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Effects of alkaline and acid pretreatment on the physical properties and nanostructures of the gelatin from channel catfish skins

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Abstract

The objective of this study was to illustrate the correlation between the physical properties and nanostructure of gelatins made of channel catfish (*Ictalurus punctatus*) skins. The gelatin samples were first pretreated with sodium hydroxide, acetic acid, or water, and then extracted with hot water before the measurement. Physical properties including the yield of protein, viscosity and textural properties were determined on gelatins obtained with different pretreatment conditions. The acid pretreatment group showed the highest gel strength and protein yield, and a reasonable viscosity. The water pretreatment group showed the lowest values for all of the physical properties. Four samples including water, 0.1 M acid and 0.25 and 1.0 M alkaline-pretreated groups' nanostructures were then studied using atomic force microscopy (AFM). The AFM images showed that the acid-pretreated gelatin was composed of sponge-like aggregates, while the others showed separated individual aggregates. Annular pores were only found in the alkaline pretreatment group. There was no significant correlation between the diameters of the spherical aggregates and the physical properties; however, the different AFM patterns may relate to the gelatin's physical properties.

Keywords: Nanostructure; Atomic force microscopy; Viscosity; Gel strength; Gelatin; Channel catfish; Nanotechnology

1. Introduction

Gelatin is a soluble polypeptide derived from collagen. It is derived from the breakdown of the cross-linkages between polypeptide chains of the collagen along with some amount of breakage of polypeptide chain bonds. When collagen is treated with alkali or acid and followed by or accompanied with heat, the fibrous structure of collagen is broken down irreversibly yielding gelatin (Zhou & Regenstein, 2004, 2005). Gelatin is now one of the most widely used ingredients in the pharmaceutical and food industries.

The gelatin industry primarily uses mammalian skins and bones as raw materials. Recently, to better use by-products from fish processing, to meet sociocultural needs and for other reasons (Montero & Gómez-Guillén, 2000), fish skins are promising alternative materials for gelatin extraction (Yang, Wang, Jiang et al., 2007). The effects of the extraction conditions on gelatin yield and the corresponding physical properties have been reported for the skins of many fish species, including sole (Devictor, Allard, Perrier, & Huc, 1995), cod (Gudmundsson & Hafsteinsson, 1997), blue shark (Yoshimura, Terashima, Hozan, & Shirai, 2000), megrim (Montero & Gómez-Guillén, 2000), tilapia (Choi & Regenstein, 2000; Jamilah & Harvinder, 2002), yellowfin tuna (Cho, Gu, & Kim, 2005), Alaska pollock (Zhou, Mulvaney, & Regenstein, 2006; Zhou & Regenstein, 2004, 2005), horse mackerel (Badii & Howell, 2006) and skate (Cho, Jahncke, Chin, & Eun, 2006).

Catfish is common farm-raised, warm-water fish supplying large amounts of fish skins year-round. The gels from catfish are relatively thermally non-degradable and show

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good gelling ability (Gómez-Guillén et al., 2002); however, gelatin gels have a complex behavior such as both concentration- and aging-time-dependent (Uricanu, Duits, Nelissen, Bennink, & Mellema, 2003). Until now, gelatin from the skins of catfish has not been systematically studied as a raw material for edible gelatin.

Determining structural information is very important for illustrating and improving the properties of fish skin gelatin. Recently, nanotechnology is receiving much attention in the agricultural and food science. For example, Atomic force microscopy (AFM) has been one of the most powerful instruments for the characterization of nanostructures of food-related materials (Yang, An, & Li, 2006; Yang, Lai, An, & Li, 2006; Yang, Wang, Lai et al., 2007). It has been applied to some biologically purified gelatins and hybrid gels (Benmouna & Johannsmann, 2004; Haugstad & Gladfelter, 1993, 1994; Lin et al., 2002; Mackie, Gunning, Ridout, & Morris, 1998; Mohanty & Bohidar, 2005; Radmacher, Fritz, & Hansma, 1995; Saxena, Sachin, Bohidar, & Verma, 2005; Uricanu et al., 2003; Yang, Wang, Regenstein, & Rouse, 2007; Yao, Liu, Lin, & Qiu, 1999).

The purpose of this research was to investigate the effects of alkaline and acid pretreatment on the physical properties and nanostructures of the gelatin extracted. AFM was used to morphologically assess the gelatin nanoparticles. The possible relationship between the nanostructure and physical properties was discussed. The results will be shown to clarify the structure and physical properties of gelatin from fish skin and can be used to direct future gelatin production.

2. Materials and methods

2.1. Gelatin extraction

2.1.1. Preparation of materials

Frozen catfish skins were provided by the Harvest Select Inc. (Uniontown, AL, USA) plant. All chemical reagents used were analytical grade. Cleaning of the fish skins used the protocol in our previous report (Yang, Wang, Jiang et al., 2007) with a maximum of 2 months frozen storage of the catfish skins. These were thawed at 4 °C for about 20 h, then cut into pieces both in length and width of 2–3 cm, and washed with tap water (1:6 w/v) at 4 °C for 10 min. Washing was repeated two more times. The fish skins were then drained, using four layers of cheesecloth for 5 min and the cheesecloth containing the skins was squeezed by hand to remove liquid. This hand squeezing was done for all cheesecloth filtrations.

2.1.2. Pretreatment on the channel catfish skin

Based on preliminary experiments, cleaned skins (ca. 30 g) were added to a flask and treated with different concentrations and times of NaOH (1:6 w/v) (0–1 M for 0–90 min), acetic acid (1:6 w/v) (0.1 M for 0–180 min) or water (50 min). Then, the samples were drained using cheesecloth and rinsed with tap water. The above procedure was repeated two times. The samples were then drained using cheesecloth and rinsed with tap water (1:6 w/v) three times. All of the solutions used in the above steps were kept at 4 °C.

