Effect of different pretreatments on functional properties of African catfish (*Clarias gariepinus*) skin gelatin

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Abstract Pretreatments with different types of alkali and acid were compared to determine their effects on gelatin extraction from African catfish (*Clarias gariepinus*) skin. The study was divided into three parts. In the first part, the skins were only treated with alkaline ($\text{Ca}(\text{OH})_2$ or NaOH) solution or pretreated with acetic acid solution. For second part, combination of alkali and acid pretreatment was carried out. For the third part, the skins were first treated with NaOH solution, followed by the treatment with acetic acid, citric acid or sulfuric acid solution. Functional properties including the yield of protein recovery, gel strength, viscosity, pH and viscoelastic properties were determined on gelatins obtained with different pretreatment conditions. Pretreatment with alkali removed noncollagenous proteins effectively, whilst acid pretreatment induced some loss of collagenous proteins. Combination of alkali and acid pretreatment not only removed the noncollagenous proteins and caused a significant amount of swelling, but also provided the proper pH condition for extraction, during which some cross-linkages could be further destroyed but with less breakage of intramolecular peptide chains. Pretreatment of catfish skins with 0.2 N NaOH followed by 0.05 M acetic acid improved yield of protein recovery, gel strength, viscosity, melting temperature and gelling temperature of gelatin extract.

Keywords African catfish · Gel strength · Gelatin · Pretreatment · Viscoelastic properties

Introduction

Gelatin refers as a high molecular weight polypeptide derived from collagen. It is also known as the denatured and partially

hydrolyzed collagen. Gelatin is obtained by partial hydrolysis of collagen derived from the skin, connective tissue and bones of animals. Gelatin is a heterogeneous protein mixture of polypeptide chains with molecular weights ranging from 80 to 250 kDa (Karim and Bhat 2009). Over the years, gelatin is used widely in the photographic, pharmaceutical and food industries. In the food industry, gelatin is an important functional ingredient, which is extensively employed to improve the elasticity, consistency and stability of foods (Jamilah and Harvinder 2002). Unlike many proteins, gelatin and collagen has little nutritional significance when consumed alone except as a source of energy. Although gelatin is deficient in essential amino acids, it is often used to supplement other proteins to give a higher protein value than any of the components. Moreover, its excellent digestibility qualify gelatin as a good protein source (Baziwane and He 2003).

Nowadays, most of the gelatins are sourced from bovine and porcine skins, and bovine bones. Fish is also a source of gelatin, but only small commercial volumes are available. According to Karim and Bhat (2009), the global demand for gelatin has been increasing over the years. The annual world output of gelatin in year 2008 was 326,000 t, with pig skin gelatin accounting for the highest (46 %) output, followed by bovine hides (29.4 %), bones (23.1 %), and other sources (1.5 %). The fish gelatin production is minor, yielding only about 1 % of the annual world gelatin production. Due to the outbreak bovine spongiform encephalopathy (BSE) and increasing demand for non-mammalian gelatin for halal and kosher food markets, greater interest and focus was generated on development of methods for efficient utilization of fish by-products to produce fish gelatin as replacements for mammalian sources. Extraction of fish gelatin has been reported for several fish species such as brownstripe red snapper and bigeye snapper (Jongjareonrak et al. 2006), cod (Gudmundsson and Hafsteinsson 1997), hake (Montero et al. 1999), megrim (Montero and Gómez-Guillén 2000), pollock (Zhou and Regenstein 2005) and salmon (Arnesen and Gildberg 2007) and tilapia (Jamilah and Harvinder 2002).

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In order to convert insoluble native collagen into soluble gelatin, it involves the treatment of destruction of the tertiary, secondary and, to some extent, the primary structure of the native collagens by breaking non-covalent bonds. Disruption of non-covalent bonds has to be accompanied by cleavage of inter- and intramolecular covalent crosslinks, ideally, without cleavage of any peptide bonds. Controlled hydrolysis is therefore needed to convert collagen (molecular weight range from 345 000 to 360 000) to gelatin (molecular weight range from 10 000 to 65 000 and in some cases up to 250 000). Continued hydrolysis will result in loss of yield and loss of desirable properties (Ockerman and Hansen 1988).

