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Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*) ☆

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ABSTRACT

Carp (*Cyprinus carpio*) is one of the main species of freshwater fish produced in China. Acid-soluble collagens (ASC) were prepared from carp skin, scale and bone. The yields of skin ASC, scale ASC and bone ASC are 41.3%, 1.35% and 1.06% (on the dry weight basis), respectively. SDS–PAGE pattern showed that ASCs of carp skin, scale and bone were all type I collagen, which were composed of two α_1 and one α_2 chains. The molecular weight of α_2 chain is 116 KDa. The amino acid composition and peptide maps of ASCs were similar to each other, but they were totally different from those of cod skin ASC. Denaturation temperatures (T_d) of ASCs were around 28 °C. Fourier transform infrared spectroscopy proved that ASCs were integrate and native. The results suggest that carp skin, scale and bone collagens have potential as an alternative source of collagen for use in various fields.

1. Introduction

China is the largest freshwater fish producer in the world. The yield of aquatic products in 2005 was 51,000,000 tons, accounting for 1/3 of the worldwide total production, and that of the freshwater products was up to 19,000,000 tons. The main species of freshwater fish are carp, grass carp, silver carp and bighead carp. With the rapid development of fishery, some processing companies were established. However, in the harvest season, many fish also have to be discarded on the bank of pool, due to not-processed on time. On one hand, they may cause pollutions and emit offensive odors; on the other hand, it brought big economic lost to the producers. Hence, comprehensive utilisation of freshwater fish, especially the production of value-added products is a promising mean to increase revenue for the producers and accelerate the development of aquatic culture industry.

Collagen is one of the major proteins in the living body. Collagen and gelatin are widely used in the food, pharmaceutical, cosmetic, biomedical materials and leather industries. The main sources of industrial collagen are those from pig and bovine skin and bones. But the outbreaks of mad cow disease have resulted in anxieties amongst users of cattle collagen. Besides, the collagen from pig's skin and bone is not allowed to use in some regions due to religious reasons. Fish offal, such as bones, scales, as well as skins is very rich in collagen (Gomez-Guillen et al., 2002). Many papers dealt with the practical utilisation of marine animals to produce collagen (Koladziejaka, Silorski, & Niecikowska, 1999; Nagai, Araki, & Suzuki, 2002; Nagai & Suzuki, 2000a; Ogawa et al., 2004; Sadowska, Koladziejaka, & Niecikowska, 2003). However, a few concerned collagen from freshwater animals (Kimura, Miyauchi, & Uchida, 1991; Zhang et al., 2007). Since the production of freshwater cultures in China is quite large, the study of freshwater fish has great significance. Therefore, the objective of this investigation was to isolate and characterise acid-soluble collagens from skin, scale and bone of carp (*Cyprinus carpio*).

2. Materials and methods

2.1. Raw materials

Live cultured carps were obtained from a free market in Lianyungang, Jiangsu province. The scales, skins and bones were removed manually and washed with chilled tap water. The samples were then placed in polyethylene bags and stored at −25 °C until used.

2.2. Chemicals reagents

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

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2.3. Preparation of collagen from scale, bone and skin

The collagens were prepared by the method of Nagai and Suzuki (2000a) with a slight modification. All the preparation procedures were performed at temperature no higher than 4 °C.

2.3.1. Skin collagen

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. The mixture was stirred for 6 h. The alkali solution was changed every 3 h. Then the samples were washed with cold distilled water, until neutral pH of washing water was obtained.

Deproteinised skins were soaked in 1.0% detergent (dish drops, Amway Inc., Guangzhou, China) at sample/detergent solution ratio of 1:10 (w/v) overnight to extract fat, and then the samples were washed with cold distilled water repeatedly. The treated skins were cut into small pieces by scissor 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 were 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 was collected by centrifuging at 20,000g for 30 min. The pellet was dissolved in 0.5 M acetic acid, dialysed against 0.1 M acetic acid and distilled water, respectively, and then lyophilised (ASC from skin).

The acid-soluble collagen was also extracted from Atlantic cod (*Gadus morhua*) skin (Taiyuan Food Ltd., Qingdao, China) in the same way (ASC from cod skin).

