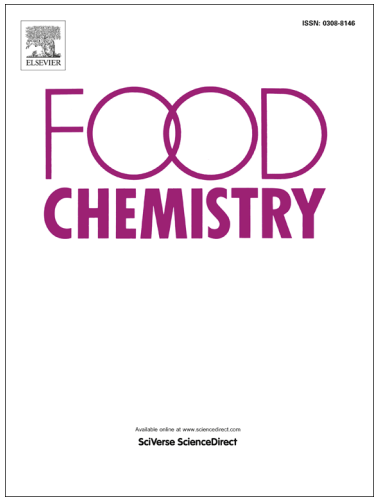

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Yuqing Tan, Sam K.C. Chang

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Isolation and Characterization of Collagen Extracted from Channel Catfish (*Ictalurus punctatus*) Skin

Author names:

Yuqing Tan[1], Sam K. C. Chang[1*]

Affiliation:

[1]Department of Food Science, Nutrition and Health Promotion, Mississippi State University, Mississippi State, MS, 39762, United States

[*] **Corresponding Author**:

Sam K. C. Chang, PhD, Email: sc1690@msstate.edu

Telephone: 662-325-5014

Email Address:

Yuqing Tan: yt142@msstate.edu

Sam K. C. Chang: sc1690@msstate.edu

Address: Department of Food Science, Nutrition and Health Promotion

945 Stone Blvd, Herzer Building, Mississippi State, MS, 39762, USA

Abstract

Channel catfish skin is a by-product from catfish fillet production. Collagens were extracted from catfish skins by: (1) acid extraction; (2) homogenization-aided; and (3) pepsin-aided extraction methods. Kinetic analysis of extraction was performed. SDS-PAGE was carried out for all collagens extracted under different conditions. Protein solubility, zeta potential, circular dichroism and gel strength methods were used to characterize the collagen extracted by three methods to determine optimal conditions. Protein recovery rate from minced skins extracted with pH 2.4 HCl containing 23.6 KU/g pepsin was the highest (64.19%). SDS-PAGE showed that collagens extracted with different methods had different proteins ratio patterns, even though the molecular mass of collagen subunits were similar, 123 and 113 KDa for α_1 and α_2 chains, 226 KDa for β chain and 338.5 KDa for γ chain, respectively. Channel catfish skin collagens were typical type I collagens and could have applications in food, medical and cosmetic industries.

Keywords: channel catfish skin, collagen, homogenization, pepsin, kinetic analysis

Research Highlights

1. Collagen from channel catfish skin was isolated and characterized.
2. Collagen extraction methods were improved for practical use.
3. Kinetic analysis was performed for collagen extraction processing.
4. Gel strength of collagen extracted by different methods were compared.

1. Introduction

Collagen is a long cylindrical protein, it is the major fraction and contributes to the unique physiological function of connective tissue in skin. Up to 27 types of collagen have been identified, and type I collagen exists the most widely in connective tissues. Type I collagen is made up of three polypeptide chain, two of the polypeptides are designated α_1, and α_1 bonded to another chain to form a third chain α_2 through hydrogen bond. Total molecular mass of collagen is about 300 KDa with each chain has a molecular mass of about 100 KDa, and it has a wide range of application of pharmaceutical, leather, biomedical and film industries (Ogawa, Portier, Moody, Bell, Schexnayder, & Losso, 2004). Collagen usually extracted from porcine skins and bones, however, it is not acceptable by Judaism and Islam due to religious restrictions (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007). In addition, collagen extracted from bovine might be contaminated with bovine spongiform encephalopathy and transmissible spongiform encephalopathy (Choi & Regenstein, 2000). Therefore, aquatic sources for collagen production is a substitution for mammalian sources even though the yield of collagen from aquatic sources is much lower than that from mammalian sources. However, the yield of collagen extracted from fish skin was greatly improved in recent years (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). Researchers have been reported that around 50% of collagen can be isolated from fish skin, and 45% of collagen from fish bone (Nagai & Suzuki, 2000).

Channel catfish aquaculture is an important aquaculture in the United States, and Mississippi State rank No. 1 in catfish production with annual production of $185 million in 2015 (MSU extension data). Catfish fillet processing contributes to the vitality of the local economy. The fish skins account for about 10% of the by-products and can be used

as a potential collagen source. More and more studies about different alternative sources and new functionalities of collagen had been reported in the last 10 to15 years. However, most studies used a single set of extraction solvent conditions. There is a lack of a systematic approach for collagen extraction, particularly for catfish collagen extractions. And the extraction yield of collagen from fish skins still remained low, and the extraction method have less practical application for food industries since most of them required long time, high energy input and dialysis processing. In addition, no systematic kinetic analysis of extraction yield has been performed. Our objectives were to use a systematic approach to optimize the extraction condition and modify the extraction method to recover the maximum yield, and to characterize the properties of collagen extracted with different extraction conditions.

2. Material and method

2.1 Materials

Catfish skin was collected from a local catfish fillet processing (Country Select, Isola, MS). The catfish skin was buried in crushed ice during transportation to our laboratory and stored at -80°C until use.

2.2 Chemicals

All chemicals, reagents and porcine gastric pepsin (EC 3.4.23.1) were analytical grade and obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A).

2.3 Proximate analysis

Moisture, protein, ash and total fat content were determined by AOAC International methods 934.01, 955.04, 942.05 and 2003.06, respectively (AOAC International, 2012). A conversion factor 5.4 was used for calculating the protein content

from nitrogen content. The hydroxyproline contents in skin material and extracted collagen were determined according to the method of Bergman and Loxley (1963). The protein recovery rate (%) was obtained by the following equation:

$$\text{Protein recovery rate (\%)} = \frac{\text{hydroxyproline content in extracted collagen sample}}{\text{hydroxyproline content in skin material}} \times 100\%$$

2.4 Extraction of collagen with acids (ASC)

Catfish skins were pretreated by washing with iced water containing 1% NaCl (1:6 W/V) and ground into small pieces, then passed through 35 mesh sieve. One hundred grams of catfish skins were mixed with 4 different acids (acetic acid, hydrochloric acid, citric acid and lactic acid, W:V = 1:50) with varies of pH (1.8, 2.1, 2.4, 2.7 and 3.0), and shaken for 48 hr (100 rpm) using an orbital shaker at 4°C, then centrifuged at 15000 *g* for 20 min at 4°C to collect the supernatant, the residue was re-extracted with the corresponding acid (W/V = 1:50) for another 12 hr. After the desire time was achieved, the mixture was centrifuged at the same condition, the supernatant was combined and salted out by adding NaCl to a final concentration of 0.9 M, the precipitated collagen was separated by centrifugation at 15,000 g for 15 min at 4°C. The resultant precipitate was washed quickly by type-I water (ultrapure water) (W/W = 1:2) for 3 times to remove NaCl and then lyophilized.

2.5 Extraction of collagen with homogenization-aided (HSC) method

Preliminary experiments showed that homogenization (Model: GLH 580, Spindle: 30*195 mm, Omni International, Kennesaw, GA, USA) at solid-to-liquid ratio (1: 50, w/v) for 5 min was the optimal condition for extraction. Therefore, catfish skins were mixed with hydrochloric acid (store in 4°C for 6 hours in advance) at pH 2.3, 2.4 and 2.5 with solid-to-liquid ratio varying from 1:15 to 1:50 (W/V). The mixture was then

homogenized at 7000 rpm for 5 s and stop 5s, the cycle was repeated until 5 min was achieved, the mixture was kept in a foam box which filled with ice during homogenization processing. After homogenization, the mixture was stirred for 1 hr in 4°C. After the desired time was achieved, the mixture was centrifuged at the same condition in Section of 2.4, and the following procedure was the same as described in Section of 2.4.

2.6 Extraction of collagen with pepsin and homogenization aided (PHSC) method

Catfish skins and hydrochloric acid (pH 2.4) were mixed with solid to liquid ratio varying from 1:5 to 1:20, and pepsin concentrations from 0.118 to 23.6 KU/g skin. The mixture was homogenized at 7000 rpm for 5 s and stop 5s, the cycle was repeated until 5 min was achieved. The mixture was centrifuged at the same conditions as described in Section 2.4, the following procedures was the same as described in 2.4.

