Preparation and Characterization of Alkali-Soluble Collagen from Pigskin Shavings

by

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Abstract

The leather industry discharges large quantities of solid wastes containing high-value native collagen. In this study, the pigskin shavings, generated from the shaving of pelts which had been dehydrated with anhydrous sodium sulfate after deliming, were pretreated to remove most of salt and fat. Then alkaline treatment method was used to extract collagen from the pigskin shavings. 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). Scanning electron microscopy showed that ASPC sponges had porous fibrillar network structures, and the pore sizes became larger with the decrease of collagen concentrations and as the pH of the collagen solution approached the pI of ASPC.

Resumen

La industria del cuero descarga grandes cantidades de residuos sólidos que contienen colágeno nativo de alto valor. En este estudio, las virutas de piel de cerdo generadas en el rebajado de pieles que habían sido deshidratadas con sulfato de sodio anhidro luego del desencalado, fueron pretratadas para eliminar la mayor parte de la sal y grasa. A continuación, un método de tratamiento alcalino fue utilizado para extraer colágeno de las virutas de la piel de cerdo. Tanto la electroforesis por Dodecil-sulfato sódico en gel de poliacrilamida (SDS-PAGE), cómo el dicroísmo circular (CD) revelaron que los espectros del colágeno alcalino soluble de las pieles de cerdo (ASPC) retuvo la cadena polipeptídica y la conformación de la triple hélice. El perfil de aminoácidos no mostró grandes desviaciones de la composición característica del colágeno. Tanto de la temperatura de desnaturalización y el punto isoeléctrico (pI) del ASPC son inferiores al colágeno soluble obtenido por [la acción de] pepsina en piel de cerdo (PSPC). Microscopía electrónica de barrido mostró que las "esponjas" de ASPC tienen una estructura reticular de fibras porosas, y el tamaño de la porosidad fue mayor con la disminución de las concentraciones de colágeno y cuando el pH de la solución de colágeno se acercó al pI del ASPC.

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INTRODUCTION

Substantial quantities of solid wastes are produced by the leather industry every year, which causes serious environmental contamination and ecological destruction. Some data have shown that 1 metric ton of wet salted hides yield only 200 kg of available leather but over 600 kg of solid wastes.[1] Most of the solid wastes are generated from mechanical processes such as trimming, fleshing, splitting, shaving, buffing etc., and 82% of these solid wastes are from wet processes before tanning.[2] In order to eliminate the pollution, many attempts have been made to utilize these solid wastes in the areas such as glue, gelatin, animal feed, collagen hydrolysate, fertilizer and so on.[3]

In recent years in China, the treatment of pig delimed pelts with anhydrous sodium sulfate followed by shaving is a common process in tannery, which can eliminate the variance in thickness of pigskins and finally produce the garment leather with high quality. However, this produces 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,[4] weak antigenicity[5] and biocompatibility.[6] 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.

Because the high content of Na_2SO_4 would block collagen extraction, the pigskin shavings are not suitable for collagen extraction directly. 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 does acetic acid treatment or pepsin treatment.[6] The physicochemical properties of the alkali-soluble pigskin collagen were characterized in detail.

EXPERIMENTAL

Materials

The pigskin shavings, generated from the shaving of the pelts which had been treated with anhydrous sodium sulfate after deliming, 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.).

Pretreatment of the pigskin shavings

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

Moisture, ash, fat and protein content of the pigskin shavings before and after pretreatment were determined according to the methods of AOAC.[7] Na_2SO_4 content was determined with the following method. A weighed dried sample was carbonized at 200°C for 3 hours and then incinerated at 600°C for 4 hours. After the ash was dissolved in 50 ml distilled water, the amount of Na_2SO_4 was measured by gravimetric analysis.[8]

Extraction of alkali-soluble pigskin collagen (ASPC)

ASPC was extracted according to the methods of Shunji Hattori et al.[9] 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.

Determination of the collagen yield

The collagen yield was determined indirectly from the hydroxyproline content, which was analyzed by the method of Bergman and Loxley.[10] The collagen sample was hydrolyzed in 6 M HCl at 110°C for 24 hours, and after HCl had been evaporated, the hydrolysate was dissolved in acetate/citrate buffer (pH6.8) for the assay, and the collagen content was calculated by multiplying the hydroxyproline content by 9.64.

