

Comparison on the Stability of Collagens from Skin and Bone of Carp (*Cyprinus carpio*) Caught in Winter and Summer

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Abstract: The acid-soluble collagens (ASC) were isolated from skin and bone of carp (*Cyprinus carpio*) caught in the winter (February in 2008) and summer (July in 2008). Thermal stability of collagens was studied by the increased pepsin digestibility upon heating and by circular dichroism (CD) spectroscopy. Collagens from skin and bone in the winter (w-skin ASC and w-bone ASC) exhibited transition curves with midpoints of 31.0 °C and 32.8 °C, respectively, which were 1.0 °C and 0.6 °C below their equivalents from summer season fish. The CD spectrum changes of collagens upon heating were in accordance with the results of pepsin digestion. The results indicated that collagens from skin and bone of summer carp were more stable than their equivalents of winter.

Key words: collagen; thermostability; pepsin digestion; circular dichroism

冬夏鲤鱼鱼皮和鱼骨胶原蛋白稳定性比较

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摘要: 从冬季(2008年2月)和夏季(2008年7月)的鲤鱼鱼皮和鱼骨中分别提取酸溶性胶原蛋白,在一定温度下加热,通过蛋白酶的酶解和圆二色谱(CD)法研究其热稳定性。结果显示:冬季鱼皮和鱼骨酸溶性胶原蛋白的变性温度分别为31.0℃和32.8℃,比夏季鱼皮和鱼骨酸溶性胶原蛋白的变性温度分别低1.0℃和0.6℃。通过圆二色谱(CD)法得到了与此一致的结果。测定结果显示,来源于夏季鱼皮和鱼骨胶原蛋白的热稳定性要优于冬季鱼皮和鱼骨胶原蛋白。

关键词: 胶原蛋白; 热稳定性; 胃蛋白酶酶解; 圆二色谱

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Collagens have been used extensively as materials in foods, pharmaceutical industries and biomedicines. However, the outbreaks of bovine spongiform encephalopathy (BSE) and foot and mouth disease (FMD) have caused the restriction of cattle collagens due to the anxieties among users for possible transmission of those diseases to human beings. 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. Carp (*Cyprinus carpio*) is one of the most abundant species of freshwater fish. The utilization of fish processing wastes is of great significance for increasing the income of the producers and for environmental protection.

A typical feature of collagen is its three polypeptide chains twisted together into a right-handed triple helix. Each polypeptide chain consists of a regular arrangement of amino acid sequence, triplet (Gly-X-Y), where X and Y are often proline (Pro) and hydroxyproline (Hyp) residues [1].

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Thermal stability is an essential prerequisite for any protein^[2]. It is recognized that collagen molecules in solution denature close to the upper limit of the physiological temperature or the maximum body temperature of the animal species from which the collagen is extracted^[3]. Therefore, environmental conditions have great influence on the collagen stability. However, seasonal difference in the thermal stability of collagen, especially collagens from freshwater fish has not been reported. In this study, the acid-soluble collagens (ASC) were isolated from skin and bone of carp caught in the winter (February in 2008) and summer (July in 2008). We hypothesized that collagens extracted from skin and bone of carp caught in winter may have lower denaturation temperatures than the equivalents in summer. The purpose of this research is to obtain fundamental information for the effective use of freshwater fish.

1 Materials and Methods

1.1 Materials, reagents and instruments

Live carp (*Cyprinus carpio*) were obtained from a free market in Lianyungang, Jiangsu. The skins and bones were removed manually and washed with chilled tap water. The samples were then placed in polyethylene bags and stored at $-40\text{ }^{\circ}\text{C}$ until used.

Pepsin Sigma(USA); Acrylamide, Bis-acrylamide Tosoh Co.(Japan).

Densitometer Fuji Inc.(Japan); Jasco J-725 Spectropolarimeter Jasco Inc.(Japan); Circulator CTE 42W Yamato Scientific Co. Ltd. (Japan).

1.2 Methods

1.2.1 Preparation of collagen from bone and skin

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

1.2.2 Thermal denaturing experiments

1.2.2.1 Denaturation temperature judged by pepsin digestion of collagens

Estimation of the denaturation temperature of the collagen was performed on the basis of the method described by Trueb et al^[5]. Lyophilized material was dissolved in 0.1 mol/L acetic acid and then heated by water bath at 26, 28, 30, 31.5, 33, 35, 40 $^{\circ}\text{C}$ and 50 $^{\circ}\text{C}$ respectively. Then the collagens were digested with pepsin. Digestion was terminated by addition of sample buffer (5% SDS, 20% glycerol and 10% β -ME). Thereafter, the samples were heated immediately in boiling

water for 2 min. The digests were resolved on 7.5% polyacrylamide gels and the band intensities of β components were quantified by densitometry.

