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# Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*)

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**Abstract**

Pepsin-soluble collagen was extracted from the skin of grass carp (*Ctenopharyngodon idella*) with a yield of 46.6%, on a dry weight basis. Electrophoretic patterns showed that the collagen contained α1 and α2 chains, similar to those of calf skin collagen. The imino acid content of the collagen from grass carp skin was much lower than those of mammalian's collagens, as also were the transition temperature and denaturation temperature which were only 24.6 °C and 28.4 °C respectively. Peptide maps of the collagen digested by trypsin and V8 protease showed different peptide fragments from those of calf skin collagen and other fish skin collagens, suggesting differences in amino acid sequences and collagen conformation. In addition, the lyophilized collagen sponge from grass carp skin had a uniform and regular network structure, just like calf skin collagen sponge. These results suggest that grass carp skin has potential for use as a supplementary source of collagen.
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*Keywords:* Skin collagen; Grass carp; Freshwater fish; Isolation; Characterization

## 1. Introduction

Collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein. At least nineteen variants of collagen have been reported, named types I-XIX (Bailey, Paul, & Knott, 1998). Collagen has very broad industrial applications, such as edible and photographic gelatin, leather industry and sausage casings. Furthermore, soluble collagen serves in cosmetics, pharmacy and as supports of enzymes or biologically active compounds due to its special characteristics, including biodegradability and weak antigenicity (Kolodziejska, Sikorski, & Niecikowska, 1999; Zhang, Li, & Shi, 2006).

Skins and bones of cow and pig are generally the main sources of collagen and gelatin; however, the outbreaks of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD) have resulted in anxiety among users of collagen and collagen-derived products from these land animals. There is a strong need to develop new collagen sources as a result. Fish offals, such as skins, bones, scales and fins, are also tissues that are mainly structured by collagen. Although the physical and chemical properties of fish collagen are different from those of mammalian collagens (Bailey & Light, 1989), collagens from fish offals are unlikely to be associated with infections such as BSE, TSE and FMD. Therefore, the fish offals may be effective alternative sources.

The skin and bone collagens from several fishes have been extracted and characterized and, except for the Nile perch (*Lates niloticus*), most studied fishes were marine species, such as bigeye snapper (*Priacanthus tayenus*), Brownstripe red snapper (*Lutjanus vitta*), paper nautilus (*Argonauta argo*) and ocellate puffer (*Takifugu rubripes*) (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Kolodziejska et al., 1999;

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Muyonga, Cole, & Duodu, 2004; Nagai, Araki, & Suzuki, 2002; Nagai & Suzuki, 2002; Yata, Yoshida, Fujisawa, Mizuta, & Yoshinaka, 2001). Grass carp (*Ctenopharyngodon idella*) is a long slender member of the minnow family native to large rivers in Asia. It is one of the most abundant freshwater fishes in China, with a production of more than 3 million tons per year, which is 20% of the total yield of freshwater fishes. Grass carp has been mainly used as fish meals and the researches on it have mainly been concentrated on its breeding and histopathology (Pipalova, 2003; Riaz & Mughal, 2002; Wang et al., 2006). Thus, isolation of collagen from grass carp skin will not only greatly promote the value of the fish, but also partly resolve the collagen resource problems. The objective of this study was to extract and characterize pepsin-soluble collagen from grass carp skin.

## 2. Materials and methods

### 2.1. Fish skin preparation

Three live grass carp (*Ctenopharyngodon idella*) with average weights of 1.5–2.0 kg were procured from a local market, and were kept in a tank with water and air for 24–36 h after being caught, and then were anaesthetized in iced water. The skins were denuded, descaled and washed with distilled water. Cleaned samples were cut into small pieces ($0.2 \times 0.2$ cm) and frozen at −20 °C within a week prior to collagen extraction.

