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Analytical Methods

# Isolation and characterization of fish scale collagen from tilapia (*Oreochromis* sp.) by a novel extrusion–hydro-extraction process

Chun-Yung Huang [a,*], Jen-Min Kuo [a], Shu-Jing Wu [b], Hsing-Tsung Tsai [a]

[a] Department of Seafood Science, National Kaohsiung Marine University, No. 142, Hai-Chuan Rd., Nan-Tzu, Kaohsiung 811, Taiwan, ROC
[b] Department of Health and Nutrition, Chia Nan University of Pharmacy and Science, No. 60, Sec. 1, Erh-Jen Rd., Jen-Te District, Tainan 717, Taiwan, ROC

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ABSTRACT

Collagen is highly valued both as a food additive and a functional food ingredient. It is generally extracted by treatments with acid or alkali, enzyme, and microorganisms. However these methods are generally batch type, time-, energy-, reactant-, and cost-consuming. Extrusion is widely used in the food industry, and offers many advantages, such as ease of operation, continuous production, high yield, and little waste. In this study, we developed a novel extrusion–hydro-extraction (EHE) process for extraction of collagen from tilapia fish scale. Extruded scale samples had a 2–3 times higher protein extraction yield than that of non-extruded scale samples. All extracts contained hydroxyproline (61–73 residues/1000 residues) and hydroxylysine (5–6 residues/1000 residues) and were identified as type-I collagens by FTIR, SDS–PAGE, and molecular weight distribution analyses. The physicochemical studies revealed that extracted collagens could have promising applications in the food, medical, and cosmetic industries.
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## 1. Introduction

Collagen is the most abundant protein in vertebrates and constitutes about 25% of vertebrate total proteins (Ogawa et al., 2004). Collagen is able to form insoluble fibers that have high tensile strength and a right-handed triple superhelical rod, consisting of three almost identical polypeptide chains. Nondenatured collagen can be utilized in cosmetics, biomedical, and pharmaceutical industry applications. Denatured collagen, known as gelatin, is water-soluble and finds broad applications in the food, pharmaceutical, photographic, cosmetic, and packaging industries (Ogawa et al., 2004). In the food industry, collagen is widely used as an ingredient to enhance the elasticity, consistency, and stability of food products. Collagen may also be hydrolyzed by protease to obtain peptides with the functionalities of skin-lightening (Zhuang & Sun, 2012), antioxidant (Yang, Ho, Chu, & Chow, 2008), decreasing blood pressure (Morimura et al., 2002), and increasing bone mineral density (Wu, Fujioka, Sugimoto, Mu, & Ishimi, 2004). The global demand for collagen has been steadily increasing over the years, and thus simple, sufficient, steady, and cheaper supplies of collagen are crucially needed.

The major source of collagen, traditionally, is isolated from the skins of land-based animals, such as cow and pig. However, these sources of collagen encounter religious (e.g., both Judaism and Islam forbid consuming any pork-related products, while Hindus do not consume any cow-related products) and safety (bovine spongiform encephalopathy [BSE] of cattle and foot-and-mouth disease [FMD] of pig) oriented concerns (Ogawa et al., 2004). Therefore, alternative sources, especially fish processing waste including skin, bone, or scale, have received increasing attention. These sources are good substitutes for mammalian collagen. The waste from fish processing plants after filleting can account for as much as 75% of the total catch weight. Accordingly, preparations of collagen from aquatic by-products not only satisfy kosher (Judaism) and halal (Islam) requirements and alleviate consumers' concerns about FMD and BSE, but also increase economic returns for the fishery industry.

Collagen from aquatic by-products is generally obtained by the following methods: (1) Acid and alkali extraction: The prepared skin was mixed with NaOH and stirred for 2 h to remove non-collagenous proteins. The mixture was then washed with tap water until neutral or faintly basic pH was obtained. The deproteinized skin was subsequently demineralized using HCl for 1 h. Then, the residue was washed thoroughly with tap water until wash water became neutral or faintly basic in pH. Thereafter, the demineralized skin was swollen by mixing the skins with acetic acid and stirred for 15 min. Finally, the swollen skin was washed thoroughly with tap water until neutral or faintly acidic pH of wash water was obtained. The swollen skin was mixed with distilled water at different temperatures (45–75 °C) for various times (6–12 h). The mixtures were then filtered and collagen was

* Corresponding author.
*E-mail address:* cyhuang@webmail.nkmu.edu.tw (C.-Y. Huang).

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obtained (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2010). This method is complicated and generally requires several preparatory steps, higher temperatures (up to 75 °C), and longer times (up to 16 h) to obtain collagen. (2) Acid and enzyme extraction: The bones and scales were soaked in NaOH for 24–48 h with stirring, and the alkali insoluble components were filtered and rinsed with distilled water until a neutral pH was reached. The insoluble components were extracted with acetic acid or acetic acid containing pepsin for 3–6 d. The solution was centrifuged and the supernatants were salted-out by adding NaCl overnight, and the resulting precipitates were collected by centrifuging and dissolved in acetic acid. The resulting solution was dialyzed against acetic acid and collagen was obtained (Ogawa et al., 2004). This method particularly requires a very long time (up to 192 h) and a large amount of chemicals (NaOH, acetic acid, pepsin, and NaCl) to obtain collagen. In summary, the methods generally used for extraction of collagen are complicated, time-, reactant-, and cost-consuming, and sometimes harmful to the environment. Therefore, a better method for the extraction of collagen is critically needed.

Extrusion cooking has been used for many years to produce several types of animal feed and human food. It is characterized by high temperature short time (HTST) and high shear force processes with many present and possible future applications in the food industry, especially for cooking, forming, and expanding cereals, as well as for texturizing proteins. Processing and other related parameters (such as composition, moisture content, and additives) have the ability to affect the degree of expansion and physical or chemical modifications of raw materials, which ultimately influence the characteristics of extrudates. During the extrusion process, various reactions occur including thermal treatment, gelatinization, protein denaturation, hydrolysis of protein, shearing, mixing, grinding, hydration, shaping, expanding, texture alteration, partial dehydration, and destruction of microorganisms and other toxic compounds (Nwabueze & Iwe, 2010). Most importantly, extrusion cooking offers numerous advantages, such as easy operation, little required labor, continuous production, high yield, limited waste, low labor cost, and multiplicity of products. Extrusion is successfully employed for the pretreatment of rice straw and expedites the saccharification of rice straw by enzymatic hydrolysis (Chen, Xu, Hwang, & Wang, 2011). Thus, it is worthwhile to use extrusion for the pretreatment of fish scale and examine the influence of extrusion parameters on the assistance of collagen extraction from fish scale extrudates.

Tilapia is the most widely cultured fish in Taiwan and mainland China. Taiwan is an important supplier of whole-frozen tilapia to the U.S. and also exports high quality chilled tilapia fillets to the Japanese sashimi market. Numerous fish scales which are regarded as waste are produced from fish fillet processing factories. Generally, collagen extracted from fish scale has a less malodorous smell than that from fish skin and bone. Fish scale is known composed of collagen (generally type I) and hydroxyapatite, and both are tightly linked together (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003) and difficult to separate (Stepnowski, Ólafsson, Helgason, & Jastorff, 2004). The aim of this work was to develop a novel extrusion–hydro-extraction (EHE) process which utilizes the extrusion process to decompose the intimate linkage between collagen and hydroxyapatite, and facilitates the release of collagen from fish scale extrudates by water extraction. Moreover, the extrusion process may also alleviate the unpleasant smell of fish scale and obtain a weaker collagen odor. To the best of our knowledge, this is the first study to use the combination of an extrusion and a water extraction process to facilitate collagen extraction from fish scale. In addition, the physicochemical properties of collagens extracted by the EHE process were also examined and their potential industrial applications were identified.

