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Article

Acid-Pepsin Soluble Collagen from Saltwater and Freshwater Fish Scales

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Abstract. Extraction and characterization of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) from scales of Giant groupers (saltwater fish) and Nile tilapia (freshwater fish) were carried out in this research. Due to a higher protein content in scales, collagen yield extracted from the Giant groupers scales was higher than that of the Nile tilapia scales. The yield increased as extraction time increased for both ASC and PSC and pepsin extraction resulted in higher yields than acid extraction. Even though there were differences in collagen yields, collagen characteristics were independent of the scale sources but some differences were observed for the ASC and PSC. The peptide hydrolysis patterns of the ASC showed a wide range of molecular weights whereas all of the PSC had similar molecular weight of around 42 kDa. FTIR spectra showed that all the collagens remained the triple helical structure though ASC might be self-aggregated. From zeta potential analysis, net charge of zero was found at pH 3.2-4.0 and the dynamic light scattering suggested that the average particle sizes at pH 11-12 were around 100-200 nm. The denaturation temperatures (T_ds) in a range of 35-42^{o}C indicated that the collagens were considerably thermally stable.

Keywords: Collagen, fish scales, enzymatic extraction, acid extraction, seawater fish, freshwater fish.

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1. Introduction

Collagen is the most abundant protein in our bodies, especially type 1 collagen. It makes up approximately 30% of the proteins within the body and 70% of the proteins within human skin. It is found in muscles, bones, skin, blood vessels, digestive system and tendons. Its role is to support structures and anchor cells to each other. It's very important for wound healing and holding together tissue so it doesn't tear. It also gives the skin strength and elasticity, along with replacing dead skin cells. It can be produced naturally; however, collagen production declines with aging and exposure to factors such as smoking and UV light, causing wrinkles, sagging skin and joint pains.

Since collagen can be found in human and animal tissues, collagen extraction from animal sources, especially from skins and bones of cows and pigs, has been established [1-3]. However, bovine spongiform encephalopathy and foot-and-mouth disease have led to safety concerns among consumers. Furthermore, the religious constraints of some groups of people, i.e. Islamic and Jewish communities, also limited its use. Recently, fish has been focused as a potential alternative source of collagen. Collagen extraction from many parts of fish, such as skins, scales, bones and swim bladders, has been investigated [4-7]. Traditionally, type I collagen can be extracted from different tissues of marine fish using acetic acid and the obtained collagen is normally called acid soluble collagen (ASC). Several research found that mainly collagen yields depended on fish species, their habitat, their environment as well as parts of tissues whereas collagen characteristics were considerably similar [1, 6, 8]. It was reported that ASC from scales of fish inhabiting in a warmer temperature contained a higher imino acids, resulting in a higher denaturation temperature [1, 2, 9]. On the other hand, Duan et al. [10] did not observe noticeable differences in amino acid compositions between collagens extracted from winter and summer carp and silver carp scales though the summer collagens had higher thermostability. They suggested that the seasonal difference in the thermostability of scale collagens was attributed to the subunit changes of the collagens. Even though fish skins provided the highest collagen yields compared to other parts of fish bodies (fins, scales, and bones), the collagens extracted from internal tissues (swim bladders and bones) were more thermally stable than those extracted from external tissues [5, 6, 8]. Though acid extraction has been found to be successful for the marine fish collagen, low yields were serious drawbacks of the process. Enzymatic hydrolysis has been widely used to increase collagen yields. Pepsin is the most commonly used enzyme and hence the collagen extracted by pepsin is called pepsin soluble collagen (PSC). It was found that pepsin digestion could increase the yield of collagen without affecting their physiochemical properties [7, 11, 12]. The PSC were found to be type I collagen and the triple helical structure remained substantially unaffected. ASC and PSC from fish scale and skin were found to be biocompatible with no signs of cytotoxic effect [13, 14].

Due to the fact that eastern and southern parts of Thailand are coastal areas, marine and aquaculture are common fishery products. The abundant fishery wastes can potentially be a large quantities of protein-rich by-products. Among the principal fish species, Giant groupers and Nile tilapia are largely consumed in Thailand on account of their very popular in Thai cuisines based on FAO statistics [15]. Giant groupers belong to the family of Epinephelus Ianceolatus, a large bony seawater fish while Nile tilapia is in the family of Oreochromis niloticus, a freshwater fish widely farmed in Thailand and exported overseas. The record also showed that the global aquaculture production of Nile tilapia in 2016 was 232,129 tons. Regarding the literature, it can be estimated that collagen from different fish species differs in molecular composition and functional properties. In addition, environmental and body temperatures are also presumed to affect the fish collagen properties. There is, however, a lack of information exists on the scale collagen of commercial fish species in Thailand. Hence, this work aimed to extract the ASC and PSC from the scales of Giant groupers and Nile tilapia and to compare the extracted scale collagen characteristic as well as collagen yield between the representative freshwater fish (Nile tilapia) and saltwater fish (Giant groupers).

