Effects of Acid Pretreatment and Extraction Temperature on the properties of gelatin

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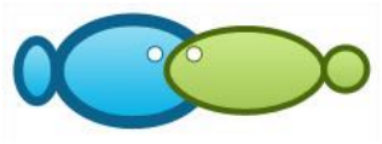
Submission date: 02-Jan-2023 09:06AM (UTC+0300)
Submission ID: 1987887994
File name: ment_and_Extraction_Temperature_on_the_properties_of_gelatin.pdf (524.13K)
Word count: 4330
Character count: 23444



Effects of acid pretreatment and extraction temperature on the properties of gelatin from striped snakehead (*Channa striata*) scales

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Abstract. The gelatin properties can be influenced by the source materials and the way it is extracted. Acid pretreatment can increase the production of gelatin from raw material by disrupting the collagens helical structure. The purpose of this research was to study how acid pretreatment and extraction temperature can influence the gelatin properties from striped snakehead (*Channa striata*) scales. *C. striata* scales were pretreated with 0.05 M acetic acid and elicited at 60, 70, and 80°C for 12 h. Experiments were based on a completely randomized design and data were analyzed with Duncan's multiple range test. The measurements were triplicated. The results showed that pretreatment with acetic acid had produced a higher yield of gelatin compared to no acid pretreatment. Rising extraction temperature increased the extraction yield, although at 70 and 80°C the yields were not significantly different ($p>0.05$) anymore. The SDS-PAGE electrophoresis banding pattern indicated that all gelatins consist of α- and β-chain components. Increased extraction temperature resulted in degraded peptides with smaller molecular weights. The degradation of protein occured at a higher temperature, reducing the gel strength and viscosity of *C. striata* scales gelatin. The spectra in FTIR spectrometer displayed a significant degree of gelatins' triple helical structure loss, due to the high temperatures of the protein degradation, which indicates that, in this state, collagen is transformed into soluble gelatins. This research concluded that *C. striata* scales could be a source of gelatin, even though the gelatins' attributes can easily be affected by the extraction process.

Key Words: collagen hydrolisis, extraction process, gelatin yield, gel strength, fish scale.

Introduction. The fibrous structural protein that comes from partial hydrolysis in collagen is called gelatin. The gelatin has been commonly used in the food processing, pharmaceutical, biomedical, cosmetic, and photographic industries (Tan et al 2019). The main sources of gelatins are the bone and skin of cows and pork, which raises serious concerns from the customers, involving social stigma, mad cow disease, and religious beliefs (Karim & Bhat 2009). In the last ten years, fish became the alternate source of gelatin, avoiding the use of cow and pork as raw materials (Shyni et al 2014; Dincer et al 2015).

Striped snakehead (*Channa striata*), locally referred to as "haruan," is a freshwater fish indigenous to Indonesia. Public interest in consuming *C. striata* meat is increasing because of its advantages for wound-healing therapy in post-operative patients and processed products such as salted fish and crackers (Mustafa et al 2012; Mustafa et al 2013; Muthmainnah 2013). However, increased utilization of *C. striata* meat will produce large quantities of waste, including scales. Scales cover nearly 5% of the edible fish meat, which makes a lot of concern for the environment because most of them are thrown away (Dincer et al 2015). Pamungkas et al (2019) successfully extracted collagen from *C. striata* scales and reported that the yield of collagen was 1.44-2.94%. Therefore, it was concluded that *C. striata* scales could be used as possible sources for gelatin production.

The main compound responsible for the triple helical structure stabilizing and for the high thermal stability of collagen is the hydroxyproline (Matmaroh et al 2011). Haug & Draget (2009) demonstrate that the extraction temperature could not optimize the

extraction process of gelatin. Using mild acid pretreatment could produce sufficient swelling and cleavage of the noncovalent intramolecular and intermolecular bonds, which increases the solubility of collagen, thereby increasing the yield of gelatin (Norland 1990; Niu et al 2013). Tan et al (2019) showed that using thermal treatment above 45ºC can cleave hydrogen and many covalent bonds, which influence the properties of the gelatin. Dincer et al (2015) reported an enhancement of the functional characteristics of gelatin from scales of sea bass following acid pretreatment. Hue et al (2017) concluded that the physicochemical characteristics of gelatin produced from the scales of sea bream are close to commercial gelatin produced from pig skin. However, there is a lack of research and available information about using the acid pretreatment method at various extraction temperatures in gelatin production from the *C. striata* scales. This study aimed to investigate the effects of acid pretreatment and extraction temperature on the properties of gelatin from *C. striata* scales.