## 2. Materials and methods

### 2.1. Materials

Tilapia fish scale (TFS) was obtained from a fishery factory in southern Taiwan. Fresh fish scale was kept on ice and transported to the laboratory immediately. TFS was mixed with 0.1 N NaOH to remove non-collagenous proteins, and the mixture was then washed with tap water until neutral or faintly basic pH was obtained. The resultant TFS was dried at 50 °C until the moisture content was less than 10%. The dried TFS was then milled into powder (<20 mesh) and stored at room temperature until use. Sodium carbonate ($Na_2CO_3$), citric acid, and acetic acid were obtained from Nihon Shiyaku Industrial, Ltd. (Tokyo, Japan). Potassium bromide (KBr), bovine serum albumin (BSA), apoferritin, myosin, pepsin, and indium were purchased from Sigma–Aldrich (St. Louis, MO, USA). Folin–Ciocalteu's phenol reagent was obtained from Merck (Darmstadt, Germany). Sephadex 200 was purchased from GE Healthcare UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents if not declared were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were all of analytical grade.

### 2.2. Extrusion cooking

TFS powder was used as raw material and mixed with double-distilled water ($ddH_2O$), 1.26% citric acid (pH 2), or 9.37% acetic acid (pH 2) at a ratio of 4.7:1 (w/v) as a preconditioning step. Extrusion cooking was performed using a model single screw extruder equipped with a screw diameter of 74 mm, a screw length to diameter (L/D) ratio of 3.07:1, and a rounded die opening at the end of the extruder (Tsung Hsing Food Machinery, Kaohsiung, Taiwan). Heating of the barrel was controlled by electric heating element jacketing the barrel and thermal probe, and the barrel temperature was set at 135 °C. Screw speed was kept constant at 360 rpm. The extrudates were collected at the die end and kept at 50 °C in a hot air oven for 30 min to remove extra moisture. Preconditioning of TFS powder and extrusion process variables used for EHE process are given in Table 1.

### 2.3. Extraction of collagen from fish scale extrudate

Tilapia fish scale extrudate (TFSE), after dried and ground into powder, was soaked in $ddH_2O$ with a sample ratio of 1:10 (w/v) and shaken in a water bath at 25 °C or 50 °C for 1 h. The mixture was centrifuged at 10,200*g* for 10 min, and the supernatant was collected for protein concentration analysis. The supernatant was lyophilized for further analyses. A flowchart of the procedures used for the extraction of collagen from TFSE in the EHE process is presented in Fig. 1. For comparison purposes, the yield of collagen was calculated by Eqs. (1) and (2) below:

$$\begin{aligned}\text{Yield }(\%) = [&(\text{hydroxyproline content of supernatant }(\text{g/ml}) \\ &\times \text{volume of supernatant }(\text{ml})) \\ &/(\text{hydroxyproline content of TFS, dry basis }(\text{g/g}) \\ &\times \text{weight of TFS used }(\text{g}))] \times 100 \end{aligned} \quad (1)$$

$$\begin{aligned}\text{Yield }(\%) = [&(\text{protein content of supernatant }(\text{g/ml}) \\ &\times \text{volume of supernatant }(\text{ml})) \\ &/(\text{crude protein content of TFS, dry basis }(\text{g/g}) \\ &\times \text{weight of TFS used }(\text{g}))] \times 100 \end{aligned} \quad (2)$$

**Table 1**
Process variables and extraction yields of water soluble collagen for FS1–FS14 at extraction temperatures of 25 and 50 °C.

| Treatments | Parameters | FS1 | FS2 | FS3 | FS4 | FS5 | FS6 | FS7 | FS8 | FS9 | FS10 | FS11 | FS12 | FS13 | FS14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Preconditioning | Solvent | – | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | $ddH_2O$ | Citric acid | Citric acid | Acetic acid | Acetic acid |
| Extrusion | Feed supply (kg/h) | – | 11.4 | 14.8 | 18.3 | 11.4 | 14.8 | 18.3 | 11.4 | 14.8 | 18.3 | – | 11.4 | – | 11.4 |
| | Die diameter (mm) | – | 3 | 3 | 3 | 4 | 4 | 4 | 5 | 5 | 5 | – | 3 | – | 3 |
| Yield* | 25 °C | $2.0 \pm 0.1^{b}$ | $7.6 \pm 0.2^{fg}$ | $6.6 \pm 0.5^{e}$ | $6.7 \pm 0.6^{ef}$ | $8.1 \pm 0.5^{g}$ | $7.2 \pm 0.3^{efg}$ | $6.7 \pm 0.5^{ef}$ | $5.6 \pm 0.6^{d}$ | $4.4 \pm 0.4^{c}$ | $4.2 \pm 0.4^{c}$ | $0.3 \pm 0.2^{a}$ | $6.9 \pm 0.7^{ef}$ | $2.1 \pm 0.2^{b}$ | $15.0 \pm 0.8^{h}$ |
| | 50 °C | $5.1 \pm 0.3^{b}$ | $14.9 \pm 0.9^{g}$ | $11.9 \pm 0.3^{ef}$ | $10.9 \pm 1.1^{def}$ | $12.1 \pm 0.5^{f}$ | $10.5 \pm 0.6^{cde}$ | $12.4 \pm 0.8^{f}$ | $10.3 \pm 0.4^{cd}$ | $9.7 \pm 0.9^{cd}$ | $9.0 \pm 1.2^{c}$ | $3.0 \pm 0.2^{a}$ | $9.9 \pm 1.3^{cd}$ | $5.1 \pm 0.4^{b}$ | $16.6 \pm 0.2^{h}$ |
| Yield** | 25 °C | $1.6 \pm 0.1^{b}$ | $6.3 \pm 0.2^{fg}$ | $5.5 \pm 0.4^{e}$ | $5.5 \pm 0.5^{ef}$ | $6.7 \pm 0.4^{g}$ | $6.0 \pm 0.2^{efg}$ | $5.5 \pm 0.4^{ef}$ | $4.6 \pm 0.5^{d}$ | $3.7 \pm 0.3^{c}$ | $3.5 \pm 0.3^{c}$ | $0.3 \pm 0.2^{a}$ | $5.9 \pm 0.6^{ef}$ | $1.5 \pm 0.2^{b}$ | $10.7 \pm 0.5^{h}$ |
| | 50 °C | $4.2 \pm 0.3^{b}$ | $12.3 \pm 0.8^{g}$ | $9.8 \pm 0.2^{ef}$ | $9.0 \pm 0.9^{def}$ | $10.0 \pm 0.4^{f}$ | $8.6 \pm 0.5^{cde}$ | $10.3 \pm 0.7^{f}$ | $8.5 \pm 0.3^{cd}$ | $8.0 \pm 0.8^{cd}$ | $7.5 \pm 1.0^{c}$ | $2.5 \pm 0.1^{a}$ | $8.4 \pm 1.1^{cd}$ | $3.6 \pm 0.3^{ab}$ | $11.8 \pm 0.1^{g}$ |

–, not adopted.
a–h Values are mean ± SD ($n = 3$); values with different letters within the same row differ significantly ($P < 0.05$).
* Expressed by g hydroxyproline/100 g hydroxyproline content in TFS, dry basis.
** Expressed by g protein/100 g crude protein content in TFS, dry basis.

