Preparation of Collagen from the Pigskin Shavings by Alkali Extraction

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Abstract: Alkaline treatment method was used to extract collagen. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and circular dichroism (CD) spectra revealed that the alkali-soluble pigskin collagen (ASPC) retained the polypeptide chains and triple helix conformation. The amino acid profiles showed no major deviation from the characteristic collagen composition. Both of the denaturation temperature and the isoelectric point (pI) of ASPC were lower than those of pepsin-solubilized pigskin collagen (PSPC).

Key words: collagen; pigskin; shavings; alkali extraction

1 Introduction

In recent years in China, the treatment of pig delimed pelts with anhydrous sodium sulfate followed by shaving is a common process in tannery. However, this produces another kind of shavings containing a large amount of Na_2SO_4. As is known to all, the major composition of the shavings is collagen, which has many attractive properties such as biodegradability, [1] weak antigenicity[2] and biocompatibility.[3] Extraction of collagen from the shavings is not only a solution to the pollution of solid wastes, but also a novel approach to utilize hide collagen, the natural biomass.

In this study, the pigskin shavings were pretreated to remove most of Na_2SO_4 and fat, and then an attempt was made to extract native collagen by alkaline treatment, which might gain higher yield than do acetic acid treatment or pepsin treatment. [3] The physicochemical properties of the alkali-soluble pigskin collagen were characterized in detail.

2 Experimental

2.1 Materials

The pigskin shavings were procured from a local commercial tannery. Tris and methylenebisacrylamide were supplied by Life Technologies, Inc. (U.S.A.). TEMED was produced by Cibcobrl (Grand Island, N.Y., U.S.A.). Coomassie brilliant blue R-250 and Ammonium persulfate were provided by Bio-Rad Co. (U.S.A.).

2.2 Pretreatment of the Pigskin Shavings

The pigskin shavings were soaked in water with a ratio of 1:6 (w/w) for 30 min at the first time and for 20 min at the other two times to remove Na_2SO_4. All above procedures were performed at 30°C with continuous stirring.

Fat was removed firstly in distilled water containing 0.5% nonionic detergent for two periods of 6 hours each, and then in isopropyl alcohol solvent for 10 hours. Defatted pigskin shavings were thoroughly washed with distilled water and then frozen at -20°C prior to collagen extraction. A weighed dried sample was carbonized at 200°C for 3 hours and then cinerated at 600°C for 4 hours. After the ash was dissolved in 50 ml distilled water, the amount of Na_2SO_4 was measured with gravimetric analysis. [4]

2.3 Characterization

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2.3.1 Extraction of Alkali-soluble Pigskin Collagen (ASPC)

ASPC was extracted according to the methods of Shunji Hattori et al.[5] with some modification. The pigskin shavings were treated with a solution of 3.0% NaOH (w/v) and 1.9% monomethylamine (v/v) at 20°C for one week. ASPC was precipitated by adjusting the pH value to 4.6~4.7 with 5 M hydrochloric acid. The precipitate was collected by centrifugation at 10,000 rpm for 30 min and then dissolved in 0.5 M acetic acid, followed by dialyzing against 0.1 M acetic acid at 4°C for 72 hours.

2.3.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight distribution of ASPC was analyzed with SDS-PAGE, and pepsin-solubilized collagen from fresh pigskin (PSPC) was used as a control.

SDS-PAGE was performed by the method of Laemmli[6]. 1% SDS, 1% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue were added to ASPC and PSPC samples respectively, and each mixture was heated immediately at 100°C for three minutes and then analyzed by SDS-PAGE using 7.5% gel. The gel was stained with 0.25% Coomassie Brilliant Blue R-250 for 45 min and destained with 7.5% acetic acid/5% methanol solution.

2.3.3 Amino Acid Analysis

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

2.3.4 Circular Dichroism (CD)

The collagen concentration was adjusted to 0.5 mg/ml before CD analysis. The solution was scanned at the wavelength range from 190 to 250 nm at 25°C. The molar ellipticity [θ] was recorded using a circular dichroism apparatus (Jasco J-500C, Japan).

2.3.5 Denaturation Temperature

The denaturation temperature was measured from the viscosity changes using an Ubbelohde viscosimeter, according to the methods of Zhang et al.[7] with some modification. 15 ml of collagen solution (0.5 mg/ml) was incubated for 20 min at the given temperature from 24 to 46°C, and the efflux time (t) was recorded. 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: $F(T)=(\eta_{sp(T)}-\eta_{sp(46°C)})/(\eta_{(24°C)}-\eta_{(46°C)})$, where η_{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.