2.1.3. Gelatin extraction

After the above pretreatment, the washed skin samples were put into flasks, and ion-free water (D4641 4 module Barnstead E-pure water system, Van Nuys, CA, USA) was added. Then Parafilm (Structure Probe, Inc/SPT Supplies, West Chester, PA, USA) and aluminum foil were used to cover the flasks and the flasks were heated at 50 °C in a water bath (Model 86, Precision Scientific Co., Chicago, IL, USA) for 3 h. Finally, the gelatin solutions were filtered through four layers of cheesecloth prior to further work.

2.2. Determination of physical properties of gelatin

2.2.1. Yield of protein

The soluble protein concentration of the extracted solutions was determined by the Biuret method (Yang, Wang, Jiang et al., 2007) using a spectrophotometer at 540 nm (Milton–Roy Spectonic 20D +, Spectronic Instrument Inc., Rochester, NY, USA) with bovine serum albumin (BSA, standard grade, Equitech-Bio Inc., Kerrville, TX, USA) as a standard. The concentration of BSA was increased from 5 to 10 mg/mL to obtain a better linear relation for the standard curve.

The yield of protein (YP) was calculated using the following equation:

$$\mathrm{YP}\ (\%) = (\text{protein concentration[g/mL]} \times \text{volume of extract [mL]/weight of sample} \times \text{(wet skins after pretreatment) [g]}) \times 100\%.$$

2.2.2. Viscosity

Distilled water was used to adjust the concentration of the extracted gelatin solutions to 3.3% protein (BSA equivalent) and then a Cannon–Fenske routine viscometer (Cannon Instrument Co., State College, PA, USA) was used to determine the viscosity (V, cP) of the gelatin in a 60 °C water bath (Yang, Wang, Jiang et al., 2007). The efflux time was recorded using a stopwatch. The density of the 3.3% gelatin was 1.020 g/ml. Thus the viscosity can be calculated from the equation below. Viscosity (cP) = Efflux time (s) × Viscometer constant (cSt/s) × Density of the measured solution (g/mL) = Efflux time (s) × 0.01430 × 1.020.

2.2.3. Gel strength

Distilled water was used to dilute the extracted solutions to a protein concentration of 3.3% (the solution was used as is when the concentration was below 3.3%). The solutions were heated in a 50 °C water bath for 30 min and then added to plastic bottles (Wheaton Industries Inc.,

Millville, NJ, USA), which are cylindrical-shaped, and flat-bottomed with an average 31-mm internal diameter × 25-mm height, with the largest diameter being 33 mm and the smallest 29 mm. After being matured at 4 °C for 17 ± 1 h, the gel strength was determined using a TA.XTPlus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA), using a 12.5 mm diameter flat plastic plunger pressing 4 mm into the gelatin gel with a 5 kg load cell at a speed of 1 mm/s at 4 °C (Yang, Wang, Jiang et al., 2007).

2.2.4. Texture profile analysis

The gelatin samples for texture profile analysis (TPA) were prepared like those used for gel strength. After being matured at 4 °C for 17 ± 1 h, the samples were removed from the bottle as one whole piece using a thin bladed knife, and TPA tests were performed with the TA. XTPlus Texture Analyzer using a 75-mm-diameter aluminum plate.

Based on preliminary experiments and another report (DeMars & Ziegler, 2001), reasonable results were obtained using a 20% compression for the TPA with the detailed test settings being: Pre-test speed: 1.0 mm/s; Test speed: 0.5 mm/s; Post-test speed: 0.5 mm/s; Target mode: Distance; Distance of compression: 5.0 mm (the height of the gel is 25 mm); Time: 10.0 s; Trigger type: Auto (Force); Trigger Force: 0.05 N; Tare Mode: Auto; and Advanced Options: On.

The samples were lubricated on their top and bottom with mineral oil (Paraffin oil, Matteson Coleman & Bell Manufacturing Chemists, Norwood, OH, USA) just prior to measurement, and the testing was done immediately after each sample was removed from the 4 °C refrigerator. Hardness, cohesiveness, springiness and chewiness were calculated from the TPA curve as shown in Yang, Wang, Jiang et al. (2007).

2.3. AFM determination of the nanostructure of the gelatin

2.3.1. AFM determination

The solution of fish gelatin was taken from the refrigeration and allowed to equilibrate to room temperature (approximately 120 min), then disrupted using a Vortex mixer (Fisher Scientific, Pittsburgh, PA, USA) to disaggregate conglomerates resulting from the lower temperature during storage and to obtain a homogeneous mixture. The solution was then diluted to about 10–40 μg/ml and a small volume (about 20 μl) was pipetted rapidly onto a piece of freshly cleaved mica sheets (ca. $1.0 \times 1.0\ cm^2$) (Muscovite Mica, Electron Microscopy Sciences, Hatfield, PA, USA). The mica surface was then air dried. Each sample was freshly prepared just before AFM imaging to minimize possible contamination of the sample. The concentration of gelatin solution can be adjusted to obtain the best images (Yang, Wang, Regenstein et al., 2007).

A Nano-R2^{TM} AFM (Pacific Nanotechnology, Inc., Santa Clara, CA, USA) was used to determine the nanostructure of gelatin in non-contact mode. The non-contact mode in this AFM is similar to the commonly called tapping mode found with other AFM equipments. The term "tapping mode" was registered by another company. However, in this paper the term "tapping mode" is used to describe this format as this is the term commonly used in the literature (Yang, Wang, Regenstein et al., 2007). The NSC 11/no Al (MikroMasch, Wilsonville, OR, USA) tip with a resonance frequency of 330 KHz and a scan speed range of 0.5–2 Hz was used. Detailed experimental information was reported by Yang, Wang, Regenstein et al. (2007).

