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Comparison Study on the Extraction of Gelatin from Nila Fish (*Oreochromis nilotichus*) Skin Using Acetic Acid and Citric Acid

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Abstract: Methods of gelatin extraction from nila fish (*Oreochromis nilotichus*) skin were studied using acid treatment (acetic acid or citric acid) at various concentrations (0, 0.05, 0.10, 0.15, 0.20 and 0.25 M) and soaking times (0, 1, 2, 3, 4 and 5 h). Gelatin extracted from nila skin treated with 0.10 M acetic acid for 2 h produced the highest yield (25.33%) with a gel strength of 346.16 g bloom and a molecule size distribution of 38-241 kDa. Gelatin extracted from the skin treated with 0.05 M citric acid for 1 h resulted in a yield of 21.09% with a gel strength of 134.52 g bloom and a molecule size distribution of 23-145 kDa. Gelatin extracted by both acids had similar infra-red absorbance spectra, ranging from 1640 to 1239 cm^{-1}. However, gelatin from nila skin treated with 0.10 M acetic acid for 2 h produced gelatin with a more homogenous molecular microstructure than nila skin treated with 0.05 M citric acid for 1 h.

Key words: Gelatin, fish, acid

INTRODUCTION

Gelatin is a protein that is produced from the hydrolysis of collagen from the skin, bone and muscle tissues of animals. The process of gelatin production from collagen occurs in threesteps: (1) removing fat and mineral components from the raw materials using acid or alkaline solutions (degreasing and demineralization); (2) converting the collagen into gelatin by heating in water (extraction) and (3) obtain in gelatin in its final form (Ward and Courts, 1977), by drying and flouring. Gelatin has functional properties that allow it to act as a water binder, thickener, gel-forming agent, stabilizer and emulsifier in the food, cosmetic and pharmaceutical industries (Jamilah *et al*., 2011). The need for gelatin increase every year and this need is often met by importing foreign products.

Based on statistical data of imported goods from the Ministry of Trade of Indonesia, annual imported gelatin from 2007 to 2011 ranged between 10, 618, 600 and 25, 036, 100 USD with an average increase 20.26% per year (Anonymous, 2016). Approximately 68.1% of gelatin is produced in Europe and is sourced from pig skin on the other hand, 9.24% of gelatin originates from cattle hide split and the remaining 22.70% of gelatin is extracted from pig and cattle bones. Schrieber and Garies (2007) stated that gelatin was produced by many countries in Europe, America, Africa and Asia with a total production in 2005 of 137,000 tons (from pig-skin), 85,000 tons (from cow hide) and 83,000 tons (from pig and cattle bones). These sources of gelatin have long been a problem for those on a halal diet and there are concerns related to poisoning from the cattle such as bovine spongiform encephalopathy (BSE). To overcome these problems, alternative sources of gelatin, such as fish skin need to be explored.

An important commodity in the aquaculture of Indonesiais nila fish (*Oreochromis niloticus*). Nila fish have been exported to European countries and Japan in the form of fresh frozen filets. The nila fillet industry produces waste that is nearly 60% head, bones, skin, guts and scales (Peranginangin *et al*., 2006). Until now, the utilization of this waste product was limited to animal feed or cracker food products; however, fresh nila skin has begun to be exported. The protein in the fish waste, especially the skin can be processed into gelatin with very high yield.

Gelatin has been well studied and its properties are ideal for application in the food industry to improve quality. Gelatin can act as a water-binding agents, thickener and stabilizers. Gelatin has also been used as a binder in syrup and a stabilizer in jelly candy, as reported by Ayudiarti *et al*. (2007) and Setiyowati (2014). Extraction of gelatin from fish skins and bones been reported, for example, from Patin (*Pangasius sp*) bone (Peranginangin *et al*., 2005), red snapper (*Lutjanus* sp) bone (Suryanti *et al*., 2006) and freshwater fish skin (See *et al*., 2010). These extractions generally produced yields of <20% (wet basis). For example, gelatin yields from red nila fish skin were 12.94% (Jamilah *et al*., 2011); gelatin from nila fish skin and perch bones were 16 and 2.4%, respectively (Muyonga *et al*., 2004a).