There are two types of pretreatment for production of gelatin: the acid pretreatment and the alkali pretreatment. The gelatins prepared by the acid pretreatment are called type A gelatin, whereas those produced by the alkali pretreatment are called type B gelatin. The alkali process refers to a pretreatment with an alkali solution, in most cases followed by neutralization with an acid solution. Therefore, the following water extraction may be carried out in an alkali, neutral, or acid medium (Montero and Gómez-Guillén 2000; Jamilah and Harvinder 2002). Generally, alkali pretreatment is used mainly for hides and bones, which are stable and highly crosslinked materials. Gelatins derived from alkali pretreated sources are almost fully deamidated and have isoelectric point close to pH 5, called Class B gelatin. It was because the ammonia was lost due to its release from glutamine and aspartamine residues. The acid pretreatment is applied to the less mature materials containing a low concentration of intra- and intermolecular crosslinks such as pig skin, bone of young cattle, fish skin and fish bone. The class A gelatin obtained from acid pretreatment have isoelectric point of pH 6–9 since there is little or no deamidation of glutamine and aspartamine residues. During fish gelatin extraction, the acid process refers to the extraction that is carried out in an acid medium (Gómez-Guillén and Montero 2001) and in some cases an acid pretreatment before extraction is applied.

Catfish is a common farm-raised, warm-water fish, supplying large quantity of fish skins annually. The gels prepared from catfish skin are relatively thermally non-degradable and show good gelling ability (Gómez-Guillén et al. 2002). Until now, gelatin from the skins of African catfish has not been systematically studied as a raw material for edible gelatin. According to Department of Fisheries Malaysia (2007), the total amount of catfish production in year 2007 was 21,891.55 metric tons. The sale of catfish produced in Malaysia in year 2007 earned RM 107 million out of the total aquaculture fish production of RM 481 million. Catfish skin, comprising about 5 % of the whole fish, has become an interesting raw material for gelatin production.

The purposes of this study were to determine the effects of alkali and/or acid pretreatments on gelatin extraction from

African catfish (*Clarias gariepinus*) skin and to compare the effectiveness of several types of alkali and acid used in pretreatment for gelatin extraction from fish skins by evaluating gel strength, viscosity, pH, viscoelastic and gelling properties of the resultant gelatins.

Materials and methods

Preparation of materials Frozen African catfish skin was obtained from a local supplier in Penang, Malaysia. After filleting, the fish skin was cleaned and frozen at -20°C until use. Cleaned catfish skins with a maximum of 2 months frozen storage were thawed at 4°C for overnight, 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 two layers of cheesecloth and the cheesecloth containing the skins was squeezed by hand to remove liquid. All chemical reagents were analytical grade.

Gelatin extraction To determine the effects of alkali and acid pretreatments on African catfish skin gelatin extraction, the cleaned fish skins were treated with different pretreatment steps. The study was divided into three parts. In the first part, the cleaned skins were only treated with 0.2 N NaOH or 0.2 N $\text{Ca}(\text{OH})_2$ solution (1:6 w/v) with different concentration ranging from 0.01, 0.05, 0.1, 0.2 and 0.5 N or pretreated with 0.05 N acetic acid solution (1:6 w/v) for 60 min at room temperature. For second part, combination of alkali and acid pretreatment was carried out. The cleaned skins were first treated with 0.2 N NaOH or 0.2 N $\text{Ca}(\text{OH})_2$ solutions (1:6 w/v) for 60 min at room temperature, followed by the treatment of 0.05 M acetic acid (1:6 w/v) for 60 min. For the third part, the cleaned skins were first treated with 0.2 N NaOH (1:6 w/v) for 60 min at room temperature, followed by the treatment with 0.05 M of several acids (1:6 w/v): acetic acid, citric acid or sulfuric acid for 60 min at room temperature. After the above pretreatments, the pretreated fish skins were drained and rinsed with tap water. The gelatin extractions were carried out by mixing the pretreated samples with distilled water (1:10 w/v) and extracted at 50°C for 180 min. The extracted gelatin solutions were concentrated and then freeze dried and kept for analysis.

Protein and hydroxyproline content Protein content of pretreatment solutions and extracted gelatins was determined by Kjeldahl method (AOAC International 2005). Hydroxyproline content of pretreatment solutions was determined according to AOAC method (2005) with modification. The samples were first hydrolyzed with 6 N HCl at 110°C for 16–18 h. The hydrolyzed sample solution was filtered through Whatman no. 4 filter paper. The filtrate was neutralized with 2 N and 0.1 N

NaOH to pH 6. The neutralized sample was diluted and transferred into a test tube. 2 ml of oxidant solution containing 1.4 % (w/v) chloroamine-T, 1-propanol, at the ratio of 1:10 (v/v) and acetate/citrate buffer, pH 6, at a ratio of 1:8 (v/v) was mixed with the diluted sample and allowed to stand for 20 min at room temperature. After that, 2 ml of colorimetric reagent solution (a mixture of 10 % of p-dimethylaminobenzaldehyde in 35 ml of 60 % (v/v) perchloric acid (w/v) and 65 ml of 2-propanol) were added. The mixture was mixed and heated at 60 °C for 15 min and then cooled for 2–3 min in running water. Absorbance of the sample was measured against blank

at 558 nm using a spectrophotometer (UV-16001, Shimadzu, Kyoto, Japan). Hydroxyproline standard solutions, with concentrations ranging from 1 to 6 ppm, were also run simultaneously. The blank was prepared in same manner by using distilled water instead of sample.