2.7 Kinetic analysis of collagen extraction

All the extraction curves could be described by the model developed by Peleg (1988):

$$C(t) = C(0) + \frac{t}{K_1 + K_2 \cdot t} \quad (1)$$

Where C(t) (%) is the protein recovery rate at time t, t is the extraction time (hours), C_0 (%) is the protein recovery rate at time t = 0, K_1 is Peleg's rate constant and K_2 is Peleg's capacity constant. C_0 in all experimental was zero, the above equation was rearranged in the following form:

$$C(t) = \frac{t}{K_1 + K_2 \cdot t} \quad (2)$$

The Peleg rate constant K_1 relates to extraction rate (B_0) at the very beginning：

$$B_0 = \frac{1}{K_1} \quad (3)$$

The Peleg's capacity constant K_2 relates to maximum of protein recovery rate. When $t \rightarrow \infty$, the following equation describes the relations between protein recovery rate and K_2 constant:

$$C|t \rightarrow \infty = \frac{1}{K_2} \quad (4)$$

Therefore, equation (1) can be transformed to a linear relationship in the final form:

$$\frac{t}{C(t)-C(0)} = K_1 + K_2 t \quad (5)$$

2.8 Sodium-dodecyl-sulfate gel electrophoresis (SDS-PAGE)

Electrophoresis was carried out according to the method of Laemmli (Laemmli, 1970). Collagen samples were dissolved in 0.02 M sodium phosphate (pH 7.2) containing 0.5 M urea. Electrophoresis was performed on a 6% resolving gel and a 4% stacking gel. Proteins were stained with 0.1% Commassie Brilliant Blue R-250 dissolved in water, methanol and acetic acid (9:9:2, v/v/v) for 30 min, then destained using a solution containing water, methanol and acetic acid (17:1:2, v/v/v). For the quantification of α and β chains, gels were scanned and analyzed by a Molecular Imager (Bio-Rad Chemidoc™ XRS+, Hercules, CA, USA) equipped with Image Lab™Analysis Software (version 5.2).

2.9 Effect of pH on solubility

Collagen extracted with different conditions were dissolved in 0.5 M acetic acid to obtain a final concentration of 3 mg/mL. The pH of protein solution was adjusted with 2 mol/L HCl or 2 mol/L NaOH to 1 to 10. The solution was centrifuged at 10,000 *g* for 20 min at 4°C. Protein content in the supernatant was determined by the method of Bradford (1976).

2.10 Effect of NaCl on solubility

Collagen extracted with different conditions were dissolved in 0.5 M acetic acid to obtain a final concentration of 6 mg/mL. Five milliliters of protein solution were mixed with 5 mL of 0.5 M acetic acid containing a serious concentration of NaCl to make a final concentration of 0%, 1%, 2%, 3%, 4%, 5% and 6%. Protein solutions were centrifuged at 10,000 *g* for 20 min at 4°C. The protein concentration in the supernatant was determined by the method of Bradford (1976).

2.11 Zeta (ζ) potential

Collagen extracted by the above three methods with optimal conditions was used. Zeta potential of collagen samples were determined by a Zeta potential analyzer (Zetasizer Nano ZS90, Malvern Instr., UK). Collagen extracted with different conditions were dissolved in 0.1 M acetic acid to make a final concentration of 1 mg/mL. One milliliter collagen solution was transferred to a capillary cell, and the pH of the collagen solutions were adjusted to 2-6 using 1 M nitric acid or 1 M KOH. The Zeta potential of each solution was measured in triplicate.

2.12 Circular dichroism (CD)

Collagen extracted by the above three methods with optimal conditions was used. The prepared collagen samples were dissolved in 0.1 M acetic acid and diluted to a concentration of 0.2 mg/mL with 0.1 M acetic acid solution. CD were measured using a Jasco 810 spectropolarimeter (Jasco International Co., Easton, MD) with a Peltier jacketed software-controlled multiple cuvette holder. The CD spectrum of the samples were measured using a 0.1 cm path length quartz cell for far-UV measurement. The spectrum was recorded from 190-250 nm in 0.1 nm steps with response times of 1 s. Five

scans were averaged for each sample.

2.13 Differential scanning calorimetry (DSC)

Collagen extracted by the above three methods with optimal conditions was used. Differential scanning calorimetry of collagen was analyzed according to the method reported previously with some modifications (Singh, Benjakul, Maqsood, & Kishimura, 2011). Collagen was rehydrated by 0.05 M acetic acid at a solid to liquid ratio of 1:40 (w/v). The mixtures were allowed to stand at 4°C for 2 days. DSC was carried out using a differential scanning calorimetry (Perkin Elmer, Model DSC8000, Norwalk, CA, USA). Forty µL of samples were accurately pipetted into aluminum pans and sealed, an empty pan was used as reference. The sample was scanned at 1°C/min over the range of 20-50°C in a nitrogen atmosphere.

2.14 Gel strength

The bloom strength of the collagen extracted by the above three methods with optimal conditions was determined according to the British standard BS 757 (BSI, 1975). The gels were formed in a standard bloom bottle by mixing 7.5 g of dry collagen samples with 105 mL distilled water (6.67%, W/V), and stir 1 h then bring to 60°C water bath for 30 min, then cooling down the solution in 4°C refrigerator for 12-16 hr. Bloom gel strength was determined by TA.XTplus Texture Analyzer (Stable Microsystems, Godalming, Surrey, UK) equipped with 0.5 inch diameter plunger with cross-head speed 0.5 mm/s. The standard glass bloom bottle was placed on the central of the plate. The maximum force (g) was determined when the plunger penetrate into 4 mm of the gel. Three replications were averaged for each collagen sample.

2.15 Statistic analysis

185 Experiments were performed based on a completely randomized design.
186 Extraction was performed in triplicate. Data were analyzed by ANOVA using 2015 SAS
187 (version 9.3, SAS Inc., Cary, NC, USA). Duncan's multiple range test was carried out to
188 determine any significant differences between different extraction conditions.

189 **3 Results and discussion**

190 **3.1 Proximate analysis of catfish skin**

191 Several studies had been reported the protein content in fish skin, Nile perch skin
192 contained 21.6% protein (Muyonga, Cole, & Duodu, 2004a) and bigeye snapper skin
193 possessed 32% of protein (Kittiphattanabawon, Benjakul, Visessanguan, Nagai, &
194 Tanaka, 2005). Protein content in catfish skin was determined in this study by the
195 Kjeldahl method and a conversion factor 5.4 was used. The protein content in catfish skin
196 was 28.88% based on the wet weight basis, and 81.21% on the dry weight basis, which is
197 comparable to the above fish species. Lipid, ash and carbohydrate contents in catfish skin
198 are 14.29%, 1.88% and 2.62% (dry weight basis), respectively. Theoretically, the
199 collagen yield should not be higher than the total protein content in catfish skin. Taking
200 the energy, solvent and time input into consideration, an extraction method with less
201 input and higher yield is expected by food industries.

202 **3.2 Collagen extracted with acids and proximate analysis**

203 In most studies, 0.1 M acetic acid (Liu et al., 2015; Nagai & Suzuki, 2000) or 0.5
204 M acetic acid (Chen, Li, Yi, Xu, Gao, & Hong, 2016; Kittiphattanabawon, Benjakul,
205 Visessanguan, Nagai, & Tanaka, 2005; za, Cole, & Duodu, 2004a; Nagai, Araki, &
206 Suzuki, 2002) were used to extract skin collagen. In the current study, different acids,
207 liquid-to-solid ratios and pH's were used to extract catfish skin collagen to search for a

208 better extraction conditions. The yield of collagen extracted with different acids were
209 expressed as protein recovery rate (%), and results are shown in Figure 1A. The protein
210 recovery rate from minced skin extracted with pH 2.4 hydrochloric acid was the highest
211 (42.36%) followed by extraction with pH 2.7 acetic acid (39.45%). Lactic acid and citric
212 acid are less effective in terms of protein recovery rate. And this observation was in
213 conflict with the report which claimed that HCl was the least effective solvent for
214 collagen extraction from cod skin (Skierka & Sadowska, 2007). The reason might be that
215 different concentration of acids were used to extract collagen from cod skin, and the pH
216 of the mixture was not maintained the same as the beginning pH, which tended to change
217 over time of extraction.

218 Collagen was successfully isolated from several fish species with the yield
219 ranging from 2% to 51.4% (Liu, Li, & Guo, 2007;Chen, Li, Yi, Xu, Gao, & Hong, 2016;
220 Nagai, Araki, & Suzuki, 2002; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, &
221 Tanaka, 2005; Ogawa, Moody, Portier, Bell, Schexnayder, & Losso, 2003; Duan, Zhang,
222 Du, Yao, & Konno, 2009; Nagai, Yamashita, Taniguchi, Kanamori, & Suzuki, 2001;
223 Nagai & Suzuki, 2000). However, due to the impurity of the extracted collagen, some of
224 the reported yield may be higher than the real value. Though extraction for collagen from
225 fish skins have been reported in several studies, most of them used a single set of
226 extraction conditions. There is a lack of a systematic approach for collagen extraction,
227 particularly for catfish collagen extractions.