2.3.2. AFM image analysis

The AFM images were analyzed offline with NanoRule software (NanoRule+^{TM} 2.0 user's manual, 2004). The bright and dark areas in the images corresponded to peaks and troughs of the gelatin molecules and gel polymers on the mica surface. Different scales have been used for the vertical and horizontal coordinates. The AFM images of gelatin were modified through flattening for reducing the electronic noise and obtaining better quality (Yang, Wang, Lai et al., 2007). Height, error signals and phase modes were used to generate the AFM images shown in this paper. The height mode includes both three-dimensional and two-dimensional images. The error signal mode images were useful because they removed slow variations in the surface topography so as to highlight edge features (Yang, Wang, Regenstein et al., 2007). Phase mode images were obtained by measuring the phase difference between the oscillations of the cantilever driving piezo and the detected oscillations. Therefore, image contrast was derived from such image properties as stiffness and viscoelasticity. The characteristic dimensions of the samples were calculated from the AFM images (Yang, Wang, Lai et al., 2007).

2.4. Statistical analysis

All of the physical property experiments other than the AFM experiments were conducted in triplicate and the average results were reported. Dozens of parallel samples for each extraction condition were examined by AFM imaging to obtain reliable, representative and statistically valid results. ANOVA ($P<0.05$) and Duncan's multiple range test were applied to determine differences in the diameters of the nanoscale structure of gelatin particles using SAS (Version 9.1.3, Statistical Analysis Systems, Cary, NC, USA). The data are reported as mean ± standard deviation. Comparisons that yielded P values <0.05 were considered significant.

3. Results and discussion

3.1. Effects of different pretreatments on viscosity, yield of protein and textural properties of extracted gelatin

Pretreatment is an important step in preparing collagen for a successful gelatin extraction. The degree of conversion of collagen into gelatin is related to both the

pretreatment and the extraction processes, in which, pH, temperature, and time are three major factors for both processes (Montero & Gómez-Guillén, 2000; Zhou & Regenstein, 2004). In this research, only pH and treatment time during the pretreatment step were studied.

Physical properties of gelatins are influenced more by extraction conditions than by imino acid composition (Montero & Gómez-Guillén, 2000). The most important physical properties of gelatin are gel strength and viscosity (Wainewright, 1977). Commercially, gelatin with high viscosity and gel strength are preferred and are most expensive (Badii & Howell, 2006), while a reasonable yield of protein is necessary for efficiency of commercial production and economic viability.

The effects of acid pretreatment time, alkaline pretreatment time and alkaline concentration on the yield of protein and viscosity are shown in Figs. 1–3, respectively. The values for the water pretreatment group (50 min) can be obtained from Fig. 3 when the concentration of NaOH is zero. Generally, both the acid and alkaline pretreatments resulted in higher yields of protein. This result is slightly different from Alaskan pollock, where high yields can only be obtained using neutral and acid pretreatments (Zhou & Regenstein, 2005). The yield of protein increased with alkaline concentrations in the range of 0–1 M. However, for pretreatment time, the rate for protein yield decreased

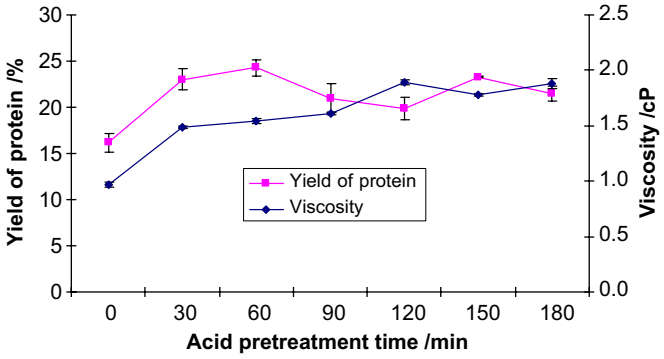

Fig. 1. Effect of acid pretreatment time on the yield of protein and on viscosity. *Note*: The acetic acid concentration for the pretreatment was 0.1 M and the water extraction was for 3 h in a 50 °C water bath.

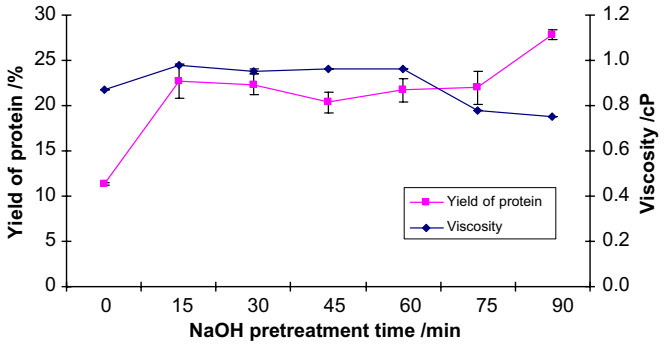

Fig. 2. Effect of alkaline pretreatment time on the yield of protein and on viscosity. *Note*: The NaOH concentration for the pretreatment was 0.3 M and the water extraction was for 3 h in a 50 °C water bath.

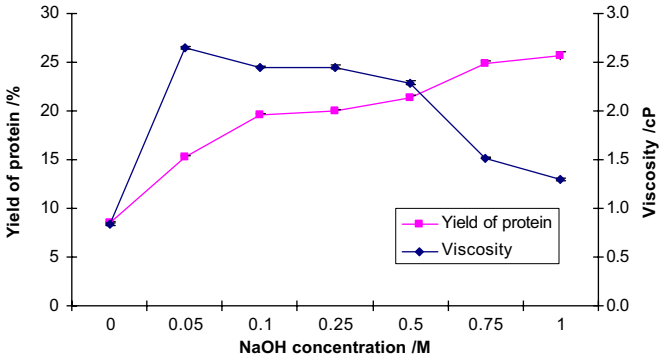

Fig. 3. Effect of alkaline concentration on the yield of protein and on viscosity. *Note*: The pretreatment time with NaOH was 50 min and the water extraction was for 3 h in a 50 °C water bath.

rapidly after 15 and 60 min for 0.3 M sodium hydroxide and 0.1 M acetic acid, respectively. Both the acid and alkaline results can be explained by a conflict between two effects: First, the acid or alkali facilitated the fragmentation of the collagen chain, but second, any collagen that dissolved in the pretreatment solution resulted in a corresponding loss in gelatin yield (Yoshimura et al., 2000). This can be seen in Figs. 1 and 2. Comparing Figs. 1 and 2, it is seen that with acetic acid solubilization is faster. In each case, time zero, is the time immediately after the alkaline or acid solution was added to the cleaned fish skins. However, the yields were different (16.2% for acetic acid and 11.3% for alkaline) in the pretreatment groups because of a several minute delay in stopping the reactions.