2.3.2. Bone collagen

The bones were broken by a hammer, and then washed with cold distilled water for 3 h. The bones were extracted with 0.1 M NaOH for 24 h at a sample/alkali solution ratio of 1:5 (w/v) to remove non-collagenous proteins, changing the solution every 6 h. The residue was washed with cold distilled water until neutral pH value water was obtained.

The treated bones were decalcified with 0.5 M EDTA–2Na solution (pH 7.5) for 5 days. Fat was removed with detergent at sample/detergent solution ratio of 1:10 (w/v) overnight, after that, the samples were washed fully with cold distilled water. The residue was extracted with 0.5 M acetic acid at sample/acid ratio of 1:4 (w/v) for 3 days. The insoluble matter was re-extracted with the same solution at sample/acid ratio of 1:2.5 (w/v) for 2 days. Each extract was filtered by cotton cloth and then centrifuged at 20,000*g* for 1 h. The supernatants were combined. Following procedures were performed essentially as described for skin (ASC from bone).

2.3.3. Scale collagen

Fish scales were extracted with 0.1 M NaOH for 6 h at a sample/alkali solution ratio of 1:8 (w/v) to remove non-collagenous proteins, washed fully with cold distilled water. The scales were decalcified with 0.5 M EDTA–2Na (pH 7.5) at sample/EDTA solution ratio of 1:10 (w/v) for 24 h, and then washed with cold distilled water. The residue was extracted with 0.5 M acetic acid at sample/acid ratio of 1:2.5 (w/v) for 4 days. Following procedures were performed essentially as described for skin (ASC from scale).

2.4. Amino acid analysis and UV–Vis spectra

Acid-soluble collagen samples from skin, scale and bones were hydrolysed respectively in 6 M hydrochloric acid 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.

The UV–Visual absorption spectra of collagens were recorded by a shimadzu spectrophotometer (Model UV-754). 1 mg ASC samples were dissolved in 2 mL 0.5 M acetic acid and the collagen solutions were centrifuged at 5000*g* for 10 min at 4 °C. The clarified samples were determined absorbance at different wavelength (from 200 nm to 460 nm) to get UV–Vis spectra of each sample.

2.5. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970). The collagen samples were dissolved in 0.1 M acetic acid. Then the samples were mixed with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 5% SDS, 20% glycerol) at 1:2 ratio at the presence of 10% β-ME. Electrophoresis was performed on 7.5% gels. High molecular weight markers (Sigma Co, USA) were used to estimate the molecular weight of proteins. Type I collagen from calf skin (Sigma Co, USA) and acid-soluble collagen from cod skin were used as control. 10 μg of protein was loaded in each well.

2.6. Peptide mapping

The lyophilised collagens (5 mg) were dispersed in 1 ml 20 mM Tris–maleate, pH 7.0, containing 0.5% (w/v) SDS at 4 °C. The samples were heated at 100 °C for 5 min and then cooled in the ice bath. The digestion was carried out at 0 °C for 60 min by adding proteinase K (Sigma Co., USA) at a enzyme/sample ratio of 1:2500 (w/w). The digestion was stopped by adding 10 μl 10 mM PMSF (Sigma Co., USA) in each sample. After adding SDS to a final concentration of 2.5%, the reaction mixture was subjected to boiling water for 3 min. SDS–PAGE was performed by the method of Laemmli (1970), using 15% gel. Peptide mapping of cod skin ASC was conducted in the same manner.

2.7. Determination of denaturation temperature

The denaturation temperature (T_d) was measured by the method of Nagai and Suzuki (2000a) with a modification. Ten millilitres 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). 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. T_d was determined as the temperature that the change in viscosity was half completed.

2.8. Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) of ASCs of carp skin, scale and bone were recorded using FTIR (NEXUS470) spectroscopy (Nicolet Instrument Co., Madison, USA).