2. Materials and Methods

2.1. Materials

Scales of Giant groupers (Epinephelus Ianceolatus), originated from the Gulf of Thailand, were obtained from a restaurant in Chonburi, Thailand, while scales of Nile tilapia (Oreochromis niloticus), farmed in Nakornsawan, were obtained a local market in Chonburi. They were washed thoroughly with tap water and subsequently air dried. The scales were then kept in a plastic container and refrigerated at -20^{o}C prior to

experiments. Pepsin from porcine gastric mucosa (EC 3.4.23.1 activity ⩾ 2,500 units/mg protein) was purchased from Sigma-Aldrich, USA, whereas hydrochloric acid (HCl), sodium hydroxide (NaOH), acetic acid (CH_3COOH), and sodium chloride (NaCl) were supplied by QRëC, New Zealand. All chemicals and reagents were of analytical grade.

2.2. Proximate Determination and Thermogravimetric Analysis (TGA)

The proximate compositions of the scales, including moisture, ash, crude fat, and crude protein, were determined according to the AOAC 2000 methods. Moisture content was measured by oven drying. 1-3 g of samples in an oven at 105°C for 5-6 hours while ash content was evaluated by burning 2 g of samples in a furnace at 550°C. Percent crude fat was determined gravimetrically after the Soxhlet extraction with petroleum ether. Crude protein was measured by the Kjeldahl method after acid digestion and a conversion factor of 5.95 was used in a calculation. To confirm the proximate compositions determined by the AOAC 2000, Mettler Toledo TGA850 was also carried out by heating 2-3 mg of samples from 30-1000°C at a heating rate of 10°C/min under a nitrogen blanket.

2.3. Fish Scales Pretreatment

Firstly, fish scales were decalcified by soaking in a 1.2 N HCl solution at a ratio of 1:6 (w/v) at a room temperature of 27±2°C for 6 hours. The solution was stirred throughout the soaking time using a magnetic stirrer. The scales were then washed with deionized water until the pH 7 was obtained. Subsequent non-collagen protein and pigment elimination process was followed. The scales were soaked in a 0.1 N NaOH solution at a ratio of 1:5 (w/v). The NaOH solution was stirred all the time and changed every 2 hours until 6 hours. The scales were neutralized using deionized water until reaching pH 7 and finally air dried.

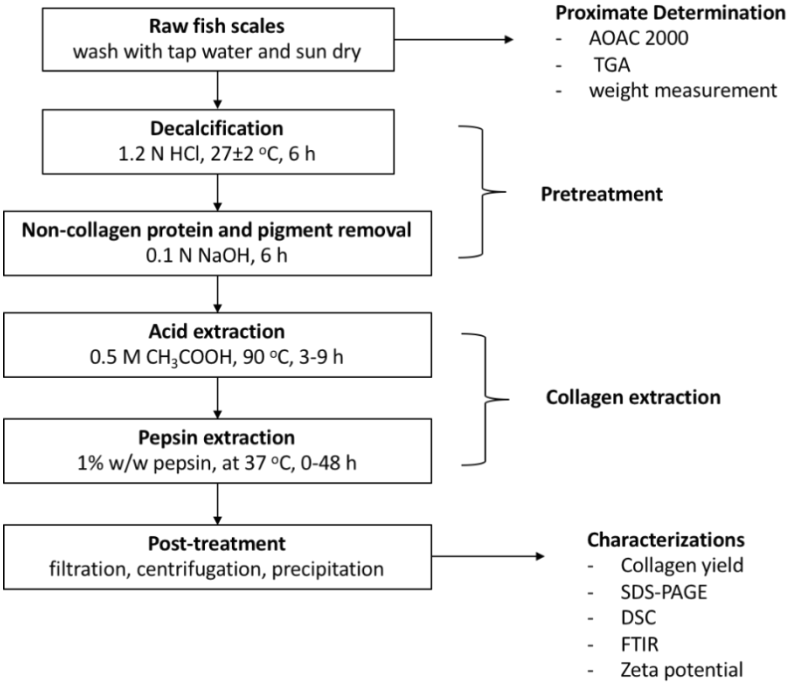

Fig. 1. Process diagram of acid-pepsin collagen extraction.

2.4. Collagen Extraction

Figure 1 illustrates a brief diagram of this experiment. 200 ml 0.5 M CH_3COOH were added into 40 g of pretreated fish scales. The solution was stirred and heated to 90°C using a hot plate for a desired period (3, 6, or 9 hours). The solution was then quickly cooled down to 37°C and adjusted to pH 2. Pepsin enzyme was added into the solution at the concentration of 1% w/w based on dry fish scale weight. The solution was again stirred for 48 hours using a magnetic stirrer. In order to terminate enzyme activity, the solution