Yield of protein recovery of gelatin The crude protein content of the extracted gelatin solutions and fish skin was determined by Kjeldahl method (AOAC International 2005). The yield of protein recovery of gelatin was calculated using the following equation:

$$\text{Yield of protein recovery(\%)} = \frac{\text{Protein content of extract} \times \text{Volume of extract}}{\text{Weight of sample(dry basis)} \times \text{Protein content of fish skin(dry basis)}} \times 100\%$$

Determination of gel strength The Bloom gel strength was determined by the British Standard 757: 1975 method (BSI 1975). A solution containing 6.67 % (w/v) gelatin was prepared in a standard Bloom jar. The mixture was later heated at 60 °C for 30 min to completely dissolve gelatin and the obtained gelatin solution was then kept in a refrigerator at 7–8 °C for 16–18 h. The gel strength was determined by TA.XT Texture Analyser (Stable Micro System, UK) equipped with a load cell of 5 kg, cross-head speed 1 mm/s and equipped with a 0.5 in. in diameter, flat bottomed plunger. The standard glass Bloom jar was placed centrally under the plunger and the penetration test was then performed. The maximum force (in g) was determined when the probe proceeded to penetrate into the gel to a depth of 4 mm. The reading was the average of three determinations.

Determination of shear viscosity The shear viscosity of 6.67 % (w/v) gelatin solution samples at 60 °C were analyzed by Rheometer Physica MCR 301 (Model Anton Paar) attached with 5 cm cone plate geometry with cone angle 2° and a gap set at 0.05 mm. Flow curves for each sample were obtained by shearing the samples at an increasing shear rate up to 1,400 s⁻¹ within 240 s.

Determination of pH The pH value of 6.67 % (w/v) gelatin solution was determined by using pH meter (Cyberscan 1000, Model RS 232 Meter) at 25 °C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Protein patterns of gelatins prepared with different pretreatment were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gelatin extracts (total protein in the gelatin extracts was 10 mg) were diluted 1:10 with sample buffer containing 0.5 M Tris-HCl, pH 6.8 containing 5 % (w/v) SDS, 20 % (v/v) glycerol and 10 % (v/v) b-mercaptoethanol at the ratio

of 1:1 (v/v) using 4 % stacking gel and 10 % resolving gel.). The samples were run in a Mini Protein II unit (Bio-Rad Laboratories) at a constant voltage of 100 V. The gel was stained with Biosafe Coomassie G250 Stain. The load volume was 20 µL in all lines. A BIO-RAD SDS-PAGE molecular weight standard, high range was used to identify the protein fractions with molecular masses ranging from 45 to 200 kDa.

Viscoelastic properties Dynamic studies were performed on a Rheometer Physica MCR 301 controlled stress rheometer rotary viscometer (Model Anton Paar) using a coneplate geometry (cone angle 2°, gap = 0.05 mm). 6.67 % gelatin solution was cooled in a refrigerator at 7 °C (maturation temperature) for 16–18 h overnight. Temperature ramps were performed at a scan rate of 1 °C/min, frequency 1 Hz, and oscillating applied stress of 3.0 Pa. The gelatin solutions were cooled from 40 to 5 °C, kept at 5 °C for 10 min and then heated from 5 to 40 °C. The elastic modulus (G'), viscosity modulus (G'') and the phase angle (δ) were represented as a function of temperature.

Statistical analyses The SAS statistical software program (SAS Institute Inc., Cary, NC, USA) was used for analyses of variance. Significance was determined at *P* < 0.05. Duncan's multiple ranges test was used to determine significant differences among means. All data reported is based on the means of three replicates (*n* = 3).

Results and discussion

Removal of protein during pretreatment Table 1 showed the protein and hydroxyproline content of pretreatment solutions, which was analyzed in order to determine the protein removed and collagen lost during pretreatment. NaOH and

Ca(OH)_2 solution with varying concentration of 0.01, 0.05, 0.1, 0.2 and 0.5 N were used for alkali pretreatments. The results presented that small quantity of proteins were extracted from catfish skin by both alkali solution with different concentration. However, hydroxyproline content was not detected in any of NaOH and Ca(OH)_2 solutions (Data not shown). This indicated that the pretreatment solution can removed noncollagenous proteins without loss of skin collagen.