2.3.6 Isoelectric Point (pI)

Collagen solution (0.5 mg/ml) was titrated with 0.25 M NaOH and the Zeta potentials at the given pH from 2.5 to 8.5 were recorded by a Zeta potential titration apparatus (Malven Zetaweight Nano ZS, UK). The titration temperature was 25℃ and the increasing pH intervals were 0.5 pH. Zeta potentials were plotted against pH and the pI of ASPC was determined at the pH value where the Zeta potential was zero.

3 Results and discussion

3.1 Effect of Pretreatment on the Chemical Compositions of the Pigskin Shavings

The chemical compositions of the pigskin shavings before and after pretreatment are shown in Table 1. Protein was still the main component except water after pretreatment. 98% of all the Na_2SO_4 in the pigskin shavings were removed by repeated washing, since most of the Na_2SO_4 were filled up among the space of the collagen fibers, and there were few chemical bonds between Na_2SO_4 and collagen molecules.

Meanwhile, the ash content of the pigskin shavings declined sharply after pretreatment, and it was due to the removal of Na_2SO_4, the main constituent in the ash. In addition, the removal percentage of fat was 81%.

Tab. 1 Chemical compositions (%) of the pigskin shavings before and after pretreatment

Pigskin shavings	Moisture	Ash	Na_2SO_4	Protein	Fat
Before pretreatment	59	9.6	9.2	29.8	1.6
After pretreatment	65	0.5	0.2	34.2	0.3

3.2 SDS-PAGE Patterns

Figure.1 shows SDS-PAGE patterns of ASPC from the pigskin shavings, along with PSPC as a comparison. It was found that both of the two collagen samples displayed two α bands and one β band . These indicated that no degradation occurred and the polypeptide chains were retained during the alkaline treatment. However, the migration of α and β bands of ASPC was slightly slower than that of PSPC, which was in agree with the previous study,[8] it was probably due to the modification of some amino acid residues during the alkaline treatment.[5]

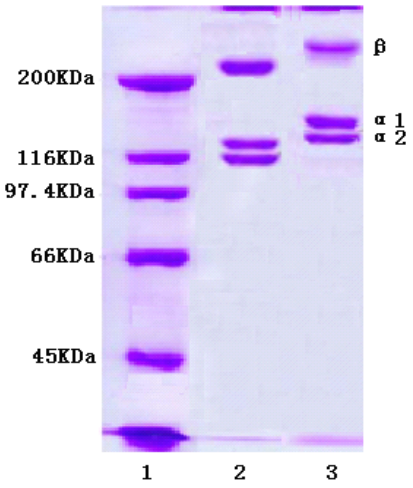

Fig.1 SDS-PAGE analysis of molecular weight standard (lane 1), PSPC from fresh pigskins (lane 2), ASPC from the pigskin shavings (lane 3) on 7.5% gel

3.3 Amino Acid Composition

Table 2 shows the amino acid composition of ASPC from the pigskin shavings. Glycine, as the major amino acid, accounted for about one third of all the amino acids. Meanwhile, ASPC had low amount of cystine, methionine, tyrosine, and histidine. Also, it consisted of proline, hydroxyproline and hydroxylysine, which are unique amino acids found in collagen. The imino acid (proline and hydroxyproline) content was 249 residues/1000 residues. These demonstrated ASPC possessed the characteristic amino acid composition of collagen. Some literatures [5, 9] have confirmed that all of the Asn and Gln residues were converted to Asp and Glu residues and some Arg residues were changed to ornithine residues as a result of the deamination during the alkaline treatment. However, we could not confirm the conversion of Asn and Gln to Asp and Glu and the existence of ornithine by present measurement techniques.

Tab. 2 Amino acid composition of ASPC from the pigskin shavings (Residues /1000 Residues)

Amino acid	ASPC

Asp	43
Thr	16
Ser	29
Glu	73
Gly	318
Ala	108
Cys	2
Met	5
Val	23
Ile	10
Leu	24
Tyr	2
Phe	13
Hyl	10
Lys	27
His	4
Arg	44
Hyp	87
Pro	162

3.4 Triple Helical Conformation

Collagen is a sort of optically active protein and adopts the polyproline II -like helical conformation[10] with a negative minimum absorption band around 190 nm and a weak positive maximum absorption band at 210-230 nm. CD spectrum of ASPC is shown in Figure 2. The collagen had a positive maximum peak at 218 nm and a negative minimum peak at 196 nm, which indicated the triple helical conformation and the helicity of the collagen molecule were maintained through the period of alkaline treatment.[11]