1.2.2.2 Circular dichroism measurement

Collagen solutions heated at different temperatures for 30 min, cooled immediately in ice bath and then centrifuged at $50000 \times g$ for 20 min. Small aliquots were taken out and transferred to a quartz cuvette (1 mm pathway), and then placed into the polarimeter to record the CD spectra. Spectra of collagen from 190 nm to 250 nm were recorded by a Jasco J-725 spectropolarimeter. The fraction of native collagen was estimated from the change in ellipticity at 220 nm. Thermal transition curves were recorded at a fixed wavelength (220 nm) by raising the temperature linearly at the rate of $1\text{ }^{\circ}\text{C/min}$ using a Gilford temperature programmer.

Sample buffer was 0.1 mol/L acetic acid. To avoid protein damage by UV, UV exposure of any sample never exceeds ten minutes.

2 Results and Discussion

2.1 Thermal denaturing experiments

2.1.1 Denaturation temperature judged by pepsin digestion of collagens

Collagens were extracted from skin and bone of carp caught in February and July 2008 as representative months of winter and summer seasons, respectively, considering for possible seasonal difference in thermal behavior. Samples in February were termed as w-skin, w-bone ASC, and those in July were s-skin, s-bone ASC. ASCs of skin and bone were heated from 26 to $50\text{ }^{\circ}\text{C}$ for 30 min, respectively and digested by pepsin.

The protein monomer of collagens consists of three polypeptide chains, which are in a unique triple-helical conformation. The triple-helical conformation of native collagen in general resists degradation by most proteinases, but specific collagenases can cleave the molecule into fragments^[6-7]. In this experiment, denaturation temperatures of collagens were studied by monitoring the protease-induced degradation of helical domains by SDS-PAGE. Seasonal differences in the thermostability of collagens were compared. The results indicated that the triple helix of collagen underwent a thermal helix to coil transition with increasing temperature, which led an increasing fraction of the collagen substrate to be susceptible to proteolysis.

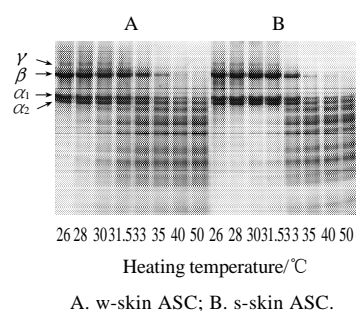


Fig.1 Pepsin digestion patterns of carp skin ASCs heated at various temperatures

As shown in Fig.1, the relative staining intensity of the β chains decreased remarkably at 30 °C for the winter samples, while the summer samples had little changes of β chain intensity at this temperature. The results showed that the winter samples denatured at lower temperatures than the summer equivalents.

The changes of β components were quantified by densitometry. Fig.2 and Fig.3 shows the relative staining intensity of the β chains plotted against the heating temperatures.

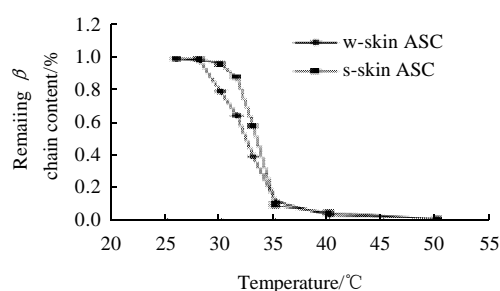


Fig.2 Determination of the transition midpoint of skin ASCs substrate using a proteolytic probe

The collagen substrate was heated at different temperatures, from 26 to 50 °C. The samples were equilibrated at any temperature for 30 min and then subjected to digestion with pepsin. Thermal transition curve of the β component was obtained from the densitometric tracing of the SDS-PAGE.

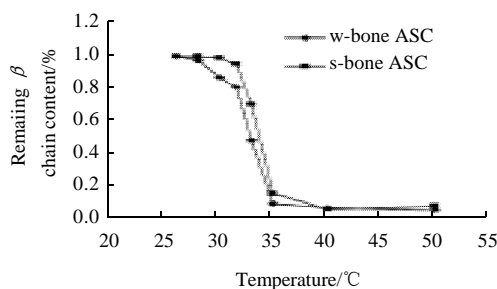


Fig.3 Determination of the T_m of bone ASCs substrate using a proteolytic probe

The bone collagens were heated at different temperatures as skin collagens. Thermal transition curve of the β component was obtained from the densitometric tracing of the SDS-PAGE.