### 2.2. Extraction of collagen

The collagen was extracted according to the method of Kittiphattanabawon et al. (2005) with some modification. All processes were performed at 4 °C with continuous stirring. Skins were soaked in 0.1 M NaOH with a sample/solution ratio of 1:30 (w/v) for 12 h with a change of solution every 4 h to remove non-collagenous proteins and pigments. To get rid of pigments more effectively from the skin, the method described by Kolodziejska et al. (1999) was used. The swollen skins were bleached with 3% $H_2O_2$ solution for 48 h and the solution was changed once. The samples were then washed with cold distilled water until the pH of the wash water became neutral or faintly basic.

Fat was removed in distilled water containing 0.5% of a non-ionic detergent for three periods of 6 h each. Defatted skins were thoroughly washed with cold distilled water. The samples were suspended in 0.5 M acetic acid with a sample/solution ratio of 1:30 (w/v), containing 1% (w/w) pepsin (EC 3.4.23.1, 1:10,000, Sigma Chemical Co.) for 24 h with continuous stirring. The mixture was then centrifuged at 9000*g* for 30 min. The pepsin-soluble collagen (PSC) was salted-out by adding NaCl to the supernatant to a final concentration of 1.5 M. The sediment, by centrifugation at 9000*g* for 20 min, was dissolved in 0.5 M acetic acid, and again precipitated by the addition of NaCl to a final concentration of 0.7 M. The resultant precipitate was collected by centrifugation at 9000*g* for 30 min and then re-dissolved in 0.5 M acetic acid, followed by dialyzing against 0.1 M acetic acid. The resultant collagen was lyophilized in a freeze dryer (Labconco Freeze Dryer FreeZone 6 Liter, USA) and its morphology was observed by scanning electron microscopy (SEM) (JEOL JSM-5900LV, Japan).

### 2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed by the method of Laemmli (1970), using the discontinuous Tris–HCl/glycine buffer system with 7.5% resolving gel and 4% stacking gel.

The collagen sample was dissolved in a sample buffer (0.5 M Tris–HCl, pH 6.8, containing 2% SDS, 25% glycerol) with 10% β-ME, to reach a final collagen concentration of 1 mg/ml, and then boiled for 3 min. The gel was stained for 30 min with 0.25% Coomassie brilliant blue R-250 solution and distained with 7.5% acetic acid/5% methanol solution until the bands were clear.

### 2.4. Amino acid analysis

The collagen sample (10–20 mg) was mixed and hydrolyzed in 6 M HCl at 110 °C for 24 h. The hydrolysate was vaporized and the remaining matter was dissolved in 25 ml citric acid buffer solution. An aliquot of 50 μl was applied to an amino acid analyzer (HITACHI 835-50 Amino Acid Analyzer, Japan).

### 2.5. Thermal transition of collagen

The maximum transition temperature was determined by differential scanning calorimetry (DSC) (Netzsch DSC 200PC, Germany). The collagen sample was dissolved in 0.05 M acetic acid, with a concentration of 5 mg/ml. Approx. 2 mg samples were sealed in an aluminium pan and an empty pan was used as the reference. The endothermal curve of the sample was recorded from 20 to 40 °C at a heating rate of 2 °C/min in a nitrogen atmosphere.

### 2.6. Viscosity of collagen solution

The denaturation temperature was measured from the viscosity changes, using an Ubbelohde viscosimeter, according to the method of Zhang et al. (2006) with some modification. Dried collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 0.5 mg/ml. A 15 ml collagen solution was heated from 15 to 40 °C, and was held for 30 min prior to efflux time ($t$) determination at the designated temperature. The measurement was carried out three times at each point. The efflux time ($t_0$) of the collagen solvent (0.5 M acetic acid) was also determined under the same conditions. The fractional viscosity at the given temperature was calculated with the equation: $F(T) = (\eta_{sp(T)} - \eta_{sp(40\,°C)})/(\eta_{sp(15\,°C)} - \eta_{sp(40\,°C)})$, where $\eta_{sp}$ is the specific

viscosity and is calculated by $(t-t_0)/t_0$. The fractional viscosities were plotted against the temperatures and the denaturation temperature was taken to be the temperature where fractional viscosity was 0.5.