228 **3.3 Collagen extracted aided by homogenization with or without pepsin**

229 Since pH 2.4 HCl gave the highest protein recovery rate in Section 3.2, therefore,
230 pH interval was decreased to investigate the optimal pH in this step. The results of

protein recovery rate of collagen extracted with homogenization-aided method are shown in Figure 1B. The protein recovery rate from minced skin extracted with pH 2.4 hydrochloric acid at solid-to-liquid ratio of 1:50 (W/V) was the highest (60.38%) but with no significant differences from that extracted by pH 2.4 hydrochloric acid at solid-to-liquid ratio of 1:40 (w/v) (57.68%). Taking the solvent input into consideration, minced skin extracted with pH 2.4 hydrochloric acid with the solid-to-liquid ratio of 1:40 (w/v) was an optimal condition for scale up in the food industries. Studies had reported collagen extracted with homogenization aided method. Collagen from Plaice skin was extracted with homogenization aided method in 0.2 and 0.4 M NaCl solution. Unfortunately, no yield data was provided in this study (Montero, Alvarez, Marti, & Borderias, 1995). An extrusion-hydro extraction method had been used to extract collagen from tilapia scale with an yield of 16.9% (Huang, Kuo, Wu, & Tsai, 2016).

Peptides in the telopeptide region can be cleaved by pepsin, with the limited concentration of pepsin digestion, the cross-linked molecules at the telopeptide region can be cleaved by pepsin but without altering the triple helix structure of collagen (Liu, Li, Miao, & Wu, 2009). Therefore, the extraction of collagen partly cleaved by pepsin gave a higher yield (Chen et al., 2016; Nagai & Suzuki, 2002; Nagai, Yamashita, Taniguchi, Kanamori, & Suzuki, 2001). Although pepsin had been used widely for the collagen extraction, most the researchers reported pepsin added on a weight basis instead of enzyme activity unit basis (Chen et al., 2016; Liu, Li, & Guo, 2007; Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007). However, only one report used enzyme activities for calculating pepsin concentration (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007) for collagen extraction from the Bigeye snapper. Results of protein

recovery rate of collagen extracted with different concentration of pepsin and liquid-to-solid ratio are shown in Figure 1C. The protein recovery rate from minced skin extracted with pH 2.4 hydrochloric acid at the solid-to-liquid ratio of 1:30 (W/V) with pepsin content 23.6 KU/g was the highest (59.03%) but with no significant differences from that extracted by pH 2.4 hydrochloric acid at the solid-to-liquid ratio of 1:20 (W/V) and with pepsin content 5.90 KU/g (56.77%). In most collagen extraction study with pepsin presented, longer time (24-48 hr) was used to extract collagen at a low pH range (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007). However, we are the first to shorten the extraction time to less than 1 hr without compromising collagen yield. Comparing the extraction method aided by homogenization with or without pepsin, the highest protein recovery rate was not significant different (64.19% and 61.80%, respectively); however, the time and solvent input of extraction aided with pepsin was lower than that of extraction without pepsin. Therefore, homogenization-aided extraction with pepsin addition could be more practical for the food industries in terms of economy.

3.4 Kinetic analysis of collagen extraction

Kinetic analysis was carried out for collagen extraction at different extraction conditions (different acids, pH, liquid to solid ratio, particle size and enzyme concentration). The Peleg model was used for the kinetic analysis. Since most studies used 0.5 M acetic acid (pH around 2.6) to extract skin collagen, therefore, we compared four different acids to extract catfish skin collagen at this pH. The effect of different acids (pH 2.6) on collagen extraction is shown in Figure 2A. As expected, protein recovery rate showed an overall increasing pattern with time extended. The extraction curves indicated that hydrochloric acid gave the highest protein recovery rate and the lowest in the case of

277 citric acid extraction. All of these four types of acids exhibited a sharp increasing protein
278 recovery rate in the first 12 hrs, and then reached a plateau-like pattern. Figure 2B-F
279 showed the effect of pH of HCl, solid-to-liquid ratio (without homogenization), particle
280 size of catfish skin, solid-to-liquid ratio (with homogenization aided) and pepsin
281 concentration (with homogenization aided) on protein recovery rate. Figure 2B exhibited
282 the influence of pH on protein recovery rate, the highest protein recovery rate occurred at
283 pH 2.4 followed by pH 2.1. A significant low protein recovery rate was observed at pH
284 3.0, and the increasing patterns were similar with that in Figure 2B. Therefore, HCl with
285 pH 2.4 was used for the following experiments. Figure 2C showed the effect of different
286 solid-to-liquid ratios on protein recovery rate, and the highest protein recovery rate was
287 obtained at the highest solid-to-liquid ratio (1:50, w/v). Figure 2D showed the influence
288 of particle size on protein recovery rate, the highest protein recovery rate was obtained at
289 particle size less than 0.5 mm, and the lowest protein recovery rate was obtained at whole
290 skins. However, no other paper studied particle size effect, this makes comparing our data
291 with reports in the literature difficult. Protein recovery rate first exhibited a sharp
292 increasing in the first 48 hrs, and asymptotically approaching the equilibrium protein
293 recovery rate. Our objective was to develop a method with less extraction time and liquid
294 input, that would be useful for scale-up commercialization; therefore, homogenization
295 aided method was applied. Figure 2E showed the effect of different solid-to-liquid ratio
296 on protein recovery rate when homogenization aided. The highest protein recovery rate
297 was obtained at solid-to-liquid ratio of 1:50 (w/v), but not significant higher than 1:40.
298 However, the solvent input was still high for the scale-up extraction in the food industries.
299 Therefore, enzyme-aided extraction method was applied as described in Section 2.6.

Figure 2F exhibited that the effect of pepsin concentration on protein recovery rate when aided by homogenization. The highest protein recovery rate was obtained at pepsin concentration of 23.6 KU/g, but not significantly higher than that of pepsin concentration at 5.9 KU/g. There are no reported studies to compared with our results since this study is the first to conduct kinetic analysis on fish skin collagen extraction. Compare to other fish skin collagen extraction studies, 51.4% of collagen (based on dry weight) was extracted from Japanese sea-bass with extraction for totally 5 days but with no solid-to-liquid ratios reported (Nagai & Suzuki, 2000); 27.2% of collagen was extracted from tilapia (based on dry weight) with extraction for over 24 hours but with no solid-to-liquid ratios provided (Chen, Li, Yi, Xu, Gao, & Hong, 2016); 46% of collagen was extracted from carp skin with extraction for over 24 hrs and 40 volumes of liquid input (Liu, et al., 2015). The Peleg extraction constants are shown in Table 1. K_1 relates to extraction rate at the very beginning, and Peleg capacity constant K_2 relates to maximum of extraction yield. As shown in Table 1, the lowest K_1 and K_2 were found in pepsin-aided extraction with enzyme activity of 23.6 KU/g. The values of extraction rate constant (K_1) and constant of extraction extent (K_2) exhibited a tendency of decreasing with the increase of solid-to-liquid ratio and pepsin concentration. The lower the extraction rate constant and constant of extraction extent suggested a higher yield with less time.

3.5 SDS-PAGE of collagen extracted with different conditions

Figure 3A-B showed SDS-PAGE patterns of collagen extracted with different conditions. All collagen products extracted with different conditions had two different α chains (α_1 and α_2) and their cross-linked chains (dimer is referred to as β chain, trimer is referred to as γ chain). The molecular weight of α_1 chain is 123 KDa and 113 KDa for α_2

chain. The α_1 chain might contain a α_3 chain that has molecular weight close to α_1 chain, resulting a indistinguishable band on a SDS-PAGE gel (Kimura, Zhu, Matsui, Shijon, & Takamizawa, 1988). And the molecular mass of subunit was 226 KDa for β chain and 338 KDa for γ chain, respectively. These values were similar to the molecular mass of black drum and sheepshead seabream skin collagen (Ogawa, Moody, Portier, Bell, Schexnayder, & Losso, 2003), carp skin collagen (Duan, Zhang, Du, Yao, & Konno, 2009) and tilapia skin collagen (Chen, Li, Yi, Xu, Gao, & Hong, 2016). However, extracted collagens described in the above literature were not from the channel catfish species. *Channel* catfish skin collagen was successfully isolated but with no molecular mass of the subunits reported (Liu, Li, & Guo, 2007). In addition, skin collagen of striped catfish was isolated and characterized before, however, no molecular mass of each subunits provided (Singh, Benjakul, Maqsood, & Kishimura, 2011). The existence of two different α chains confirmed that the major collagen from catfish skins was type-I collagen (Sato, 1993). Band intensity ratio of $\beta/(\alpha_1+ \alpha_2)$ chains in acid-solubilized collagen (ASC), homogenization-solubilized collagen (HSC) and pepsin-homogenization solubilized collagen (PHSC) were 1.94, 1.72 and 0.41, respectively. The β chain was richer in acid-extracted collagen than that in pepsin-aided extracted collagen, and 55.5% of the β chain degraded into α_1 and α_2 chains. The reason might be that some β chains were converted to α chains by pepsin, this observation was similar to the study of skin collagen from channel catfish, black drum and sheepshead seabream (Ogawa, Moody, Portier, Bell, Schexnayder, & Losso, 2003; Zhang et al., 2016). As mentioned above, pepsin removes the cross-linked containing telopeptide, and one β chain is converted to two α chains (Kaori Sato, Ebihara, Adachi, Kawashima, Hattori, & Irie, 2000). Since no

studies had used homogenization-aided method to extract fish skin collagen, therefore, our studies presented the first homogenization effect on catfish collagen.

