

# Circular Dichroism and Chromatographic Studies of Fish Skin Collagens

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**Abstract:** Acid soluble collagens (ASC) were isolated from carp (*Cyprinus carpio*) skin and cod (*Gadus morhua*) skin. Partial biochemical properties of both ASCs were investigated and compared. Denaturation temperatures, measured by melting point using circular dichroism (CD), was 36 °C for carp skin ASC and 19.0 °C for cod skin ASC, which were in agreement with the amino acid contents of the two collagens. SP-Toyopearl column chromatographic analysis showed that both skin collagens consisted of three kinds of  $\alpha$  chains ( $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$ ), which may exist in ( $\alpha_1$ )<sub>2</sub>  $\alpha_2$  and  $\alpha_1$   $\alpha_2$   $\alpha_3$  hetero-trimer. The pepsin digestion patterns of  $\alpha$  chain of carp and cod skin ASCs were considerably different from each other.

**Key words:** fish skin; collagen; chromatography; circular dichroism

## 鱼皮胶原蛋白性质的圆二色性和色谱法研究

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**摘要:** 从鲤鱼(*Cyprinus carpio*)和鳕鱼(*Gadus morhua*)鱼皮中提取酸溶性胶原蛋白(ASC), 并对两种胶原蛋白的性质进行研究和比较。通过圆二色性(CD)分析鲤鱼鱼皮ASC的变性温度为36℃, 鳕鱼鱼皮ASC的变性温度为19.0℃, 与胶原蛋白亚氨基酸含量的分析结果一致。柱色谱层析结果显示, 两种胶原蛋白都由3种不同的 $\alpha$ 链组成( $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ), 3种单链形成的聚合体含有( $\alpha_1$ )<sub>2</sub>  $\alpha_2$ 和 $\alpha_1$   $\alpha_2$   $\alpha_3$ 两种不同的形式。鲤鱼和鳕鱼鱼皮 $\alpha$ 链的胃蛋白酶酶解图谱显示两种胶原蛋白之间存在明显的差异。

**关键词:** 鱼皮; 胶原蛋白; 层析; 圆二色性

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Collagen is the most abundant biological macro-molecule protein in vertebrates, constituting about a quarter of the total. It is the major structural element of skin, bone, tendon, cartilage, blood vessels, and teeth of vertebrates<sup>[1]</sup>. Collagen structure is distinguished by the formation of a right-handed superhelix consisting of three polypeptide chains. These peptides are extremely rich in proline and glycine, both of which are important for the formation of the collagen-specific helical structure<sup>[2]</sup>. It has been widely used in pharmaceutical, artificial organism material.

At present, the main sources of type I collagen are

limited to those of bovine or porcine dermis, which has been broadly employed in functional food, cosmetics, and pharmaceuticals. However, due to the emergence of bovine spongiform encephalopathy (BSE), the foot-and-mouth disease (FMD) crisis, today's health-conscious consumers are reluctant to try collagen extracted from land animals. As a consequence, increasing attention has been paid to alternative collagen sources, especially fish skin and bone from seafood processing wastes. About 30% of these wastes consist of skin and bone, which are very rich in collagen<sup>[3]</sup>.

Some reports have been dealt with collagens extracted

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from the skin of aquatic species, eg. jellyfish, starfish, octopus, paper nautilus, cuttlefish, purple sea urchin, and others<sup>[4-6]</sup>. Atlantic cod (*Gadus morhua*) is a kind of marine fish which is used in surimi industry and cod skin is one of the major wastes. On the other hand, China is the largest freshwater fish producer in the world and carp (*Cyprinus carpio*) is one of the most abundant species of freshwater fish. However, there were a few papers concerned collagens from freshwater animals<sup>[7-8]</sup>.

Thermal stability is an essential prerequisite for any protein. It is well recognized that collagen from cold water fish skin is more unstable than that from warm water fish skin. However, few report could be found concerning on the relations between the subunit and thermal stability. It is worthy to further study the properties of collagens to reveal the differences of collagens from the marine and fresh water fish.

## 1 Materials and Methods

### 1.1 Materials

Live cultured carps were obtained from a free market in Lianyungang, Jiangsu province (February, 2008). The skins were removed manually. Atlantic cod (*Gadus morhua*) skin was offered by Taiyuan Food Ltd., Qingdao, China.

The samples were washed with chilled tap water and then placed in polyethylene bags and stored at  $-25\text{ }^{\circ}\text{C}$  until used.

All reagents were of analytical grade.

### 1.2 Preparation of skin collagen

All the preparation procedures were performed at  $4\text{ }^{\circ}\text{C}$ . The collagens were prepared by the method of Nagai et al.<sup>[6]</sup> with a slight modification.