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. PSPC was prepared by the following method. Briefly, a small piece of fresh pigskin was chosen, and the hair was scraped off with a knife, then the pigskin was cut into small pieces. After the fat was removed by nonionic detergent and isopropyl alcohol, the cleaned pigskin was suspended in 0.5 M acetic acid containing 1% pepsin, followed by centrifugation and salting-out. The precipitate was dissolved in 0.5 M acetic acid and dialyzed against 0.1 M acetic acid.

SDS-PAGE was performed by the method of Laemmli[11]. 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 until the bands were clear.

Amino acid analysis

The lyophilized collagen sample was hydrolyzed in 6 M HCl at 110°C for 24 hours. The HCl was evaporated 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).

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).

Denaturation temperature determined by viscosity method

The denaturation temperature was measured from the viscosity changes using an Ubbelohde viscometer, according to the methods of Zhang et al.[12] 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 H_{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.

Isoelectric point (pI) of ASPC

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°C 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.

Preparation of ASPC sponges with different concentrations

10 mg/ml collagen samples (pH2.5) were diluted with 0.5 M acetic acid to different concentrations: 2.5 mg/ml, 3.75 mg/ml, 5 mg/ml, 7.5 mg/ml. Each solution was frozen at -20°C for 24 hours and lyophilized in a freeze dryer (Labconco Freeze Dryer FreeZone 6 Liter, USA) at -40°C for 48 hours.

Preparation of ASPC sponges with different pH

5 mg/ml collagen samples (pH2.5) were dialyzed against different buffers respectively: 0.1 M acetic acid (pH3.0), 0.05 M acetic acid (pH3.5), 0.05 M Tris-HCl buffers with different pH (7.2, 8.1, 9.0). All the dialysis were performed at 4°C for 72 hours, then each solution was freeze-dried under the same conditions stated above.

Morphologies of ASPC sponges

The morphologies of different ASPC sponges were observed by a scanning electron microscopy (S-800, HITACHI, Tokyo, Japan). Specimens were coated with gold-palladium in an ion sputter (E1010, HITACHI, Tokyo, Japan). The average pore size of each sponge was analyzed by the manual measurement of the image analyzer program and at least 20 pores were assessed.

RESULTS AND DISCUSSIONS

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 I. Protein was still the main component except for water after pretreatment. 98% of all the Na_2SO_4 in the pigskin shavings was removed by repeated washing, since most of the Na_2SO_4 filled up the space between 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%.

TABLE I

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

Extraction of alkali-soluble pigskin collagen (ASPC)

ASPC was easily extracted from the pigskin shavings by alkaline treatment and the yield was very high, reaching 85%. Meanwhile, almost all the proteins other than collagen were hydrolyzed,[9] which simplified the collagen purification procedure. These indicated that alkaline treatment method was a powerful way to extract collagen. It has been reported in the literature[9] that alkaline treatment can not only remove the telopeptides of the collagen molecules, but also break off additional crosslinking in the triple helical regions without damaging the integrity of the triple helix.

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 (100 Kda for α1 and α2) and one β band (200 Kda). 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 agreed with the previous study,[13] and it was probably due to the modification of some amino acid residues during the alkaline treatment.[9]

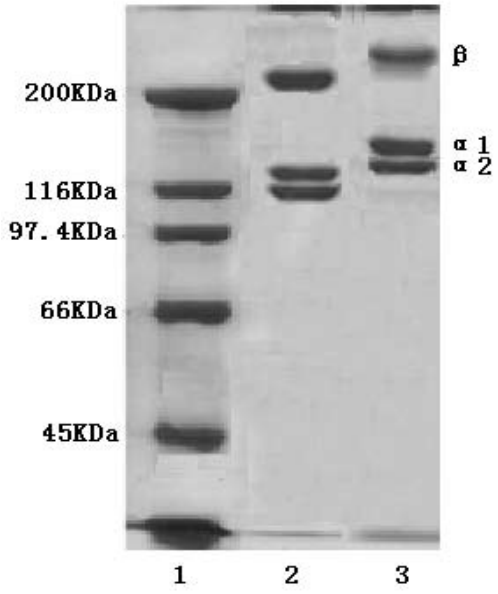

Figure 1. – SDS-PAGE analysis of molecular weight standard (lane 1), PSPC from fresh pigskins (lane 2), ASPC from the pigskin shavings (lane 3) on 7.5% gel.