### 2.7. Peptide mapping of collagen

The collagen sample was dissolved in 0.5 M acetic acid, pH 2.5, at a concentration of 5 mg/ml. After adding trypsin (EC 3.4.21.4, 1:250, Sigma Chemical Co.) with an enzyme/substrate ratio of 1/2 (w/w) to the collagen solution, the reaction mixture was incubated at 37 °C for 3 h. In order to stop the reaction, the SDS–PAGE sample buffer was added to the digestion sample, and the mixture was heated immediately at 100 °C for 3 min. SDS–PAGE was performed to separate peptides generated by the protease digestion, using 7.5% gel. The pepsin-soluble collagen from calf skin was also digested by trypsin in the same manner and the peptide maps were compared.

Another collagen sample was dissolved in 0.1 M sodium phosphate buffer, pH 7.8, at a concentration of 6 mg/ml. After adding trypsin or *Staphylococcus aureus* V8 protease (EC 3.4.21.19, 541units/mg solid, Sigma Chemical Co.) with an enzyme/substrate ratio of 3/100 (w/w) to the collagen solution, both the reaction mixtures were incubated at 37 °C for 2 min. The reaction was terminated by adding the same SDS–PAGE sample buffer to the mixture and subjecting it to boiling water for 3 min. SDS–PAGE was also performed to separate peptides using 7.5% and 10% gels.

## 3. Results and discussion

### 3.1. Extraction of collagen from grass carp skin

The collagen of grass carp skin was easily solubilized by limited pepsin and the yield was very high, reaching 46.6% on a dry weight basis. This result was similar to those of paper nautilus (50%) and ocellate puffer (44.7%) (Nagai et al., 2002). However, the yield of acid-soluble collagen (ASC) that was extracted according to the initial experiment plan was only about 8%, on a dry weight basis. This was different from that of Nile perch (Muyonga et al., 2004) whose ASC yield was more than 50%, but also in agreement with those of paper nautilus (5.2%) and ocellate puffer (10.7%). The different yields of ASC and PSC from grass carp skin might suggest that there were many interchain crosslinks at the telopeptide region of the collagen that resulted in low solubility in acid. After adding pepsin, the crosslinked regions at the telopeptide were cleaved without damaging the integrity of the triple helix, leading to the high solubility of collagen in acid. In addition, from the different yields of ASC of grass carp skin and Nile perch skin, it might be concluded that there were more cross-links at the telopeptide region of collagen from grass carp skin than from Nile perch skin, although both are freshwater fishes, but the degree of cross-linking at the telopeptide region of the collagen from grass carp skin might accord with those of collagen from the skins of paper nautilus and ocellate puffer, considering the similar ASC and PSC yields.

### 3.2. SDS–PAGE patterns of collagen

Fig. 1 shows SDS–PAGE patterns of collagen from grass carp skin, along with type I collagen from calf skin as a comparison. It was found that the collagen consisted of α chains ($\alpha_1$ and $\alpha_2$) and their dimers (β chains), and small amounts of γ components were also found. These patterns were similar to those of calf skin collagen, and agreed with those of collagens from most other fishes studied (Jongjareonrak et al., 2005; Kittiphattanabawon et al., 2005; Kolodziejska et al., 1999; Muyonga et al., 2004; Nagai et al., 2002; Nagai & Suzuki, 2000; Yata et al., 2001).

From the patterns of the collagen of grass carp skin, it could not be concluded whether the collagen contained α3 chains or not. Piez (1965) first reported the isolation and characterization of an α3 chain from the soluble collagen of cod skin, indicating the existence of a unique $\alpha_1\alpha_2\alpha_3$ heterotrimer containing three non-identical α chains. After that, more fish skin collagens were found to have α3 chains, such as Alaska pollack (Kimura & Ohno, 1987) and carp (Miyauchi & Kimura, 1990). Kimura, Ohno, Miyauchi, and Uchida (1987) reported that the α3 chain was widely distributed in the teleost. But the α3 chain could not be separated under the electrophoretic conditions employed, because it showed similarity in chemical nature to α1 and migrated electrophoretically to the same position as α1 (Kimura, 1992; Miyauchi & Kimura, 1990).