As shown in Fig.2 and Fig.3, the transition curves of summer collagens exhibited sharper changes than that of the winter ones. The transition midpoint (T_m) of w-skin ASC was 31 °C and that of w-bone ASC was 32.8 °C, while the T_m of s-skin and s-bone collagens were higher than those of winter ones by 1 °C and 0.6 °C, respectively. Namely, the T_m of s-skin ASC was 32 °C and s-bone ASC was 33.4 °C, respectively. The results indicated that ASCs from skin and bone in summer were slightly more stable than those of winter ones.

2.1.2 CD spectrum changes of skin and bone collagens

To verify the difference of thermal stability between collagens from winter and summer fish, CD spectrum changes of the samples were studied. After incubated at different temperatures for 30 min, the collagen solutions were centrifuged at $50000 \times g$ for 20 min. Native collagen from skin and bone gave a characteristic CD spectrum with a positive extreme at 220 nm and negative peaks that appeared at 198 — 209 nm.

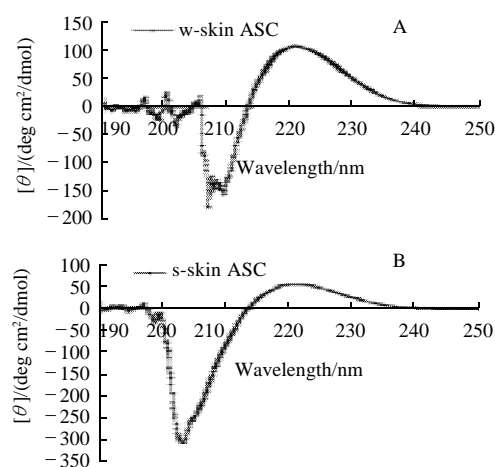


Fig.4 CD spectrum of winter and summer skin ASCs in 0.1 mol/L acetic acid solution

As shown in Fig.4, w-skin collagen had two negative peaks at 207 nm and 209 nm, while s-skin ASC had only one negative peak at 202 nm. As shown in Fig.5, The spectrum of w-bone ASC showed there was only one negative peak at 202 nm, while the peaks of s-bone collagen were at 199, 201, 203 nm. The results were consistent with the reports of Piez et al^[8]. Positive band around 223 nm and a stronger negative band near 198 nm characterize the CD spectrum of triple-helical collagen.

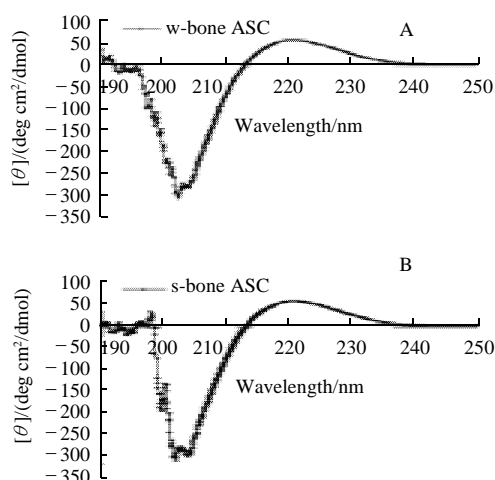


Fig.5 CD spectrum of winter and summer bone ASCs in 0.1 mol/L acetic acid solution

The melting characteristics of dissolved collagen molecules were studied by monitoring the ellipticity (220 nm) which is proportional to the degree of helix-coil transition. The deviations in ellipticity suggested that there were discrepancies in structures between winter and summer samples. Some researchers reported the existence of bi-phase thermal transitions of collagen^[9-11]. In this case, the collagens possessed at least two inner domains with diverse stabilities or two different collagen molecules with diverse stabilities^[12].

The collagens were dissolved in 0.1 mol/L acetic acid. Ellipticity at 220 nm was monitored by circular dichroism spectroscopy as the temperature was increased by increments of 1 °C/min.

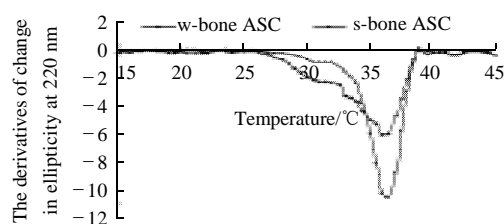


Fig.6 First-derivative plots of melting curves of ASCs from bones

Fig.6 presents different thermal denaturation profiles of w-bone and s-bone collagens. Although they had the same T_m -CD with 36.5 °C, the helix to coil conversion of w-bone ASC occurred at lower temperature than s-bone ASC. The results were in accordance with that of Fig.3. Moreover, w-bone collagen also had a bi-phase thermal transition with the transition temperatures at 32.5 °C and 36 °C, respectively. Although it has often treated collagen melting as a single phase

transition, implying that a molecule is either in the triplehelical structure or is isolated as a single strand in an unordered conformation, in reality, one might expect the path from triple helix to single unordered strand to involve multiple steps. The derivatives of the melting curves clearly suggest a two-stage thermal transition for collagen in a dilute acetic acid solution^[10].