NaOH and Ca(OH)_2 are two alkalis that are often used for gelatin extraction. Generally, the NaOH pretreatment solution had higher protein content than Ca(OH)_2 pretreatment solution. This suggested NaOH showed better ability to remove noncollagenous proteins (Table 1) than Ca(OH)_2 . By observation, NaOH and Ca(OH)_2 pretreatment solution presented different effects on the catfish skin: NaOH caused significant swelling of catfish skin during pretreatment, whereas Ca(OH)_2 did not. This observation was also noticed in a study of pollock skins (Zhou and Regenstein 2005) and sturgeon skin (Hao et al. 2009).

During pretreatment, the alkali solution caused noncollagenous components likes mucopolysaccharide, keratin, globulins, elastin, mucins, and albumins were converted to a more soluble product and some of the fat was changed into polar form so that they can be eliminated by subsequent washing. For the acid pretreatment, acetic acid with concentration of 0.05 M was used. The total protein content in the acid pretreatment solution was higher than that of both alkali pretreatment solutions. A very small amount of hydroxyproline content (0.05 ± 0.01 %) was observed in the acid pretreatment solution (Data not shown). This indicated that acid pretreatment could remove a small amount of fish collagen. This might be due to the acid lability of crosslinking in the less fully crosslinked material found in the catfish skin.

Table 1 Protein content of different pretreatments solution

Pretreatment solution	Protein content (%)
0.01 N NaOH	$0.09 \pm 0.05^{\text{cd}}$
0.05 N NaOH	$0.17 \pm 0.06^{\text{bc}}$
0.1 N NaOH	$0.20 \pm 0.01^{\text{b}}$
0.2 N NaOH	$0.18 \pm 0.04^{\text{bc}}$
0.5 N NaOH	$0.21 \pm 0.08^{\text{b}}$
0.01 N Ca(OH)_2	$0.08 \pm 0.02^{\text{cd}}$
0.05 N Ca(OH)_2	$0.09 \pm 0.01^{\text{cd}}$
0.1 N Ca(OH)_2	$0.07 \pm 0.02^{\text{d}}$
0.2 N Ca(OH)_2	$0.09 \pm 0.01^{\text{cd}}$
0.5 N Ca(OH)_2	$0.07 \pm 0.03^{\text{d}}$
0.05 M acetic acid	$0.39 \pm 0.12^{\text{a}}$

Values represent means $\pm$ standard deviation ($n=3$)

Values in same columns with different superscripts are significantly different at $\alpha=0.05$

Visually, acetic acid also caused swelling effect on the catfish skin during pretreatment. Swelling is important because it favors protein unfolding by disruption of noncovalent bonding, exposing the collagen to subsequent extraction and solubilization (Stainsby 1987).

Characteristics of gelatin prepared by different pretreatments Characteristics of catfish skins gelatin prepared by various pretreatments prior to extraction were presented in Table 2. Control was gelatin extracted from African catfish skins without any pretreatment. It was found that the control showed a very low yield of protein recovery (5.86 %) and viscosity (0.89 mPa.s). Furthermore, gelatins solution prepared without pretreatment could not form the gel under temperature of 10 °C. Without pretreatment, hot water extraction at temperature of 50 °C only was not enough to break the covalent crosslink found in catfish skin collagen and denatures the collagen into gelatin. This showed that pretreatment was very important for gelatin extraction. Pretreatment could break intra- and intermolecular bonds to disorganize the protein structure, thus producing adequate swelling. Swellings have the important effect of lowering the collagen denaturation temperature (helix-to-coil transition) and thus permit the gelatin to be extracted using relatively mild condition (Stainsby 1987).

The most important physical properties of gelatin are gel strength and viscosity. Commercially, gelatin with high viscosity and gel strength are preferred and are most expensive, while a reasonable yield of protein recovery is necessary for efficiency of commercial production and economic viability. For alkali pretreatment, yield of protein recovery of the gelatin prepared by pretreatment with NaOH solution (25.12–34.73 %) was higher than that from pretreatment with Ca(OH)_2 solution (20.98–24.10 %). Pretreatment with combination of alkali and acid pretreatment provided higher yield of protein recovery (48.86–58.79 %) than both alkali pretreatment but lower than those pretreated with 0.05 M acetic acid (68.97 %).