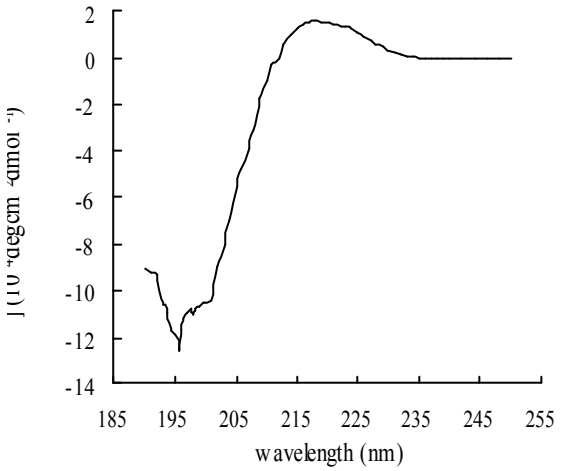

Fig.2 CD spectra of ASPC from the pigskin shavings

3.5 Thermal Stability

The heat transformation of collagen is interpreted as disintegration of the collagen triple helical structure into random coils. These are accompanied by a change in physical properties such as viscosity,

sedimentation, diffusion, light scattering and optical activity.[12] The curve of the fractional viscosities plotted against the temperatures is given in Figure 3. The denaturation temperature of ASPC from the pigskin shavings was approx. 34.5°C. Nagai *et al.*[13] reported the denaturation temperature of PSPC was 37°C, which was higher than that of ASPC, and this would be interpreted as follows, deamination of the acid amide groups of the Asn and Gln residues resulted in the appearance of more negative carboxyl groups and stronger electronstatic repulsion, therefore, the collagen helices might be less stable and disintegrate at a lower temperature.

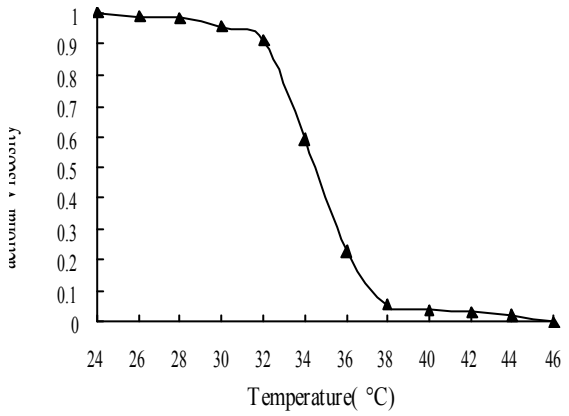

Fig. 3 Thermal denaturation curve of ASPC from the pigskin shavings

3.6 Isoelectric Point (pI)

The curve of Zeta potential plotted against the pH is given in Figure 4, the pI of ASPC from the pigskin shavings was 4.68, which was lower than that of PSPC (5.02).[14] The fall of the pI was attributed to the decrease of some basic amino acids (Arg residues) and the increase of carboxyl groups as a result of deamination of the acid amide groups of the Asn and Gln residues[9, 15]

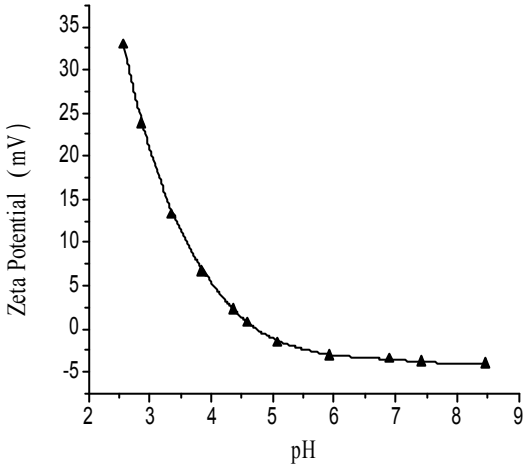

Fig.4 Isoelectric point titration graph of ASPC from the pigskin shavings

4 Conclusions

Most of the Na_2SO_4 and fat were firstly removed, and then the collagen was extracted from the pretreated shavings with alkaline treatment method. The alkali-soluble pigskin collagen (ASPC) retained

polypeptide chains and triple helical conformation. The amino acid analysis showed no major deviation from the characteristic collagen composition. The denaturation temperature and the isoelectric point (pI) of ASPC were 34.5°C and 4.68 respectively. The extraction of ASPC from the pigskin shavings might be a high value route for the utilization of leather wastes.

Acknowledgements

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