The scaled skins were suspended in  $0.1\text{ mol/L}$  NaOH to remove non-collagenous proteins. Then the samples were rinsed with cold distilled water repeatedly until a neutral pH of washing water was obtained.

Deproteinised skins were soaked in butyl alcohol overnight to remove 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  $0.5\text{ mol/L}$  acetic acid with stirring. The extract was centrifuged at  $20000\times g$  for 1 h. The supernatant were salted-out by adding NaCl to a final concentration of  $2.5\text{ mol/L}$  at the presence of Tris. The resultant precipitate was collected by centrifuging at  $20000\times g$  for 30 min. The pellet was dissolved in  $0.5\text{ mol/L}$  acetic acid, dialyzed against  $0.1\text{ mol/L}$  acetic acid and distilled water, respectively, and then lyophilized.

### 1.3 Amino acid composition

Acid-soluble collagen samples from skins were hydrolyzed respectively in  $6\text{ mol/L}$  hydrochloric acid at  $110\text{ }^{\circ}\text{C}$  for 24 h in the absence of oxygen<sup>[9]</sup>. The hydrolysates were analyzed on a Hitachi 835-50 amino acid analyzer.

### 1.4 Circular dichroism(CD) measurement

Collagens dissolved in acetic acid was centrifuged. Small aliquots were taken out and transferred to a quartz cuvette, and then placed into the polarimeter to record the CD spectra. CD spectra of collagen samples were recorded on a Jasco J-725 spectropolarimeter (Jasco Inc., Japan). Five scans were averaged for the wavelength  $250 - 190\text{ nm}$ .

The melting curve of collagen was determined by monitoring  $[\theta]_{220\text{nm}}$  at the wavelength of a positive extreme at  $220\text{ nm}$ . The transition temperature was determined as the midpoint temperature between native-folded and completely unfolded forms. The analyses were repeated twice.

### 1.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli<sup>[10]</sup>. The collagen samples and lyophilized  $\alpha$  chains fractioned by chromatography were dissolved in acetic acid. Then the samples were mixed with the sample buffer ( $0.5\text{ mol/L}$  Tris-HCl, 20% glycerol). Electrophoresis was performed on 7.5% gels.

### 1.6 SP-Toyopearl column chromatography

To separate the subunits of each collagen sample, the sample was applied to a column chromatography (Tosoh Co., Tokyo, Japan). The collagen sample were dissolved in sodium acetate, and the solution was centrifuged at  $50000\times g$ . The supernatants were applied to a SP-Toyopearl 650M column ( $1.5\text{ cm}\times 10\text{ cm}$ ). Appropriate fractions were pooled. The fractions indicated by the numbers were examined by SDS-PAGE.

The subunit  $\alpha$  chain of collagens fractioned by chromatography were collected and lyophilized.

### 1.7 Pepsin digestion of $\alpha$ chain

Pepsin digestion was carried out as Trueb et al<sup>[11]</sup>, reported. At intervals aliquots were withdrawn and terminated. The digests were resolved on 7.5% polyacrylamide gels containing SDS (SDS-PAGE)<sup>[10]</sup>.

## 2 Results and Analyses

### 2.1 CD measurement

Collagen solution in cuvette was heated from  $10$  to  $45\text{ }^{\circ}\text{C}$ . The collagens showed a rotatory maximum at  $220\text{ nm}$  and minimum at  $191\text{ nm}$  and a consistent cross over point (zero

rotation) at about 213 nm, which was characteristic of the triple helical conformation of the protein<sup>[12]</sup>. Fig. 1 showed the corresponding mean molar ellipticities,  $[\theta]_{220\text{nm}}$ , as a function of temperature.

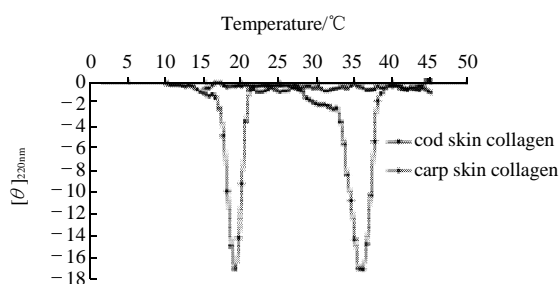


Fig.1 Thermal unfolding of carp and cod skin ASC as expressed as the first - derivative plots

For carp skin collagen, the flatness of the curves below 20 °C and above 40 °C suggested that these regions of the curve represent completely folded and completely unfolded protein, respectively. The  $[\theta]_{220\text{nm}}$  values decreased with temperature due to decomposition of the collagen triple helical structure, and indicated denaturation temperatures of 36 °C and 19 °C for carp and cod skin collagen, respectively. carp and cod skin collagens had different amino acid profile, although glycine is the major amino acid in every collagen<sup>[13]</sup>. Table 1 showed the imino acid contents of the collagens per 1000 total residues.