was cooled down to 4°C for 30 min. Subsequently, the solution was filtered using cheese cloth and centrifuged at 5,500 rpm at 4°C for 50 min. Then, NaCl was added into the supernatant to a final concentration of 1.5 mol/L and the solution was again centrifuged at 4°C using a high speed centrifuge at 10,000 g for 90 min. The precipitate of scale extraction was dissolved in a 0.5 M CH_3COOH at a ratio of 1:4 (w/v) and the solution was dialyzed with deionized water. Once pH 7 was achieved (around 24 hours by changing deionized water every 6 hours), the solution was frozen in a freezer at -80°C prior to freeze drying using a FreeZone Plus 4.5 L Cascade Freeze Dryer System, Labconco, USA. The dry collagen was stored at -4°C prior to its characterization. In order to examine the effects of an enzymatic extraction, a blank test (without enzymatic extraction) as well as an enzymatic extraction time of 24 hours was carried out with a 3 hour acid extraction. Thereafter, the collagen samples were symbolized as GX-Y or NX-Y where G means the collagen from the scales of Giant groupers, N means the collagen from the scales of Nile tilapia, X means an acetic acid extraction period (in a unit of hour), and Y means a pepsin enzyme extraction period (in a unit of hour). Table 1 summarizes the collagen extraction conditions.

Table 1. The collagen extraction parameters.

Samples	Fish scales	Acid extraction time (h)	Enzymatic extraction time (h)
G3-0	Giant groupers	3	-
G3-24	Giant groupers	3	24
G3-48	Giant groupers	3	48
G6-48	Giant groupers	6	48
G9-48	Giant groupers	9	48
N3-0	Nile tilapia	3	-
N3-24	Nile tilapia	3	24
N3-48	Nile tilapia	3	48
N6-48	Nile tilapia	6	48
N9-48	Nile tilapia	9	48

2.5. Yield

According to Huang et al. [16], calculation of yields of collagen was recommended to follow Eq. (1) rather than the calculation of hydroxyproline content which was more difficult to evaluate and more deviation in the results.

$$\textbf{\textit{collagen yield}}\ (\%) = \frac{\left(\textbf{\textit{protein content of supernatant}},\frac{g}{ml}\right)\times(\textbf{\textit{volume of supernatant}},ml)}{(\textbf{\textit{dry basis crude protein content}},\frac{g}{g})\times(\textbf{\textit{weight of scale}},g)} \times \mathbf{100} \qquad (1)$$

2.6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Collagen Hydrolysate

Electrophoresis (SDS-PAGE) was carried out following the method of Laemmli [17]. The solubilized sample was mixed with the NuPAGE LDS sample buffer (Invitrogen, USA) at the ratio of 1:3 (v/v) and incubated using a Major Science Heat Box, USA, at 100°C for 10 min. SDS-PAGE gel was prepared using a 12% separation gel (NuPAGETM 4-12% Bis-Tris Gel, Invitrogen, USA) and 4% stacking gel and subjected to electrophoresis at a constant 200 V for 45 min. After electrophoresis, the gel was rinsed with 100 ml ultrapure water 3 times for 5 min. Finally, the gel was stained at room temperature for 1 hour using a 20 ml SimplyBlueTM SafeStain, Invitrogen, USA, with gentle shaking and destained using distilled water for 1 hour. The molecular weight was estimated using a Blueye prestained protein ladder (GeneDirex, Taiwan).

2.7. Differential Scanning Calorimetry (DSC)

Collagen samples were rehydrated by 0.05 M CH_3COOH at a solid to solution ratio of 1:40 (w/v). The mixtures were allowed to stand for 2 days at 4°C. About 3-5 mg of the lyophilized collagen was packed into an aluminum pan, sealed, and performed the test using a Mettler Toledo DSC822. The sample was then heated from 20°C to 60°C with a heating rate of 1°C/min under a nitrogen blanket. An empty pan was used

as the reference. The denaturation temperature (T_d) was recorded as the peak temperature of each endothermic peak.

2.8. FTIR

Thermo Scientific Nicolet iS50 FT-IR, USA, equipped with a horizontal Smart ARK flat ZnSe crystal cell was used to carry out FTIR experiments. The crystal window of the size of 80×10 mm where the lyophilized collagen was placed onto allowed 12 reflections per measurement, enhancing consistency and accuracy. The spectra were acquired over the range of 4000-650 cm^{-1} with a resolution of 4 cm^{-1} for 32 scans against a background spectrum recorded from the clean empty cell. The operations were done at a room temperature of 21°C.

2.9. Zeta Potential

A four digit balance was used to prepare 0.1 g of collagen and then the sample was dissolved with deionized water to a final concentration of 0.1% (w/v). The solution was stirred using a magnetic stirrer for a couple hour. 20 ml of each solution was adjusted pH to 3-12 using either 0.1 M HCl and 0.1 M KOH under constant stirring and titrated using an automatic EUTECH titrator. The zeta potential of the solution was performed according to electrophoretic light scattering using Zetasizer NanoZS instrument (Malvern Instruments Ltd., United Kingdom).

3. Results and Discussion

3.1. Appearance

The appearances of collagen extracted from both Giant groupers and Nile tilapia fish scales are similar except for the color. The collagen obtained was in a form of fluffy fibrils but they are very tough and strong. While the collagen extracted from Giant groupers scales was white, the collagen extracted from Nile tilapia scales was yellowish as shown in Fig. 2. This is might be due to the fact that Nile tilapia scales contain more pigments than those of Giant groupers scales.