Gel strength analysis and TPA provide two different texture measurements. Gel strength is a major physical property of gelatin gels, and the commercial value of gelatin is mainly based on its bloom value (Zhou et al., 2006). TPA is useful for gel texture analysis because the textural parameters obtained from the TPA curves have been well correlated with sensory evaluation of textural parameters and it provides more information than traditional gel strength measurements (Lau, Tang, & Paulson, 2000). TPA tests are done to simulate the action exerted on the gel by the tongue and teeth. The force/time curves for the two cycles resemble isosceles triangles, similar to the curves obtained from gel strength tests (Yang, Wang, Jiang et al., 2007). Generally, the higher the gel strength and TPA values (for hardness, cohesiveness, springiness, and chewiness), the better the gelatin qualities (Yang, Wang, Jiang et al., 2007).

Tables 1 and 2 show the effects of acid pretreatment time and alkaline concentration on the textural properties of extracted gelatin, respectively. The water pretreatment group can be observed as treatment no. 1 in Table 2. The results indicated that the gel strength with water extraction (4.6 g) was lower than those for the acid or alkaline extractions. Gel strength reached its maximum with a reasonable alkaline or acid concentration and intermediate times were used. When concentration and time were either increased or decreased from the optimum, the gel strength

Table 1
Effect of acid pretreatment time on the textural properties of gelatin

No.	Gel strength (g)	Hardness (g)	Cohesiveness	Springiness	Chewiness (g)
1	27.3 ± 6.4^{e}	–	–	–	–
2	178 ± 10^{d}	202 ± 8^{a}	0.95 ± 0.01^{b}	1.00 ± 0.01^{a}	191 ± 6^{a}
3	167 ± 3^{d}	171 ± 14^{b}	0.94 ± 0.03^{b}	0.99 ± 0.03^{a}	159 ± 22^{b}
4	217 ± 8^{a}	202 ± 4^{a}	0.96 ± 0.00^{ab}	1.00 ± 0.01^{a}	194 ± 3^{a}
5	203 ± 6^{ab}	204 ± 5^{a}	0.95 ± 0.01^{b}	0.98 ± 0.02^{a}	189 ± 1^{a}
6	184 ± 6^{cd}	182 ± 7^{b}	0.97 ± 0.00^{a}	1.00 ± 0.01^{a}	177 ± 6^{ab}
7	199 ± 4^{bc}	197 ± 8^{a}	0.96 ± 0.01^{ab}	0.99 ± 0.01^{a}	187 ± 10^{a}

Note: The TPA test was a 20% compression test. The gelatin from no. 1 did not form a gel so the TPA was not determined. Nos. 1–7: the times of 0.1 M acetic acid pretreatments were 0, 30, 60, 90, 120, 150 and 180 min, respectively. The acetic acid concentration for the pretreatment was 0.1 M and the water extraction was for 3 h in a 50 °C water bath.
Different letters in the same column indicate significant ($P<0.05$) differences among different pretreatment times.

Table 2
Effect of alkaline concentration on the textural properties of gelatin

No.	Gel strength (g)	Hardness (g)	Cohesiveness	Springiness	Chewiness (g)
1	4.6 ± 3.9^{e}	–	–	–	–
2	82.1 ± 1.0^{a}	72.6 ± 6.0^{a}	0.94 ± 0.03^{a}	1.00 ± 0.00^{a}	68.2 ± 7.1^{a}
3	46.6 ± 0.5^{b}	54.5 ± 3.5^{b}	0.95 ± 0.01^{a}	0.96 ± 0.09^{a}	49.8 ± 7.2^{b}
4	48.0 ± 1.1^{b}	45.8 ± 1.1^{c}	0.95 ± 0.00^{a}	0.98 ± 0.01^{a}	42.5 ± 1.3^{b}
5	29.6 ± 1.0^{c}	30.2 ± 0.4^{d}	0.94 ± 0.00^{a}	0.97 ± 0.01^{a}	27.6 ± 0.4^{c}
6	17.6 ± 2.7^{d}	19.7 ± 1.8^{e}	0.81 ± 0.02^{b}	0.93 ± 0.02^{a}	14.9 ± 0.7^{d}
7	8.7 ± 0.1^{e}	–	–	–	–

Note: The TPA test was a 20% compression test. The gelatin from no. 1 and 7 did not form a gel so the TPA was not determined. The NaOH concentrations of no. 1–7 were 0, 0.05, 0.1, 0.25, 0.5, 0.75 and 1 mol/L, respectively. The pretreatment time with NaOH was 50 min and the water extraction was for 3 h in a 50 °C water bath.
Different letters in the same column indicate significant ($P<0.05$) differences among different alkaline concentrations.

of the extracted gel were lowered. Higher concentrations of both alkali and acid, or longer pretreatment times resulted in lower gel strength, which showed that the gel-forming ability of the gelatin was sensitive to acid and alkali hydrolysis as both affected the cross-linking in the collagen as reported by Zhou and Regenstein (2005). From Tables 1 and 2, the gel strengths and TPA values of the acid pretreatment groups were higher than those of the alkaline pretreatment groups. For acid pretreatment, 0.1 M acetic acid for 90 min and for alkaline pretreatment, 0.05 M NaOH for 50 min would produce the maximum gel strength of 217 and 82.1 g, respectively. The higher gel strength in the acid pretreatment group than that in alkaline is consistent with that report by Zhou and Regenstein (2005).