### 2.4. Chemical composition analyses

The determinations of the moisture, fat, ash, and crude protein were carried out using the following AOAC (1984) procedures: moisture (%) was measured by drying samples in an oven at 103 °C for 8 h; crude fat (%) was determined gravimetrically after the Soxhlet extraction with petroleum ether; crude ash (%) was obtained by incineration in a muffle furnace at 580 °C for 8 h; and crude protein ($N \times 5.95$) (%) was measured by the Kjeldahl method after acid digestion.

### 2.5. Sensory evaluation

Sensory evaluation was conducted using a 24-member panel. Only individuals who were able to detect off-odor in samples having a slightly putrid odor were selected. Samples for sensory evaluation were prepared using lyophilized collagen powder. The samples (in solution 6.67%; w/v or in powder) were placed in test tubes with screw caps, and the samples in solution were held in a water bath at 50 °C, with the screw caps lightly closed. Panelists were instructed to remove the screw caps, smell the contents, and identify the odor that they perceived, as well as indicate the odor intensity. The nine-point hedonic scale (1 = no odor; 5 = middle odor; 9 = very strong and very offensive odor) was used for the analysis of odor intensity (Muyonga, Cole, & Duodu, 2004b).

### 2.6. Determination of hydroxyproline

Hydroxyproline content was determined using the colorimetric method described elsewhere (Wang et al., 2008). A calibration curve was performed using five standard solutions of hydroxyproline. Hydroxyproline content in the sample was calculated from the standard curve.

### 2.7. Determination of protein concentration

The Lowry assay was performed according to previous work (Lowry, Rosebrough, Farr, & Randall, 1951). Varying concentrations of stock BSA protein solution (1 mg/ml) were utilized for calibration.

### 2.8. Fourier transform infrared (FTIR) spectroscopy

Two milligram of protein powder were ground evenly with approximately 100 mg KBr until particles measured <2.5 μm in size. The transparent KBr pieces were made at 500 kg/cm$^2$. The FTIR spectra were obtained using a FT-730 spectrometer (Horiba, Japan). The signals were automatically collected using 32 scans over the range of 4000–400 cm$^{-1}$ at a resolution of 2 cm$^{-1}$ and were compared to a background spectrum collected from the KBr alone at room temperature.

### 2.9. Determination of gel strength

Bloom gel strength was determined by the British Standard 757: 1975 method (BSI, 1975) with a texture analyzer (Stevens LFRA TA 1000, England). A solution containing 6.67% (w/v) collagen was prepared by mixing 7.5 g of collagen and 105 ml of $ddH_2O$ in a Bloom bottle. The mixture was stirred and allowed to stand for 30 min at room temperature to let the collagen to absorb water and swell. The Bloom bottles were then transferred to a water bath maintained at 42 °C and held for 30 min, during which they were stirred intermittently. The samples were then transferred to a cold water bath maintained at 10 ± 0.1 °C and held at this temperature for 16–18 h before determination of gel strength. The Bloom gel

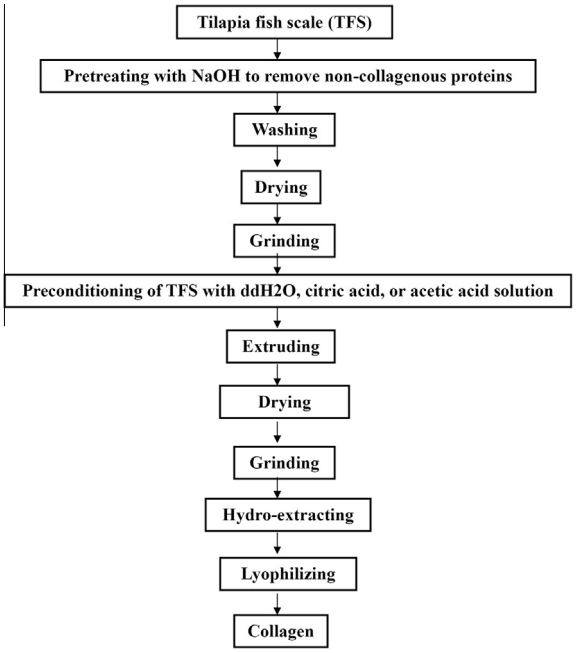


**Fig. 1.** Flowchart of the novel extrusion–hydro-extraction (EHE) process for extraction of collagen from tilapia fish scale.

strength (in g) was determined with the texture analyzer set to make a 4 mm depression at a rate of 0.5 mm/s.

### 2.10. Differential scanning calorimetry (DSC)

The studies of DSC were performed using a DSC 200 F3 calorimeter (Netzsch-Gerätebau GmbH, Germany). The instrument was calibrated for temperature and enthalpy using indium as the standard, and the measurements were done while samples were constantly purged with ultrahigh-purity nitrogen at 50 $cm^3$/min. The lyophilized protein sample (5 mg) was accurately weighed into aluminum pans, and hermetically sealed and scanned from 0 to 200 °C at a heating rate of 5 °C/min. An empty sealed aluminum pan was used as the reference. The maximum denaturation temperature ($T_{max}$) was recorded by the software as the peak temperature of each endothermic peak, and the total denaturation enthalpy (ΔH) (J/g protein sample) for each peak was determined by measuring the corresponding area under each endothermic peak (Liu, Liang, Regenstein, & Zhou, 2012).

### 2.11. Determination of viscosity

Protein sample (6.67%, w/v) was melted in a water bath maintained at 45 °C and then poured into the viscometer. The viscometer was held in a water bath maintained at 60 °C for 15 min before the viscosity was determined. Viscosity was recorded using a LVD-V viscometer (Brookfield, Massachusetts, USA) equipped with a BL rotor No. 862 at 100 rpm at 60 °C. The viscosity was read and reported in terms of centipoise (cP).

### 2.12. Sodium dodecyl sulfate gel electrophoresis

Protein patterns of collagen were determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970). The solubilized protein samples were mixed at a ratio of 1:1 (v/v) with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS and 20% glycerol) in the presence of 10% 2-mercaptoethanol and then boiled for 10 min. A portion of sample (20 μg) per well was loaded onto polyacrylamide gels consisting of a 8% running gel and a 4% stacking gel, and subjected to electrophoresis at a constant 100 V/gel using a mini-vertical electrophoresis system (Hoefer, GE Healthcare, USA). Gels were stained for 3 h using 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid. The gel was finally destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Wide range molecular weight markers (Sigma–Aldrich, St. Louis, MO, USA) were used to estimate the molecular weight of proteins.

### 2.13. Measurement of water absorption capacity (WAC)

Lyophilized protein samples were put into desiccators, which contained saturated $Na_2CO_3$ solution (43% relative humidity). The desiccators were stored at 30 °C in a thermostatic chamber for 24 h. The moisture absorption was evaluated by the percentage of weight increase of the dry sample:

$$\text{WAC}\ (\%) = [(W_n - W_0)/W_0] \times 100\%,$$

where $W_0$ and $W_n$ were the weights of sample before and after they were put into the desiccators for 24 h.