3.6 Effect of pH and NaCl on collagen solubility

3.6.1 Effect of pH on collagen solubility

Collagen extracted with pH 2.4 HCl for 48 hrs (ASC), homogenized with pH 2.4 HCl for 5 min (HSC) with (PHSC, pepsin concentration 11.8 KU/g) or without pepsin were used for solubility characterization. The effect of pH on the solubility of collagen extracted with different methods are shown in Figure 4A. ASC, HSC, and PHSC showed the highest solubility at pH 2 ($p < 0.05$). All the collagens were solubilized at low pH range (1 to 4), and had a sharp solubility decreasing was observed when pH higher than 5 ($p < 0.05$). When the pH was not equal to pI, the net charge of the protein molecules was larger than zero, therefore, the solubility of the protein was increased by the repulsion forces between chains. In contrast, when the pH was equal or close to pI, the total net charge of the protein molecules approached zero and resulting the precipitation. It had been reported that the pI of collagen is varying from 6 to 9 (Foegeding, Lanier, & Hultin, 1996). The minimum solubility of collagen is around pH 6 in the current study, and this observation was similar with the solubility of collagen from brown-striped red snapper skin (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). Acid-extracted collagen exhibited higher solubility than the other two collagens at pH 1 to 4 but with no significant differences ($p < 0.05$). However, collagen extracted with pepsin from bigeye snapper had been reported to have the highest solubility at pH 5 (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2007), which is more close to isoelectric point (6 to 9).

3.6.2 Effect of NaCl on collagen solubility

Solubility of ASC, HSC and PHSC in 0.5 M acetic acid remained consistent at NaCl concentration at 0-2% ($p > 0.05$), and the results are shown in Figure 4B. A slight decrease in solubility was observed at 3% NaCl. A sharp decrease was observed when the concentration of NaCl increased to 4% and above ($p < 0.05$). Solubility of collagen extracted from skin of bigeye snapper, striped catfish, tilapia and brown stripe red snapper (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Singh, Benjakul, Maqsood, & Kishimura, 2011; Chen, Li, Yi, Xu, Gao & Hong, 2016) also decreased with the increasing concentration of sodium chloride. When higher concentration of sodium chloride (> 3%) presented, an increasing ionic strength might lead to a reduction of protein solubility by improving the interaction between protein chains. Thus, solubility of protein might be decreased by salting out effect via increasing hydrophobic interaction and aggregation, which competing with the protein for water. Collagen extracted by homogenization method with pepsin addition showed a higher solubility than the other two collagens, and this observation was in accordance with the collagen extracted with acid and pepsin from striped catfish skin (Singh, Benjakul, Maqsood, & Kishimura, 2011). Greater solubility of PHSC is due to the action of pepsin in altering the collagen structure and altered the compositions of α, β and γ chains as discussed in section 3.3. Therefore, collagen extracted by different methods might have slightly different molecular properties. Comparing Figure 3A and Figure 3B, pepsin-added extraction produced a high band intensity of α_1 and α_2 chains, which were derived from the β chain in non-pepsin extracted collagen (Figure 3A).

3.7 Zeta (ζ) potential

Zeta potential is used for colloidal dispersions, it is the e^- potential difference between dispersion medium and the slipping plane of the dispersed particles. A high zeta potential will give high stability to resist aggregation. In contrast, when the zeta potential is small or close to zero, attractive forces may be larger than repulsion force, and the dispersion may form aggregates. The zeta potentials of ASC, HSC and PHSC solutions at different pHs are shown in the Figure 5A. The zero surface net charge of ASC, HSC and PHSC was observed at pH of 5.34, 5.73 and 5.42, respectively. The total net charge of protein would be zero when the pH of protein close to zero (Vojdani, 1996). The results of effect of pH on protein solubility indicated that PHSC had the lowest solubility at pH 6, which suggested that the isoelectric point of ASC, HSC and PHSC was around 6. Thus, the results of zeta potential and solubility test were mutual supportive. And the differences in surface charge of ASC and HSC might be due to the differences in acidic and basic amino acid residues, which were more likely dominated by the removal of telopeptides by high-speed homogenization. The zero surface charge of collagen extracted by acid and pepsin from striped catfish were 4.72 and 5.43, respectively (Singh, Benjakul, Maqsood, & Kishimura, 2011). Collagen extracted with acid and pepsin from brown-banded bamboo shark skin were reported had a zero surface charge at 6.21 and 6.56, respectively (Kittiphattanabawon, Benjakul, Visessanguan, Kishimura, & Shahidi, 2010). In this study, PHSC showed a higher zero surface net charge than ASC, and this observation was in accordance with other studies which described above.

3.8 Circular dichroism (CD)

CD spectra of collagen extracted with different methods are shown in Figure 5B. CD spectrum with positive peaks at 221 nm for both ASC and PHSC and 222 nm for

HSC were observed; and negative extreme for HSC and PHSC were observed at 199 nm, ASC showed a negative extreme at 200 nm. Slight deviations in ellipticity were observed among the three collagen products extracted with different methods, which indicated that there was a minor alternation in the structures of these three collagens. The spectra characteristics in the three collagens are typical of the collagen triple helix structure (Engel, 1987), which suggested that the triple helical structure of collagen was not destroyed during extraction processing. CD spectra of collagen extracted from *Pagrus major*, *oreochromis niloticas*, *Labeo rihita*, *Catla* scale, eel-fish skin, black drum and sheepshead seabream skin, bone and scale (Ogawa, Moody, Portier, Bell, Schexnayder, & Losso, 2003; Ogawa, Portier, Moody, Bell, Schexnayder, & Losso, 2004) are all exhibited a similar pattern with our study as well.

3.9 Differential scanning calorimetry (DSC)

DSC thermograms of collagen extracted by different methods are shown in Figure 5C. Endothermic peaks with T_{max} of collagens extracted by three different method are ranging from 35.57 to 36.12°C. The ΔH and T_{max} of ASC and HSC was slightly higher than that of PHSC. This might be caused by the different α and β chains composition since ASC and HSC are exhibited higher ratio of $\beta/(\alpha_1+ \alpha_2)$ chains (discussed in section 3.5). Higher levels of cross-linkage of collagens are more likely to contribute to the higher T_{max} (Singh, Benjakul, Maqsood, & Kishimura, 2011). The results of DSC were in agreement with a previous report on the collagens extracted from channel catfish skin (T_{max} = 32.5°C) (Liu, Li, & Guo, 2007). The minor difference might be caused by the different determination methods. DSC was used in the current study. However, denaturation temperature in the cited reference was determined by monitoring the

438 viscosity change of collagen solution under a temperature range. Therefore, different
439 denaturation temperatures might be resulted from different analytical methods.

440 **3.10 Gel strength**

441 Gel strength is one of the most important index for collagen quality and it can by
442 classified into 3 levels: low (<150 g), medium (150-220 g) and high (220-300 g)
443 (Johnston-Bank, 1983). The prepared collagen gels were used to test the gel strength, and
444 ASC, HSC and PHSC exhibited gel strength of 223.13, 220.48 and 73.59 g, respectively.
445 This result was expected since pepsin-added extraction produced more smaller $\alpha_1 + \alpha_2$
446 molecules, which would produce weaker gels. Collagen induced gel with the same
447 concentration has been reported for tilapia scale (157- 260 g) (Huang, Kuo, Wu, & Tsai,
448 2016). The discrepancy in gel strength among species might due to the different amino
449 acid composition and molecular size of protein chains (Muyonga, Cole, & Duodu, 2004b).
450 When gel strength was measured at low temperature (< 10 °C), some short chain peptides
451 present in low viscosity gelatins tend to strengthen the gel (Montero & Gómez-Guillén,
452 2000). In addition, the thermal shrinkage, denaturation temperature of collagens and
453 melting temperature of gelatins isolated from cold-water fish are significantly lower than
454 that collagens and gelatins isolated from fish living in warm waters, the reason of the
455 differences might be due to decreased proline hydroxylation degree of cold-water fish
456 (Karim & Bhat, 2009).

457 **4 Conclusion**

458 In summary, collagens were extracted and characterized from catfish skin
459 successfully. Protein recovery rate from minced skins extracted with pH 2.4 HCl
460 containing 23.6 KU/g pepsin was the highest (70.15%). Kinetic analysis of collagen yield

during extraction was performed. Pepsin-extracted collagen produced more smaller size chains (α chains). Gel strengths of ASC, HSC are significantly higher than that of PHSC. HCl extraction with homogenization-aided method can be used for the industries to improve collagen yield in terms of extraction efficiency and economy.