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Niu *et al*. (2013) found that gelatin extraction using an alkaline (NaOH 0.3 M) degreasing solution followed by soaking in an acid solution (acetic or citric acid at concentrations ranging from 0.05-0.2 M for 1 h) could increase the gelatin protein yield, ranging from 10 to 22.4% for extractions with citric acid and 1.92-21.5% for extractions with acetic acid, with a molecular weight distribution of ≤116 kDa to ≥200 kDa. Gelatin extracted from nila skin with a degreasing process involving soaking in warm water (60-70°C) for 10s, followed by soaking in 0.05 M acetic acid for 10 h, yield 29.44% and the molecular weight of the product ranged from 97-206 kDa (Setiyowati, 2014). Including the acid process in gelatin extraction from animal skin can remove most minerals, so it is referred to as the demineralization process. Bonds between the molecules in collagen from fish skin are not very strong compared to gelatin from fish bones, so it is easily broken downin an acidic solution (Ward and Courts, 1977). Acetic and citric acid are organic acid that are often used as flavor (sourness) enhancers in foods, so they are safe for gelatin preparation intended for application in the food industry. The objective of this research was to compare gelatin extracted from nila fish (*Oreochromis nilotichus*) skin using acetic acid and citric acid at various concentrations (0, 0.05, 0.10, 0.15, 0.20 and 0.25 M) and soaking times (0, 1, 2, 3, 4 and 5 h).

MATERIALS AND METHODS

Raw material: The skin waste of nila fish (*Oreochromis niloticus*) was obtained from an industrial fishery in Central Java, Indonesia. Fresh nila skin was cleaned of scales and meat and washed with water to obtain clean fresh nila fish skin.

Extraction of gelatin from nila skin: The skin was first degreased by soaking it in warm water (60-70°C) for 10 s. The skin was then reduced in size to4 x 4 cm. Demineralization was performed by soaking the skin in a solution of citric acid or acetic acid (0.05, 0.10, 0.15, 0.20 or 0.25 M) at a ratio of 1:8 w/v for 1, 2, 3, 4 or 5 h. The pH was brought close to neutral by washing with water. Skin size was reduced by a homogenizer (10s). Gelatin extraction was performedusing distilled water at a ratio of 1:3 (w/v) at a temperature of 80°C (3 h), followed by filtration. The gelatin was then dried at a temperature of 21-25°C for 72-96 h and milled to obtain the gelatin powder.

Analysis procedures

Yield of gelatin: The yield of the gelatinwas based on the wet weight (Jamilah *et al*., 2011):

$$\% \text{ yield of gelatin (wb)} = \frac{\text{Weight of dry gelatin}}{\text{Weight of wet skin}} \times 100\%$$

Proximate composition: The moisture, ash, fat and crude protein content of the fish skins and the gelatin were determined according to the AOAC method (1995) and a nitrogen conversion factor of 5.4 was used for calculation of the crude protein content (Muyonga *et al*., 2004a).

Gel strength: A 6.67% (w/w) gelatin solution was prepared at 60°C, placed in a glass bloom and cooled to 10°C (18 h). Gel strength was determined according to the Gelatin Manufacture Institute of America (GMIA) testing standard using a Stable Micro System Texture Analyzer TA XT plus HD (Stable Micro System Ltd., UK) with a cylindrical probe P/0.5, a 5 kg load cell and a 1.0 mm/s test speed at distance of 4 mm.

Molecular weight distribution: Separating and stacking gel concentrations were 10 and 5%, respectively,and contained 80 µg of gelatin (Setiyowati, 2014). The protein molecular weight marker was from Bio-Rad Laboratories (10-250 kDa). The electrophoresis was conductedat 120 V and 40 mA using a Mini Protein Unit. The protein bands were stained with 0.1% Coomassie Brilliant Blue R-250 and destained using water: methanol: acetic acid (8:1:1).