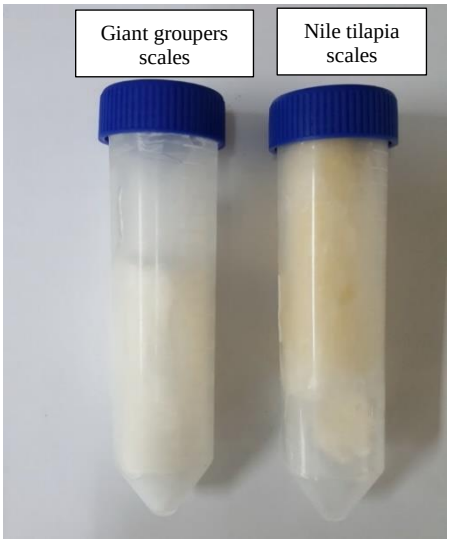

Fig. 2. Collagen extracted from Giant groupers and Nile tilapia fish scales.

3.2. Raw Material Compositions

Calcium, non-protein collagen and pigments contents as well as proximate compositions were shown in Table 2. Moisture contents of the two fish scales were similar and are around 10% w/w as measured both by AOAC 2000 and TGA methods. The results showed that Giant groupers scales which are the scales of deep

saltwater fish contained a higher amount of both protein and fat than the Nile tilapia scales which are scales from freshwater fish, and hence a higher collagen yield would be expected for the Giant groupers scales. Previous research has shown that the amount of fish scale compositions varied depending on the type and origins of the fish, but mostly the protein contents in fish scale are in the range of 35-55% [1]. Since the amounts of protein and fat of the Nile tilapia were lower, the ash content determined by AOAC 2000 which includes calcium, non-protein collagen and pigments, was higher. The results of calcium and non-protein collagen and pigments contents calculated based on weight loss during the sample pretreatment process showed that scales of Giant groupers contained a higher amount of calcium at around 40% whereas the contents of non-protein collagen and pigments were as low as 3%. Oppositely, scales of Nile tilapia composed of a lower calcium content of 30% but a noticeable higher amount of non-protein collagen and pigments at 15%. Once the calcium and non-collagen protein and pigments were removed, the ash contents decreased down to 0-10% as calculated from weight measurements and the TGA technique. In addition to calculation based on weight loss during pretreatment, the amount of fish scales' calcium can be measured using TGA. The results shown in Fig. 3 reveal that the amounts of calcium calculated from differences in ash remaining between raw fish scales and pretreated (decalcified) fish scales were 35 and 25% for Giant groupers and Nile tilapia, respectively. Even though TGA results were slightly lower (about 5%) than those of weight loss calculation, they confirmed that scales of Giant groupers contain a higher amount of calcium than scales of Nile tilapia.

Table 2. Proximate compositions and calcium and non-collagen protein and pigment contents.

Compositions	Giant groupers scales	Nile tilapia scales
Moisture (AOAC 2000)	12.58%	13.31%
Moisture (TGA)	~ 8-10%	~ 8-10%
Protein (AOAC 2000)	41.49%	31.05%
Fat (AOAC 2000)	0.90%	0.53%
Ash (AOAC 2000)	44.93%	55.11%
Ash (weight measurement, after calcium removal)	1.89%	10.20%
Ash (TGA, after calcium removal)	0%	5.03%
Calcium (TGA)	35.14%	24.65%
Calcium (weight measurement)	39.95%	29.94%
Non-collagen protein and pigments	3.09%	14.97%

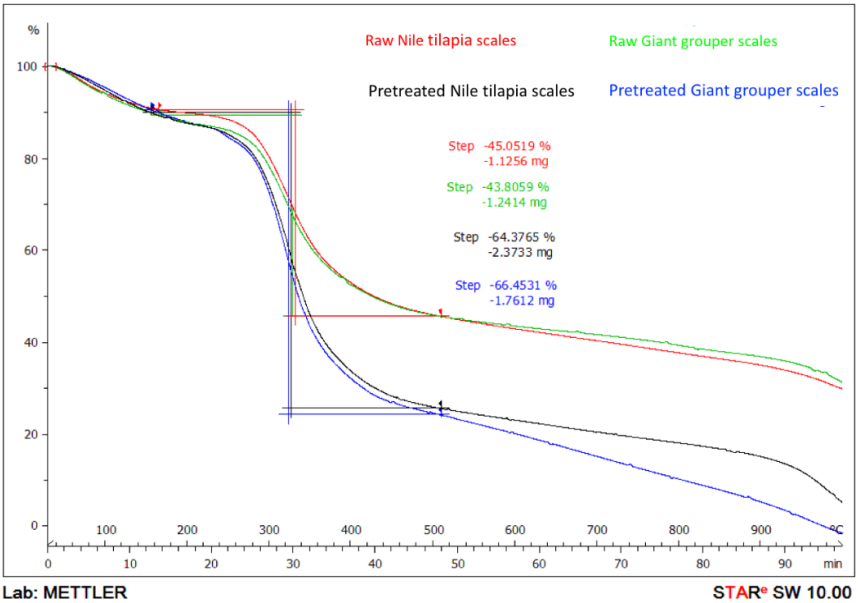

Fig. 3. TGA analysis of fish scales as raw and pretreated (decalcified).