3.2. Effects of pretreatments on nanostructure changes of the extracted gelatin

Generally, the softness of biological samples makes it difficult to obtain high-resolution AFM images without damaging the sample. In tapping mode the cantilever is vibrated externally and largely reduces the lateral forces. Another advantage of the tapping mode is that it is not sensitive to cantilever drift (Radmacher et al., 1995). Imaging under a liquid has been used to decrease the adhesive forces that generally damage the sample (Mackie et al., 1998). However, the liquid may alter the gel structure (Yang, Wang, Lai et al., 2007; Yang, Wang, Regenstein et al., 2007). Therefore, the AFM experiments were conducted in air.

The nanostructures of gelatins obtained with water, acid and alkaline (including 0.25 M and 1.0 M NaOH) pretreatments (from pretreatment no. 1 in Table 2, no. 3 in Table 1, no. 4 and no. 7 in Table 2, respectively) are shown in Figs. 4–7. Gelatin with water pretreatment shows large aggregates and some partially hydrolyzed segments as shown in Fig. 4a. Figs. 4a–d show the different mode images for the same scanning area. Separate aggregates can be found in the gelatin products (Figs. 4e and f). The relatively large aggregate of water-pretreated gelatin (Fig. 4a) shows that the gelatin is not as extensively hydrolyzed as the acid or alkaline pretreatments. Gelatin with acid pretreatment (Fig. 5) shows coacervates of dense matter with a large heterogeneity without definite geometric structures in some parts, and fibril structures in other parts. The coexisting coacervates and fibril structures are very similar to those of an acid-treated pig gelatin (Uricanu et al., 2003), and an alkaline-treated gelatin (Mohanty & Bohidar, 2005). However, the pretreatment information for these latter two gelatins has not been reported although the results suggest that the two gelatins may have been pretreated with acid before extraction. Gelatins with alkaline pretreatments (Figs. 6 and 7) show that both separate aggregates and annular pores are seen. Annular pores were not observed in the water and acid pretreatment groups, which suggests that the effect happens only with the alkaline pretreatment samples. Previously, it was reported that gelatin from a pretreatment with acid after alkali also had such a structure and the reason suggested was that during hydrolysis the acid and alkaline solutions penetrate into the gelatin molecules unevenly (Yang, Wang, Regenstein et al., 2007). Figs. 6 and 7 also show that the aggregates of gelatin with alkaline pretreatment were inclined to form separate aggregates, which is different from the more continuous coacervate that formed with acid pretreatment. It seems that there is no fundamental difference between the aggregates from the alkaline pretreatment group with the two different concentrations (0.25 M and 1.0 M NaOH). The results indicate that the pH of the pretreatment (neutral, acid or alkaline) influenced the nanostructure of the gelatin much more than the ionic strength.

Cross-sectional views of the images can be used to determine the dimensions of the aggregates and the annular pores (Haugstad & Gladfelter, 1993). All of the geometrical dimensions of the aggregates and pores can be calculated assuming the results can be transferred into circular objects for the convenience of comparison. The statistical results

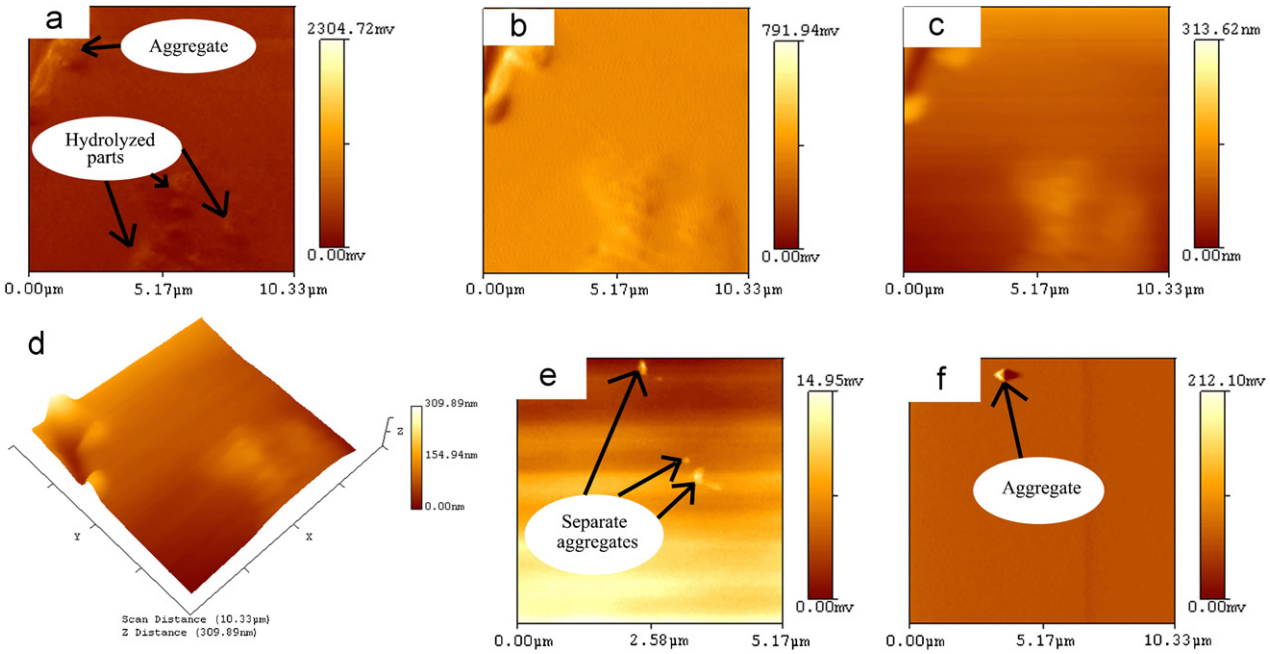

Fig. 4. AFM images of gelatin from the water pretreatment group. (a) Phase mode image; (b) corresponding error signal mode image; (c) corresponding plane height mode image; (d) corresponding 3D height mode image; (e) separate particle images as seen in the phase image; (f) another separate particle of error signal mode image. *Note*: The pretreatment time was 50 min and the water extraction was for 3 h in a 50 °C water bath.