### 2.14. Measurement of water retention capacity (WRC)

Wet protein samples were prepared by adding $ddH_2O$ to each lyophilized protein sample (w/w = 1:1) and waited for the water was absorbed into the samples. The wet samples were put into desiccators which contained saturated $Na_2CO_3$ solution (43% relative humidity). The desiccators were stored at 30 °C in a thermostatic chamber for 24 h. The water retention was evaluated by the percentage of residual water of the wet sample:

$$\text{WRC}\,(\%) = (H_n/H_0) \times 100\%,$$

where $H_0$ and $H_n$ were the weights of water in the sample before and after they were put in the desiccators for 24 h.

### 2.15. Molecular weight analysis

The molecular weight analysis of the collagen samples was conducted using a size exclusion HPLC column Superdex 200 (300 mm × 10 mm ID, GE Healthcare, USA) using a SHIMADZU HPLC system (Shimadzu, Kyoto, Japan). The chromatography conditions were: eluent 0.1 M $Na_2SO_4$, 0.02 M $NaH_2PO_4{\cdot}2H_2O$ and 0.5% SDS, at pH 5.30; flow rate 0.2 ml/min, sample concentration 1%; injection volume 0.5 ml; temperature 25 °C; and wavelength 280 nm. Standard proteins used for calibration of MW are apoferritin (443 kDa), myosin (200 kDa), BSA (67 kDa), and pepsin (35 kDa).

### 2.16. Determination of amino acid composition

A 0.01 g sample of protein was hydrolyzed in 0.6 ml of 6 M HCl in an evacuated and sealed tube at 110 °C for 24 h. The hydrolysate was dried at 65 °C under vacuum. Dry hydrolysate was dissolved in 2 ml of 0.25 M sodium citrate buffer (pH 2.2) and then filtered through 0.45-μm PVDF filters. A Shimadzu LC-10A high performance liquid chromatography system (Shimadzu, Kyoto, Japan) instrument equipped with a dual-pump LC-10AT binary system (Shimadzu, Kyoto, Japan), a fluorescence detector RF-10AXL (Shimadzu, Kyoto, Japan), and a Shim-pack AMINO-Na column (100 mm × 6.0 mm) was used to conduct the analysis. Amino acid analysis was done using pre-column fluorescence derivatization with *o*-phthaldialdehyde. The individual amino acid content was based on the area of the corresponding peak on the elution curves of the samples and standards, as determined by software (Sigma–

Aldrich, St. Louis, MO, USA), and the sample's amino acid composition was expressed on the basis of residues per 1000 total residues. The contents of methionine and cystine and/or cysteine were determined by HPLC analysis after converting them into methionine sulfone and cysteic acid, respectively (AOAC, 1984). Determination of tryptophan was also performed by HPLC analysis after alkaline hydrolysis (AOAC, 1984).

### 2.17. Statistical analysis

All experiments were performed in triplicate, and the results were the average of three independent experiments. Measurements were presented as means ± standard deviation. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS). The results obtained were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range tests. A probability value of $P < 0.05$ was considered statistically significant.

## 3. Results and discussion

### 3.1. Effects of parameters of EHE on extraction yield of water soluble collagen from TFS

The TFS, obtained from a fishery factory in the south of Taiwan, possessed the chemical composition of 49.42% protein, 0.02% lipid, 45.18% ash, and 5.38% carbohydrate on the basis of dry weight. TFS had similar protein content as compared to other fish scales, such as spotted golden goatfish (45.2% protein, dry basis) (Matmaroh, Benjakul, Prodpran, Encarnacion, & Kishimura, 2011) and deep-sea redfish (56.9% protein, dry basis) (Wang et al., 2008), indicating that TFS is also a good source for extraction of collagen. Since collagen is tightly linked to hydroxyapatite in fish scale (Ikoma et al., 2003), a reactant- and time-consuming demineralization process is necessary to loosen the matrix of scale, and thus collagen can be readily extracted from the scale matrix (Matmaroh et al., 2011). In the present study, a novel EHE process described in Fig. 1 was developed to extract collagen from scale without the necessity of a demineralization process. The EHE process is mainly composed of preconditioning, extrusion, and hydro-extraction steps. The designed variables used for the EHE process are listed in Table 1. TFS powder was previously preconditioned with $ddH_2O$, citric acid solution (pH 2), or acetic acid solution (pH 2) at a ratio of 4.7:1 (w/v), which wetted TFS powder and made it suitable for extrusion. Preliminary study showed that constant screw speed at 360 rpm and constant barrel temperature at 135 °C obtained steady operation for extrusion. Therefore, effects of different preconditioning conditions ($ddH_2O$, citric acid solution, and acetic acid solution), variables in feed supply (11.4, 14.8, and 18.3 kg/h), variables in die diameter (3, 4, and 5 mm), and different hydro-extraction temperatures (25 and 50 °C) on the extraction yield of water soluble collagen from TFS were examined, and the results were presented in Table 1. Previous studies showed the yield of collagen can be expressed by g hydroxyproline extracted/100 g hydroxyproline content in raw material used (Kolodziejska, Skierka, Sadowska, Kolodziejski, & Niecikowska, 2008) or g protein extracted/100 g crude protein content in raw material used (Zhang, Duan, Ye, & Konno, 2010). We found that in all sample groups the yields expressed as g hydroxyproline/100 g hydroxyproline content in TFS were higher than those expressed as g protein/100 g crude protein content in TFS (Table 1). However, the differences were not remarkable. Since the measurement of protein content in extracts is relatively simple and fast, with less deviation as compared to the measurement of hydroxyproline content in extracts, we therefore recommend using the expression of g protein/100 g crude protein content in TFS to determine the extraction yield. Extruded samples (FS2-FS10, FS12, and FS14) had significantly higher extraction yields as compared to non-extruded samples (FS1, FS11, and FS13) despite hydro-extraction temperatures at 25 or 50 °C (Table 1). Studies suggested that fish scales were biocomposites of highly ordered collagen fibers with numerous cross linked regions (Zylberberg, Bereiter-Hahn, & Sire, 1988). Moreover, the collagen was tightly linked to hydroxyapatite in the matrix of scale, and therefore it is difficult to isolate collagen from scales (Ikoma et al., 2003; Stepnowski et al., 2004). In the present study, the non-collagenous proteins of TFS were previously removed by treatment of 0.1 N NaOH, and then the TFS was subjected to extrusion. The high heat, high pressure, and high mechanical force produced during the extrusion process (Yeh, Hwang, & Guo, 1992) may aid to decompose the matrix of scales and weaken the tight linkage between collagen and hydroxyapatite, and thus facilitate the solvent to enter and increase the extraction yield of collagen from TFS. Due to the step of removing non-collagenous proteins, it may be supposed that the extracted protein by the EHE process is mainly water soluble collagen. Although the extrusion technique was widely utilized to facilitate oil extraction from seeds (Sriti et al., 2012), facilitate alginate extraction from *Laminaria digitata* (Vauchel, Kaas, Arhaliass, Baron, & Legrand, 2008), pretreat rice straw for producing cellulosic ethanol (Chen et al., 2011), and extract proteins from crops (Colas, Doumeng, Pontalier, & Rigal, 2013), to the best of the authors' knowledge, this is the first study to report using extrusion to decompose fish scale matrix and then facilitate collagen extraction from fish scale. For the samples in the same preconditioning ($ddH_2O$) and same feed supply (11.4 kg/h) group (FS2, FS5, and FS8), it was found that elevated die diameter (3, 4, and then 5 mm) tended to decrease the extraction yield of water soluble collagen for extraction temperature at either 25 or 50 °C (Fig. 2A). For the samples in the same preconditioning ($ddH_2O$) and same die diameter (3 mm) group (FS2, FS3, and FS4), it was found that elevated feed supply (11.4, 14.8, and then 18.3 kg/h) tended to decline the extraction yield of water soluble collagen for extraction temperature at either 25 or 50 °C (Fig. 2B). Studies suggested that both increasing the feed supply (Yeh et al., 1992) and die diameter (De Ruyck, 1997) reduced the mean residence time (MRT) of extrudate. In the present study, elevated levels of feed supply and die diameter may reduce the MRT, diminish the effects of heat, pressure, and mechanical force on FS, and then result in a lower extraction yield of water soluble collagen from TFS. Accordingly, among FS2 to FS10, the FS2 (feed supply at 11.4 kg/h; die diameter at 3 mm) possessed the highest extraction yield of water soluble collagen at 50 °C and might be the best condition for extraction of collagen from TFS (Table 1). Certain studies utilized acid to isolate collagen from FS (Matmaroh et al., 2011; Wang et al., 2008). In the present study, we used citric acid solution (FS12) and acetic acid solution (FS14) to precondition and extrude TFS, and then compared the extraction yields of water soluble collagen from FS12 and FS14 with that of FS2 (preconditioning with $ddH_2O$). Results suggested that for hydro-extraction at a temperature of 25 °C, FS14 (10.7%; expressed by g protein/100 g crude protein content in FS, dry basis) had the highest extraction yield of collagen, followed by FS2 (6.3%), and then FS12 (5.9%). For hydro-extraction at a temperature of 50 °C, FS2 (12.3%) possessed the highest extraction yield of water soluble collagen, followed by FS14 (11.8%), and then FS12 (8.4%) (Table 1 and Fig. 2C). Thus, preconditioning by two types of acid solution did not obviously affect the extraction yield of water soluble collagen from TFS. Nevertheless, the process of acid preconditioning may alter the physicochemical properties of extracted collagen, and this issue requires further clarification. In comparison with the extraction yield of collagen from FS reported by other researchers, it was