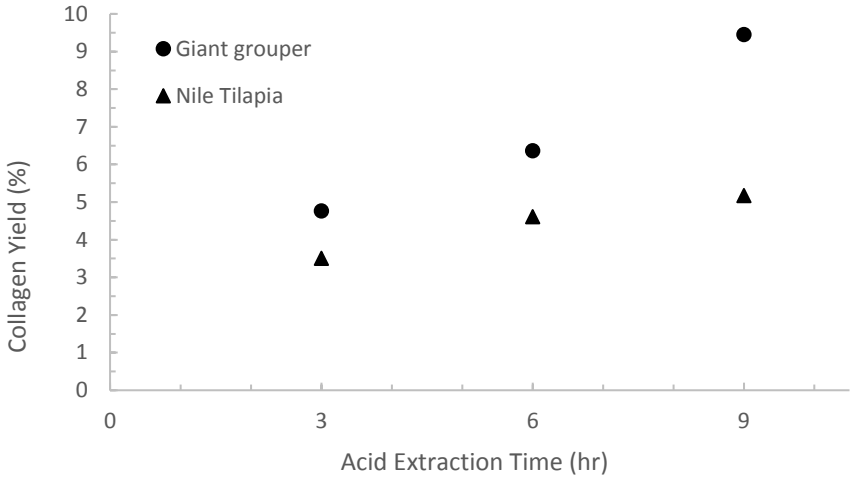

Fig. 4. Collagen yields extracted from fish scales of Giant groupers and Nile tilapias at different acid extracting periods with the same enzymatic extracting time of 48 hours.

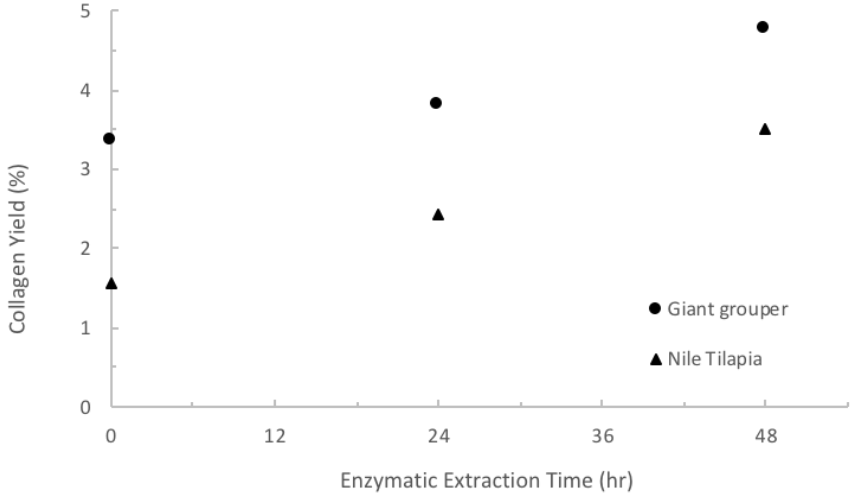

Fig. 5. Collagen yields extracted from fish scales of Giant groupers and Nile tilapias at different enzymatic extracting periods with the same acid extracting time of 3 hours.

3.3. Collagen Yield

Figure 4 shows the effects of acid extracting time on collagen yields from fish scales of Giant groupers and Nile tilapias at an enzymatic extracting time of 48 hours while Fig. 5 shows the effects of enzymatic extracting time on collagen yields from fish scales of Giant groupers and Nile tilapias at an acid extracting time of 3 hours. Both figures show the low yields of fish scales collagen due to the fact that they contain a high amount of cross-linked structures at the telopeptide region. These interchain cross-linking hinders the solubility of collagen in acids. It was found that the scales of Giant gropers offered higher collagen yield than those of Nile tilapia. This might due to the fact that there were more protein contents in the Giant groupers scales than the Nile tilapia scales as shown in Table 3. However, few research has reported that the collagen yields of fish scales are not always depended on the amount of protein in the scales but rather depended on fish species, sources of fish, biological conditions, and extraction procedures [1, 5]. The collagen yields found in this work (1.5-9.5%) are in the ranges of the yields from fish scales reported by others as can be seen in Table 3. By increasing acid extracting time twice, the yields increased around 1.3 times for both fish scales. 1.5 to 2-fold of collagen yields were obtained if the acid extracting time increased to 9 hours, equivalent to 3-fold. Once the acid extraction collagen was followed with pepsin enzyme extraction at a very low concentration of 1% pepsin, an increase in collagen yield by 1.5-fold (50%) was obtained at a 24 hour enzymatic extracting time and by 2-fold (100%) was achieved at a 48 hour enzymatic extracting time. This is because pepsin has

an ability to cleave the crosslinkages in those telopeptide regions without damaging the integrity of the triple helix [18].

Table 3. Percent yield of collagen extracted from different types of fish scales.