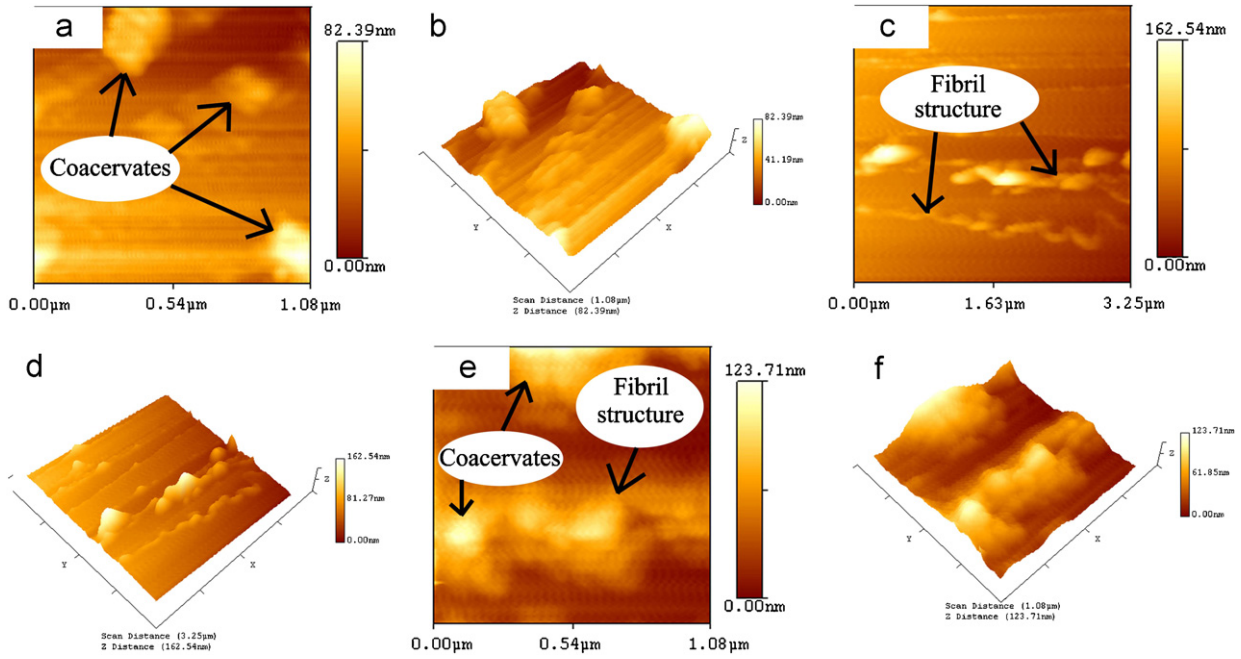

Fig. 5. AFM images of gelatin from the 0.1 M acid pretreatment group. (a) Coacervates as observed in the plane height mode; (b) corresponding 3D height mode image; (c) fibril structure as observed in the height mode; (d) corresponding 3D height mode image; (e) coacervates and fibril structure as observed in the height mode; (f) corresponding 3D height mode image. *Note*: The pretreatment time was 60 min and the water extraction was for 3 h in a 50 °C water bath.

generated for the spherical aggregates and annular pores of gelatins are shown in Table 3. Haugstad and Gladfelter (1993) reported that AFM images show the dependence of the morphology of a photographic-grade gelatin film on the pH of the gelatin solution, and that lower-pH gelatin solutions resulted in a more spatially uniform film, which is consistent with the results reported here (Table 3), where the standard deviation for the aggregates was 219.0 nm, the lowest value of all the four groups. The aggregates of gelatin pretreated under these four different conditions (water, 0.1 M acid, 0.25 and 1.0 M alkaline) are not as smooth as those in the previous report of gelatin samples

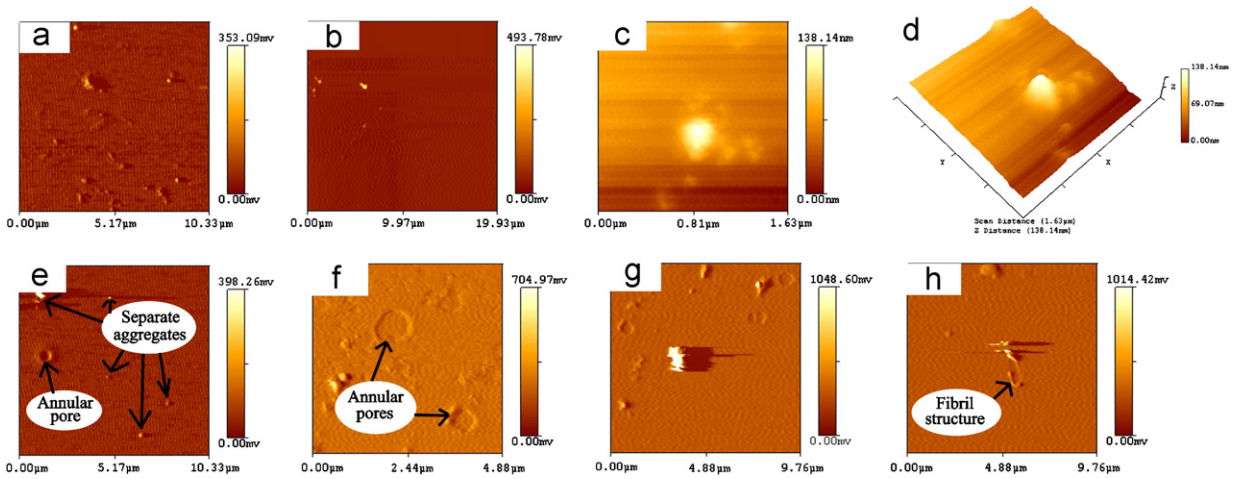

Fig. 6. AFM images of gelatin from the 0.25 M NaOH pretreatment group. (a) a typical image in the phase mode; (b) error signal image of the separate aggregates; (c) detailed hydrated plane height mode image; (d) corresponding 3D height mode image; (e–g) error signal mode image of the annular pores as well as separate aggregates; (h) error signal mode image of the fibril structure that occasionally appeared. *Note*: The pretreatment time was 50 min and the water extraction was for 3 h in a 50 °C water bath.