Material and Method

Materials. The *C. striata* scales used in this research were collected from a conventional market in Samarinda, Indonesia. Before the extraction, the scales were cleaned with cold water to remove impurities, then packed in polyethylene bags and stored at -18°C till extraction.

Chemicals and reagents. The chemicals for electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA, USA) excepted tthree chemicals: NaOH, HCl, and CH_3COO, which were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All reagents used in this research were at the analytical grade.

The gelatin extraction. The method used for the gelatin extraction was based on Kittiphattanabawon et al (2016), with slight changes. First, the fish scales were submerged in NaOH with a proportion of 1:10 (w/v) and stirred for six hours to remove the non-collagen proteins. Every 3 hours, the alkaline water was replaced. Then the scales were cleaned with water until the pH of the washed water became neutral. Afterwards, a demineralization of the *C. striata* scales was carried out using HCl with a proportion of 1:5 (w/v), then the mixture was stirred for half an hour and washed again with tap water. Acid pretreatment was done by soaking the scales in 0.05 M acetic acid (1:2 w/v) at 25°C for three hours, and then extraction was performed with distilled water at different temperatures (60, 70, and 80°C) for 12 h. Gelatin without acid pretreatment was directly extracted from demineralized scales with distilled water at the same temperatures as described previously. The extracted gelatin was filtered with two layers of cheesecloth and grade 3 Whatman paper, using a Buchner funnel. Then the filtrate was dried, using a freeze drier. The samples extracted with acid pretreatment at 60, 70, and 80°C were labeled individually as A60, A70, and A80. The extracted samples without acid pretreatment at 60, 70, and 80°C were labeled individually as W60, W70, and W80. The result of the comparison between the freeze-dried gelatin with the dry mass of the sample is referred to as gelatin yield.

The gel strength of gelatin. To determine the gel strength of gelatin, the methodof Kittiphattanabawon et al (2010) was used. The dried gelatin was dissolved and stirred in boiled water (6.67%; w/v) at 60°C for 30 min. Then the mixture was stored in a cold chamber (4°C) for 18 h. After that, the mixture was measured using a TA.XTPlus texture analyzer (Stable Micro Systems Ltd., UK) with a plunger of 1.27 cm in diameter, a penetration depth of 4 mm, and a probe speed of 1 mm sec^{-1}.

The viscosity of gelatin. The viscosity of the gelatin was determined by using Niu et al (2013) method. The gelatin at a concentration of 6.67% (w/v) was prepared with water and heated at 60°C for 30 min. Then the viscosity of *C. striata* scale gelatin was measured using a Brookfield viscometer with spindle no. 6, at 25°C and 60 rpm.

The molecular weight distribution of gelatin. SDS-PAGE gel electrophoresis was conducted by Laemmli (1970) method with a slight modification by using 10% separating gel and 5% stacking gel. The gelatin (10 mg) was dissolved in 0.1 mL of 10% (w/v) SDS, heated at 85°C for 1 hour, and then centrifuged at 10,000 x g for 15 min (Eppendorf Centrifuge 5417 R, Hamburg, Germany). The supernatants were mixed with a sample buffer containing βME as a reducing agent and then heated for 3 min. Protein samples were loaded into the polyacrylamide gel and submitted to an electrophoresis at 120 V.

FTIR of gelatin. Fourier transform infrared spectroscopy of the gelatin was performed using Shimadzu PC-8201. The produced gelatin (2 mg) was then mixed with potassium bromide, and then the mixture was placed into a disk for spectrum recording. Measurements were taken in the 4,000-500 cm^{-1} (mid-IR) region, at 25°C.

The statistical analysis of the gelatin. Statistical analysis used in this research was the SPSS statistical program Version 20. All analyses were triplicated, then the data were analyzed by using a one-way analysis of variance and Duncan's multiple range test at $p<0.05$.

Results and discussion

Gelatin yield. The effect of acid pretreatment and extraction temperatures on the gelatin yield of *C. striata* scales is shown in Figure 1.

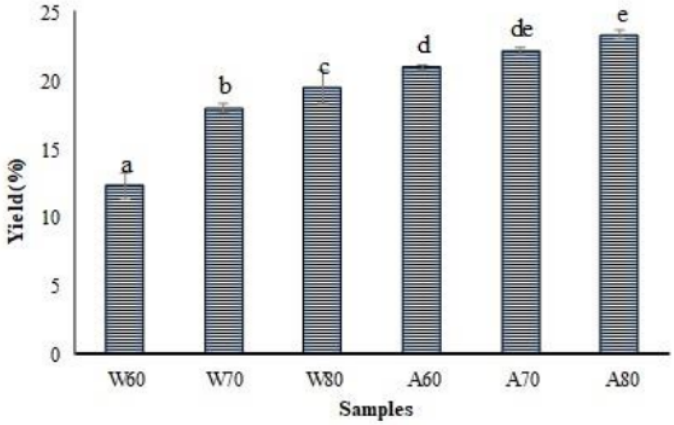

Figure 1. Gelatin yields from *Channa striata* scales. W (without acid pretreatment); A (acid pretreatment); 60, 70, 80 (extraction temperatures in °C). Bars indicate the standard deviations and different letters indicate statistically significant differences ($p<0.05$).