Fourier transform infrared (FTIR) spectroscopy: Dried gelatin samples and KBr powder were mixed and pressed into sheets. The FTIR spectra of samples were scanned on a Nicolet 5700 FTIR Spectrometer at wave numbers ranging from 400 to 4000 cm^{-1} in 32 scans. The data were plotted as absorbance vs wavenumber (cm^{-1}) (Sinthusamran *et al*., 2014).

Molecular microstructure: Dry gelatin powder was coated with gold using a vacuum smart coaterand placedonto the specimen holder. The specimen of gelatin microstructure was then imaged with a scanning electron microscope (JEOL JCM 6000, Tokyo, Japan).

Statistical analyses: All data analyses were performed twice using SPSS software system. The statistical analyses were performed by a completely randomized factorial design using ANOVA and a Duncan advanced test to determine if the differences were statistically significant between treatments.

RESULTS AND DISCUSSION

Yield of gelatin: Treatment with different acids at various concentrations and soak times resulted in different gelatin yields from nila fish (*Oreochromis niloticus*) skin (Fig. 1). The yield of gelatin extracted using acetic and citric acid ranged from 11.53-25.33% and 2.89-24.30%, respectively. Using acetic acid, the yield of gelatin first increased and then decreased with increasing concentration of acid. In contrast, the use of citric acid caused a decrease in yield as the concentration was increased ($p<0.05$). The highest

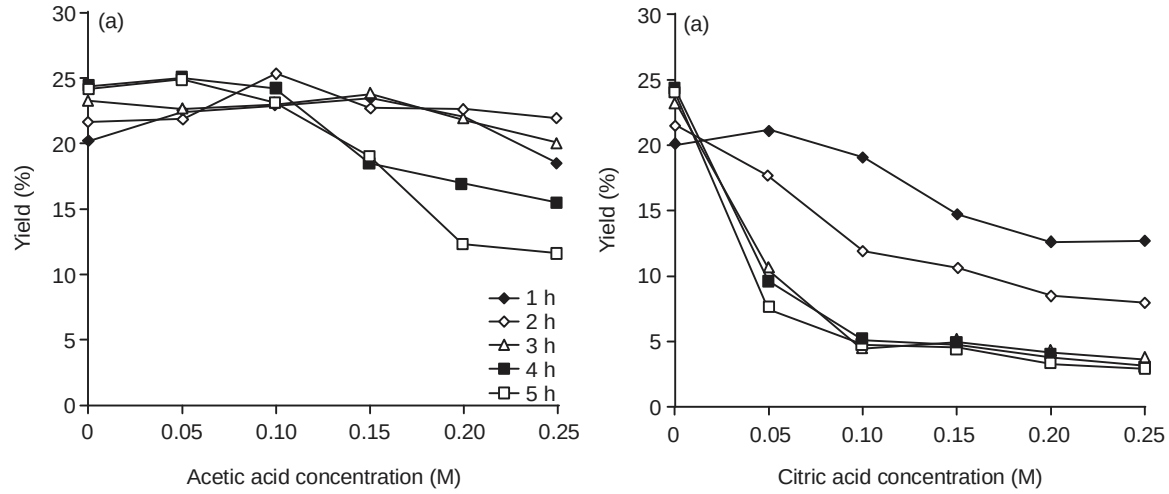

Fig. 1: Effect of acetic acid (a) and citric acid (b) treatments on the yield of gelatin extracted from nila fish (*Oreochromis nilotichus*) skin