3. Results and discussion

3.1. Preparation of collagens

3.1.1. Skin collagen

Because of collagen characteristics, fish skins are difficult to mince in a meat grinder. For carp skin, the extractability of acid soluble collagen was 41.3% (on a dry weight basis), which is quite different from some marine species (Nagai, Yamashita, Taniguchi, Kanamori, & Suzuki, 2001; Nagai et al., 2002). The yields of ASCs from the skin of ocellate puffer fish and cuttlefish were low (10.7% and 2%, respectively, on a dry weight basis). However, for some other species, the yields of ASC were high and the value were about 51.4% (Japanese sea-bass), 49.8% (chub mackerel), and 50.1% (bullhead shark), respectively (on the basis of dry weight) (Nagai et al., 2000). Also in this experiment, the yield of acid soluble collagen from cod skin was around 42.5%.

This result might suggest that there are some discrepancies in the construction of collagens among different species. For collagen molecule, the two terminal ends are non-helical parts, which play an important role in the cross-linked structure. If the molecules are highly cross-linked at the telopeptide region, the solubility of collagen in acid solution will decrease (Foegeding, Lanier, & Hultin, 1996). Therefore, in the case of carp and cod skin, the degree of cross-linking among collagen molecules might be weaker than that of some marine fish (such as ocellate puffer fish and cuttlefish), which makes ASC could be extracted readily and the yield was rather high.

It suggested that carp skin had abundant collagen and a large amount of collagen can be obtained.

3.1.2. Bone collagen

The bones were decalcified with 0.5 M EDTA–2Na and were not easily solublilized with 0.5 M acetic acid. The collagen in 0.5 M acetic acid was precipitated by addition of solid NaCl to a final concentration of 2.5 M in the presence of 0.05 M tris (hydroxymethyl) aminomethane, pH 7.0. The yield of ASC from bone was 1.06%, much lower than that of collagens from bones of some marine fish: skipjack tuna (42.3%), yellow sea bream (40.1%), horse mackerel (43.5%) (Nagai & Suzuki, 2000b). The results may be attributed to the different structures of bone collagens between freshwater fish and marine fish. But the exact reason should be studied further.

3.1.3. Fish scale collagen

Under the conditions, the yield of scale ASC was 1.35%, which is similar to that of total collagen from fish scales of Pagrus major and Oreochromis niloticas (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003). For scale and bone, the yields of pepsin-soluble collagens were all higher than those of acid-soluble collagens (data did not show here).

3.2. Amino acid composition

The amino acid composition of acid-soluble collagens of carp skin, scale and bone, as well as cod skin ASC and calf skin collagen, is presented in Table 1. Glycine is the major amino acid in every collagen. The imino acid contents (hydroxyproline and proline) of ASCs of carp skin, scale and bone were 190, 192 and 192 residues/1000 residues, whilst that of cod skin ASC was only 154 residues/1000 residues. Calf skin collagen contained 215 residues/1000 residues (Giraud-Guille, Besseau, Chopin, Durand, & Herbage, 2000). Therefore, carp ASCs had lower imino acid contents than mammalian collagens, which was in accordance with the report from Jongjareonrak, Benjakul, Visessanguan, Nagai, and Tanaka (2005). However, the contents of imino acid in collagens from carp offal were much higher than that of cod skin ASC. The results suggested ASCs of carp skin, scale and bone may have higher thermal stability than cod skin ASC, whilst lower than mammalian collagens.

The degree of hydroxylation of proline was calculated to be 40.0% in ASC from carp skin, 40.1% in ASC from scale, 41.7% in ASC from bone, and 33.1% in cod skin ASC. Hydroxylated proline plays an important role in stabilizing the triple helix of collagen (Ramachandran, 1988). From the result, hydroxylation of proline in collagen from carp bone was slightly higher than that in collagens from skin and scale, which suggests that collagen from bone may have a slightly more complex structure than that from skin and scale as shown by the higher degree of hydroxylation (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005).

Since tryptophan did not exit in the collagens from carp skin, bone or scale, also the contents of tyrosine and phenylalanine were very low, all ASCs do not have the absorption peak at 280 nm, but at 223 or 224 nm, which is accordance with results from Li, Liu, Gao, and Chen (2004). A positive peak at around 223 nm and a negative peak near 204 nm is characteristic for triple-helical collagen (Piez, 1984).

The UV–Vis spectra of cod skin ASC had the similar absorption peak (Figure did not show here).