Amino acid composition

Table **II** 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 researchers [9, 14] 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.

TABLE II

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

Triple helical conformation

Collagen is an optically active protein and adopts the polyproline **II** –like helical conformation[15] with a negative minimum absorption band around 190 nm and a weak positive maximum absorption band at 210-230 nm. The CD spectrum of ASPC from the pigskin shavings 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.[16]

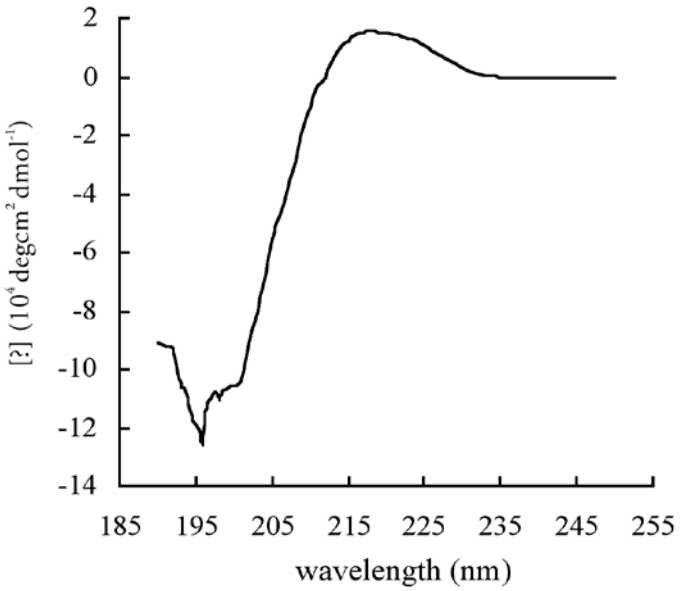

Figure 2. – CD spectra of ASPC from the pigskin shavings.

Viscosity and thermal stability

The heat transformation of collagen is interpreted as disintegration of the collagen triple helical structure into random coils. The helix-coil transition of collagen involves the disintegration of the trimers (γ) of collagen molecules into individual chains (α) or dimmers (β) and the breakage of hydrogen bonds between the adjacent polypeptide chains. These are accompanied by a change in physical properties such as viscosity, sedimentation, diffusion, light scattering and optical activity.[17] Viscosity changes are widely used to determine the denaturation temperature of collagen. The curve of the fractional viscosities plotted against the temperatures is given in Figure 3. The viscosity of the collagen solution began to decrease at about 28°C and reached a platform at about 38°C, and the denaturation temperature of ASPC from the pigskin shavings was approx. 34.5°C. Nagai *et al.*[18] 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 lower temperature.

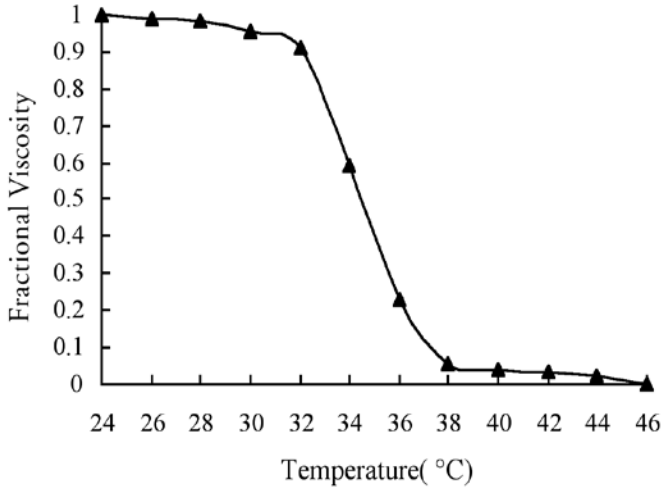

Figure 3. – Thermal denaturation curve of ASPC from the pigskin shavings.