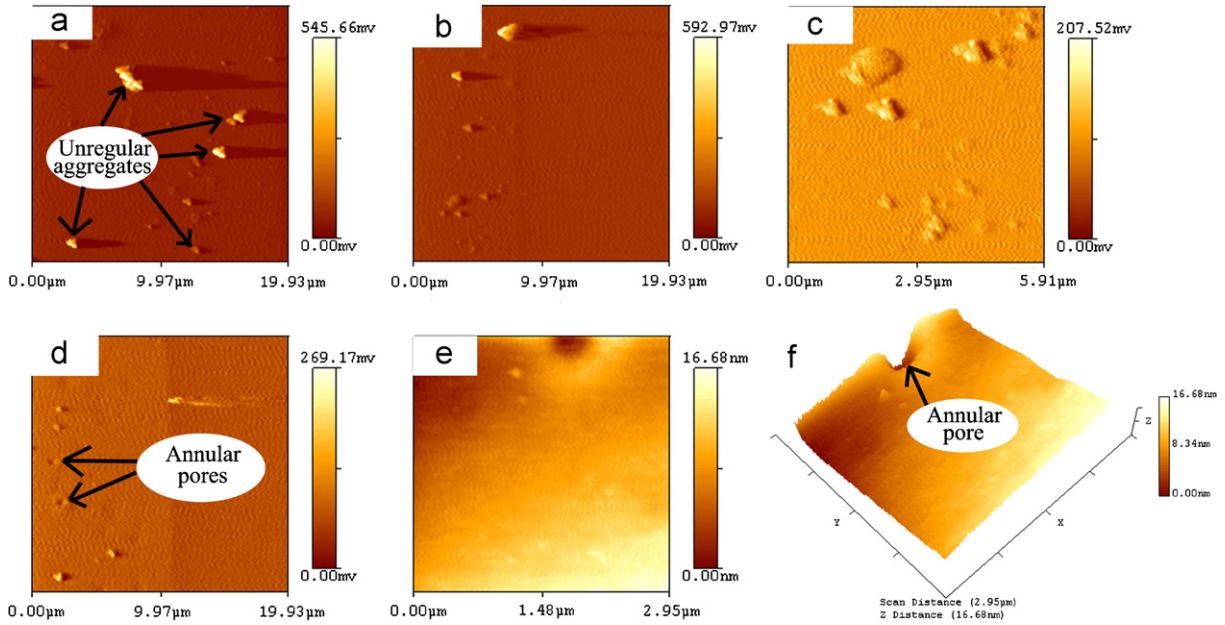

Fig. 7. AFM images of gelatin from the 1.0 M NaOH pretreatment group. (a–c) Typical structures observed in the error signal mode; (d) annular pores as observed in the error signal mode; (e) detailed height mode image of an annular pore; (f) corresponding 3D height mode image of an annular pore. *Note*: The pretreatment time was 50 min and the water extraction was for 3 h in a 50 °C water bath.

Table 3
Effects of pretreatment on the dimension of the nanostructure gelatins

Pretreatment media	Diameter of spherical aggregates (nm)	Diameter of annular pores (nm)
Water	485 ± 533 (8)	–
0.1 M acetic acid	307 ± 219 (25)	–
0.25 M NaOH	309 ± 347 (46)	549 ± 150 (6)
1.0 M NaOH	499 ± 423 (43)	409 ± 71 (2)

Note: The data are reported as means ± standard deviations (replicates) if applicable.

with an acid pretreatment after the alkaline pretreatment (Yang, Wang, Regenstein et al., 2007). The dimensions of the annular pores reported here (549.1 and 409.4 nm for 0.25 and 1.0 M alkaline pretreatment groups, respectively) are larger than that of the Type 2688 Kind and Knox gelatin studied by Haugstad and Gladfelter (1993), which were in the range of 10–100 nm. The pore size is important for gelatin's application in the photographic industry (Haugstad & Gladfelter, 1993).

Table 3 also shows that there are no significant differences for the spherical nanoparticle aggregates in

the different pretreatment groups. Since the gelatin nanoparticles were formed largely through inter- and intra-molecular interactions (Saxena et al., 2005), it suggests that there are no significant differences in the inter- and intra-molecular electrostatic interactions of the gelatins from the different groups. Lin et al. (2002) proposed that gelatin aggregation occurred as a multimeric association (or cluster association) process, where multimers of any size can form the cluster structures that then associate further into spherical aggregates. Micrographs obtained by both light and transmission electron microscopy suggested that gelatin was concentrated to form a gel in a mixed gel system (including gelatin, pectin, water, corn syrup and sucrose) (DeMars & Ziegler, 2001). Saxena et al. (2005) believed that gelatin molecules have both positive and negatively charged segments at all pH values that differ at the varying pHs, with the net charge being the key difference. Their results showed the significant difference in nanoparticle size at two pH levels, which is different from the current results. One of the possible reasons for the difference is that catfish gelatin showed greater heterogeneous structure and a larger standard deviation, which could mask any significant difference among the different groups. The results also show that the extracted catfish gelatin sample is more heterogenous than those for the biological-grade gelatin (Haugstad & Gladfelter, 1993). It should be noted that Table 3 may not clarify all of the gelatin structural information. Mackie et al. (1998) mentioned that sometimes molecules are still in the random coil state and imaging individual molecules can be unsuccessful due to the small height of the molecules during scanning. The other possible reason is that some small-dimension aggregates were ignored due to the difficulty in calculating their parameters.