### 3.3. Amino acid composition

Table 1 shows the amino acid composition of the collagen from grass carp skin, compared with those of mamma-

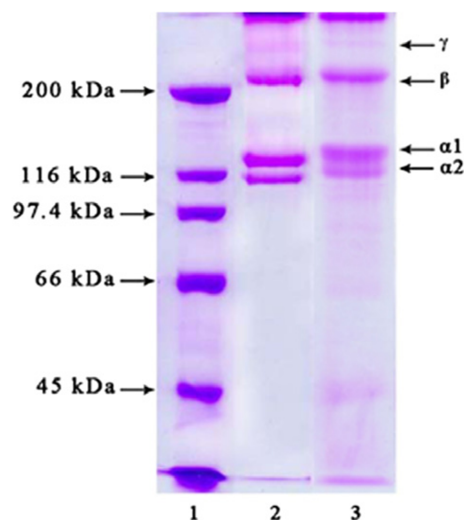


Fig. 1. SDS–PAGE pattern of collagen from the skin of grass carp on 7.5% gel. Lane 1: protein markers; lane 2: calf skin collagen; lane 3: grass carp skin collagen.

Table 1
Amino acid composition of collagen from grass carp skin (residues/1000 residues)

| Amino acid | Grass carp skin collagen | Pig skin collagen | Calf skin collagen | Bigeye snapper skin collagen | Ocellate puffer collagen |
|---|---|---|---|---|---|
| Hyp | 65 | 97 | 94 | 77 | 67 |
| Asp | 42 | 44 | 45 | 51 | 50 |
| Thr | 24 | 16 | 18 | 29 | 25 |
| Ser | 39 | 33 | 33 | 36 | 48 |
| Glu | 61 | 72 | 75 | 78 | 87 |
| Pro | 121 | 123 | 121 | 116 | 103 |
| Gly | 334 | 341 | 330 | 286 | 351 |
| Ala | 135 | 115 | 119 | 136 | 106 |
| Cys | 4 | 0 | 0 | 0 | 2 |
| Val | 31 | 22 | 21 | 22 | 17 |
| Met | 10 | 6 | 6 | 12 | 14 |
| Ile | 10 | 10 | 11 | 5 | 12 |
| Leu | 22 | 22 | 23 | 24 | 23 |
| Tyr | 2 | 1 | 3 | 4 | 4 |
| Phe | 17 | 12 | 3 | 15 | 10 |
| Hyl | 8 | 7 | 7 | 10 | – |
| Lys | 23 | 27 | 26 | 31 | 19 |
| His | 5 | 5 | 5 | 10 | 8 |
| Arg | 57 | 48 | 50 | 60 | 54 |
| Total | 1000 | 1000 | 1000 | 1000 | 1000 |
| Imino acid | 186 | 220 | 215 | 193 | 170 |

lian collagens, such as calf skin collagen (Giraud-Guille, Besseau, Chopin, Durand, & Herbage, 2000) and pig skin collagen (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003), and with those of two other fish skin collagens (Kittiphattanabawon et al., 2005; Nagai et al., 2002). The collagen from grass carp skin was found to have glycine (nearly 1/3) as the major amino acid and to be very low in methionine, tyrosine and histidine like other collagens. Also, the collagen consisted of proline, hydroxyproline and hydroxylysine, which are unique amino acids found in collagen. The imino acid (proline and hydroxyproline) content of the collagen from grass carp skin was 186 residues/1000 residues, which was lower than those of pig skin collagen (220 residues/1000 residues) and calf skin collagen (215 residues/1000 residues), but was similar with those of several fish skin collagens. This result underlined the conclusion reached by Foegeding, Lanier, and Hultin (1996): fish collagens have lower imino acid contents than have mammalian collagens. The higher the imino acid content, the more stable are the helices, because the molecular structure of collagen is maintained mainly by restrictions on changes in the secondary structure of the polypeptide chain, imposed by the pyrrolidine rings of proline and hydroxyproline, and also maintained partially by the hydrogen bond ability through the hydroxy group of hydroxyproline (Gustavson, 1955; Piez & Gross, 1960). Therefore, the collagen helices of grass carp skin might be less stable than those of mammalian skins, due to the lower imino acid content.