5 Acknowledgements

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References

AOAC International (2012). Official methods of analysis of AOAC international (19th ed.). Gaitherburg, MD: Association of Official Analytical Chemists.

Bergman, I., & Loxley, R. (1963). Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Analytical Chemistry*, *35*, 1961-1965.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248-254.

BSI (British Standards Institution). (1975). Methods for sampling and testing gelatin (physical and chemical methods). London: BSI.

Chen, J., Li, L., Yi, R., Xu, N., Gao, R., & Hong, B. (2016). Extraction and characterization of acid-soluble collagen from scales and skin of tilapia (*Oreochromis niloticus*). *LWT - Food Science and Technology, 66*, 453-459.

Chen, S., Chen, H., Xie, Q., Hong, B., Chen, J., Hua, F., Bai, K., He, J., Yi, R., & Wu, H. (2016). Rapid isolation of high purity pepsin-soluble type I collagen from scales of red drum fish (*Sciaenops ocellatus*). *Food Hydrocolloids, 52*, 468-477.

Choi, S. S., & Regenstein, J. M. (2000). Physicochemical and Sensory Characteristics of Fish Gelatin. *Journal of Food Science, 65*, 194-199.

Duan, R., Zhang, J., Du, X., Yao, X., & Konno, K. (2009). Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*). *Food Chemistry, 112*, 702-706.

Engel, J. (1987). Folding and unfolding of collagen triple helices. *Advances in meat research, 4*, 145-161.

508 Foegeding, E., Lanier, T., & Hultin, H. (1996). Characteristics of edible muscle tissues.
509 *Food Chemistry, 3*, 879-942.
510 Gómez-Guillén, M. C., Giménez, B., López-Caballero, M. E., & Montero, M. P. (2011).
511 Functional and bioactive properties of collagen and gelatin from alternative
512 sources: A review. *Food Hydrocolloids, 25*, 1813-1827.
513 Huang, C. Y., Kuo, J. M., Wu, S. J., & Tsai, H. T. (2016). Isolation and characterization
514 of fish scale collagen from tilapia (*Oreochromis sp.*) by a novel extrusion-hydro-
515 extraction process. *Food Chemistry, 190*, 997-1006.
516 Johnston-Bank, F. (1983). From tannery to table: an account of gelatin production.
517 *Journal of the Society of Leather Technologists and Chemists, 68*, 141-145.
518 Jongjareonrak, A., Benjakul, S., Visessanguan, W., Nagai, T., & Tanaka, M. (2005).
519 Isolation and characterisation of acid and pepsin-solubilised collagens from the
520 skin of Brownstripe red snapper (*Lutjanus vitta*). *Food Chemistry, 93*, 475-484.
521 Karim, A. A., & Bhat, R. (2009). Fish gelatin: properties, challenges, and prospects as an
522 alternative to mammalian gelatins. *Food hydrocolloids*, 23, 563-576.
523 Kimura, S., Zhu, X. P., Matsui, R., Shijoh, M., & Takamizawa, S. (1988).
524 Characterization of fish muscle type I collagen. *Journal of Food Science, 53*,
525 1315-1318.
526 Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Kishimura, H., & Shahidi, F.
527 (2010). Isolation and characterisation of collagen from the skin of brownbanded
528 bamboo shark (*Chiloscyllium punctatum*). *Food Chemistry, 119*, 1519-1526.

529 Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., Nagai, T., & Tanaka, M. (2005).
530 Characterisation of acid-soluble collagen from skin and bone of bigeye snapper
531 (*Priacanthus tayenus*). *Food Chemistry, 89*, 363-372.
532 Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head
533 of bacteriophage T4. *nature, 227*, 680-685.
534 Liu, D., Wei, G., Li, T., Hu, J., Lu, N., Regenstein, J. M., & Zhou, P. (2015). Effects of
535 alkaline pretreatments and acid extraction conditions on the acid-soluble collagen
536 from grass carp (*Ctenopharyngodon idella*) skin. *Food Chemistry, 172*, 836-843.
537 Liu, H., Li, D., & Guo, S. (2007). Studies on collagen from the skin of channel catfish
538 (*Ictalurus punctaus*). *Food Chemistry, 101*, 621-625.
539 Liu, W., Li, G., Miao, Y., & Wu, X. (2009). Preparation and characterization of pepsin-
540 colubilized Type I collagen from the scales of Snakehead (*Ophiocephalus argus*).
541 *Journal of food biochemistry, 33*, 20-37.
542 Montero, P., Alvarez, C., Marti, M., & Borderias, A. (1995). Plaice skin collagen
543 extraction and functional properties. *Journal of Food Science, 60*, 1-3.
544 Montero, P., & Gómez-Guillén, M. C. (2000). Extracting conditions for megrim
545 (*Lepidorhombus boscii*) skin collagen affect functional properties of the resulting
546 gelatin. *Journal of Food Science, 65*, 434-438.
547 Muyonga, J. H., Cole, C. G. B., & Duodu, K. G. (2004a). Characterisation of acid soluble
548 collagen from skins of young and adult Nile perch (*Lates niloticus*). *Food*
549 *Chemistry, 85*, 81-89.

Muyonga, J. H., Cole, C. G. B., & Duodu, K. G. (2004b). Extraction and physico-chemical characterisation of Nile perch (*Lates niloticus*) skin and bone gelatin. *Food Hydrocolloids, 18*, 581-592.

Nagai, T., Araki, Y., & Suzuki, N. (2002). Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chemistry, 78*, 173-177.

Nagai, T., & Suzuki, N. (2000). Isolation of collagen from fish waste material- skin, bone and fins. *Food Chemistry, 68*, 277-281.

Nagai, T., & Suzuki, N. (2002). Preparation and partial characterization of collagen from paper nautilus (*Argonauta argo*, Linnaeus) outer skin. *Food Chemistry, 76*, 149-153.

Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N., & Suzuki, N. (2001). Isolation and characterisation of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). *Food Chemistry, 72*, 425-429.

Nalinanon, S., Benjakul, S., Visessanguan, W., & Kishimura, H. (2007). Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). *Food Chemistry, 104*, 593-601.

Ogawa, M., Moody, M. W., Portier, R. J., Bell, J., Schexnayder, M. A., & Losso, J. N. (2003). Biochemical properties of black drum and sheepshead seabream skin collagen. *Journal of Agricultural and food Chemistry, 51*, 8088-8092.

Ogawa, M., Portier, R. J., Moody, M. W., Bell, J., Schexnayder, M. A., & Losso, J. N. (2004). Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*). *Food Chemistry, 88*, 495-501.

573 Peleg, M. (1988). An empirical model for the description of moisture sorption curves.
574 *Journal of Food Science, 53*, 1216-1217.
575 Sato, K. (1993). Comparative biochemistry of molecular species of fish and mammalian
576 collagens. *Trends in Comparative Biochemistry Physiology, 1*, 557-567.
577 Sato, K., Ebihara, T., Adachi, E., Kawashima, S., Hattori, S., & Irie, S. (2000). Possible
578 involvement of aminotelopeptide in self-assembly and thermal stability of
579 collagen I as revealed by its removal with proteases. *Journal of Biological*
580 *Chemistry, 275*, 25870-25875.
581 Singh, P., Benjakul, S., Maqsood, S., & Kishimura, H. (2011). Isolation and
582 characterisation of collagen extracted from the skin of striped catfish
583 (*Pangasianodon hypophthalmus*). *Food Chemistry, 124*, 97-105.
584 Skierka, E., & Sadowska, M. (2007). The influence of different acids and pepsin on the
585 extractability of collagen from the skin of Baltic cod (*Gadus morhua*). *Food*
586 *Chemistry, 105*, 1302-1306.
587 Vojdani, F. (1996). Solubility. *Methods of testing protein functionality*, 11-60.
588 Zhang, Q., Wang, Q., Lv, S., Lu, J., Jiang, S., Regenstein, J. M., & Lin, L. (2016).
589 Comparison of collagen and gelatin extracted from the skins of Nile tilapia
590 (*Oreochromis niloticus*) and channel catfish (*Ictalurus punctatus*). *Food*
591 *Bioscience*, *13*, 41-48.
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Figure Captions

Figure 1A. Protein recovery rate of collagen extracted with different acids. Figure 1B. Protein recovery rate of collagen extracted by HCl with homogenization aided method. Figure 1C. Protein recovery rate of collagen extracted by HCl with pepsin aided method.