Acid pretreatment resulted in a higher gelatin yield than no acid pretreatment. Increased extraction temperature resulted in increased gelatin yield. The highest gelatin yield was achieved at an extraction temperature of 80°C (23.26%), but this was not significantly different ($p>0.05$) than the yield at 70°C (22.11%). Acid pretreatment has an important role in breaking both the intramolecular and intermolecular bonds of collagen without altering its original triple-helical configuration, which makes the collagen to dissolve easily in water (Mulyani et al 2017). After the thermal treatment breaking the hydrogen and covalent bonds, the triple helical structure becomes unstable, the collagen ending up as gelatin (Djabourov et al 1993). Sinthusamran et al (2014) also explain that when the heat increases with treatment temperatures, there more energy is applied to the extraction system and the disruption of the triple helix into random coil structures is

amplified. As the extraction temperature increased, more gelatin could be extracted into the aqueous medium. This result was higher than gelatin yields from farmed sea bass (*Dicentrarchus labrax*) scales (Dincer et al 2015), bighead carp (*Hypophthalmichthys nobilis*) scales (Tu et al 2015), spotted golden goatfish (*Parupeneus heptacanthus*) scales (Chuaychan 2016), and seabream (*Sparus latus* Houttuyn) scales (Hue et al 2017). The variations in gelatin yield might be attributed to the differences in the preparation method for extracting the gelatin out of the scales (heat, duration, pH, raw material to water proportion) and the protein content in the raw material, which may differ among the species (Koli et al 2012).

The protein patterns of gelatin. Gelatin from *C. striata* scales extracted with acid pretreatment at different temperatures had protein patterns, as presented in Figure 2. The electrophoretic banding pattern showed that the extracted gelatin consisted of two chains, namely α1 and α2, as the major protein components and had a high molecular weight β-chain. The protein pattern, which resulted from acid pretreatment showed significant low molecular weight components compared to no acid pretreatment. Gelatin extracted at temperatures of 60 and 70°C had similar migration bands, whereas, at an extraction temperature of 80°C, the two α-chains and the β-chain bands completely disappeared and resulted in the highest portion of low molecular weight peptides. This result suggests that acid pretreatment and extraction temperatures affect the protein structure of gelatin. Kim et al (2012) show that acid pretreatment weaken the cross-linked collagen by disrupting the noncovalent intra- and intermolecular bonds. Also, an increased extraction temperature resulted in the changes of the triple-helical integrity of collagen and in the formation of low molecular weight peptides in the *C. striata* scales gelatin patterns (Liu et al 2017).

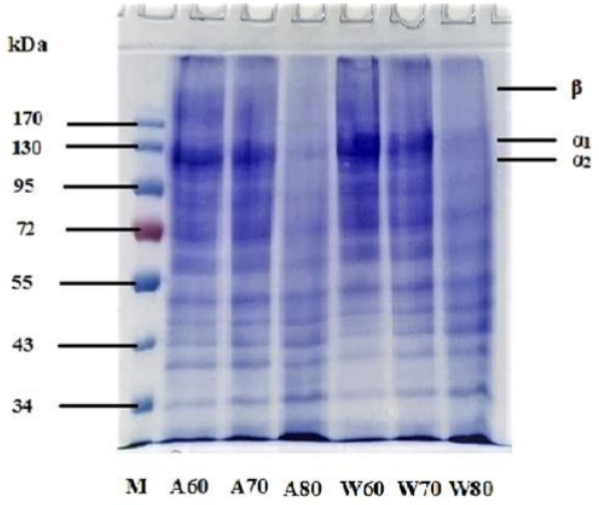

Figure 2. SDS-PAGE patterns of gelatins from *Channa striata* scales. M (marker); W (without acid pretreatment); A (acid pretreatment); 60, 70, 80 (extraction temperatures in °C).