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). The gelatin extracts pretreated with NaOH solution showed gel strength ranging from 179.42 g to 187.76 g whereas those pretreated Ca(OH)_2 solutions presented gel strength of 136.56–151.85 g. Pretreatment with 0.05 M acetic acid solution produced the gelatin extracts with gel strength of 202.68 g. Combination of alkali and acid pretreatment improved the gel strength up to 218.40–265.60 g.

Besides that, combination of alkali and acid pretreatment also improved the viscosity of the gelatin solution up to 3.56–3.62 mPa.s compared with those pretreated with alkali solution only (range from 1.72 to 2.96 mPa.s) or acid solution (1.78 mPa.s) only. Viscosity is the second most important

Table 2 Characteristics of gelatin prepared by various pretreatments of catfish skins

Pretreatment	Yield of protein recovery (%)	Gel strength (g)	Viscosity (mPa.s)	pH
Control ^a	5.9	N/A ^b	0.89±0.11 ^c	7.01±0.02 ^h
0.01 N NaOH	25.1	181.1±3.62 ^d	2.09±0.23 ^{cd}	8.48±0.02 ^f
0.05 N NaOH	31.0	187.2±0.92 ^d	1.98±0.07 ^{cd}	9.19±0.01 ^c
0.1 N NaOH	34.6	185.5±4.01 ^d	2.51±0.08 ^{bc}	9.45±0.04 ^b
0.2 N NaOH	34.7	186.7±2.95 ^d	2.96±0.17 ^b	9.69±0.03 ^a
0.5 N NaOH	32.4	179.4±1.75 ^d	2.87±0.09 ^b	9.74±0.02 ^a
0.01 N Ca(OH) ₂	21.0	136.6±0.89 ^g	1.72±0.13 ^d	8.18±0.01 ^g
0.05 N Ca(OH) ₂	23.0	137.1±4.71 ^g	1.91±0.18 ^d	8.91±0.04 ^c
0.1 N Ca(OH) ₂	23.5	149.8±4.23 ^{ef}	2.01±0.21 ^{cd}	9.01±0.05 ^{de}
0.2 N Ca(OH) ₂	24.1	151.9±1.22 ^c	2.19±0.19 ^{cd}	9.11±0.02 ^{cd}
0.5 N Ca(OH) ₂	23.1	141.1±1.95 ^{fg}	2.09±0.21 ^{cd}	9.23±0.01 ^c
0.05 M acetic acid	69.0	202.6±5.24 ^c	1.78±0.15 ^b	4.45±0.02 ^j
0.2 N NaOH-0.05 M acetic acid	58.8	265.6±4.22 ^a	3.62±0.01 ^a	6.26±0.03 ⁱ
0.2 N CaOH-0.05 M acetic acid	48.8	218.4±3.75 ^b	3.56±0.21 ^a	6.22±0.03 ⁱ

Values represent means ± standard deviation ($n=3$)

Values in same columns with different superscripts are significantly different at $\alpha=0.05$

^aControl–Gelatin extraction from African catfish skins without any pretreatment

^bN/A–Not detected

parameter of gelatin. The differences in viscosity of those gelatins could be the result of different average molecular weights and molecular size distribution of the protein molecule. Higher molecule weight subunits give an increase in viscosity (Gudmundsson and Hafsteinsson 1997).

It was found that differences in yield of protein recovery and gel strength of the gelatin obtained from various types of pretreatment could be explained by the pH attained after extraction. Based on the result showed in Table 2, gelatin extracts with only alkali pretreatment had final pH values of 8.48–9.74 (NaOH solutions) and 8.18–9.23 (Ca(OH)₂ solutions). The gelatin extract with only 0.05 M acetic acid pretreatment gave the final pH value of 4.45, while that with alkali pretreatment followed by acid pretreatment showed the final pH value ranging from 6.26 to 6.62. This suggested that the lower the final pH value of the gelatin extract, the higher the yield of protein recovery. However, the high gel strength can only be obtained around neutral or weak acid condition. At the alkali condition, gelatin with the lower yield of protein recovery and gel strength was produced. These results were consistent with the result reported by Zhou and Regenstein (2005). The gel strength and viscosity of the gelatin were at a distinct maximum at the neutral condition and showed minimum values at low and high pH. Besides that, a low pH can favor a maximum extraction rate but is detrimental to the physical properties because it produces more degradation and proliferation of lower-molecular weight peptides. Combination of alkali and acid pretreatment provided optimum pH for extraction to improve the yield protein recovery of gelatin and major physical properties of gelatin, which were gel strength and viscosity.