### 3.4. Thermal stability of collagen

The heat transformation of collagen is interpreted as disintegration of the collagen triple helical structure into random coils. This is accompanied by a change in physical properties, such as viscosity, sedimentation, diffusion, light scattering and optical activity (Usha & Ramasami, 2004). DSC and viscosity measurements are usually used to investigate the thermal stability of collagen. The former is a well-developed analytical tool used for measurement of transitions in polymers, and the latter is used for learning about the loss of viscosity with heating, attributed to denaturation of collagen.

The thermal transition curve of the collagen from grass carp skin in 0.05 M acetic acid is shown in Fig. 2, with the peak maximum temperature ($T_{max}$) of 24.6 °C. Fig. 3 shows the changes in fractional viscosity with increasing temperature and the denaturation temperature ($T_d$) was calculated to be 28.4 °C. Both the temperatures were much

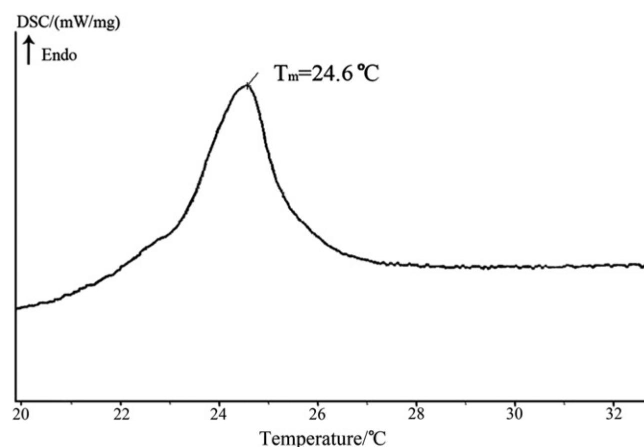


Fig. 2. Thermal transition curve of collagen from grass carp skin, as shown by DSC.

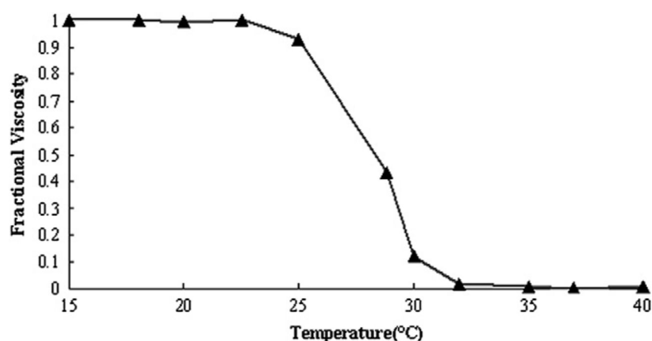


Fig. 3. Thermal denaturation curve of collagen from grass carp skin, as shown by changes in fractional viscosity.

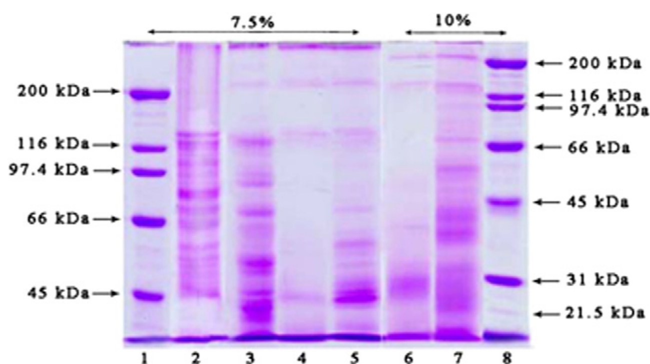


Fig. 4. Peptide maps of collagen from grass carp skin digested by trypsin and V8 protease. Lanes 1 and 8: protein markers on 7.5% and 10% gels, respectively; lanes 2 and 3: peptide fragments of collagen from calf skin and grass carp skin with trypsin digestion at pH 2.5, respectively; lanes 4–7: peptide fragments of grass carp skin collagen with trypsin (lanes 4 and 6) and V8 protease (lanes 5 and 7) digestion at pH 7.8, on 7.5% gel (lanes 4–5) and on 10% gel (lanes 6–7), respectively.