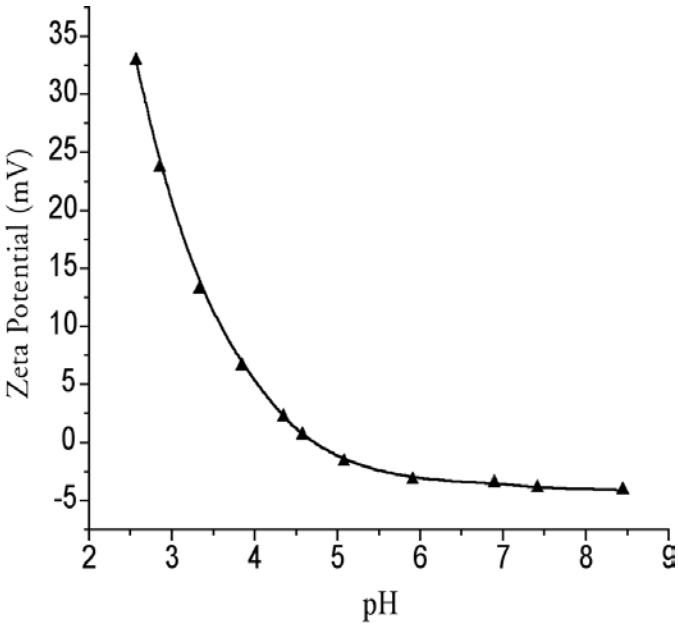

Figure 4. – Isoelectric point titration graph of ASPC from the pigskin shavings.

Isoelectric point (pI)

pI is an important parameter of protein, which is related to the proportion of acidic amino acid residues and basic amino acid residues in protein. The curve of Zeta potential plotted against the pH is given in Figure 4, and the pI of ASPC from the pigskin shavings was 4.68, which was lower than that of PSPC (5.02).[19] 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.[14, 20]

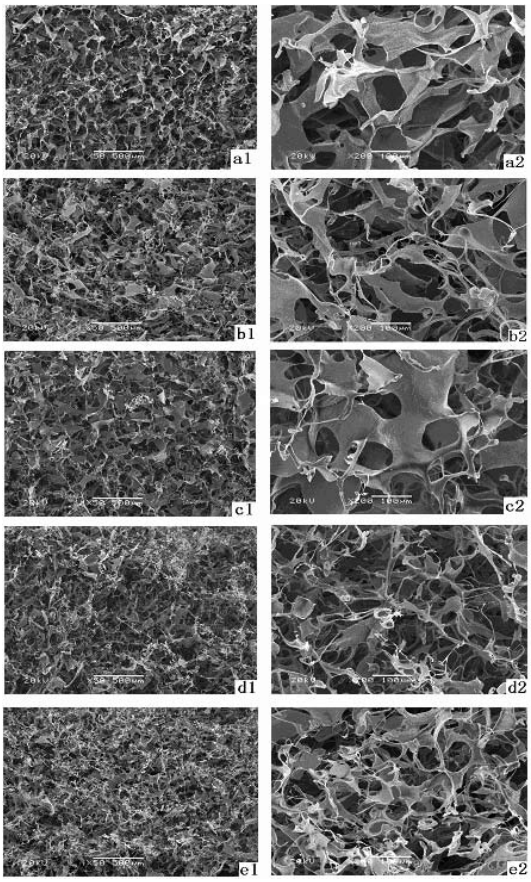

Figure 5. – SEM images of ASPC sponges with various concentrations at a pH of 2.5. 2.5 mg/ml (a1, a2), 3.75 mg/ml (b1, b2), 5 mg/ml (c1, c2), 7.5 mg/ml (d1, d2), 10 mg/ml (e1, e2), at the magnification of x50 (a1,b1,c1,d1,e1,bars:500 μm) and x200 (a2,b2,c2,d2,e2, bars:100 μm).