3.3. Electrophoresis

The ASC samples from carp skin, scales and bones were analysed by polycrylamide gel electrophoresis in the presence of SDS, using 7.5% gel (Fig. 1).

SDS–PAGE pattern showed that all collagens were composed of at least two different αchains, $\alpha_1$and α_2, and the density of α_1 is higher than α_2. Based on electrophoretic mobility and subunit composition, it was suggested that collagens from carp skin, scale

Table 1
Amino acid composition of the acid-soluble collagen from skin, scales and bones (results are expressed as residues/1000 residues)

Amino acid	Calf skin collagen[a]	Cod skin collagen	Skin collagen	Scale collagen	Bone collagen
Aspartic acid	45	53	49	48	47
Threonine	18	23	24	23	25
Serine	33	59	35	35	33
Glutamic acid	75	80	76	76	76
Glycine	330	342	332	336	334
Alanine	119	107	118	119	122
Cysteine	–	–	–	–	–
Valine	21	19	19	19	19
Methionine	6	15	14	14	13
Isoleucine	11	12	10	9	10
Leucine	23	22	22	21	21
Tyrosine	3	4	3	2	3
Phenylalanine	3	12	13	12	13
Hydrolysine		7	7	8	8
Lysine	26	29	28	26	26
Histidine	5	8	5	5	4
Arginine	50	54	55	55	54
Tryptophan	–	–	–	–	–
Hydroxyproline	94	51	76	77	80
Proline	121	103	114	115	112
Imino acid	215	154	190	192	192

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

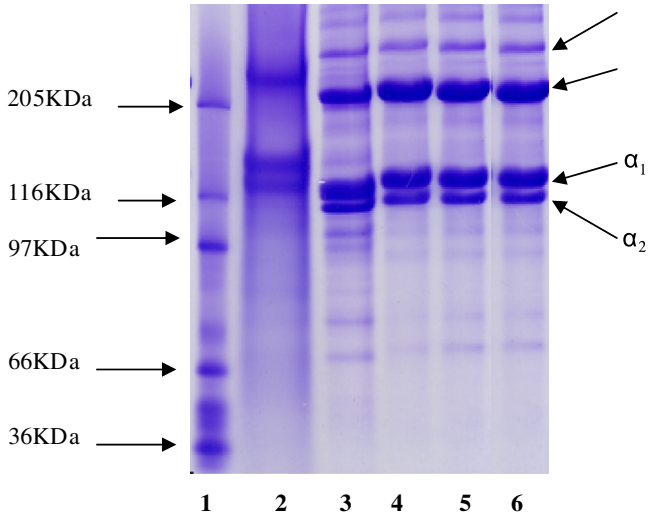

Fig. 1. SDS–polyacrylamide gel eletrophoresis of ASC from carp skin, scales and bones on 7.5% gels. Lane 1: high molecular weight marker; lane 2: calf skin ASC; lane 3: cod skin ASC; lane 4: carp skin ASC; lane 5: carp scale ASC and lane 6: carp bone ASC.

and bone were type I collagens, and were composed of two α_1 and one α_2 chains. The results were in agreement with the previous report (Kimura et al., 1991). The mobility of α chains of the collagens from skin, scale and bone was almost the same, which means these α chains may have similar primary structures. The molecule weight of α_2 was exactly 116KDa. A great amount of β chain can be observed in the pattern of all these collagens. From the pattern, subunit molecular weights (α_1, α_2 chain, β chain) of carp collagens were lower than those of calf skin collagen, but higher than those of cod skin ASC. The discrepancy may be due to the different resources of collagen, namely, resources from mammalian animals and aquatic animals. It seemed that the molecular weight of collagen had some relations with the thermal stability. Collagen with higher molecular weight may have higher thermal stability. There is no report on it. Further researches are needed to confirm the hypothesis.

3.4. Peptide mapping

Lysyl endopeptidase from Achromobacter lyticus and Staphylococcus aureus V8 protease were usually employed to study the peptide mapping of collagen. And the digestion were carried out at 37 °C for 15 min or 5 min (Kittiphattanabawon et al., 2005; Nagai & Suzuki, 2002b). In this experiment, proteinase K was used to degrade collagen at the ratio of enzyme to collagen at 1:2500. We once performed the digestion at 15 °C. The results showed that the rate of digestion was too fast and the clear bands could not be seen. In this case, we lowered the digestion temperature rather than reduce the amount of proteinase K. Clear pattern of peptide mapping was achieved by digestion at 0 °C.