Fish scales	Extraction method	Collagen yield (%)	Reference
Giant groupers	Acid-pepsin	3.4-9.5	This work
Nile tilapia	Acid-pepsin	1.5-5.2	This work
Seabass	Acid	0.38	[19]
Seabass	Pepsin	1.06	[19]
Red drum	Acid	0.61	[3]
Red drum	Acid-pepsin	4.32	[3]
Bighead carp	Acid-pepsin	1.10	[5]
Spotted golden goatfish	Acid	0.46	[7]
Spotted golden goatfish	Acid-pepsin	1.20	[7]
Deep sea redfish	Acid	6.80	[6]
Tilapia	Acid	3.20	[20]
Silver carp	Acid	1.45	[21]
Horse mackerel (Japan)	Acid	1.51	[1]
Horse mackerel (Vietnam)	Acid	0.64	[1]
Lizard fish (Japan)	Acid	0.79	[1]
Lizard fish (Vietnam)	Acid	0.69	[1]
Grey mullet	Acid	0.43	[1]
Flying fish	Acid	0.72	[1]
Yellowback seabream	Acid	0.90	[1]

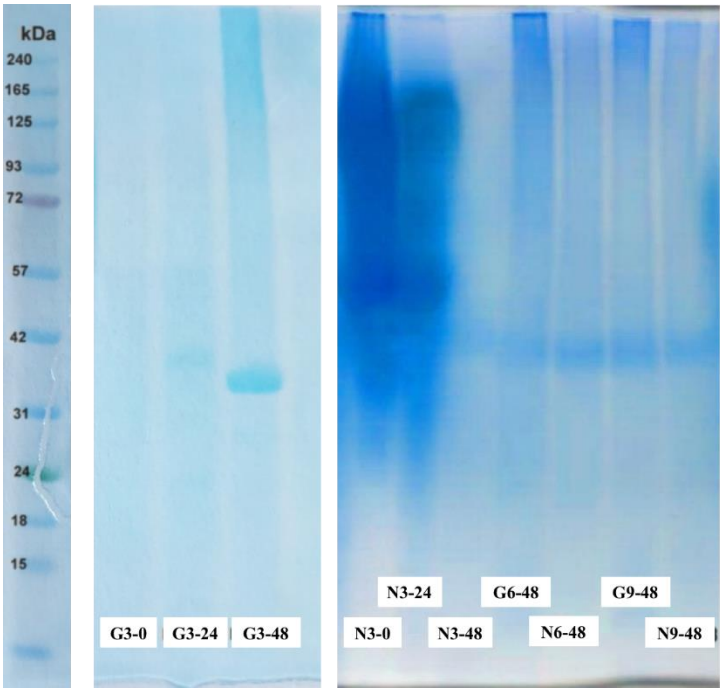

Fig. 6. Gel images of SDS-PAGE of collagens extracted from Giant groupers (G) and Nile tilapia (N) fish scales at different acid-pepsin conditions.

3.4. Collagen Protein Pattern

Figure 6 shows the electrophoretic patterns of collagen extracted from Giant groupers and Nile tilapia fish scales at different extraction conditions. For the acid soluble collagen (G3-0 and N3-0), the peptide hydrolysis patterns of the collagen showed a wide molecular weight peptide fragments of around 31-200 kDa as seen

from diffusing bands. These high molecular weight crosslinked components entirely disappeared for the pepsin soluble collagens except for the collagen of the Nile tilapia fish scaled at a 3 hour acid extraction followed by a 24 hour pepsin extraction (N3-24) in which the molecular weight was in the range of 57-165 kDa. It can be seen that as the pepsin extraction time increased from 24 to 48 hours, the collagen molecular weight decreased from 40 to 34 kDa for the Giant groupers scales (G3-24 and G3-48) and from 57-165 to 40 kDa for the Nile tilapia scales (N3-24 and N3-48). However, at the pepsin extraction time of 48 hours, an increase in acid extraction time from 3 to 9 hours did not affect the size of collagen molecular weight fragments. The collagen extracted from the two types of fish scales was approximately 42 kDa for all samples which was closed to the results from Veeruraj et al. [22], Liu et al.[23], Tang et al. [24], and Liu et al. [25, 26] although Tang et al. [24] reported that peptide maps of collagens were dependent on sources and species of fish.

3.5. Denaturation Temperature

Collagen structures can be changed when subjected to temperature. Thermal conversion of collagen is attributed to the disintegration of the triple helical structures into random coils and hence the denaturation temperature (T_d) is an important indication for evaluating the thermal stability of collagen. It was well known that collagen from aquatic animals usually has lower T_d than that of mammalian collagen and this become one of the limiting issues for the application of collagen from marine life. Table 4 illustrates T_d and total denaturation enthalpy (ΔH) of acid-pepsin fish scale collagens extracted at different conditions. The T_ds from our experiments were in the range of 35-42^{o}C. Several research reported different T_ds for different sources of collagen and suggested that T_d depended on fish species, fish tissues used for extraction, ages, habitat temperature and environments, and seasons [1, 19, 24, 27]. While Gao et al. [28], Matmaroh et al.[7], and Tan et al. [11] disclosed that ASC has a slightly higher T_d than that of PSC, the study from Chuaychan et al. [19] showed the opposite result. It was found that collagen extracted from internal tissues like swim-bladders and bones had higher T_d than that extracted from external tissues like skins and scales [5, 26, 27, 29]. Even though many previous works have found that fish collagen, which has T_d in a range of 18-37^{o}C, is less thermally stable than mammalian collagen, which has T_d higher than 41^{o}C, few reports including our experiments have found comparable T_ds which were above 39^{o}C [7, 27]. Denaturation enthalpy was the energy required for destabilization the triple helix structure; however, it was very sensitive to impurities and difficult to use as a representative for real variations. The effects of fish scale types as well as extraction times could not be clearly seen from the results in Table 4 and the conclusive summary could not be made.