lower than the $T_m$ of calf skin collagen (40.8 °C) (Komsa-Penkova, Koynova, Kostov, & Tenchov, 1999) and the $T_d$ of pig skin collagen (37 °C) (Nagai et al., 2002). These results further proved that the helices of collagen from grass carp skin were less stable than those of mammalian collagens. Compared to the transition temperatures of the collagens from other fish skins, such as bigeye Snapper (28.7 °C) (Kittiphattanabawon et al., 2005) and Brownstripe red snapper (30.52 °C) (Jongjareonrak et al., 2005), the $T_m$ of the collagen from grass carp skin was also a little lower; however, its $T_d$ was equal to those of collagens from the skins of paper nautilus (27 °C) (Nagai et al., 2002) and ocellate puffer (28 °C) (Nagai et al., 2002). These results also agreed with the imino acid contents of the fishes. The increasing imino acid content resulted in increased denaturation temperature of the collagen (Piez & Gross, 1960). Besides, the thermal stability of collagen was associated with the environmental and body temperatures of the fishes. Bigeye snapper and Brownstripe red snapper are tropical fish, while grass carp, paper nautilus and ocellate puffer might be temperate fish. Also, the $T_d$ of the collagen from grass carp skin was higher than those of cold-water fish collagens, such as cod (15 °C) (Rigby, 1968) and Alaska pollack (16.8 °C) (Kimura et al., 1987).

### 3.5. Peptide mapping of collagen

Fig. 4 shows the peptide maps of the collagen from grass carp skin digested by trypsin and V8 protease, in comparison with calf skin collagen digested by trypsin. For peptide maps of both collagens digested by trypsin at pH 2.5, 37 °C (lanes 2–3), there were obvious decreases in the band intensities of cross-linked components (β- and γ-components). The α-components (α1 and α2) of calf skin collagen were hydrolyzed to some extent, with the nearly even appearance of peptide fragments of molecular weight (MW) ranging from 116 to 45 kDa. But the α1 component of grass carp skin collagen was almost entirely digested and the part α2 component remained, with more fragments of MW values around 45 kDa. This result suggested that α-components of grass carp skin collagen were less tolerant to digestion by trypsin than those of calf skin collagen under this condition.

From the peptide maps of collagen from grass carp skin digested by trypsin and V8 protease at pH 7.8, 37 °C (lanes 4–7), it could be conlcluded that the fish skin collagen was more susceptible to hydrolysis by trypsin than by V8 protease as the collagen was mostly degraded into 31 kDa fragments by trypsin. But, for the maps digested by V8 protease, many more fragments of larger MW, ranging from 65.4 to 21.5 kDa, were observed. Compared to the skin collagens of bigeye snapper (Kittiphattanabawon et al., 2005) and Brownstripe red snapper (Jongjareonrak et al., 2005) digested by V8 protease, the peptide maps were different. The collagen from bigeye snapper was degraded into three major peptide fragments with MW values of 53.3, 36.9 and 33.8 kDa, while collagen from Brownstripe red snapper was degraded into peptides with MW ranging from 103.6 to 32.3 kDa. Since V8 protease shows a high degree of specificity for glutamic acid and aspartic acid residues of proteins (Vercaigne-Marko, Kosciarz, Nedjar-Arroume, & Guillochon, 2000) and peptide maps of collagens were reported to differ among sources and species (Mizuta, Yamasa, Miyagi, & Yoshinaka, 1999), there might be some differences in their primary structures, in terms of the sequence and the composition of amino acids.