Molecular weight distribution In gelatin manufacture, the conversion of collagen to gelatin yields molecules of varying

mass, due to the cleavage of interchain chemical cross-linkages and some unfavorable breakage of intrachain peptide linkages. The basic element of gelatin is α chain with a molecular weight of 95000. Gelatin is composed of α chains, β chains (α chains dimmers), higher molecular weight polymer including γ components (α chains trimers) and some lower molecular weight fragments. The molecular weight distribution of gelatin extract was an important factor affecting the gel strength of gelatine. High gel strength of gelatine contains a high proportion (usually more than 50 %) of molecules in the form of α - and β -chains (Sims et al. 1997). According to Zhou and Regenstein (2005), the band at 250 kDa is the β -chain and the band at 120 kDa is the α -chain.

Figure 1 showed SDS–PAGE profiles of gelatin extracts from African catfish skin using different pretreatments. After pretreatment with 0.2 N NaOH or 0.2 N Ca(OH)₂, protein species with higher molecular weight were observed in the gels of the resultant gelatin samples, however, most fractions had molecular-weight components lower than α -chain (lane 1 and 2). This suggested that alkali pretreatment might inhibit protease activity and decreased the enzymatic degradation of gelatin extracts, thus prevented autolysis by endogenous proteinases occurred. Gelatin prepared by alkali pretreatment exhibited very weak and broad bands around 120 kDa, indicating the α -chains might be broken to smaller sizes during extraction. This caused the gelatin pretreated with alkali presented low gel strength.

Furthermore, gelatin pretreated with 0.05 M acetic acid (lane 5) alone exhibited higher molecular weight compared with that without any pretreatment and those pretreated with alkali solution. It was dominated by peptide around 97.4 kDa. This indicated that pretreatment with acetic acid not only inhibited protease activity and decreased the enzymatic degradation of gelatin extracts, but also destroyed

Fig. 1 Sodium dodecyl sulfate-polyacrylamide gel (10 %) electrophoresis (SDS-PAGE) patterns of gelatin extracts from African catfish skin with different pretreatments. M: Molecular weight standard; lane 1: 0.2 N $\text{Ca}(\text{OH})_2$; lane 2: 0.2 N NaOH; lane 3: 0.2 N $\text{Ca}(\text{OH})_2$ –0.05 M acetic acid; lane 4: 0.2 N NaOH–0.05 M acetic acid; lane 5: 0.05 N acetic acid; lane 6: Control. The α and β refer to the α and β chains of African catfish gelatin

intermolecular crosslink in collagen with less breakage of intramolecular peptide chains.

Gelatin processed with acid pretreatment exhibited higher gel strength than that pretreated with alkali solutions (Table 2) and this could be explained by their molecular distribution. It was found that pretreatment with alkali cause the gel strength to decrease markedly with decreasing molecular weight compared with acid pretreatment. According to Ledward (1986), acid hydrolysis occurs preferentially in the polar regions, whilst alkali degradation hydrolyses the gelatin less specifically, including bonds in the apolar regions. If the hydrolysis occurs in the apolar, pyrrolidine-rich regions, imino residues at the chain end unable to take part in poly-L-proline II type configuration. If the hydrolysis occurs

in the polar region, spiralisation of the polypeptide chain should be unaffected and hence yielding high gel strength.

African catfish skin pretreated with 0.2 N NaOH or 0.2 N $\text{Ca}(\text{OH})_2$, followed by 0.05 M acetic acid (lane 3 and 4) increased the molecular weight of the extracted protein species significantly. This is shown by the presence of high molecular weight proteins including α and β bands in the lanes of the gel. These gelatins exhibited two major protein bands at approximately 200 kDa and 116.25 kDa and several minor bands above the MW of 200 kDa. The higher amount of α -chains, but especially of β - and γ -components found in gelatin prepared with combination of alkali and acid pretreatment, would allow a more organized structure with higher gel strength. Lower molecular weight fragments

Fig. 2 Storage modulus (G') of gelatin extract prepared with various pretreatments as a function of temperature in (a) cooling (from 40 °C to 5 °C) and (b) subsequent heating (from 5 °C to 40 °C)

Fig. 3 Loss modulus (G'') of gelatin extract prepared with various pretreatments as a function of temperature in (a) cooling (from 40 °C to 5 °C) and (b) subsequent heating (from 5 °C to 40 °C)

presented in gelatins prepared by other pretreatments need more cross-links per unit in order to form a gel, thus exhibiting lower gel strength. This is also in agreement with Stainsby (1987), who stated that a greater presence of β -chains and γ -chains will encourage better ability of renaturation to the fully collagen native form.

