 mM pepstatin (Sigma Chemical Co., St. Louis, Mo., USA) in each sample. After adding 1/4 of sample buffer (5% SDS, 0.5 M Tris–HCl, pH 6.8, 20% glycerol and 10% b-mercaptoethanol (ME)), the reaction mixture was subjected to boiling water for 2 min. The digests were resolved on 7.5% polyacrylamide gels containing 0.1% SDS (SDS–PAGE) (Laemmli, 1970). Protein (10 μg) was loaded in each well. High molecular weight markers (Sigma Chemical Co., St. Louis, Mo., USA) were used to estimate the molecular weight of proteins.

3. Results and discussion

3.1. The proximate composition of skins of silver carp, carp and cod

The proximate analyses of carp, silver carp and cod skin are shown in Table 1. Silver carp and cod skin contained almost the same moisture content (65.3% and 66.2%, respectively), which was higher than that of carp skin. Among the three samples, silver

Table 1
Proximate analyses and hydroxyproline content of skins of cod, carp and silver carp.

Sample	Proximate compositions[a] (% wet wt.)				Hydroxyproline (mg/g sample)
	Moisture	Protein	Fat	Ash	
Carp skin	52.7 ± 0.39	31.4 ± 0.26	17.9 ± 0.15	0.38 ± 0.09	24.2 ± 0.28
Silver carp skin	65.3 ± 0.08	34.6 ± 0.31	1.53 ± 0.04	0.40 ± 0.07	27.1 ± 0.39
Cod skin	66.2 ± 0.14	31.9 ± 0.43	0.89 ± 0.05	0.93 ± 0.10	21.6 ± 0.23

[a] Average ± SD from triplicate determinations.

carp skin and cod skin had the highest contents of protein and ash, respectively. It was obvious that the fat content of carp skin was much higher than that of the other two fish skins. The hydroxyproline content of cod skin was 21.6 mg/g sample, which was lower than that of carp and silver carp skin. It is noteworthy that the ratios of hydroxyproline to protein were 7.83%, 7.69% and 6.77% for silver carp, carp and cod skin, respectively. Different hydroxyproline contents among species might depend upon the species, environment and body temperature of fish (Rigby, 1968). The above results were in accordance with the previous reports (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005).

3.2. Amino acid composition

The amino acid composition of ASCs from silver carp, carp and cod skin, as well as calf skin collagen (Giraud-Guille, Besseau, Chopin, Durand, & Herbage, 2000), expressed as residues per 1000 total residues is shown in Table 2.

Glycine was the major amino acid in each kind of collagen. The hydroxyproline and proline content of ASC from silver carp skin is 192 residues/1000 residues, which is quite similar with that of ASC from carp skin at 190 residues/1000 residues. The results implied that the two kinds of collagens may have similar thermal stabilities due to analogical living conditions. On the other hand, the hydroxyproline and proline content of ASC from silver carp skin was much higher than that of cod skin collagen (154 residues/1000 residues) and lower than that of calf skin collagen (215 residues/1000 residues) (Giraud-Guille et al., 2000). The amino acid (hydroxyproline and proline) content of collagen is closely related to thermostability (Privalov, 1982). The results suggested ASC from silver carp skin may have higher thermal stability than cod skin ASC, while lower than mammalian collagens.

Table 2
Amino acid composition of the acid-soluble collagens from skins of silver carp, carp and cod (results are expressed as residues/1000 residues).

Amino acid	Silver carp skin collagen	Carp skin collagen	Cod skin collagen	calf skin collagen[b]
Aspartic acid	51	49	53	45
Threonine	20	24	23	18
Serine	32	35	59	33
Glutamic acid	77	76	80	75
Glycine	329	332	342	330
Alanine	112	118	107	119
Cystine	–	–	–	–
Valine	20	19	19	21
Methionine	16	14	15	6
Isoleucine	14	10	12	11
Leucine	23	22	22	23
Tyreonine	5	3	4	3
Phenylalanine	14	13	12	3
Hydrolysine	8	7	7	
Lysine	28	28	29	26
Histidine	5	5	8	5
Arginine	54	55	54	50
Tryptophan	–	–	–	–
Hydroxyproline	78	76	51	94
Proline	114	114	103	121
Imino acid	192	190	154	215

[b] Giraud-Guille et al. (2000).