Table 1 Imino acid content and thermal transition temperature of skin collagens

Collagen	Hydrolysine/ (residues/1000residues)	Proline/(residues/ 1000residues)	Imino acids(Pro+Hyp)/ (residues/1000residues)	$t_m$ /°C
Carp skin ASC	76	114	190	36
Cod skin ASC	51	103	154	19

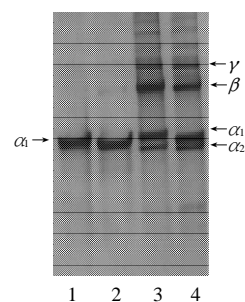
Carp ASCs had higher imino acid contents (192 residues/1000 residues) than cod skin collagen (154 residues/1000 residues). The degree of hydroxylation of proline was calculated to be 40.0% in carp skin ASC and 33.1% in cod skin ASC. In general, collagen has a high content of imino acids, and imino acid content is closely related to thermostability<sup>[14]</sup>. The results of amino acid composition were in accordance with those of CD measurement.

Aquatic species collagen that denatures above 30 °C (measured by CD) includes skipjack (33.0 °C), eel (30.2 °C),

and Japanese sea bass (30.0 °C)<sup>[15-16,6]</sup>. Compared with cod skin ASC, carp skin ASC had higher stability, of which denaturation temperature was only 4.8 °C below  $t_m$  of calf skin collagen<sup>[17]</sup>. From this point, it is possible for carp skin collagen to substitute for the land base animal collagens in current commercial applications.

## 2.2 SDS-PAGE

Fig.2 showed the SDS-PAGE patterns of cod and carp skin collagens, along with  $\alpha_1$  chains of the two collagens fractioned by chromatography.



lane1.  $\alpha_1$  chain from carp skin ASC; lane2.  $\alpha_1$  chain from cod skin ASC; lane3.carp skin ASC; lane4.cod skin ASC.

Fig.2 SDS-PAGE patterns of carp and cod skin ASC, along with  $\alpha_1$  chains from the two samples

Gel electrophoretic patterns indicated the presence of two different subunits,  $\alpha_1$  and  $\alpha_2$ , in a molar ratio of about 2:1.  $\alpha_3$  chain, if present, could not be separated from the corresponding  $\alpha_1$  chain under the electrophoretic conditions employed. SDS-PAGE patterns showed that collagens from carp and cod skin were type I collagens. However, as reported before<sup>[13]</sup>, the subunit molecular weights ( $\alpha_1$ ,  $\alpha_2$  chain,  $\beta$  chain) of carp collagens were higher than those of cod skin ASC. From the pattern, the molecular mass of “single”  $\alpha_1$  chain fractioned by chromatography from the carp skin collagen was also higher than that from cod skin collagen, which further confirmed the discrepancy of the molecular weight of two samples.

## 2.3 Chromatography

The collagens were resolved by SP-Toyopearl 650M column chromatography to determine the subunit composition of the two species collagens.

Various chromatographic fractions were examined by SDS-polyacrylamide gel electrophoresis. Fig.3 and Fig.4 showed the chromatography and SDS-PAGE patterns of the fractions indicated by the number. The first, second and third peak contained three distinct  $\alpha$  chains designated as  $\alpha_1$ ,  $\alpha_3$  and  $\alpha_2$ , respectively<sup>[12]</sup>. As seen in the SDS-PAGE patterns, the first peak contained entirely  $\alpha_1$  chain compo-

nent (fraction A), while the second peak was as the mixtures of  $\alpha_3$  and  $\beta$  chains.  $\alpha_3$  eluted chromatographically at a position close to that of  $\alpha_1$  and migrated electrophoretically at the same position as  $\alpha_1$ . The third peak (fraction C) contained  $\alpha_2$  component, whose molecular weight was smaller than  $\alpha_1$  and  $\alpha_3$  according to their mobility on SDS-PAGE. The  $\beta$  chains were estimated to be  $\beta_{11}$ ,  $\beta_{13}$ ,  $\beta_{12}$  and  $\beta_{23}$  in the order of their elution positions<sup>[18]</sup>.