The gel strength of gelatin. Table 1 shows the gel strength of the gelatin from *C. striata* scales. Acid pretreatment and extraction temperature significantly affected ($p<0.05$) the gel strength of the *C. striata* scale gelatin. The gel strength declined with both pretreatment acid concentration and extraction temperature increase, in accordance with Kim et al (2012). Alfaro et al (2014) also demonstrate that the gel strength of the produced gelatin decreases as extraction temperature increases, especially at 85°C. Liu et al (2017) state that the molecular weight distribution of gelatin molecules significantly

affect the gelation process. Because of the high temperature in gelatin extraction, the degradation of protein produces protein fragments, lowering gel-forming ability (Normand 2000). The high temperature also affects the gelatin molecules resulting shorter peptide chains, making them unable to form a robust inter-junction zone during gelation, which lowers the gel strength (Kittiphattanabawon et al 2016). Hence, gelatin extracted at a temperature of 60 to 70°C, still contains a higher amount of α- and β-chains that could form a stronger gel than gelatin extracted at a temperature of 80°C.

Table 1

Gel strength and viscosity of gelatin from *Channa striata* scales

Samples	*Gel strength (g cm^{-2})*	*Viscosity (cP)*
W60	273.79±2.05^{f}	16.95±0.05^{f}
W70	229.22±7.72^{d}	8.65±0.05^{d}
W80	126.69±3.19^{b}	6.60±0.10^{b}
A60	264.72±2.05^{e}	12.35±0.25^{e}
A70	208.28±5.35^{c}	7.05±0.05^{c}
A80	66.33±1.99^{a}	6.15±0.15^{a}

W (without acid pretreatment); A (acid pretreatment); 60, 70, 80 (extraction temperature in °C). The values are given as the mean ± standard deviation from the triplicate determination. Values with different letters indicate statistically significant differences ($p<0.05$).

Viscosity of gelatin. Gelatin from *C. striata* scales had a viscosity in the range of 6.15 to 16.95 cP (Table 1). Acid pretreatment and extraction temperature had a significant effect ($p<0.05$) on the viscosity of the produced gelatin. The viscosity of gelatin decreased with the acid pretreatment at a high extraction temperature. Niu et al (2013) said that the molecular weight distribution of the extracted proteins was related to the viscosity of gelatin. Acid pretreatment produced peptides with significantly low molecular weight compared to the peptides produced without acid pretreatment (Figure 2). Acetic acid breaks the peptide bonds of amino acids into short-chain molecules, which reduces viscosity (Sompie & Triasih 2018), whereas the decrease in viscosity of gelatin at high extraction temperatures is due to the transformation of collagen structure, in particular the triple helix, into a single chain. Higher extraction temperatures determine the opening of amino acid chain structures, resulting in shorter chains and in a lower viscosity of the gelatin (Pradarameswari et al 2018).

Fourier transform spectroscopy of gelatin. Gelatin from *C. striata* scales had the FTIR spectra as shown in Figure 3. Every gelatin sample had similar patterns of FTIR spectra, including amide I, amide II, amide III, amide A and amide B. In all gelatin samples, Amide I was detected at 1635.64 cm^{-1}. Amide I was correlated with stretching vibrations of the C=O bond (Payne & Veis 1988). Nagarajan et al (2012) demonstrate that amide I is used for analyzing the protein's secondary structure. The research result was almost in line with Mulyani et al (2017), who show that amide I of buffalo hide gelatin pretreated with acid is also detected at 1635.64 cm^{-1}.

The amide II region in gelatin samples had the absorption bands at 1527.62 cm^{-1} for A60, A70, A80, W70, and W80, whereas W60 was detected at 1543.05 cm^{-1}. Amide II was composed of a combination of a CN stretch and NH deformation modes (Nagarajan et al 2012). The amide III region in the gelatin (1195.87-1242.16 cm^{-1}) had a lower amplitude with acid pretreatment at higher extraction temperatures. Amide III involved stretching vibrations of CN groups and deformation of NH groups from the amide bonds (Abedinia et al 2017). Lower amplitude indicated a more significant disruption of the gelatin structure, especially from an alpha helix to a random coil structure (Muyonga et al 2004).

Amide A in gelatin samples, namely A60, A70, A80, W60, W70, and W80, was detected at 3425.58, 3425.58, 3410.15, 3448.72, 3448.72, and 3410.15 cm^{-1}, respectively. The wavenumber value of amide A is usually found at 3400–3440 cm^{-1} (Sinthusamran et al 2014). The peak of amide A is correlated with the stretching

frequency of NH groups and indicates the presence of an H-bond. Gelatin with acid pretreatment had a lower wavenumber than gelatin without acid pretreatment. Mulyani et al (2017) reported that a lower wavenumber indicated bigger molecular degradation of the gelatin. Furthermore, the gelatin extracted at 80°C had an amide A region with a lower wavenumber. This result might be due to the degradation of the collagen structure by thermal hydrolysis during gelatin extraction.