The ASCs digested by protease K at 0 °C were applied to 15% SDS–PAGE gel. As shown in Fig. 2, from the peptide mapping of digests, 60 min was enough to compare the patterns of peptide fragmentation of ASCs of carp skin, scale and bone. The patterns of low molecular weight fragments were similar to each other, as well as the patterns of high molecular weight fragments.

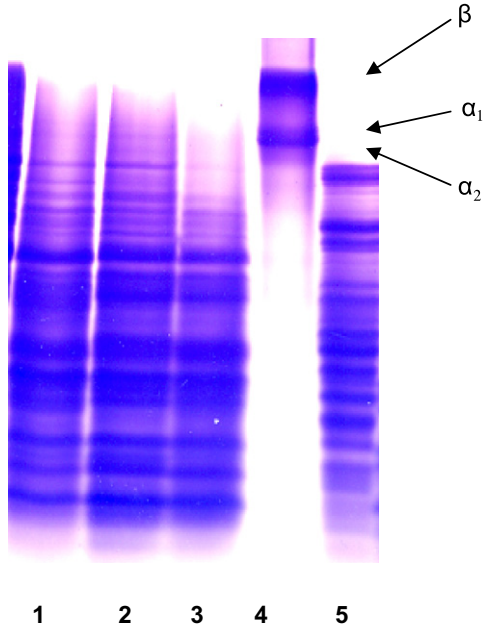

Fig. 2. Peptide mapping of protease K digests from ASC from carp skin, scales and bones at 0 °C. Lane 1: peptide fragments of carp skin ASC; lane 2: peptide fragments of carp scale ASC; lane 3: peptide fragments of carp bone ASC; lane 4: calf skin ASC and lane 5: peptide fragments of cod skin ASC.

Regarding the peptide mapping of collagens digested by protease K under the same condition, the peptide fragments of cod skin collagen were quite different from those of carp skin ASC, scale ASC and bone ASC. And at the same time, calf skin collagen could not be degraded, which meant the structure of calf skin collagen was much rigid against digestion by proteinase K. In general, peptide fragments of collagens from carp skin, scale and bone were quite similar, which could be presumed that primary structures of these collagens were very similar to each other, but different from that of cod skin ASC, in terms of amino acid sequence.

3.5. Denaturation temperature

The denaturation temperature (T_d) was determined by viscosity measurement. Relative viscosity of collagen in 0.1 M acetic acid is depicted in Fig. 3. From the temperature range of 14–23 °C, relative viscosity of carp skin, scale and bone ASCs was almost stable. However, it decreased continuously on heating up to 34 °C. The T_d value of each collagen, defined as the temperature at which the change in viscosity was half completed. The T_ds of carp skin ASC, scale ASC and bone ASC were around 28 °C. However, T_d of cod skin ASC was determined as 15.0 °C. Compared with the collagens from some marine species, the T_ds of collagens from carp skin, scale and bone were higher than those of collagens from Alaska Pollack skin (16.8 °C), swim bladder (18.4 °C) (Kimura & Ohno, 1987), common mackerel (26.1 °C), saury (23.0 °C), chum salmon (19.4 °C) (Kimura, Zhu, Matsui, Shijoh, & Takamizawa, 1988), skin of Japanese sea bass (26.5 °C) (Nagai et al., 2002). In general, T_d of collagen from cold water fish is lower than that of warm-water fish (Takahashi & Yokoyama, 1954). It suggested collagens from carp offal had higher thermal stability than those from many marine species. At the same time, the T_ds of carp ASCs were lower than that of calf skin ASC (40.8 °C) (Komsa-Penkova, Koyonava, Kostov, & Tenchov, 1999). The results are in agreement with previous reports.

3.6. Fourier transform infrared spectroscopy

The FTIR spectra of ASC from carp skin was shown in Fig. 4. The FTIR spectra of scale ASC and bone ASC were similar as that of skin ASC (Figures are not shown).