Moreover, the collagen from grass carp skin digested by trypsin at pH 2.5 and pH 7.8 (lanes 3–4) was degraded into different peptide fragments. There were major fragments with MW values of 96.3, 77.3, 54.4 and 36 kDa at pH 2.5; however, there were mostly small MW fragments, lower than 31 kDa at pH 7.8. This result is attributed to the activity of the trypsin that was greater for the collagen in neutral or faintly basic medium than in acid medium.

### 3.6. Properties of the lyophilized collagen

Freeze-drying has proven to be the most advantageous process to manufacture homogeneous porous collagen devices. As shown in Fig. 5, the lyophilized collagen sponge

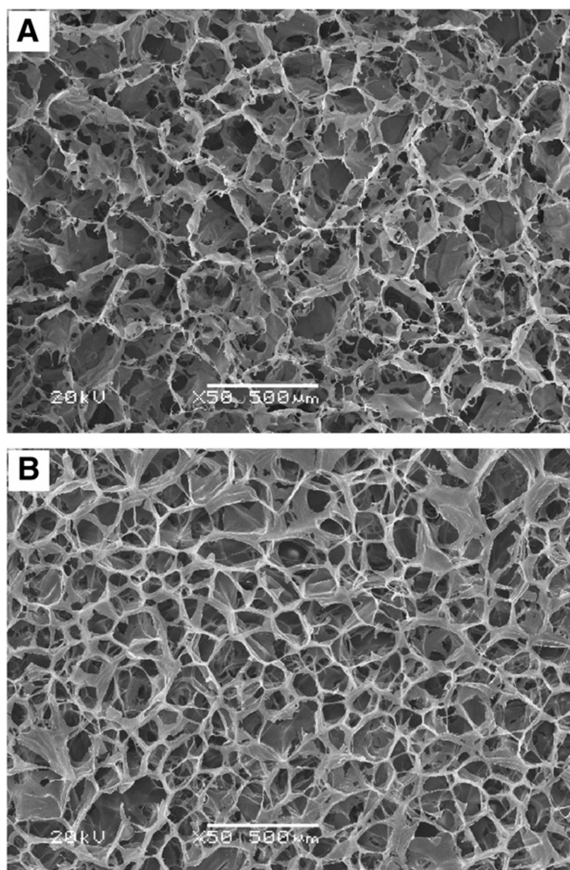


Fig. 5. SEM images of collagen sponges from grass carp skin (A) and calf skin (B).

from grass carp skin (A) was loose and porous, just like calf skin lyophilized collagen sponge (B). The lyophilization conditions (temperature, vacuum, rate of rising and falling) for the two collagens were the same and the collagen concentrations before lyophilization were different. Both the two sponges were endowed with uniform and regular alveolate pores. Uniform and regular network structures of sponges, as drug carriers are propitious, not only for well-proportioned distribution for other drugs, but also for evaporation of fluid. Taking the network structure into account, the collagen sponge from grass carp skin might also have good properties for distribution of the drugs as carriers. For the different pore sizes of the two collagen sponges, they might be attributed to the different collagen concentrations before lyophilizing. The pore size of collagen increased with the increase of water content in the preparation, and the pore size of the collagen sponge should be adjusted so that it is above the size of the implantation cells for cell incursion and growth (Ikoma et al., 2003; Yaylaoglu, Yildiz, Korkusuz, & Hasirci, 1999).

## 4. Conclusion

PSC was extracted from grass carp skin with a high yield of 46.6% and contained two α chains, α1 and α2. The collagen had a lower imino acid content than had mammalian collagens. The transition temperature or denaturation temperature of the collagen from grass carp skin was much lower than those of collagens from mammals, a little lower than those of collagens from tropical fish and relatively equal to those of collagens from temperate fish. The different peptide maps of collagens from grass carp skin and calf skin indicated that there might be some differences in amino acid sequences or conformation. The lyophilized grass carp skin collagen sponge had a uniform and regular network structure, just like calf skin collagen sponge. Therefore, there is a possibility of using the skin of grass carp as an alternative to calf skin and pig skin collagens for industrial purposes.

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