Table 4. Denaturation temperature (T_d) and total denaturation enthalpy (ΔH) of acid-pepsin collagens.

Samples	T_d (^{o}C)	ΔH (J/g)
G3-48	35.18	0.27
G6-48	40.86	0.21
G9-48	39.22	0.26
N3-48	38.17	0.26
N6-48	35.57	0.22
N9-48	42.34	0.21

3.6. FTIR Spectra

The FIIR spectra in the range of 4000-650 cm^{-1} of collagen obtained by acid extraction (G3-0) and acid-pepsin extraction (G3-48 and N3-48) were shown in Fig. 7. The amide A bands, which are commonly associated with a free N-H stretching vibration, of G3-0, G3-48, and N3-48 were found at the wavenumber of 3265, 3252, and 3255 cm^{-1} respectively, close to those reported by various research [4, 5, 19, 20]. This amide A peak normally occurs in the range of 3400-3440 cm^{-1}; however, the slight shift to a lower wavenumber found here was due to the hydrogen bonds in peptide by which collagen triple-helical structure is held together. Amide B is associated with the asymmetrical stretch of CH_2 and usually found at around 2900-3000 cm^{-1}. Even though the amide B bands of the acid and acid-pepsin extracted collagen were also observed at the same wavenumber of around 2910 cm^{-1}, the intensities of the peaks were dissimilar. The higher amplitude of amide B peak was found in acid extraction collagen, opposite to what Chuaychan et al.

[19] observed. The slight differences in the amplitude of amide B bands between the acid and acid-pepsin derived collagens might be due to the slight differences in the microstructure of the self-aggregated collagen [27]. Additionally, there were also peaks at around 2850-2860 cm^{-1}, which was generated by the symmetrical stretch of CH_2 [27]. The amide I, II and III bands are known to be responsible for the degree of molecular order in collagen, associated with the formation of its triple helical structure. The amide I, II, and III bands are originated from vibrations of C=O stretching, N-H bending coupled with C-N stretching, and C-H stretching, respectively. Wavenumbers in the range of 1600-1700 cm^{-1} of amide I are usually used as a sensitive marker of the peptide's secondary structure. The sharp amide I bands of acid extracted (G3-0) and acid-pepsin extracted (G3-48 and N3-48) were found at 1612 and 1624 cm^{-1}, respectively. A shift of amide I peak to a lower frequency indicated a decrease in molecular order [30]. Amide II bands, generally shown at 1550-1600 cm^{-1}, of G3-0, G3-48, and N3-48 were very closed at 1506, 1534, and 1533 cm^{-1}, respectively, and shifted to lower wavenumbers, indicating the existence of hydrogen bonds. Similarly, amide III bands of the three samples were detected to be in the same range of 1220-1240 cm^{-1}. The absorbance ratios of amide III and 1454 cm^{-1} bands for acid extracted Giant groupers scale collagen (G3-0), acid-pepsin Giant groupers scale collagen (G3-48), and acid-pepsin Nile tilapia scale collagen (N3-48) were 0.98, 0.90, and 0.98, respectively, indicating the triple helical structure [4, 20, 22, 27]. In conclusion, the acid and acid-pepsin collagens showed a slight difference in the secondary structure, but they remained in triple-helix, whereas the collagens extracted from two different fish scales were identical.