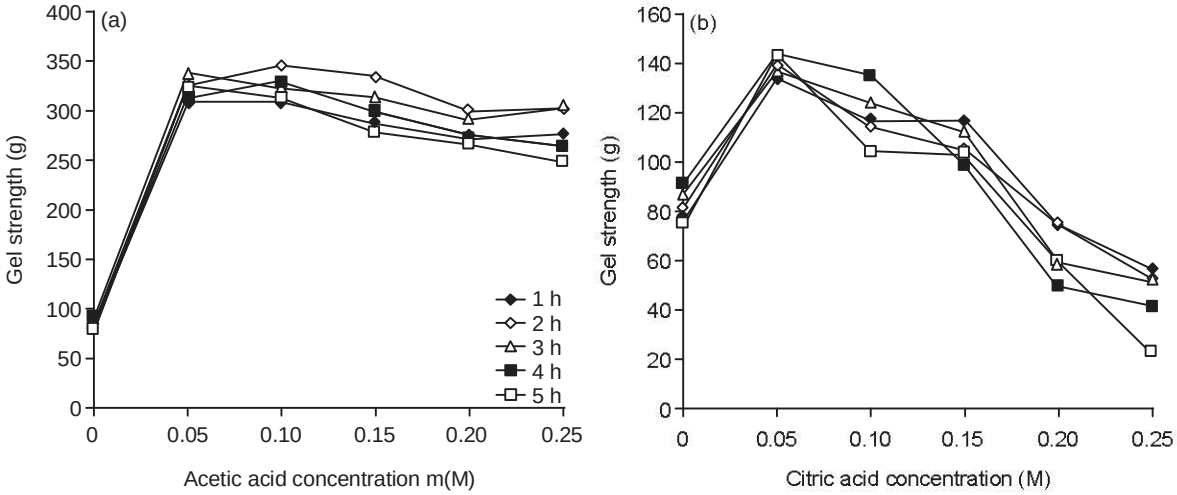

Fig. 2: Effect of acetic acid (a) and citric acid (b) treatments on the gel strength of gelatin extracted form nila fish (*Oreochromis nilotichus*) skin

gelatin yield (25.33%) was reached when soaking was performed in 0.10 M acetic acid (2 h). This result was higher than previously reported gelatin extractions using a combination of alkali and acetic acid (1.92- 21.55%) (Niu *et al*., 2013). At concentrations of acetic acid ≥0.15 M, the gelatin yield decreased with longer soaking times. Acid treatment could remove some acid-soluble proteins, lipids or other undesired components, as well as disrupt some cross-links in the collagen molecule (Ahmad and Benjakul, 2011) so that the collagen chain is cleaved and the gelatin released. During the washing process some gelatin is lost and these losses could increase with the increasing concentrations of acid. If citric acid was used, the highest yield (21.09%) was obtainedat a concentration of0.05M (1 h). This result was similar to previous nila skin gelatin extraction yields using a combination of alkali and citric acid (10.52-22.40%) (Niu *et al*., 2013). Figure 1 shows that the higher citric acid concentrations led to decreased gelatin yields ($p<0.05$). Acids with multiple ionized groups, such as citric acid, have a greater number of reactive hydrogen ions at low concentration, which could help extract gelatin (Niu *et al*., 2013).

Gel strength: Figure 2 shows the gel strength (g) of the gelatin from the nila fish (*Oreochromis niloticus*) skin extracted using acid at various concentrations and soaking times.

Acid concentrations are expected to affect the gel strength of gelatin from nila fish skin (Fig. 2). Extraction with no acid resulted in the lowest gel strength values (<100 g bloom). The highest gel strengths were 346.16 and 144.21 g when extracted with acetate at 0.10 M (2 h) and citric

Fig. 3: Infrared absorbance spectra of the molecular structure of nila fish (*Oreochromis nilotichus*) skin gelatin

acid at 0.05 M (1 h), respectively. Figure 2a shows that as the acetic acid concentration increased, the gel strength increased, reached a maximum value and then decreased ($p<0.05$). Acid treatment could disrupt some cross-links in the collagen molecules, caused skin swelling, which increased the gel strength. At a concentration of acetic acid ≥0.15 M for soaking times ≥3 h, the gel strength decreased; however, this gel strength was still relatively high (>200 g). Acetic acid is an organic acid with a weak ionization power, whereas there were many chains of collagen bonds to break. Figure 2b shows that as the concentration of citric acid increased, the gel strength decreased ($p<0.05$). Gelatin from nila fish skin extracted using citric acid at a concentration of ≥0.15 M and a soaking time of ≥3 h had a very low gel strength (<80 g). This value was lower than the standard quality gel strength for gelatin based on the GMIA, which is 50-300 g (Anonymous, 2012a).