found that acid soluble collagen (ASC) and pepsin soluble collagen (PSC) extracted from scale of spotted golden goatfish (*Parupeneus heptacanthus*) had yields of approximately 1.06% and 2.76% (expressed by g protein/100 g crude protein content in FS, dry basis), respectively (Matmaroh et al., 2011), collagen extracted from scale of deep-sea redfish (*Sebastes mentella*) had a yield of 6.8% (expressed by g hydroxyproline content in prepared collagen/100 g hydroxyproline content in FS, dry basis) (Wang et al., 2008), PSC extracted from scale of bighead carp (*Hypophth almichthys nobilis*) had a yield of ~3.2% (expressed by g protein/100 g crude protein content in FS, dry basis) (Liu et al., 2012), and ASC extracted from scale of silver carp (*Hypophth almichthys molitrix*) had a yield of 1.03% (expressed by g protein/100 g crude protein content in FS, dry basis); whereas, that of PSC was 2.79% (expressed by g protein/100 g crude protein content in FS, dry basis) (Zhang et al., 2010). In general, the present study showed that the yield of collagen extracted from TFS by the EHE process approximately ranged from 7.5 to 12.3% (expressed by g protein/100 g crude protein content in FS, dry basis, with extraction temperature of 50 °C), and this exceeded the extraction yield of collagen (1.02–6.8%) reported by other studies. It is worth noting that the samples from hydro-extraction at a temperature of 50 °C exhibited higher extraction yield of water soluble protein (approximate two-fold) as compared to hydro-extraction at a temperature of 25 °C (Table 1). In consideration of the aspect between energy consumption and the extraction yield of collagen, we recommended that extraction temperature at 50 °C was a preferable selection. The collagens from FS2 (preconditioning by $ddH_2O$), FS12 (preconditioning by citric acid solution), and FS14 (preconditioning by acetic acid solution) were selected for further examination of their physicochemical properties.

### 3.2. Identification of the extracted collagen

The collagens obtained in FS2, FS12, and FS14 were characterized using FTIR, SDS–PAGE, and HPLC gel filtration chromatography, respectively. Studies revealed that FTIR spectra of collagen possessed five major adsorption bands in the amide band region, including 1644–1653 $cm^{-1}$ (amide I), 1541–1548 $cm^{-1}$ (amide II), 1237–1239 $cm^{-1}$ (amide III), 3304–3315 $cm^{-1}$ (amide A), and 2922–2940 $cm^{-1}$ (amide B) (Kittiphattanabawon et al., 2010; Muyonga, Cole, & Duodu, 2004a). In the present study, FTIR spectra of FS2, FS12, and FS14 exhibited the characteristic peaks of amide I, II, III as well as amide A and B (Fig. 3A). The major absorption bands at 1620 $cm^{-1}$ (amide I), 1520 $cm^{-1}$ (amide II), 1220 $cm^{-1}$ (amide III), 3290 $cm^{-1}$ (amide A) and 2910 $cm^{-1}$ (amide B) were present, and were similar to the spectra exhibited by other collagens (Jackson, Choo, Watson, Halliday, & Mantsch, 1995), indicating that collagen was extracted from TFS by the EHE process. The SDS–PAGE patterns of FS2, FS12, and FS14 showed two different α chains (α1 and α2) and their cross-linked β chains (Fig. 3B). The existence of two different α subunits shows that a major collagen from the fish scale is a type I collagen (Ogawa et al., 2004). There were no significant differences in protein patterns of SDS–PAGE among FS2, FS12, and FS14 (Fig. 3B). HPLC gel filtration chromatogram was utilized for further identification of molecular weights among collagens in FS2, FS12, and FS14. Fig. 3C showed that α subunit (123.7 kDa) and β subunit (255.5 kDa) were present in FS2, α subunit (126.2 kDa) and β subunit (281.1 kDa) were present in FS12, and α subunit (116.5 kDa) and γ subunit (417.3 kDa) were present in FS14. We speculated that the slight alteration of retention time of α subunit in FS2, FS12, and FS14 might be due to the different preconditioning conditions. It is worth noting that