3.3. Electrophoresis

The skin ASC samples from sliver carp, carp and cod, along with type I collagen from calf skin were analysed by polycrylamide gel electrophoresis, in the presence of SDS using 7.5% gel (Fig. 1).

SDS–PAGE pattern showed that all collagens consisted of α chains, their dimmer (β chains), and small amounts of γ components. All collagens were composed of at least two different α chains, α_1 and α_2, and the density of α_1 is higher than that of α_2. There was a small amount of another α chain, α_3, which migrated to the same position as α_1, and could not be separated under the electrophoretic conditions employed (Kimura, Ohno, Miyauchi, & Uchida, 1987b). No difference in the mobility of the α chains between silver carp and carp skin collagens could be observed, which meant they had similar molecular masses. On the other hand, subunit molecular weights (α_1, α_2 chain, β chain) of silver carp skin collagen were lower than those of calf skin collagen, but higher than those of cod skin ASC, which property was the same as that of ASC from carp skin (Duan et al., 2009). A great amount of β chains could be seen in the patterns of all collagens, which meant the collagens were rich in inter- and intra-molecular crosslinks.

3.4. Isolation of collagen components

To determine the subunit compositions of ASC from silver carp skin, the collagen was further fractionated on a column of SP-Toyopearl under denaturing conditions. Fig. 2 shows the chromatography and SDS–PAGE patterns of the fractions indicated by the number. As shown in Fig. 2, silver carp skin collagen eluted three major peaks. The distinct α chain designated as α_1, α_3 and α_2 (fraction number 44–49, 50–55, and 63–72, respectively) according to Piez (1965) were observed in the first, second and third peak,

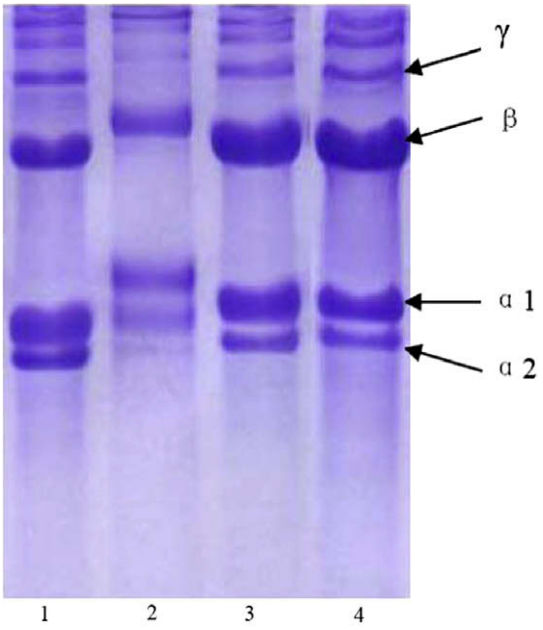

Fig. 1. SDS-polyacrylamide gel electrophoresis of ASC from silver carp skin, carp skin, cod skin and calf skin on 7.5% gels. lanes1: cod skin ASC; lanes2: calf skin ASC; lanes3: silver carp skin; lanes4: carp skin ASC.