Proximate composition: The approximate composition of gelatin from the skin of nila fish (*Oreochromis nilotichus*) prepared from the best extraction conditions found above are shown in Table 1.

As shown in Table 1, the approximate composition of fresh nila fish (*Oreochromis nilotichus*) skin is significantly different from the approximate composition of the gelatin from the nila skin treated by using acetic acid 0.10 M (2 h) (GAs) and citric acid 0.05 M (1 h) (GSi). These results show the effect of the acid treatment during the gelatin extraction; ash, fat and carbohydrate content are reduced, but the protein content is increased. GAs had higher ash

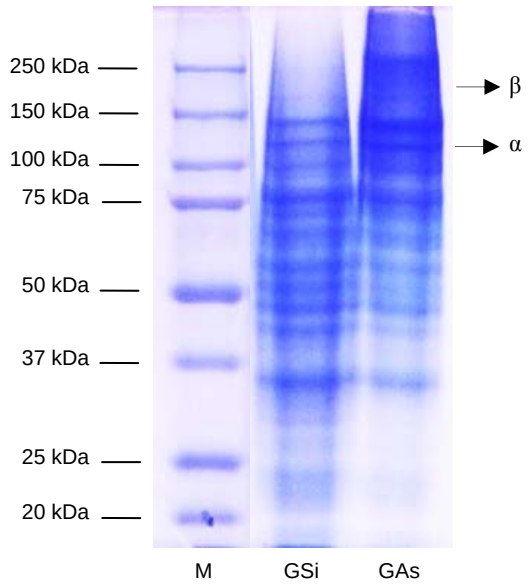

Fig. 4: Molecular weight of nila fish (*Oreochromis nilotichus*) gelatin components (GAs, soaked in 0.10 M acetic acid; GSi, soaked in 0.05 M citric acid)

and protein content compared to GSi, but the fat was lower in GAs than in GSi. Ash content was likely higher because this mineral was trappedin thecollagen. The GSi had a lower protein content than the gelatin of GAs, but it was still higher than the gelatin from walking catfish (77.88%), striped catfish (80.02%) (Jamilah *et al*., 2011),

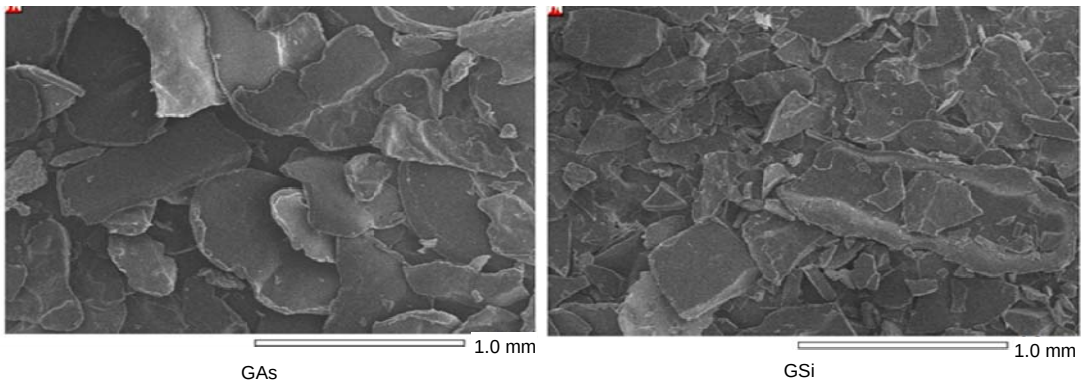

Fig. 5: Microstructure molecule of nila fish (*Oreochromis nilotichus*) gelatin (GAs, soaked in 0.10 M acetic acid; GSi, soaked in 0.05 M citric acid)