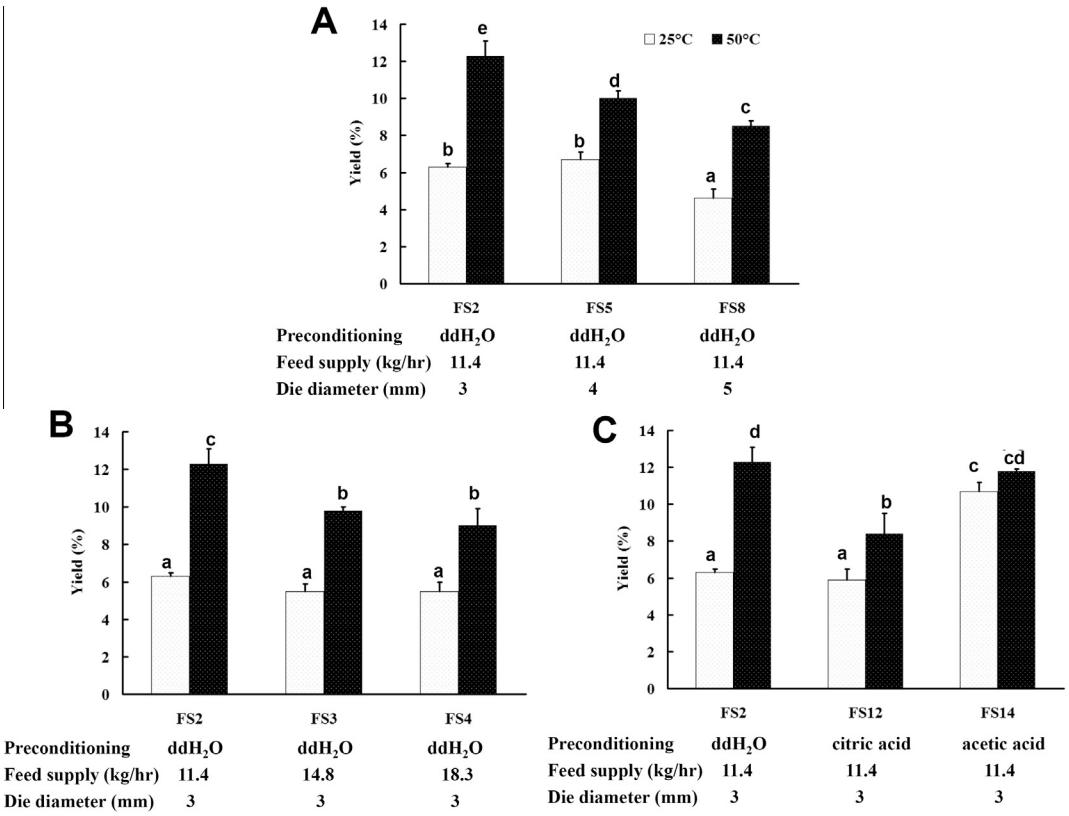


**Fig. 2.** Influence of die diameter (A), feed supply (B), and preconditioning (C) on extraction yield of water soluble collagen from TFS by the EHE process. Yields were expressed by g protein/100 g crude protein content in TFS, dry basis. Results are shown as means ± SD ($n = 3$) and letters indicate significant differences in yields among samples ($P < 0.05$).

the cross-linked γ subunit (trimer) obviously existed in FS14, suggesting that a trimer (triple-helix) form of collagen could be obtained from FS14, and might be a good candidate for medical and therapeutic uses (Ruggiero et al., 2000). In addition, as shown in Fig. 3C, there are certain peaks observed between 35 and 67 kDa, and these proteins may be the degraded fragments of collagen. The degraded fragments of collagen possess smaller molecular weight and may have certain functionalities, such as antioxidant, mineral chelating, and angiotensin I converting enzyme (ACE) inhibitory activities. However, these issues should be further addressed. In summary, we confirmed that the extracted protein was type I collagen which possessed α1 and α2 subunits at a molecular weight of approximately 116.5–126.2 kDa, β subunit at a molecular weight of approximately 255.5–281.1 kDa, and subunit at a molecular weight of approximately 417.3 kDa.

### 3.3. Amino acid composition and physicochemical properties of the extracted collagen

The amino acid composition of FS1, FS2, FS12, and FS14 is presented in Table 2. In all extracted collagens, glycine was the most abundant amino acid (about 378–390 residues/1000 residues). They were also found to contain large amounts of alanine, proline, hydroxyproline, and glutamic acid/glutamine, but tiny amounts of cysteine, tyrosine, and tryptophan. Additionally, all extracted collagens contained hydroxyproline (61–73 residues/1000 residues) and hydroxylysine (5–6 residues/1000 residues), the unique amino acids found in collagen. The amino acid profiles of all extracted collagens were quite similar to those of type I collagen isolated from fins, scales, skins, bones, and swim bladders of bighead carp (Liu et al., 2012). However, slight differences in amino acid compositions were also found among FS2, FS12, and FS14. The content of imino acids (proline and hydroxyproline) is known to contribute to the thermal stability of collagens (Liu et al., 2012). It was found that FS12 contained the largest amount of imino acids (115 + 73 = 188, residues per 1000 total residues), followed by FS2 (107 + 68 = 175, residues per 1000 total residues), and FS14 (109 + 61 = 170, residues per 1000 total residues). Therefore, further study is needed to elucidate the relationships between amino acid composition and the physicochemical properties of extracted collagens in FS2, FS12, and FS14. In Table 2, the collagens extracted from FS2, FS12, and FS14 exhibited gel strength of 260.3, 185.0, and 157.0 g, respectively. Preconditioning of TFS with either citric acid or acetic acid solution decreased gel strength as compared to that of preconditioning of TFS with $ddH_2O$ to a significant degree ($P < 0.05$). Previous research has suggested that protein degradation fragments might reduce the ability of α-chains to anneal correctly by hindering the growth of the existing nucleation sites, and thus decrease gel strength (Ledward, 1986). As a result, preconditioning of TFS with acid solutions (FS12 and FS14) may partially degrade collagen and decrease gel strength. The difference in gel strength of extracted collagen was possibly due to the fish species,