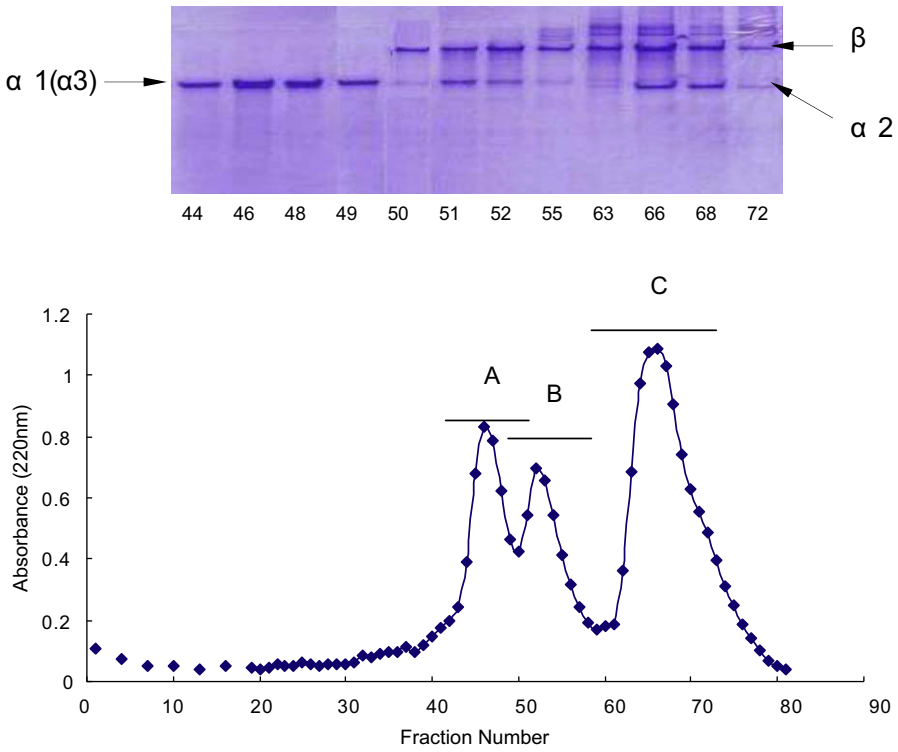

Fig. 2. SP-Toyopeal 650 M column chromatography of denatured ASC from silver carp skin and SDS-polyacrylamide gel electrophoresis from the fraction indicated by the numbers. A Toyopeal 650 M column of 1.5 × 10 cm was equilibrated with 0.06 M sodium acetate buffer, pH 4.8. 10 mg sample dissolved in 5 ml of 60 mM sodium acetate buffer, pH 4.8, then denatured at 45 °C for 30 min. Elution was achieved by the starting buffer with a linear gradient of 100–250 mM NaCl over a total volume of 300 ml at a flow rate of 90 ml/h.

respectively. α_3 eluted chromatographically at a position close to that of α_1 and migrated electrophoretically at the same position as α_1. Such a similarity between α_1 and α_3 was previously observed for Type I collagens from many teleosts (Kimura et al., 1987a). Many fish belonging to the teleost such as eel, sardine, chum salmon, etc. have a third subunit, α_3 chain (Kimura et al., 1987b). The SDS–PAGE pattern of ASC from silver carp skin also showed the existence of α_3 chain.

Crosslinked dimers of α chain, β, were eluted in the second and third peaks. The β chains were estimated to be β_{11}, β_{13}, β_{12} and β_{23} in the order of their elution positions (Kimura et al., 1987a). The first peak (fraction A) contains only the α_1 component. The second peak (fraction B) contains a β_{11} and β_{13} component and small amount of an α_3 component. The third peak (fraction c) contains an α_2 component, whose molecular weight was smaller than α_1 and α_3 according to their mobility on SDS–PAGE. The relative abundance of α_1, α_2 and small amount of α_3 strongly suggested the coexistence of a $(\alpha_1)_2\alpha_2$ and $\alpha_1\alpha_2\alpha_3$ hetero-trimer as the major and minor molecular forms of type I collagen.

The chromatography pattern of ASC from carp skin (figure not shown here) bears a strong resemblance to that of silver carp skin. From the chromatography and SDS–PAGE patterns of ASC fractions, it was suggested that the subunits of carp skin are similar to that of silver carp skin. Kimura, Zhu, Matsui, Shijoh, and Takamizawa (1988) reported $(\alpha_1)_2\alpha_2$ and $\alpha_1\alpha_2\alpha_3$ heterotrimers appeared to be major and minor components, respectively, in carp muscle collagen. Scale and bone contain a larger quantity of α_3 than skin and muscle (Kimura et al., 1991).