Mackie et al. (1998) reported on the fibrous structure of gelatin. In this research, the fibrous structure normally appeared in the acid pretreatment group and only occasionally appeared in the 0.25 M NaOH pretreatment group (Fig. 6h). Collagen in fish skin comprises a triple helix structure that forms fibers that are arranged in bundles, and constitute the connective tissue matrix. After being hydrolyzed into gelatin, cross-links or junction zones are observed through the partial formation of ordered triple helices (Badii & Howell, 2006). Montero and Gómez-Guillén (2000) also reported that certain regions of the gelatin chains may retain some helical structure.

There were many small non-regular aggregates in the alkaline pretreatment groups (Figs. 6a and 7a–c). Zhou and Regenstein (2005) reported that alkaline extraction caused some polypeptide chains of collagen to break into small pieces, while with neutral or weak acid conditions, gelatin fractions were mainly α chain, β chain and high oligomers.

3.3. Relationship between the physical, textural and nanostructures of the extracted gelatin

Tables 4 and 5 show the correlation matrices for gelatin's physical properties with the acid and alkaline pretreatment groups. From these two tables, it is observed that yield has a significant negative correlation with gel strength ($R^2 = -0.88$) and hardness ($R^2 = -0.82$), and there are significant positive correlations between hardness and chewiness for both the acid and alkaline pretreatments.

The physical properties of gelatin depend on the structure, not only the amino acid composition, specifically including the relative content of α-, β-chains or γ-components and higher-molecular-weight aggregates, as well as on the presence of lower-molecular-weight protein fragments (Gómez-Guillén et al., 2002). It is important to understand how the nanostructure of a gel network relates to the complex macroscopic physical properties (Foegeding, 2006). DeMars and Ziegler (2001) used light and transmission electronic microscopy to undertake a qualitative analysis of gel microstructure as related to the texture with the goal of establishing structure–property–perception relationships. They found that a high-molecular-weight gelatin has higher gel strength than a lower-molecular-weight gelatin. In addition, the number and distribution of polypeptides, which are influenced by the manufacturing method, and pH, also affect gelation properties. Unfortunately, these properties have only been studied to a limited extent for fish collagen (Badii & Howell, 2006). Also, there have been many light scattering studies used to characterize protein aggregates and the aggregation process, but the lack of information on intermediate structures prevents an accurate analysis (Foegeding, 2006).

Table 4
Correlation matrix of gelatin's physical properties in acid pretreatments

	YP	*V*	GS	Hardness	Cohesiveness	Springiness	Chewiness
YP	1.00						
V	−0.62	1.00					
GS	−0.88*	0.45	1.00				
Hardness	−0.82*	0.24	0.72	1.00			
Cohesiveness	−0.19	0.45	0.44	0.17	1.00		
Springiness	0.47	−0.57	−0.08	−0.09	0.47	1.00	
Chewiness	−0.75	0.24	0.77	0.96**	0.41	0.12	1.00

Note: *, **, *** indicate significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. YP: yield of protein; *V*: viscosity; GS: gel strength.

Table 5
Correlation matrix of gelatin's physical properties in alkaline pretreatments

	YP	*V*	GS	Hardness	Cohesiveness	Springiness	Chewiness
YP	1.00						
V	−0.91*	1.00					
GS	−0.98**	0.82	1.00				
Hardness	−0.97**	0.85	0.97**	1.00			
Cohesiveness	−0.74	0.95*	0.61	0.67	1.00		
Springiness	−0.92*	0.91*	0.88*	0.82	0.79	1.00	
Chewiness	−0.98**	0.87	0.98**	1.00***	0.70	0.84	1.00

Note: *, **, *** indicate significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. YP: yield of protein; *V*: viscosity; GS: gel strength.

Because of the significant differences in the gel strength of water, acid, alkaline (0.25 M and 1.0 M) pretreatment groups (4.6, 167, 48.0 and 8.7 g, respectively) and insignificant difference among the corresponding spherical aggregates of the nanostructure of gelatin (Table 3), correlation analysis of YD, *V*, GS and the diameter of the spherical aggregates (DS) was run for the four groups and the correlation coefficients are −0.32 (between YD and DS), −0.76 (between *V* and DS) and −0.77 (between GS and DS). The results show that the physical properties have no significant correlation with the diameter of the aggregates.

The irregular separate aggregates in the water and alkaline pretreatment groups and the sponge-like patterns in the acid pretreatment groups indicate that acid facilitates the swelling process and the results are consistent with the physical properties (Yao et al., 1999), including the higher gel strength and higher TPA values for the acid pretreatment group compared to those in the water and alkaline pretreatment groups. The results indicate that the continuous sponge-like patterns give higher macroscopic textural properties than that observed with the irregularly separated aggregates. The structural patterns may relate to differences in the physical properties.

4. Conclusion

The effects of alkaline and acid pretreatment on the textural, viscosity and nanostructure properties of channel catfish skin gelatin were investigated. Acid pretreatment groups showed the highest gel strength and yield of protein and a reasonable viscosity. The water pretreatment group showed the lowest corresponding physical properties. Nanostructural characterization of these groups was performed and acid group was observed to have a sponge-like aggregate composition, while the alkaline and water pretreatment groups were separate individual aggregates. Annular pores only appeared in the alkaline pretreatment group. There was no significant correlation between the diameter of the spherical aggregates and the physical properties. However, the patterns of the gelatins were perhaps related to the physical properties. The results are helpful for illustrating the mechanism of the alkaline or acid pretreatments on the gelatin's properties and for further refinement of the extraction technology. The results also suggest that AFM is a promising nanotechnique for obtaining a better understanding of gel nanostructure.

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