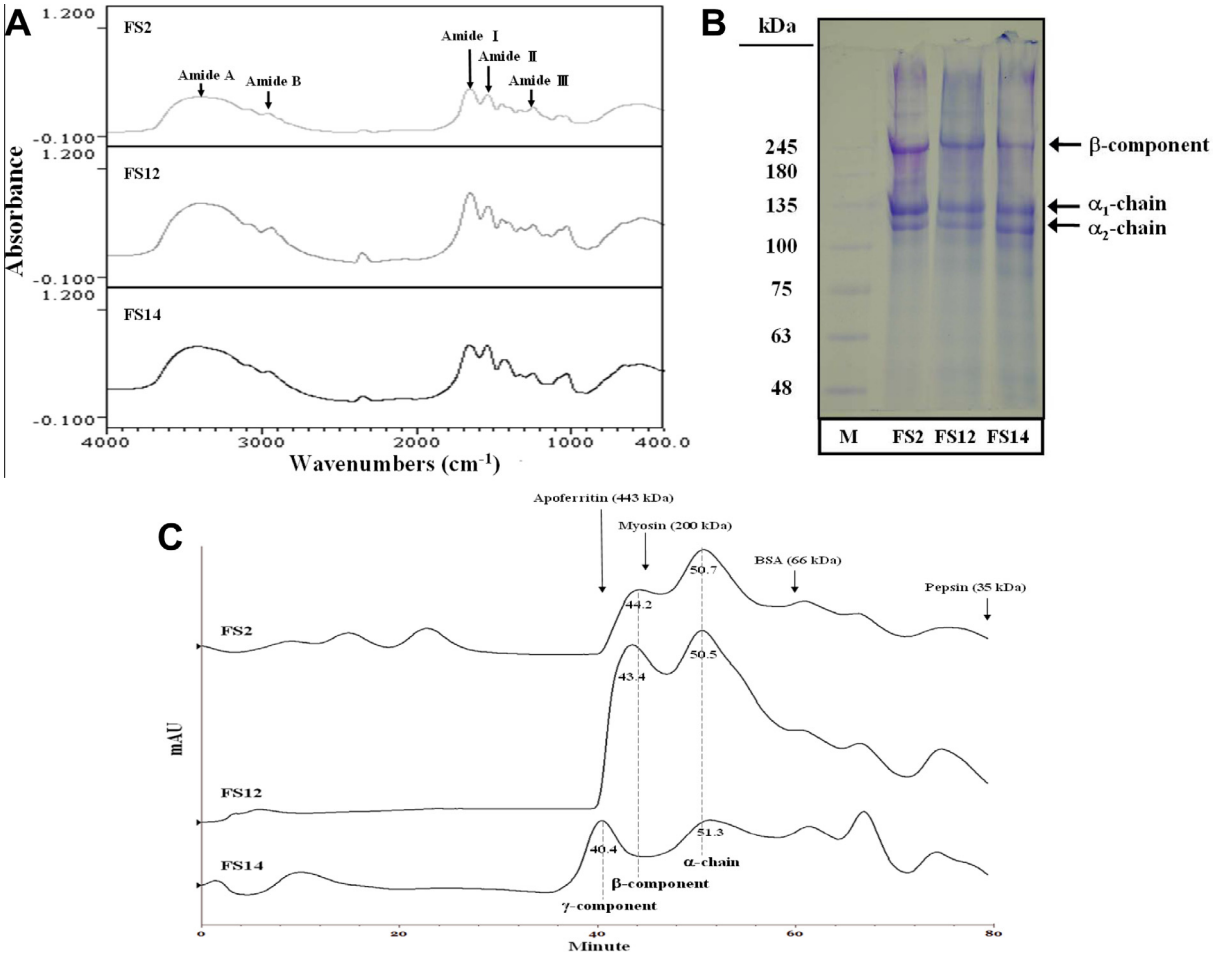


**Fig. 3.** Characteristics of extracted collagens in FS2, FS12, and FS14. (A) FTIR spectra of collagens from FS2, FS12, and FS14. (B) SDS–PAGE patterns of collagens from FS2, FS12, and FS14. The first lane is protein marker (M). (C) Size exclusion chromatographic profiles of collagens from FS2, FS12, and FS14. Apoferritin (443 kDa), myosin (200 kDa), BSA (67 kDa), and pepsin (35 kDa) were utilized as standard proteins.

**Table 2**
Amino acid composition, bloom gel strength, viscosity, denaturation temperatures ($T_{max1}$, $T_{max2}$), denaturation enthalpies (H1, H2), water absorption capacity (WAC), and water retention capacity (WRC) of collagens from FS1, FS2, FS12, and FS14.

| | | FS1 | FS2 | FS12 | FS14 |
|---|---|---|---|---|---|
| Amino acids | Aspartic acid/asparagine | 40.5 ± 0.5[a] | 35.7 ± 1.9[a] | 37.5 ± 0.7[a] | 37.1 ± 1.4[a] |
| | Glutamic acid/glutamine | 65.6 ± 0.2[b] | 64.2 ± 0.7[ab] | 64.5 ± 0.2[ab] | 63.2 ± 0.5[a] |
| | Serine | 21.1 ± 0.3[a] | 20.9 ± 0.4[a] | 21.8 ± 0.1[a] | 20.9 ± 0.0[a] |
| | Histidine | 7.8 ± 0.1[ab] | 8.0 ± 0.4[b] | 8.0 ± 0.1[b] | 6.9 ± 0.1[a] |
| | Glycine | 386.5 ± 0.6[b] | 384.9 ± 1.0[b] | 378.4 ± 1.6[a] | 390.2 ± 2.3[b] |
| | Threonine | 21.6 ± 0.1[b] | 21.1 ± 0.4[b] | 21.5 ± 0.1[b] | 19.1 ± 0.1[a] |
| | Arginine | 48.7 ± 0.1[a] | 50.6 ± 0.8[b] | 49.3 ± 0.0[ab] | 51.0 ± 0.5[b] |
| | Alanine | 128.9 ± 0.6[b] | 134.0 ± 0.9[c] | 126.4 ± 0.1[a] | 129.2 ± 0.3[b] |
| | Tyrosine | 1.4 ± 0.1[a] | 1.5 ± 0.2[a] | 1.7 ± 0.1[a] | 1.4 ± 0.1[a] |
| | Cysteine | 4.5 ± 0.3[b] | 3.7 ± 0.3[ab] | 4.7 ± 0.4[b] | 2.8 ± 0.5[a] |
| | Valine | 18.0 ± 0.3[a] | 18.0 ± 0.5[a] | 17.7 ± 0.1[a] | 16.8 ± 0.3[a] |
| | Methionine | 12.6 ± 0.9[b] | 12.8 ± 0.1[b] | 13.6 ± 0.7[b] | 8.4 ± 0.1[a] |
| | Phenylalanine | 11.1 ± 0.4[a] | 11.4 ± 0.4[a] | 11.5 ± 0.0[a] | 12.7 ± 0.1[b] |
| | Isoleucine | 8.2 ± 0.1[a] | 8.1 ± 0.3[a] | 7.7 ± 0.2[a] | 16.6 ± 0.1[b] |
| | Leucine | 19.4 ± 0.0[a] | 20.4 ± 0.5[b] | 19.1 ± 0.8[ab] | 17.6 ± 0.4[a] |
| | Lysine | 22.4 ± 0.2[a] | 24.0 ± 0.7[b] | 22.8 ± 0.1[ab] | 29.9 ± 0.0[c] |
| | Tryptophan | 0.9 ± 0.1[a] | 1.2 ± 0.2[a] | 1.1 ± 0.1[a] | 1.1 ± 0.1[a] |
| | Proline | 106.2 ± 0.2[a] | 106.5 ± 2.8[a] | 114.7 ± 1.4[b] | 108.7 ± 0.3[bc] |
| | Hydroxylysine | 5.3 ± 0.0[b] | 5.1 ± 0.0[a] | 5.2 ± 0.1[ab] | 5.5 ± 0.0[c] |
| | Hydroxyproline | 69.4 ± 0.8[bc] | 67.8 ± 0.2[b] | 73.0 ± 0.5[c] | 60.8 ± 1.9[a] |
| | Total | 1000.0 | 1000.0 | 1000.0 | 1000.0 |
| Physicochemical properties | Bloom gel strength (g) | – | 260.3 ± 1.7[c] | 185.0 ± 5.4[b] | 157.0 ± 5.1[a] |
| | Viscosity (cP) | – | 8.5 ± 0.1[a] | 10.2 ± 0.2[b] | 8.1 ± 0.2[a] |
| | $T_{max1}$ (°C) | – | 71.5 | 79 | 57.9 |
| | $T_{max2}$ (°C) | – | 173.3 | 176.1 | 182.0 |
| | H1 (J/g) | – | 21.1 | 1.4 | 8.0 |
| | H2 (J/g) | – | 239.8 | 115.6 | 166.4 |
| | WAC (%) | – | 9.8 ± 1.3[a] | 7.7 ± 0.9[a] | 13.3 ± 0.6[b] |
| | WRC (%) | – | 9.4 ± 0.4[b] | 7.8 ± 0.6[a] | 15.0 ± 0.3[c] |

–, not adopted due to the low extraction yield of FS1.
[a–c] Values are mean ± SD (amino acid composition: $n = 2$, bloom gel strength, viscosity, WAC, and WRC: $n = 3$); values with different letters within the same row differ significantly ($P < 0.05$).