3.5. Denaturation temperature

The denaturation temperature (T_d) was determined by a viscosity measurement. Collagen was dissolved in 0.1 M acetic acid and then subjected to heat treatment at different temperatures. Fig. 3 shows that the transition curve of T_d of ASC from silver carp skin was similar as that of ASC from carp skin in the temperature range of 17–40 °C, namely, the fractional changes of relative viscosity of the two fish skin collagens were almost the same. The T_d of silver carp ASC was around 29 °C, almost identical to ASC from carp skin of 28.0 °C and grass carp skin of 28.4 °C (Zhang et al., 2007). However, the T_d of cod skin ASC was only about 15.0 °C. The T_d of collagen from sliver carp skin was also higher than those of collagens from some marine species, Alaska Pollack skin (16.8 °C) and bladder (18.4 °C) (Kimura et al,1987a), chum salmon (19.4 °C) (Kimura, Zhu, Matsui, Shijoh & Takamizawa,1988). In general, T_d of collagen from cold water fish is lower than that of warm-water fish (Takahashi & Yokoyama, 1954). The results were in accordance with amino acid compositions of collagens, namely,

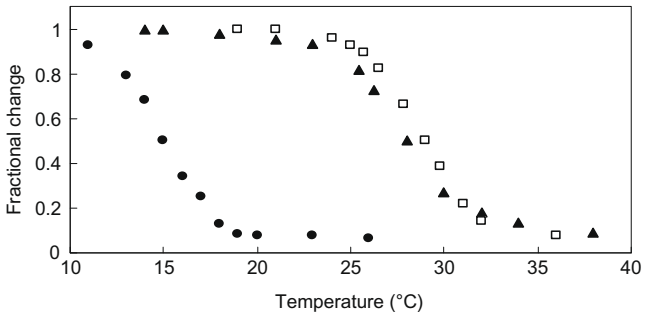

Fig. 3. Thermal denaturation curve of acid-soluble collagen solution as measured by viscosity in 0.1 M acetic acid. The incubation time at each temperature was 20 min. Collagen concentration: 0.75%. (□): sliver carp skin ASC; (▲): carp skin ASC; (●) cod skin ASC.

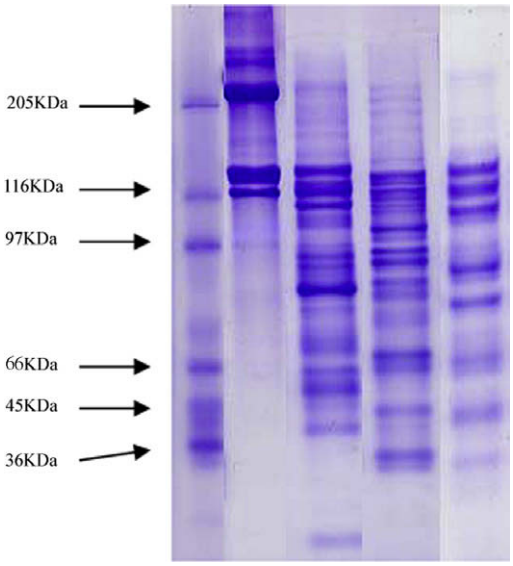

Fig. 4. Peptide mapping of ASCs digested by pepsin from silver carp skin, carp skin and cod skin at 30 °C. lanes1: high molecular weight marker; lanes2: ASC from silver carp skin; lanes3: peptide fragments of ASC from silver carp skin; lanes4: peptide fragments of ASC from cod skin; lanes5: peptide fragments of ASC from carp skin.

the amino acid (hydroxyproline and proline) contents were correlated with the differences in the T_d values.

3.6. Peptide mapping

In this experiment, the collagens were heated at 50 °C for 30 min, and then cooled immediately in an ice bath. The samples were subjected to pepsin digestion (1/100 (w/w), 30 °C, 5 h).