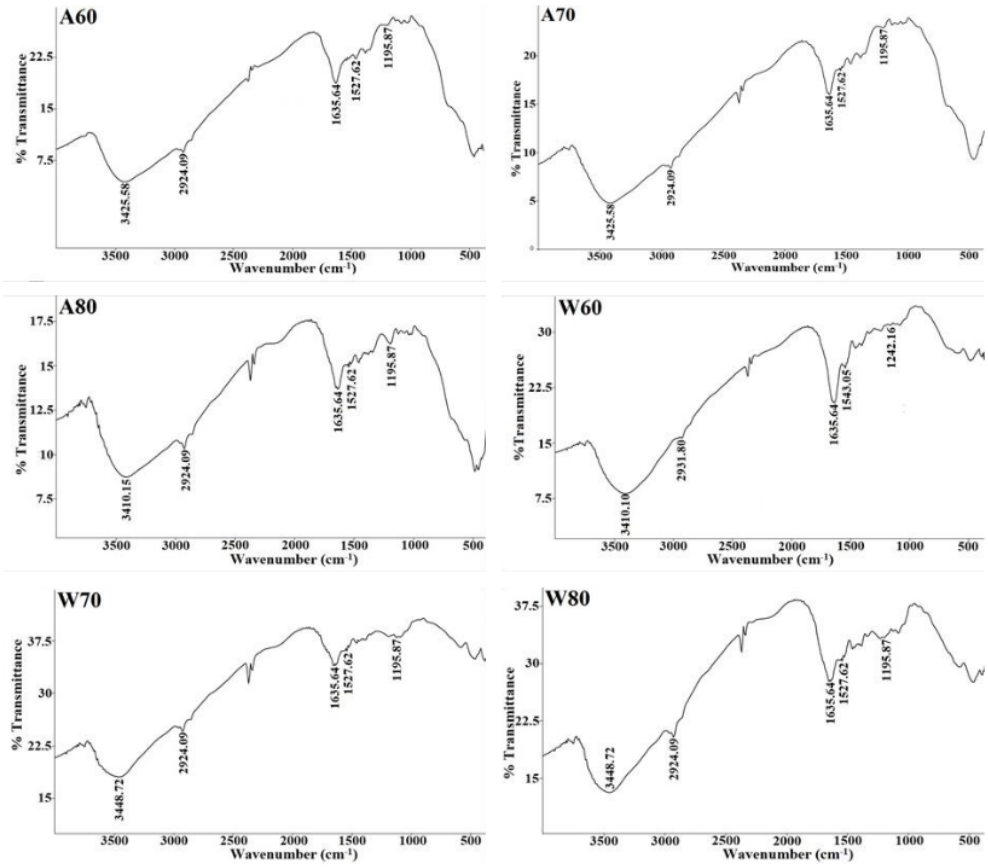

Figure 3. FTIR spectra of gelatins from *Channa striata* scales. A (acid pretreatment); W (without acid pretreatment); 60, 70, 80 (extraction temperatures in °C).

The absorption bands of amide B for A60, A70, A80, W70, and W80 were detected at 2924.09 cm^{-1}, whereas W60 was found at 2931.80 cm^{-1}. Gelatin with acid pretreatment showed a lower wavenumber for amide B compared to no acid pretreatment. Extraction at a higher temperature also showed a lower wavenumber. This result indicated that the acid pretreatment and extraction temperaturesignificantly affected the functional groups and secondary structure of gelatin from *C. striata* scales.

Conclusions. The properties of gelatin from *C. striata* scales were affected by acid pretreatment and extraction temperature. The increase of the extraction temperature determined an increase of the gelatin yield. However, acid pretreatment and high extraction temperature resulted in decreased gel strength and viscosity of gelatin. This was mostly related to the degradation of protein at higher extraction temperatures based on the SDS-PAGE electrophoresis pattern. Therefore, an acid pretreatment process

combined to a lower extraction temperature would be an optimal choice to produce superior gelatin from *C. striata* scales.

Acknowledgements. This work was supported by the Islamic Development Bank (IsDB) Project under the Development of Four Higher Education Institution Project IsDB, Mulawarman University (grant number 137/UN17.11/PL/2019).

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Received: 15 August 2020. Accepted: 20 October 2020. Published online: 29 October 2020.
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How to cite this article:
Zuraida I., Pamungkas B. F., 2020 Effects of acid pretreatment and extraction temperature on the properties of gelatin from striped snakehead (*Channa striata*) scales. AACL Bioflux 13(5):2937-2945.

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