catfish (87.81%), pangasius catfish (81.61%), snake-head (75.63%) (See *et al*., 2010), fresh yellowfin tuna skin (81%) and red snapper (71%) (Pranoto *et al*., 2011). The protein content was similar to that of gelatin extracted from red nila fish (89.70%) (See *et al*., 2010),young Nile perch skin (87.4-88.8%), adult Nile perch skin (87.9-88.7%) (Muyonga *et al*., 2004a) and Cobia skin (79.12%) (Amiza and Siti, 2011). The difference in protein content might be due to a higher loss of soluble components during the soaking treatment in citric acid, which had higher ionization power. The soaking time of the acid treatment could also reduce the mineral content by cleaving hydrogen bonds and amidebonds (Ward and Courts, 1977). Low ash content is used as a marker for gelatin quality. GAs and GSi had ash contentsof 0.94 and 0.78%, respectively, which was higher than the standardfor gelatin quality from the GMIA of 0.5% (Anonymous, 2012); however, the Indonesian National Standard (SNI, 1995) requires that the ash content of gelatin be below 3.25%. Ash content of the gelatin prepared in this study was higher than gelatin prepared from fresh yellow fin tuna skin (3.66%) and fresh red snapper skin (4.02%) (Pranoto *et al*., 2011). Low ash content could be obtained by increasing the soaking time or the acid concentration.

Carbohydrates in gelatin are associated with glycation of collagen that is required in the formation of pentosidine cross-links (Kent *et al*., 1985; Muyonga *et al*., 2004a). Glycation in collagen was damaged by acid treatment and high temperatures in these extraction conditions. It seems that the carbohydratelevels in the fresh nila fish skin (23.09%) were significantly higher than in GSi (8.24%), while GAs had no carbohydrates because they were trappedin thecollagen.

Fourier transform infrared (FTIR) spectroscopic analysis

Figure 3 shows the absorbance spectra of the nila skin gelatin using FTIR spectroscopic analysis. The molecular structure of the gelatin was detected in four regions at wavenumbers of 3600-2300 cm^{-1} (Amide A), 1656-1644 cm^{-1} (Amide I), 1560-1335 cm^{-1} (Amide II) and 1240-670 cm^{-1} (Amide III) (Muyonga *et al*., 2004b). Gelatin from nila skin treated with 0.5 M citric acid (1 h) showed no significant differences in the spectral features compared to that of 0.1 M acetic acid (2 h). In nila fish gelatin, GAs had more intense vibration bands than GSi. For all samples, vibration bands increased at wavenumbers of 3443-2926 cm^{-1} (Amide A), 1640 cm^{-1} (Amide I), 1541-1335 cm^{-1}(Amide II), 1239 cm^{-1} (Amide III) and 1100-1000 cm^{-1}.