The gelatin extract prepared without any pretreatment showed a large amount of low-molecular-weight electrophoretic pattern without α and β bands (lane 6). It was dominated by peptides below 45 kDa. This might result from the presence of some protease activity caused by endogenous proteinases. Intarasirisawat et al. (2007) reported that fish skin contained endogenous serine and metallo proteinases, which caused

autolysis of skin at 50–60 °C. The gelatin prepared without any pretreatment did not show gel forming capacity. This may be due to the absence of α -chains, along with the disability of the highly degraded peptides that compose this gelatin to establish suitable junction zones for collagen refolding to form a gel. As a consequence of the predominance of low molecular weight components in this gelatin, which leads to a limited availability of junction zones, probably the number of established cross links would not be enough to form a gel.

Sims et al. (1997) indicated that the requirements to form a stable gel is the ability of a free α -chain to make many interchain reactions, possibly generating short triple helices, rather than the ability to generate an almost complete triple

Fig. 4 Phase angle (δ) of gelatin extract prepared with various pretreatments as a function of temperature in (a) cooling (from 40 °C to 5 °C) and (b) subsequent heating (from 5 °C to 40 °C)

Table 3 Gelling and melting point of gelatin prepared with various pretreatment

Pretreatment	Gelling point (°C)	Melting point (°C)
0.2 N NaOH	12.6±0.3 ^e	20.8±0.4 ^d
0.2 N Ca(OH) ₂	13.5±0.4 ^d	21.5±0.4 ^d
0.05 M acetic acid	16.0±0.3 ^b	23.6±0.1 ^b
0.2 N NaOH-0.05 M acetic acid	15.1±0.2 ^c	22.4±0.2 ^c
0.2 N CaOH-0.05 M acetic acid	18.9±0.3 ^a	26.3±0.3 ^a

Values represent means ± standard deviation ($n=3$)

Values in same columns with different superscripts are significantly different at $\alpha=0.05$

helix. The rapid formation of a complete triple helix by registered chains may reduce the interactions necessary to form a network across the gel. Shi et al. (2002) showed that there was a strong correlation between gel strength and the content of α -chains in gelatin. Gelatin, which contains more α -chains, would show higher gel strength. On the other hand, high ratio of peptides with molecular weight higher or lower than α -chains would decrease the gel strength of gelatin. The crosslinks between α -chains seems more stable than those between other components.

Viscoelastic properties The evolution of viscoelastic properties: storage modulus (G'), loss modulus (G'') and phase angle (δ), during both cooling (from 40 °C to 5 °C) and subsequent heating (from 5 °C to 40 °C) ramps, of the gel prepared with various pretreatment were showed in Figs. 2, 3 and 4 Among gelatins prepared with various pretreatment,

gelatin pretreated with 0.2 N NaOH followed 0.05 M acetic acid presented the best gelling ability, showing a significant higher increase in G' and G'' upon cooling than other pretreatment. These were also observed when the viscoelastic properties upon heating during which this gelatin presented a significant higher decrease in G' and G'' upon heating. This indicated that gelatin pretreated with 0.2 N NaOH followed 0.05 M acetic acid had the highest thermal stability. As discussed previously in SDS-PAGE of gelatin, gelatin pretreated with 0.2 N NaOH followed 0.05 M acetic acid contained high amount of α -chain and β -chain than other pretreatments. Therefore, more cross-links between α -chains appeared in gel. Those cross-links of α -chains make gel more firm and stable.

The gelling and melting points can be judged from the sharp decrease in phase angle (Fernández-Díaz et al. 2003). The gelling and melting point of gelatin prepared with various pretreatment were indicated in Table 3. The evolution of phase angle of gelatin solution prepared with different pretreatment showed that the gelling onset arise faster in gelatin pretreated with combination of 0.2 N NaOH and 0.05 M acetic acid. This gelatin formed a gel at 18.9 °C as observed with sharp decrease in the phase angle during cooling, due to increase in amount of energy that is elastically stored in G' . Upon subsequent heating, the phase angle showed a sharp decrease and the gelatin melted at 26.3 °C. The gelatin solution prepared with 0.2 N NaOH pretreatment followed by 0.05 M acetic acid pretreatment showed the highest gelling and melting point compared with other pretreatment. The onset of gelling of gelatins prepared with 0.2 N NaOH or 0.2 N Ca(OH)₂ took place at 12.6 and 15 °C, melted at 20.8 and 21.5 °C, respectively. The gelling and melting points of

Fig. 5 Effect of different acid type on the yield and gel strength of catfish skin gelatin extracts: **a** yield; **b** gel strength. Values represent means ± standard deviation ($n=3$). For each measurement, the data marked by different letters are significantly different ($P<0.05$)

gelatin pretreated with alkali solution were lower than those pretreated with 0.05 M acetic acid or combination of alkali (0.2 N NaOH or $\text{Ca}(\text{OH})_2$), followed by 0.05 M acetic acid.