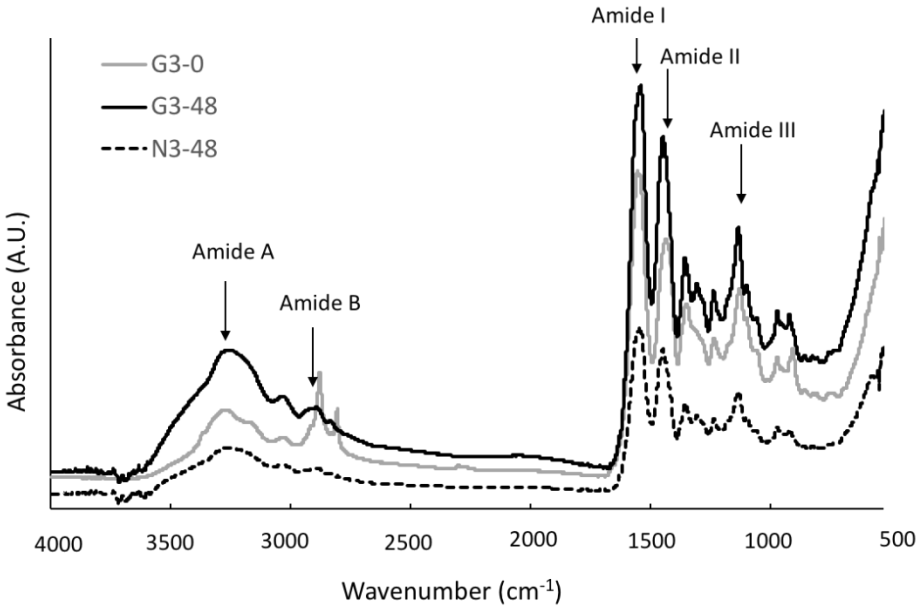

Fig. 7. FTIR of collagens extracted from Giant groupers (G) and Nile tilapia (N) fish scales at different extraction conditions.

3.7. Zeta Potential

The potential values of the collagen samples extracted from the Giant groupers' scales using an acetic acid at different extraction times (3, 6, and 9 hours) followed by 48 hour enzymatic extraction at different pH are shown in Fig. 8. Zeta potential has been used to characterize the surface charge of collagen. It is well known that a protein usually has the worst solubility when the pH of solution was at the isoelectric point (pI). Protein molecules in an aqueous system have zero net charge, where the positive charges are balanced out by the negative charges, at their pIs. At the pI, electrostatic repulsion would be minimized and the precipitation as well as an aggregation of collagen molecules would be favoured [31]. The net surface charges of collagen extracted from Giant groupers' scales at different extraction time are zero at pHs in a range of around 3.2-4.0 which are closed to those of various types of collagens. As the acid extraction time increased, the pIs slightly increased; however, they were still in the acidic range. This suggested that the collagen contained high contents of carboxyl groups and the slight differences in pI might be caused by the slight difference in their amino acid compositions and distribution of amino acid residues, particularly on the surface domains.

For molecules and particles that are small enough, a high zeta potential will confer stability. Roughly, the zeta potentials in the range of ±30 to ±40 mV as in our cases are considered to be moderate stability. From dynamic light scattering analysis at the most stable conditions (pH around 11-12 in our study), the particle sizes were in the range of 100-200 nm and varied according to the acid extraction time as seen in Table 5. It was likely that the particle sizes decreased with increasing the acid extraction time. However, the biggest particle size of G6-48 might be due to the fact that it might not be measured at the lowest zeta potential as can be seen from Fig. 8 and some agglomeration might occur.

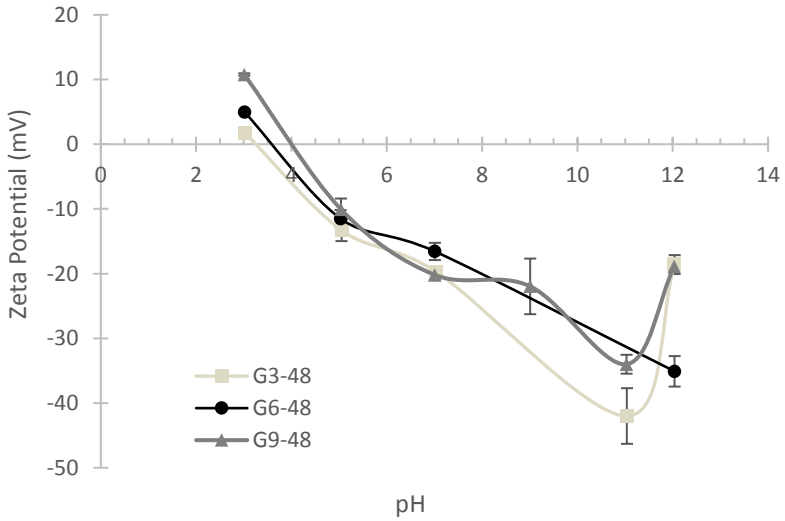

Fig. 8. Zeta potential of collagens extracted from Giant groupers (G) fish scales at different acid extraction time.

Table 5. Collagen particle sizes.

Samples	pH	Average particle size (nm)
G3-48	11.04	179.43 ± 2.37
G6-48	12.04	232.53 ± 4.90
G9-48	12.03	113.10 ± 1.41

4. Conclusions

Collagens from scales of Giant groupers (Epinephelus Ianceolatus), which is saltwater fish, and Nile tilapia (Oreochromis niloticus), which is freshwater fish, were successfully extracted. With the enzymatic extraction, the collagen yields were higher and the molecular weights were lowered compared to the acid extraction. The increase in acid extracting time from 3 to 9 hours resulted in an increase in collagen yield, a slight increase in pH at zero potential, and a decrease in average particle size. The collagen yields also increased as the pepsin extracting time increased from 24 to 48 hours. However, collagen protein pattern and structure were found to be similar for both ASC and PSC from both fish scales. Besides the differences in collagen yield due to the differences protein contents, scales of saltwater and freshwater fish provided similar collagen characteristics. The collagen molecular structures were remained to be the triple helical structure. Comparative denaturation temperatures to those of mammalian collagens suggested a high potential for a commercial alternative.

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