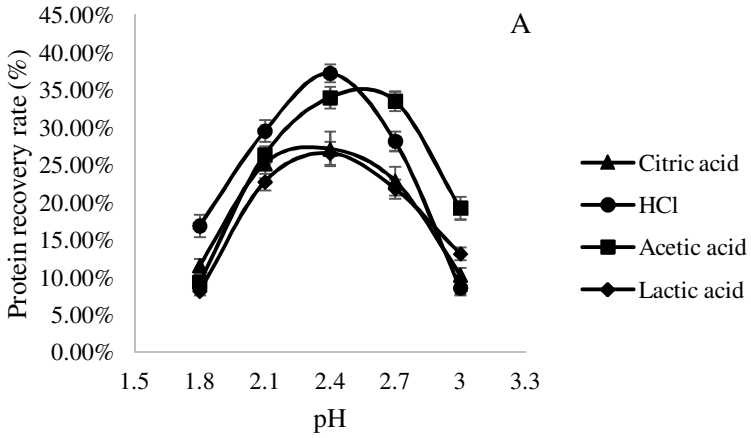
Figure 2A. Protein recovery rate of collagen extracted with different acids at pH 2.6. Figure 2B. Protein recovery rate of collagen extracted with HCl at different pH. Figure 2C. Protein recovery rate of collagen extracted by pH 2.4 HCl with different solid to liquid ratio (W/V). Figure 2D. protein recovery rate of collagen extracted by pH 2.4 HCl (W/V=1:50) with different skin particle size. Figure 2E. Protein recovery rate of collagen extracted by pH 2.4 HCl with different solid to liquid (W/V) ratio with homogenization aided. Figure 2F. Protein recovery rate of collagen extracted by pH 2.4 HCl (W/V=1:50) with different pepsin concentration.

Figure 3A. SDS-PAGE gel of collagens extracted with acid extraction. Left figure: Lane 1: HCl, pH 3 extracted collagen; Lane 2: HCl, pH 2.7 extracted collagen; Lane 3: HCl, pH 2.4 extracted collagen; Lane 4: HCl, pH 2.1 extracted collagen; Lane 5: HCl, pH 1.8 extracted collage; Lane 6: Acetic acid, pH 3 extracted collagen; Lane 7: Acetic acid, pH 2.7 extracted collagen; Lane 8: Acetic acid, pH 2.4 extracted collagen; Lane 9: Acetic acid, pH 2.1 extracted collagen; Lane 10: Acetic acid, pH 1.8 extracted collagen. Right figure: Lane 1: Lactic acid, pH 3 extracted collagen; Lane 2: Lactic acid, pH 2.7 extracted collagen; Lane 3: Lactic acid, pH 2.4 extracted collagen; Lane 4: Lactic acid, pH 2.1 extracted collagen; Lane 5: Lactic acid, pH 1.8 extracted collagen; Lane 6: Citric acid, pH 3 extracted collagen; Lane 7: Citric acid, pH 2.7 extracted collagen; Lane 8: Citric acid, pH 2.4 extracted collagen; Lane 9: Citric acid, pH 2.1 extracted collagen; Lane 10: Citric acid, pH 1.8 extracted collagen.

Figure 3B. SDS-PAGE gel of collagen extracted with different concentration of pepsin. Lane 1: HCl at pH 2.4, W/V =1:5, homogenization, pepsin concentration 118U/g; Lane 2: HCl at pH 2.4, W/V =1:5, homogenization, pepsin concentration 1180U/g; Lane 3: HCl at pH 2.4, W/V =1:5, homogenization, pepsin concentration 5900U/g); Lane 4: HCl at pH 2.4, W/V =1:5, homogenization, pepsin concentration 11800U/g; Lane 5: HCl at pH 2.4, W/V =1:5, homogenization, pepsin concentration 23600U/g; Lane 6: HCl at pH 2.4, W/V =1:20, homogenization, pepsin concentration 118U/g; Lane 7: HCl at pH 2.4, W/V =1:20, homogenization, pepsin concentration 1180U/g; Lane 8: HCl at pH 2.4, W/V =1:20, homogenization, pepsin concentration 5900U/g; Lane 9: HCl at pH 2.4, W/V =1:20, homogenization, pepsin concentration 11800U/g; Lane 10: HCl at pH 2.4, W/V =1:20, homogenization, pepsin concentration 23600U/g.

Figure 4A. Effect of pH on collagen solubility. Figure 4B. Effect of NaCl on collagen solubility.

Figure 5A. Zeta potential (ζ) of collagen extracted with different conditions. Figure 5B. CD spectra of collagen extracted with different conditions. Figure 5C. DSC thermograms of ASC (acid solubilized collagen), HSC (homogenization aided solubilized collagen) and HPSC (pepsin and homogenization solubilized collagen) from channel catfish skin dispersed in 0.05 M acetic acid.



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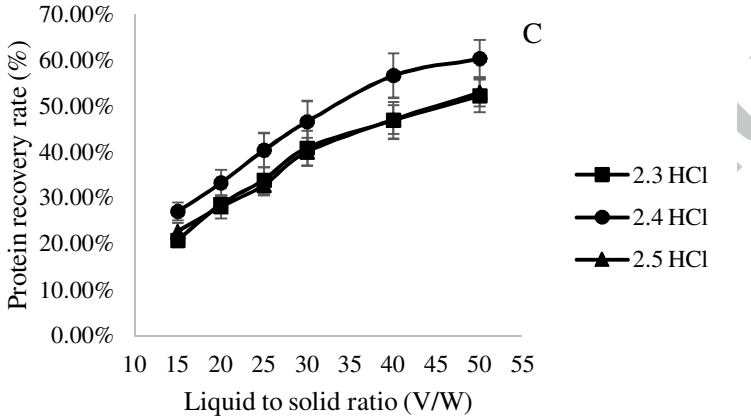

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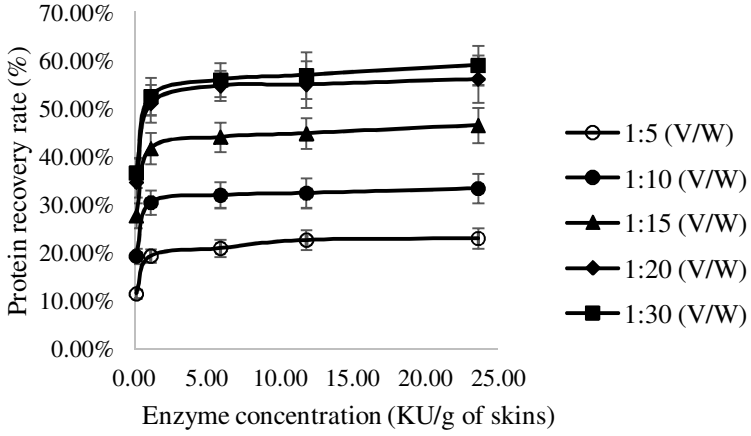

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638 Figure 1A-C

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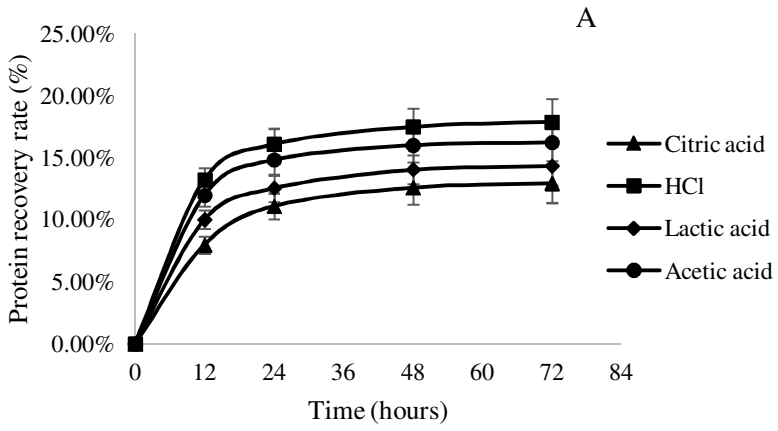
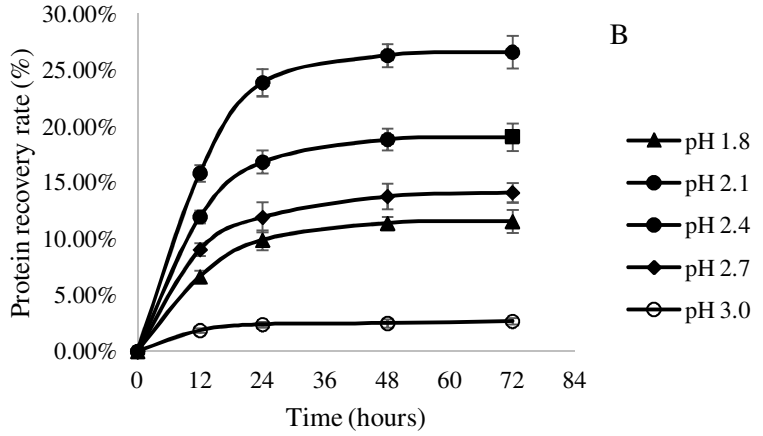
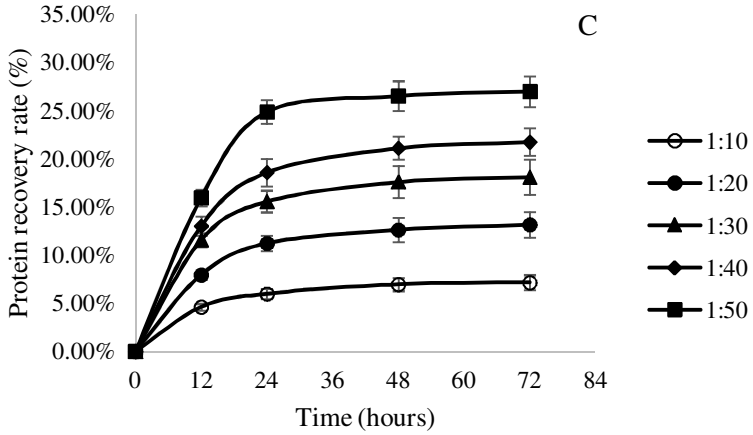
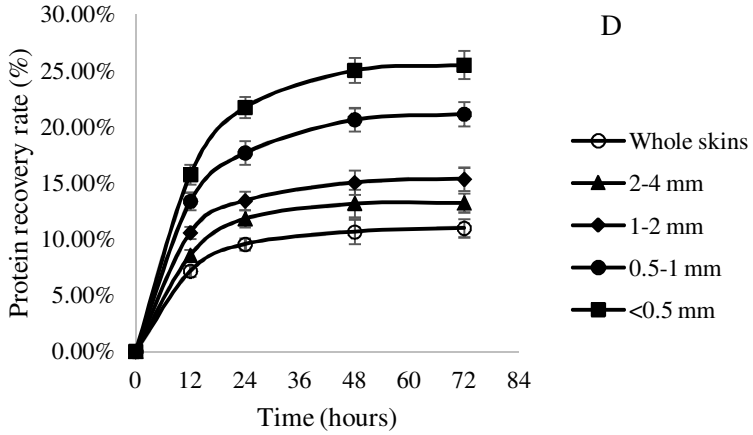