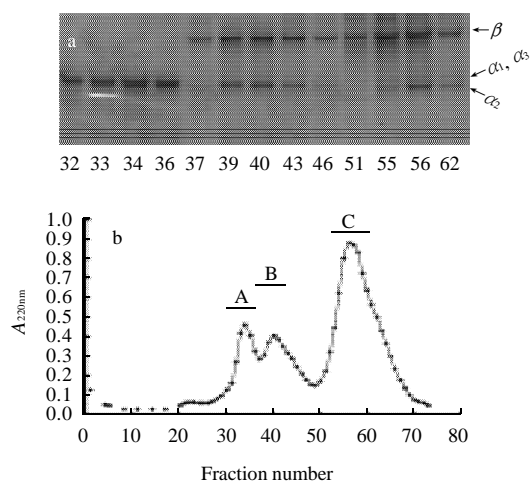


Fig.3 SP-Toyopearl chromatography of skin Type I collagen from carp

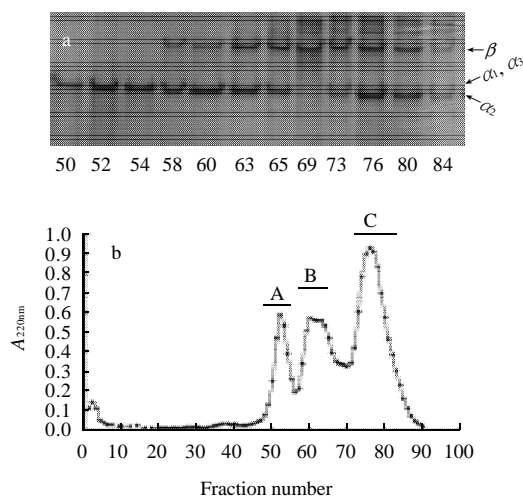


Fig.4 SP-Toyopearl chromatography of skin Type I collagen from cod

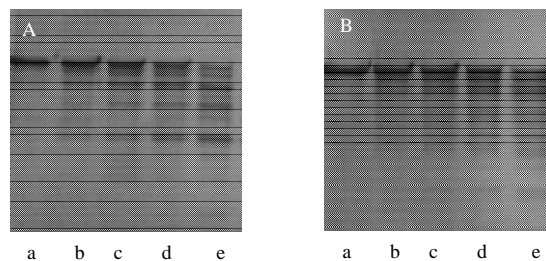
Kimura et al.<sup>[11]</sup> reported that  $\alpha_3$  chain was widely distributed in the teleost, eel, sardine, chum salmon, rainbow trout, carp, anger, Alaska Pollack, halfbeak, tilapia. It can be presumed that the differences of relative  $\alpha_3$  amount among various species should be more obvious. Therefore, from the results, it was estimated that  $\alpha_1 \alpha_2 \alpha_3$  hetero-trimer was as the major molecular forms of cod skin ASC, while ( $\alpha_1$ )<sub>2</sub>  $\alpha_2$  and

$\alpha_1 \alpha_2 \alpha_3$  hetero-trimer was as the major and minor molecular forms of carp skin collagen.

Type I collagen serves the main supporting function in all vertebrates. In addition to  $\alpha_1$  and  $\alpha_2$  subunits common to all vertebrates, many fish belonging to the Teleosts have a third subunit,  $\alpha_3$ , which has not been found in any other vertebrates<sup>[19]</sup>. That meant calf and porcine skin collagens do not possess  $\alpha_3$  chain. The properties of  $\alpha_3$  chains in general are closely related to those of  $\alpha_1$  chains. It has been suggested that the  $\alpha_3$  gene arose as a duplication of the  $\alpha_1$  gene and underwent mutation and selection<sup>[19]</sup>.

#### 2.4 Pepsin digestion patterns of $\alpha_1$ chains of carp and cod skin ASC

To further compare the properties of cod and carp skin collagens, lyophilized  $\alpha_1$  chain of collagens was dissolve and digested with the pepsin. Extensive works on vertebrate collagens have demonstrated that the triple-helical conformation of native collagen is resistant to the degradation by most proteinases, except specific collagenases<sup>[20]</sup>. In this case,  $\alpha_1$  chains were separated by column chromatography. Thus,  $\alpha_1$  chains were susceptible to pepsin and could be degraded into short peptides. The digestion patterns are shown in Fig.5.



a-e.Digestion time is 0, 5, 15, 30, 60min;Carp skin ASC (A); Cod skin ASC (B).

Fig.5 Pepsin digestion patterns of  $\alpha_1$  chains of carp and cod skin ASC

With duration, the  $\alpha_1$  chains of carp and cod skin collagen were remarkably degraded into low molecular weight fragments. As the major subunit composition, the digestion pattern of  $\alpha_1$  chain from carp skin collagen was quite distinguished with that of cod skin collagen. Duan et al.<sup>[13]</sup> reported the proteinase K digestion patterns of the two samples were different from each other. This result further indicated that the primary structure of the collagen from carp skin was different from that of cod skin ASC in terms of amino acid sequence, which is also in accordance with the results of SP-Toyopearl chromatography and CD measurement.

### 3 Conclusion

Although a few researchers reported the extraction and properties of collagen from cod and carp skin, in this paper, the two collagens were studied by circular dichroism and chromatography. We got clear chromatography profiles with three peaks which have not been reported before. The results were accordant with the results of CD and previous reports.

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