The melting curves of collagens from skins in winter and summer season were very similar with the T_m at 36 °C (Figure 6 not shown). No bi-phase thermal transitions were observed in the two samples, which might suggest the discrepancy in the structures of two samples were very small.

The collagen triple helix differs in thermal stability along its length^[13]. Consequently, thermal unfolding does not occur simultaneously along the whole molecule but starts in the most thermally labile regions, which are in a constant flux of hydrogen-bond breakage and formation. Initially there is a process of activation that involves the unfolding of a critical length of the chain called the thermally labile unit. Once the alpha chains in this unit are uncoupled and moving randomly under the influence of thermal agitation, the whole molecule becomes unstable and unzips^[2]. Therefore, for winter collagens, the position or the number of the thermally labile units as well as inter or intra crossing-links are different from the summer ones. Collagens from skin and bone of carp caught in winter may have more labile domains than the summer equivalents, which results in their less thermal stability.

3 Conclusions

The acid-soluble collagens (ASC) were isolated from skin and bone of carp (*Cyprinus carpio*) caught in the winter and summer. The results of pepsin digestion and CD measurement showed the seasonal differences in the thermal stability of collagens from skin and bone of carp. Collagens from skin and bone in the winter (w-skin ASC and w-bone ASC) exhibited transition curves with midpoints of 31.0 °C and 32.8 °C, respectively, while T_m s of collagens from skin and bone in the summer were 32.0 °C and 33.4 °C. Collagens from summer fish had higher stability than winter ones. The discrepancy may be attributed to the different living environmental conditions.

References:

- [1] SENARATNE L S, PARK P J, KIM S K. Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin[J].

- Bioresource Technology, 2006, 97: 191-197.
- [2] MILES C A, KNOTT L, SUMNER I G, et al. Differences between the thermal stabilities of the three triple-helical domains of type IX collagen[J]. Journal of Molecular Biology, 1998, 277: 135-144.
- [3] PRIVALOV P L. Stability of proteins: Proteins which do not present a single cooperative system[J]. Advances in Protein Chemistry, 1982, 35: 1-104.
- [4] NAGAI T, SUZUKI N. Isolation of collagen from fish waste material: skin, bone and fins[J]. Food Chemistry, 2000, 68: 277-281.
- [5] TRUEB B, SCHREIER T, BRUCKNER P, et al. Type VI collagen represents a major fraction of connective tissue collagen[J]. European Journal of Biochemistry, 1987, 166: 699-703.
- [6] GROSS J, LAPIERE C M. Collagenolytic activity in amphibian tissue: a tissue culture assay[J]. The Proceedings of the National Academy of Sciences Online, 1962, 48: 1014-1022.
- [7] SAKAI T, GROSS J. Some properties of the products of reaction of tadpole collagenase with collagen[J]. Biochemistry, 1967, 6(2): 518-528.
- [8] PIEZ K A, REDDI A H. Extracellular matrix biochemistry[M]. New York: Elsevier, 1984.
- [9] OGAWA M, MOODY M W, PORTIER R J, et al. Biochemical properties of black drum and sheepshead seabream skin collagen[J]. Journal of Agricultural and Food Chemistry, 2003, 51: 8088-8092.
- [10] BROWN E M, FARRELL H M, WILDERMUTH R J. Influence of neutral salts on the hydrothermal stability of acid-soluble collagen[J]. Journal of Protein Chemistry, 2000, 19(2): 85-92.
- [11] SATO K, EBIHARA T, ADACHI E, et al. Possible involvement of aminotelopeptide in self-assembly and thermal stability of collagen I as revealed by its removal with proteases[J]. Journal of Biological Chemistry, 2000, 275: 25870-25875.
- [12] OGAWA M, PORTIER R J, MOODY M W, et al. Biochemical properties of bone and scale collagen isolated from the subtropical fish black drum and sheepshead seabream[J]. Food Chemistry, 2004, 88: 495-501.
- [13] MILES C A, BURJANADZE T V, BAILEY A J. The kinetics of the thermal denaturation of collagen in unrestrained rat tail tendon determined by differential scanning calorimetry[J]. Journal of Molecular Biology, 1995, 245: 437-446.