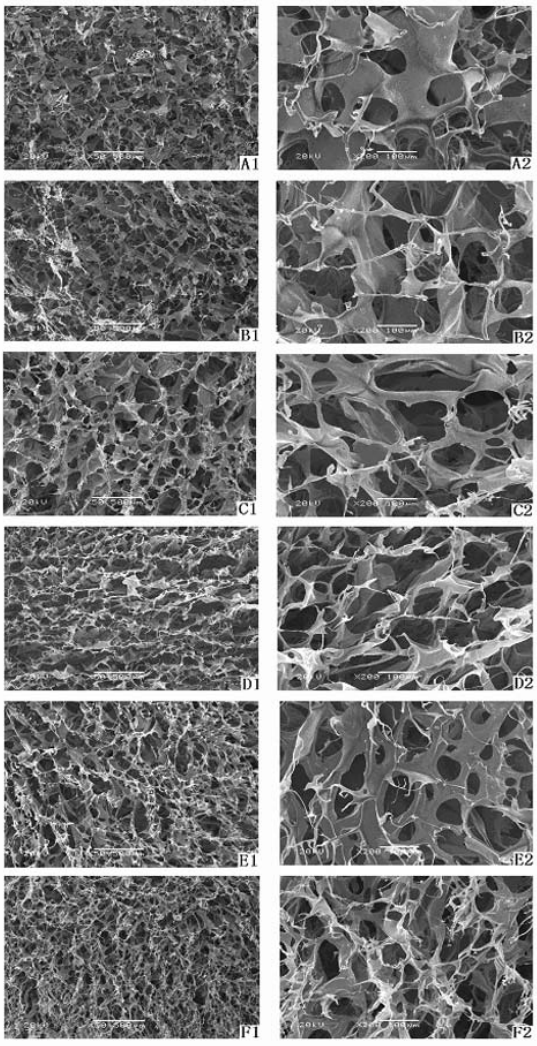

Figure 6. – SEM images of ASPC sponges with different pH at a concentration of 5 mg/ml. pH: 2.5 (A1, A2), pH: 3.0 (B1, B2), pH: 3.5 (C1, C2), pH: 7.2 (D1, D2), pH: 8.1 (E1, E2), pH: 9.0 (F1, F2), at the magnification of x50 (A1,B1,C1,D1,E1,F1,bars:500 μm) and x200 (A2,B2,C2,D2,E2,F2,bars:100 μm)

TABLE III

Pore Sizes of ASPC Sponges with the Variation of Collagen Concentrations at a pH of 2.5 and with Different pH of Collagen Solutions at a Concentration of 5 mg/ml

	Concentration of the collagen solution (mg/ml)					pH of the collagen solution					
	2.5	3.75	5	7.5	10	2.5	3.0	3.5	7.2	8.1	9.0
Pore size (μm)	110	100	80	60	50	80	140	160	150	75	60

Morphologies of ASPC sponges

All the ASPC sponges had regular network structures and consisted of a large amount of pores, channels, fibers and sheet-like structure. The upper surface of the sponges in direct contact with the atmosphere during the freeze-drying process were found to collapse in almost all cases, which was pointed out in previous study.[21] For this reason, in this study, we concentrated on the pan side of the sponges, which were in direct contact with the petri dish and observed to exhibit a variation of structures defined below.

Effect of the concentration of collagen solution on the microstructures of ASPC sponges

The SEM images of ASPC sponges with varying concentrations at a pH of 2.5 are shown in Figure 5 and their average pore sizes are given in Table III. In association with the increase of collagen concentration from 2.5 mg/ml to 10 mg/ml, average pore sizes of ASPC sponges decreased from 110 μm to 50 μm, dropping by 55%. Water in the collagen solution is composed of water bound to the collagen molecules and free water. Free water is subject to ice crystallization, whereas bound water is normally more mobile and has lower tendency to crystallization.[21] The dense collagen solution has less free water than that of diluted collagen solution at the same pH value, therefore, increased concentration resulted in smaller pore sizes.

Effect of the pH of collagen solution on the microstructures of ASPC sponges

The SEM images of ASPC sponges with varying pH at a concentration of 5 mg/ml are shown in Figure 6 and their average pore sizes are given in Table III. With the increase of pH from 2.5 to 3.5, average pore sizes increased from 80 μm to 160 μm. When the pH fell from 9.0 to 7.2, average pore sizes increased from 60 μm to 150 μm. This indicated that the pores of the collagen sponges would become larger when the pH value of collagen solution was close to the pI of ASPC. As is known to all, collagen is a kind of ampholyte, and there are quantities of acidic amino acid residues and basic amino acid residues on the polypeptide chains. Collagen may hold different charges at different pH. When the pH of the collagen solution approached to the pI gradually, the water bonded to the collagen molecules became free water along with the decrease of the charges and increased amount of free water resulted in larger pores of the collagen sponges.

CONCLUSIONS

In order to recover high-value native collagen from the pigskin shavings, 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 lyophilized ASPC sponges had regular network structures and the average pore sizes became larger with the decrease of collagen concentrations and the approach of the pH to the pI of ASPC. Therefore, extraction of ASPC from the pigskin shavings might be a high value route for the utilization of leather wastes.

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