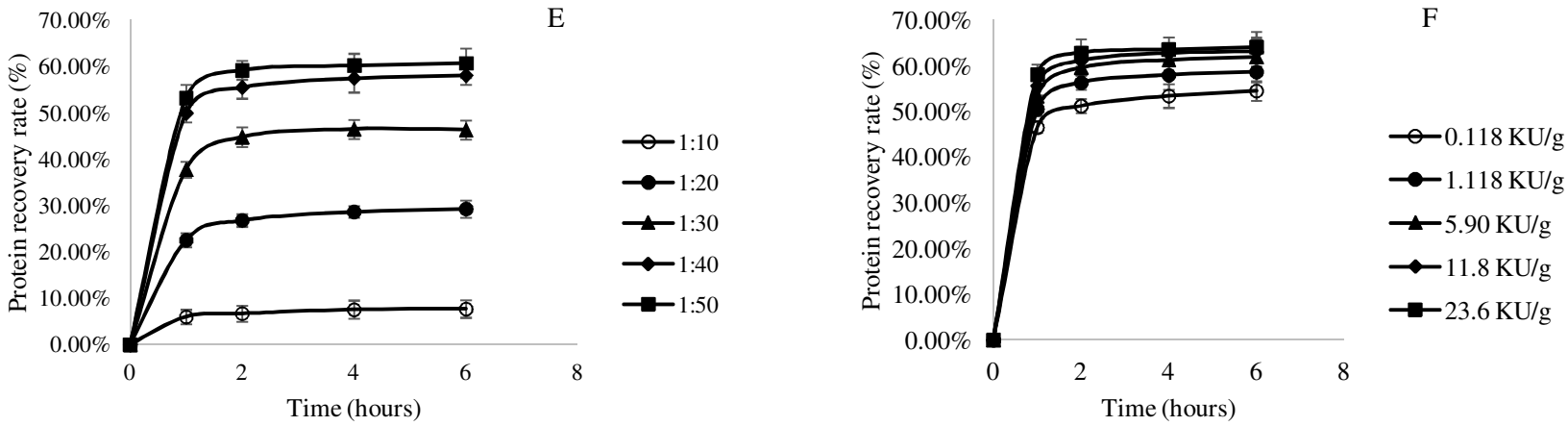


Figure 2A-F

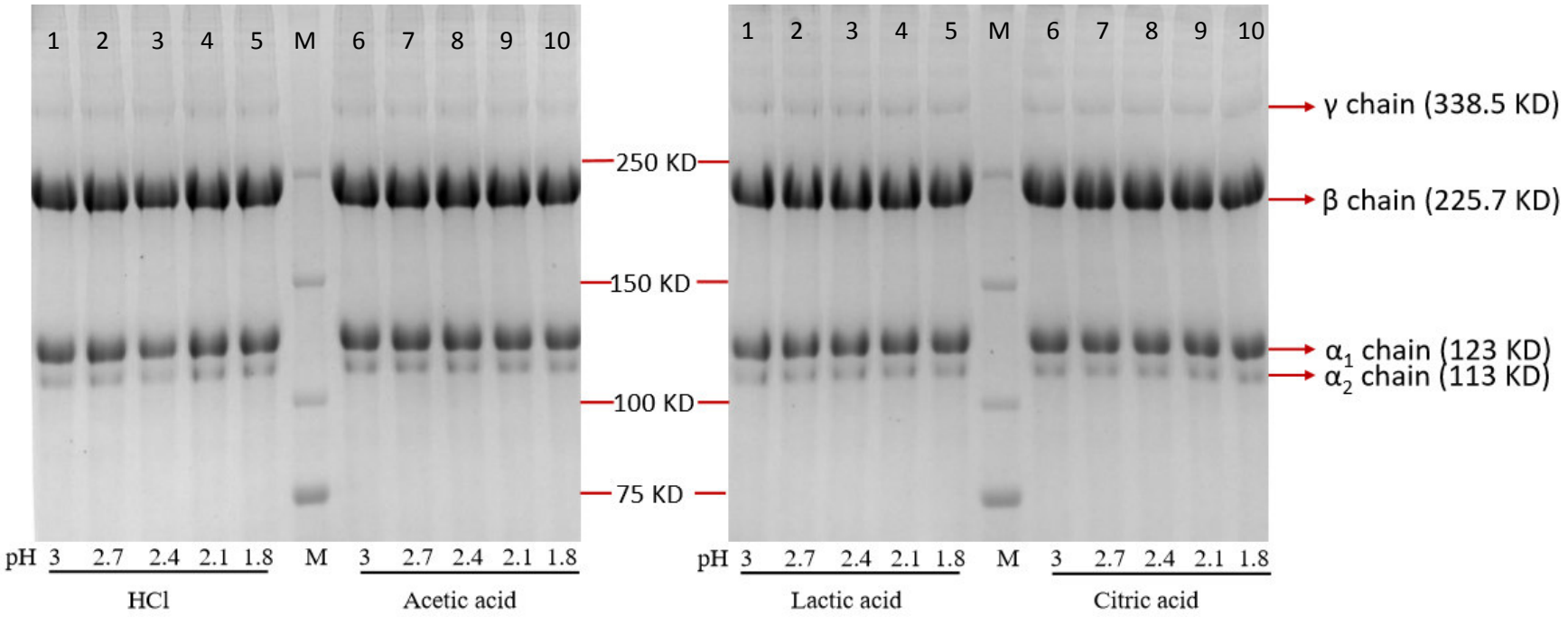


Figure 3A

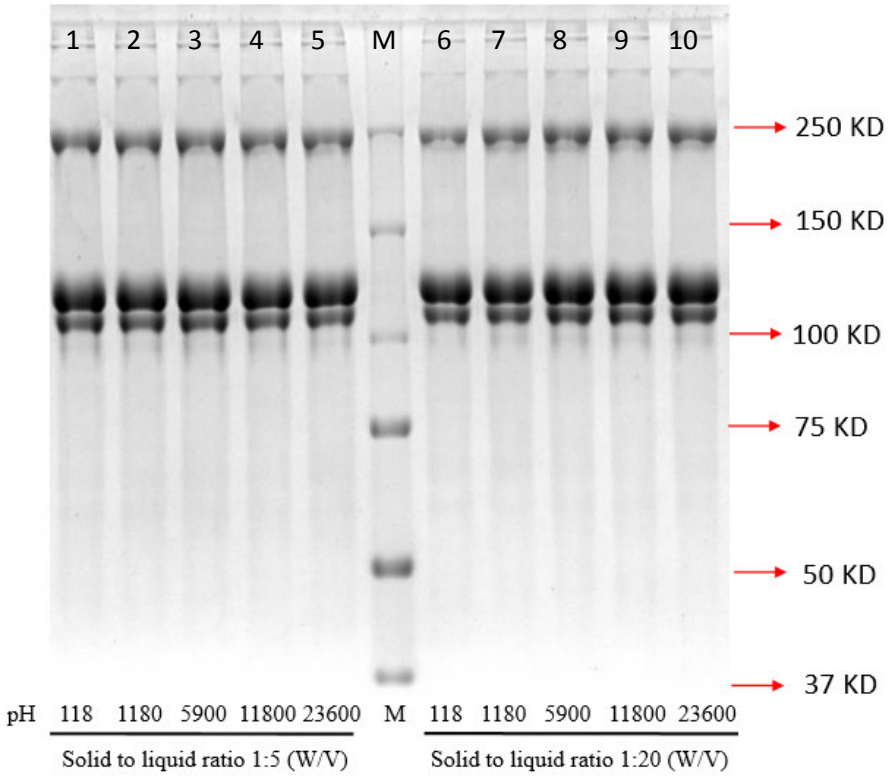
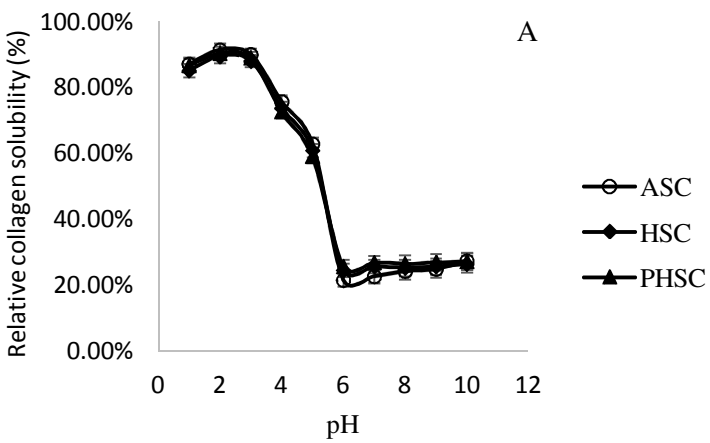


Figure 3B.



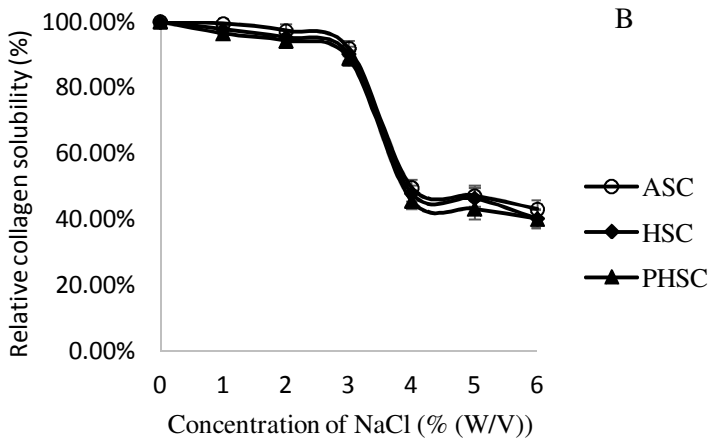
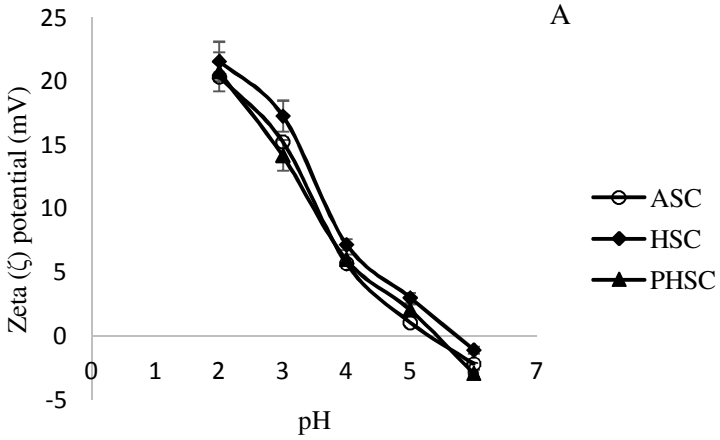


Figure 4A-B

A



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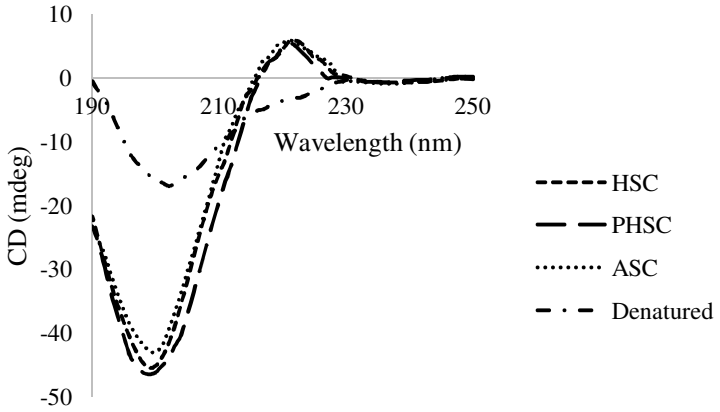

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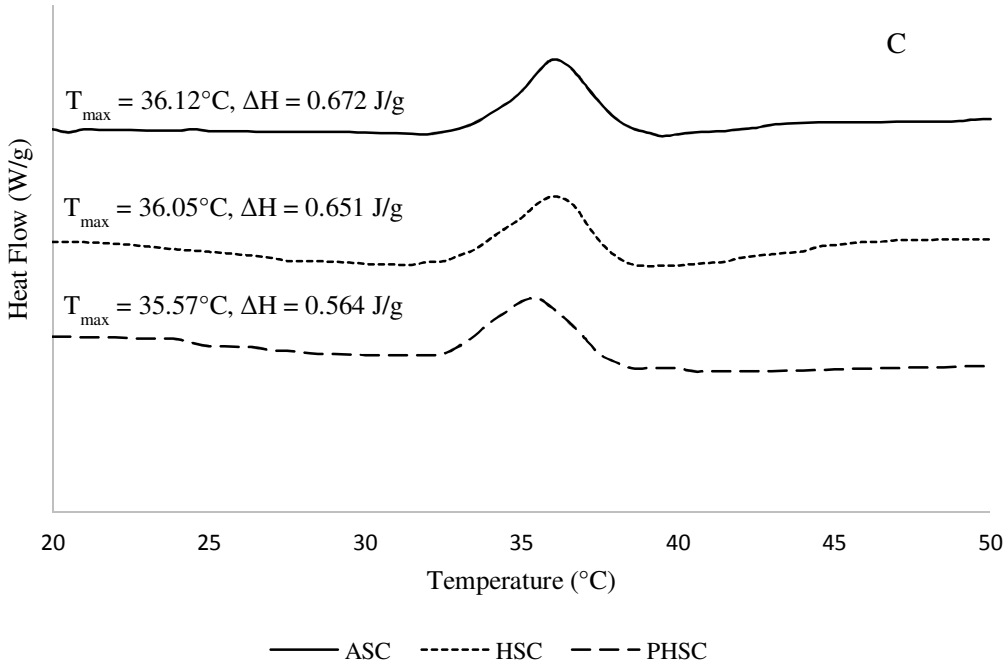

Figure 5A-C

16 Table 1. Rate constant and R^2 of protein recovery rate under different extraction
17 conditions.

	K_1	K_2	R^2
Acid			
Citric acid	30.47	7.39	0.989
Lactic acid	20.49	6.74	0.994
Acetic acid	13.91	5.99	0.997
Hydrochloric acid	13.23	5.46	0.996
pH			
pH 1.8	38.86	8.16	0.984
pH 2.1	18.69	5.00	0.990
pH 2.4	14.39	3.57	0.987
pH 2.7	26.78	6.78	0.990
pH 3.0	110.03	3671	0.994
Ratio (without homogenization)			
1:10	53.59	13.39	0.990
1:20	31.74	7.26	0.988
1:30	20.25	5.29	0.991
1:40	19.33	4.37	0.987
1:50	13.81	3.53	0.987
Particle size			
Whole skins	31.39	8.77	0.992
2-4 mm	24.47	7.23	0.992
1-2 mm	19.65	6.30	0.994
0.5-1 mm	18.64	4.52	0.989
<1 mm	15.54	3.73	0.989
Ratio (with homogenization)			
1:10	2.49	12.70	0.997
1:20	0.57	3.34	0.998
1:30	0.23	2.12	0.999
1:40	0.15	1.70	0.999
1:50	0.11	1.63	0.999
Pepsin concentration			
0.118 KU/g	0.19	1.81	0.999
1.18 KU/g	0.14	1.68	0.999
5.90 KU/g	0.14	1.59	0.999
11.8 KU/g	0.11	1.57	0.999
23.6 KU/g	0.08	1.55	0.999

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