A free N-H stretching vibration occurs in the range of 3400–3440 cm^{-1}. When the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequency, usually 3300 cm^{-1} (Li et al., 2004). For all the collagens from carp skin, scales and bones, the infrared spectrum of each sample had a shifted N-H stretching vibration (skin ASC, 3324.33 cm^{-1}; scale ASC, 3314.02 cm^{-1}; bone ASC, 3313.85 cm^{-1}), which meant hydrogen bond exited in each collagen. The amide I bands position of collagens were found around 1650 cm^{-1} (skin ASC,1649.50 cm^{-1}; scale ASC, 1650.55 cm^{-1}; bone ASC, 1650.46 cm^{-1}), fitting well the range of 1625–1690 cm^{-1} for general amide I bands position; The amide II bands

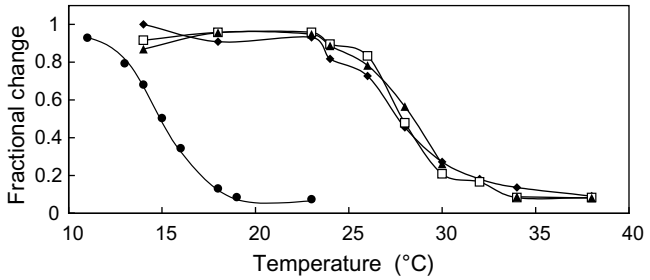

Fig. 3. Thermal denaturation curve of acid soluble collagen solution as measured by viscosity in 0.1 M acetic acid. The incubation time at each temperature was 20 min. Collagen concentration: 0.75%. (♦): skin ASC; (□): scale ASC; (▲): bone ASC; (●): cod ASC.

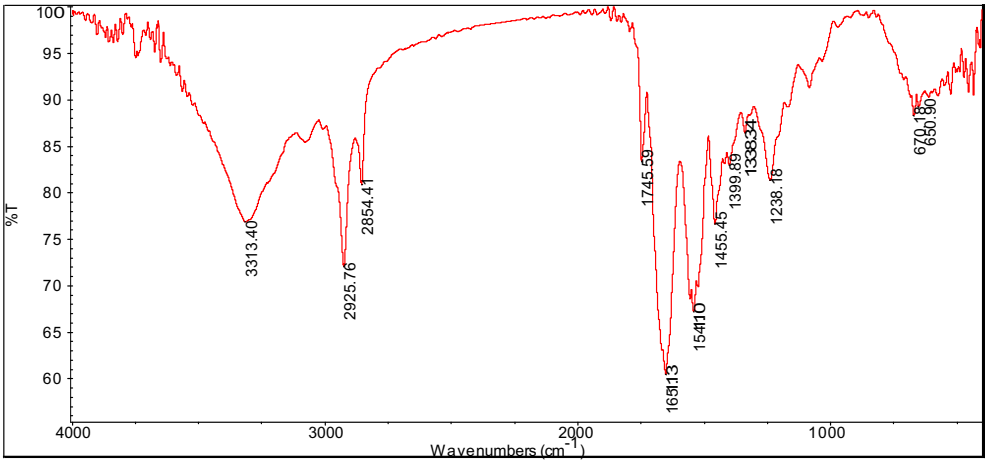

Fig. 4. Fourier transform infrared spectrum of carp skin ASC. (The unit of abscissa is cm^{-1}).

position were detected around 1540 cm^{-1} (skin ASC,1540.00 cm^{-1}; scale ASC, 1539.73 cm^{-1}; bone ASC, 1540.03 cm^{-1}). Compared to the normal absorption range of the amideII bands position (1550–1600 cm^{-1}), the position is shifted to lower frequency, 1540 cm^{-1}, which also showed the existence of hydrogen bonds in each collagen.

The helical structure of the collagen was confirmed from IR absorption ratio between the 1235 (amide III) and 1450 bands, which was approximately equal to each preparation. The results showed the helical structure of collagens were kept well. In addition, for carp skin ASC, a strong C-H stretching vibration occurs around 2854 cm^{-1} and 1745 cm^{-1}.

Fourier transform infrared spectrum of ASC from carp skin, scales and bones had great similarity to each other, which suggested their structures were quite similar.

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