The characteristicsof the gelatin spectra showed high intensities for amide I and amide II, while the amide III peak was less intense. The amide III region isassociated the N-H bond-stretching mode of hydrogen bonded amide groups, which are formed parallel to the helix axis in α-helical structures (Hashim *et al*., 2010). Thus, changes in this region occur upon denaturation of a triple helix protein tertiary structure in the gelatin at the high temperaturesexperienced during the extraction. Amide I absorption contains C=O stretching vibration of the amide group (approximately 80%) with a minor contribution froma C-N stretching vibration. The amide I band has a characteristic strong absorbance in the range wavenumbers of 1600-1700 cm^{-1}, which is the absorption band for the C=O stretching vibration along the polypeptide backbone. Amide II absorption contains N-H bending (60%) and C-N stretching (40%). The amide I and II absorptions are predicted by the strength of any hydrogen bonds involving amide C=O and N-H groups. The amide III absorption contains more C-N stretching vibrations coupled to N-H bending vibrations, with weak contributions from C-C stretching and C=O bending (Jackson and Henry, 1995). The region in between 1100-1000 cm^{-1} includes the vibrations arising from C-O and C-O-C stretching of the carbohydrate residues present in collagen (Cebi *et al*., 2016). The amide A region at wavenumbers of 3290-3280 cm^{-1} is dominated by the N-H bond stretching mode of the hydrogen bonded amide groups (Hashim *et al*., 2010) and amide A tends to merge with the CH_2 stretching when carboxylic acid groups exist in stable dimeric (inter molecular) associations (Muyonga *et al*., 2004b).

Table 1: Proximate compositions of nila fish (*Oreochromis nilotichus*) skin gelatin

Sample	Ash (%db)	Fat (%db)	Crude protein (%db)	Carbohydrate (% db)
GAs	0.94±0.37[a]	0.43±0.07[a]	99.56±0.57[a]	-
GSi	0.78±0.16[a]	1.15±0.50[a]	88.84±0.18[b]	8.24±0.49[b]
KNs	3.1±0.08[b]	5.08±0.76[b]	68.80±0.12[c]	23.09±0.35[c]

GAs: Nila skin gelatin treated with 0.10 Macetic acid (2 h)
GSi: Nila skin gelatin treated with 0.050 Mcitric acid (1 h)
KNs: Nila fish (*Oreochromis nilotichus*) skin fresh
a-c, Different letters indicate significantly different values ($p<0.05$)

Molecular weight distribution: The acid treatment in the gelatin extraction affected the molecular weight distribution of the product. The molecular weight of the gelatin treated with 0.05 M acetic acid (2 h) had a higher molecular weight ranging from 38.37 to 221.31 kDa, whereas nila skin gelatin treated with 0.10 M citric acid (1 h) had molecular weights ranging from 21.43-144.21 kDa (Fig. 4).

According to Sinthusamran *et al*. (2014) gelatin containing β-chain and α-chain molecules had molecular weights of 193 and 125-113 kDa, respectively. The α-chains and β-chains of the mother collagen were retained during degradation. The band intensity of the α-chains and β-chains in the gelatin slightly decreased with increasing extraction temperature. There are two α chains: α1 and α2. The β chain consists of two covalently bound α chains and the γ chain consists of three covalentlybound α chains. The α1 molecule contains fewer histidines, tyrosines and hydroxyl-lysines and more hydroxyl-prolinesand hydrophobic amino acids, such as valine, leucine and isoleucine, compared to α2 (Ward and Court, 1977). The acetic acid treatment could produce gelatin with higher molecular weight distributions than the citric acid treatment. Gelatin from acetic acid treatment contains more β-chains and α-chains, while gelatin from citric acid treatment only contains the α component. As a result, the gel strength of the GAs was higher than that of GSi.

Microstructure of the gelatin molecules: The microstructure of the gelatin molecues extracted using different acids is shown in Fig. 5. Gelatin from nila skin treated with 0.10 M acetic acid (2 h) had a larger microstructure and tended be uniform in shape. In contrast, the nila skin gelatin prepared using 0.05 M citric acid (1 h) had an irregular microstructure. Gelatin extracted using acetic acid had more β-chains, which consists of two α-chains; hence,this gelatin had a larger micro structure and higher gel strength.

Conclusion: The acetic and citric acid treatments at various concentrations and soaking times during the extraction of gelatin affected the gel strength and yield. Gelatin from nila skin prepared using a 0.10 M acetic acid solution and a soaking time of 2 h exhibited good gel strength and yield. In contrast, citric acid treatment at 0.05M with a soaking time of 1 h produced gelatin with a lower gel strength and yield. However, gelatin from nila skin produced with either acid could be applied in the food industry.

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