Extensive work on vertebrate collagens has demonstrated that the triple-helical conformation of native collagen is resistant to the degradation by most proteinases, except specific collagenases (Gross & Lapiere, 1962). However, after heating at 50 °C, the collagens were susceptible to hydrolysis by pepsin. As shown in Fig. 4, ASC from silver carp skin was markedly degraded. The peptide with the molecular weight between 97 kDa and 66 kDa showed high density in the pattern, which meant it was the major product of pepsin digestion of silver carp skin collagen. The pattern of ASC from silver carp skin was distinguished with that of cod skin collagen, which was degraded into three major peptide fragments with MW values around 97, 66 and 36 kDa. When comparing peptide maps between collagens from skins of silver carp and carp, different peptide fragments of MWs ranging from 116 to 36 kDa were observed. The results indicated that the primary structure of ASC from silver carp skin was different from that of cod skin collagen. Additionally, ASCs from skins of the two freshwater fish were not similar to each other in terms of amino acid sequence.

Acknowledgements

This work was supported in parts by the grants from the Bureau of Science and Technology of Lianyungang through Research Foundation Project # CG0803-3 and Japan Society for the Promotion of Science (JSPS), Japan. We would like to express our heartfelt gratitude to the Bureau of Science and Technology and JSPS.

References

Anonymous (1978). Meat and meat products-determination of L-hydroxyproline content (reference method). *International standard, ISO* 3496(E).

AOAC (1999). In K. Helrich (Ed.), *Official methods of analysis* (14th ed.). Washington, DC: Association of Official Analytical Chemists.

Duan, R., Zhang, J. J., Du, X. Q., Yao, X. C., & Konno, K. (2009). Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*). *Food Chemistry, 112*, 702–706.

Giraud-Guille, M.-M., Besseau, L., Chopin, C., Durand, P., & Herbage, D. (2000). Structural aspects of fish skin collagen which forms ordered arrays via liquid crystalline states. *Biomaterials, 21*, 899–906.

Gross, J. & Lapiere, C. M. (1962). Collagenogenolytic activity in amphibian tissue: A tissue culture assay. In *The proceedings of the national academy of sciences online (US)* (Vol. 48, pp. 1014–1022).

Kimura, S., & Ohno, Y. (1987a). Fish type I collagen: Tissue-specific existence of two molecular forms, $(\alpha_1)_2\alpha_2$ and $\alpha_1\alpha_2\alpha_3$ in Alaska Pollack. *Comparative Biochemistry and Physiology, 88B*(2), 409–413.

Kimura, S., Ohno, Y., Miyauchi, Y., & Uchida, N. (1987b). Fish skin type I collagen: Wide distribution of an α_3 subunit in teleost. *Comparative Biochemistry and Physiology, Part B, 88*, 27–34.

Kimura, S., Zhu, X.-P., Matsui, R., Shijoh, H., & Takamizawa, S. (1988). Characterization of fish muscle type I collagen. *Journal of Food Science, 53*, 1315–1318.

Kimura, S., Miyauchi, Y., & Uchida, N. (1991). Scale and bone Type I collagen of carp (*Cyprinus carpio*). *Comparative Biochemistry and Physiology, Part B, 99*(2), 473–476.

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

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

Ministry of agriculture (2006). National statistics of fishery in 2005. *Chinese Journal of Animal Science, 14*, 9–10.

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

Nagai, T., Worawattanamateekul, W., Suzuki, N., Nakamura, T., Ito, T., & Fujiki, K. (2000). Isolation and characterization of collagen from rhizostomous jellyfish (*Rhopilma asamushi*). *Food chemistry, 70*, 205–208.

Piez, K. A. (1965). Characterization of a collagen from codfish skin containing three chromatographically different α chains. *Biochemistry, 4*, 2590–2596.

Privalov, P. L. (1982). Stability of proteins. *Advances in Protein Chemistry, 35*, 1–104.

Regina, K. P., Rositza, S., & Blagovest, B. (1996). Modification of Lowry's method for collagen concentration measurement. *Journal of Biochemical and Biophysical Methods, 32*, 33–43.

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

Takahashi, T., & Yokoyama, W. (1954). Physico-chemical studies on the skin and leather of marine animals – XII. The content of hydroxypoline in the collagen of different fish skins. *Bulletin of the Japanese Society of Scientific Fisheries, 20*, 525–529 (in Japanese).

Zhang, Y., Liu, W. T., Li, G. Y., Shi, B., Miao, Y. Q., & Wu, X. H. (2007). Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). *Food Chemistry, 103*, 906–912.