The inferior viscoelastic properties and gelling and melting temperature shown by the gelatins prepared with different pretreatment could be explained by their differences in molecular weight distribution. As described by Gilsenan and Ross-Murphy (2000), low molecular weight gelatins melt at a lower temperature than high molecular weight ones, since the lower the molecular weight, the greater the number of crosslinks per unit volume needed to form a gel. Therefore, the gelatin pretreated with alkali solution that exhibited the lower molecular weight had the lower gelling ability and gelling and melting point. Besides, it was found that gelatin pretreated with 0.2 N NaOH followed by 0.05 M acetic acid that presented higher gel strength had higher gelling and melting point whilst the gelatin pretreated with only alkali solution had the lower gel strength, gelling and melting point. This was consistent with the finding of Choi and Regenstein (2000) who stated that the increase in gel strength of a gelatin gel is accompanied by an increase in melting point.

Effect of different type of acids on gelatin extraction Among the different pretreatments, the pretreatment of 0.2 N NaOH followed with 0.05 M acetic acid gave higher gelatin yield, gel strength and viscosity of gelatin. Therefore, 0.2 N NaOH was chosen for further study. Following 0.2 N NaOH pretreatment, three acids (acetic, citric and sulfuric acid) with 0.05 M concentration were used to investigate the effect of different acid types on gelatin extracts. Figure 5 showed the effect of different acid types on the yield of protein recovery and gel strength of African catfish skin gelatin extracts.

At the same concentration of 0.05 M, the yield and gel strength of the gelatin extracts pretreated with these acids was in the order of acetic acid > citric acid > sulfuric acid which is the opposite order as their ionization strength with acetic acid < citric acid < sulfuric acid. Acetic acid is a weak acid with a K_a of 1.8×10^{-5} ; citric acid is also a weak acid with a K_a of 7.1×10^{-4} for the first stage of ionization, 1.7×10^{-5} for the second stage, and 6.4×10^{-6} for the third stage; sulfuric acid, on the other hand, is a strong acid for the first stage and a weak acid for the second stage with a K_a of 1.3×10^{-2} . Thus, although the total H^+ concentration of each acid was the same, the ionized H^+ available in solution was different, which had a different effect on African catfish skins during pretreatment and resulted in a different yield and gel strength of the gelatin extracts (Chang 1991). According to Zhou and Regenstein (2005), when the pH conditions during extraction are the same, the final products will have similar yields and gel strength, no matter what type or concentration of acid is used in the pretreatments.

Alkali pretreatment of skins before acid extraction produced the highest gel strength in the case of acetic acid

extracts. Prior to acid pretreatment, the alkali pretreatment of skins may reduce protein molecular size through slight hydrolysis of the polar regions (Johnston-Banks 1990). This facilitates swelling of collagen protein by organic acids, especially in the case of the larger-molecular-size acids, improving collagen solubilization. On the other hand, in gelatin extracts with other acids, a number of negative factors converge, such as lower pH, higher ionic strength and poor swelling during extraction which may result in the poor gel strength of the gelatin extracts.

Conclusion

A high-quality gelatin was obtained from African catfish skin using a combination of alkali and acid pretreatment. During gelatin extraction, alkali and/or acid pretreatments showed effects on removing noncollagenous proteins with minimum collagen loss and inhibiting degradation caused by endogenous proteases. African catfish skin pretreated with alkali and/or acid induced removal of protein with different molecular weight distributions. Proteins with low molecular weight predominated in basic pretreatment solutions, thus producing gelatin with low gel strength, gelling and melting point. Pretreatment with a combination of alkali and acid provide a weak acid extraction medium which was the optimum gelatin extraction pH. During gelatin extraction in weak acid condition, intermolecular crosslinks in collagen might be broken with less breakage of intramolecular peptide bond and produced a gelatin containing higher molecular weight component (α and β -chains), which induced high yield of protein recovery, gel strength, viscosity, gelling and melting point. Factors such as swelling capacity of collagen, pH of extraction and ionic strength, which varies depending on the type of acid used, are important for the functional effectiveness of the extraction.

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