resources (skin, bone, or scale), amino acid composition, and size of protein chains (Muyonga et al., 2004b). The quality of collagen is generally determined by gel strength, including low (<150 g), medium (150–220 g), and high bloom (220–300 g) (Johnston-Bank, 1983). Compared to the gel strength reported by other researchers, such as in lizardfish (*Saurida* spp.) scale (103–249 g) (Wangtueai & Noomhorm, 2009), Nile perch skins (222 and 229 g, respectively, for young and adult fish) and Nile perch bone (179 and 134 g, respectively, for young and adult fish) (Muyonga et al., 2004b), giant catfish skin (153.0 g) and calf skin (134.8 g) (Jongjareonrak et al., 2010), Atlantic salmon skin (108 g) and cod skin (71 g) (Arnesen & Gildberg, 2007), bigeye snapper skin (105.7 g) and brownstripe red snapper skin (218.6 g) (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006), and sin croaker (125 g) and short-fin scad (177 g) (Cheow, Norizah, Kyaw, & Howell, 2007), it is found that the gel strength of FS2 (260.3 g) reaches the extent of high bloom (220–300 g) and generally surpasses that of most reported fish-originated collagen. The viscosity of collagen sample solutions (FS2, FS12, and FS14) is shown in Table 2. FS12 (10.2 cP) had higher viscosity than FS2 (8.5 cP) and FS14 (8.1 cP), suggesting that pre-conditioning of TFS with citric acid solution resulted in higher viscosity of collagen solution. The viscosity of collagen solutions varies with molecular weight and molecular size distribution of proteins (Sperling, 1985). Due to the same condition used in testing viscosity among collagens from FS2, FS12, and FS14, the molecular weight and molecular size distributions of extracted collagen in FS12 may change after citric acid pretreatment. Viscosity is considered the second most important commercial physical property of collagen (Johnston-Banks, 1990). The present study showed that the viscosity of the extracted collagens ranged from 8.1 to 10.2 cP (measured at 6.67% concentration and 60 °C), which exceeded the viscosity of the lizardfish scale collagen (ranged from 3.14 to 5.80 cP, measured at 6.67% concentration and 25 °C) (Wangtueai & Noomhorm, 2009). DSC results of FS2, FS12, and FS14 are shown in Table 2. Studies suggested that DSC analysis could be performed using samples under rehydrated state [rehydrated in 0.05 M acetic acid at a sample/solution ratio of 1:40 (w/v)] (Liu et al., 2012) or solid state (Safandowska & Pietrucha, 2013). Generally, in rehydrated state, the endothermal peak and enthalpy of collagen will change with different amounts of added moisture. In addition, there is normally one endothermal peak ($T_{max}$ = 41 °C) existing in rehydrated sample (Matmaroh et al., 2011); whereas, two endothermal peaks ($T_{max}$ = 77 and 121 °C) existed in solid sample (Safandowska & Pietrucha, 2013). Consistent with the results reported by Safandowska and Pietrucha (2013), we found that two different endothermal peaks existed in the DSC thermograms of FS2, FS12, and FS14. The first peak ($T_{max1}$) is related to the temperature of thermal denaturation of collagen, and the second peak ($T_{max2}$) is connected with the continued conformational changes of superhelix, and consequently with the destruction of materials (Safandowska & Pietrucha, 2013). The relatively high denaturation parameters of $T_{max1}$ and H1 may contribute to the high thermal stability of collagen. From Table 2, the $T_{max1}$ of FS2 (71.5 °C), FS12 (79.0 °C), and FS14 (57.9 °C) were obtained, and the total H1 of FS2, FS12, and FS14 were also measured as 21.1, 1.4, and 8.0 J/g, respectively. Higher $T_{max1}$ was observed in FS12 and FS2 than FS14, indicating that FS12 and FS2 possessed higher thermal stability, and their high heat resistance and greater structural stability might be beneficial as potential substitutes for mammalian collagen. Interestingly, the $T_{max1}$ trend observed in FS2, FS12, and FS14 (FS12 > FS2 > FS14) also correlated well with that of imino acid amount (an indicator for the thermal stability of collagen) in FS2, FS12, and FS14 (FS12 > FS2 > FS14), and thus it seems reasonable to suggest that amino acid composition affects the thermal

**Table 3**
Sensory evaluation of offensive odor for collagen samples (in solution 6.67%; w/v or in powder) from FS1, FS2, FS12, and FS14.

| Samples | Hedonic scale* | |
|---|---|---|
| | In solution | In powder |
| FS1 | 5.0 ± 1.3[b] | 5.9 ± 1.9[c] |
| FS2 | 5.0 ± 1.7[b] | 4.2 ± 1.8[b] |
| FS12 | 3.8 ± 1.7[a] | 2.9 ± 1.2[a] |
| FS14 | 5.0 ± 2.1[b] | 5.8 ± 2.0[c] |

[a–c] Values are mean ± SD ($n = 24$); values with different letters within the same column differ significantly ($P < 0.05$).

* Hedonic scale (1 = no odor; 5 = middle odor; 9 = very strong and very offensive odor).

properties of collagen. Moreover, FS2 had the highest value of H1, followed by FS14, and then FS12. Therefore, FS2 (without acid pretreatment) had more thermal stability than that of FS12 (pretreatment with citric acid solution), as well as FS14 (pretreatment with acetic acid solution). The water absorption properties of FS2, FS12, and FS14 were examined, and presented in Table 2. FS14 exhibited the best water absorption ability, followed by FS2 and then FS12. Moreover, the water retention capacity showed a similar tendency to water absorption ability (Table 2). Proteins with higher water absorption and retention properties are expected to have proportionally more polar residues, with the ability to form hydrogen bonds with water (Hou et al., 2012). Therefore, the extracted collagen, particularly FS14 (pretreatment with acetic acid solution), showed good moisture absorption and retention properties, and thus benefited applications in the cosmetic industry. The offensive odor of collagens from the non-extruded sample (FS1) and extruded group (FS2, FS12, and FS14) was evaluated, using a nine-point hedonic scale. Table 3 (collagens in solution) revealed that FS12 (preconditioning by citric acid solution) exhibited the least odor, followed by FS1 (non-extruded sample), FS2 (preconditioning by $ddH_2O$), and FS14 (preconditioning by acetic acid solution). Table 3 (collagens in powder) showed that FS12 exhibited the least odor, followed by FS2, FS14, and then FS1. In general, extruded sample (FS2) had less fishy odor as compared to non-extruded sample (FS1), suggesting that high temperature and high pressure extrusion process might reduce the odor of extracted collagen. Preconditioning of TFS with citric acid solution (FS12) could further decrease the odor of extracted collagen (Table 3), therefore broadening its application in the food additive and cosmetic industries. We also found that the fishy odor of FS12 was even lower than commercial fish-derived collagens. In addition, due to the distinctive pungent odor of acetic acid, it was found that preconditioning of TFS with acetic acid solution (FS14) adversely enhanced the unpleasant odor, and thus received the highest offensive odor score (Table 3). In summary, the collagens with less odor (FS2 and FS12) may benefit their applications in the food and cosmetic industries. Taken together, FS2 exhibited the highest bloom strength, higher thermal stability, and less odor, which would obviously be advantageous in food applications. FS14, which contained trimer (triple-helix) collagen component and the highest water absorption and retention capacities, could be beneficially utilized in medical, therapeutic, and cosmetic applications.

## 4. Conclusions

We successfully developed a novel EHE process for decomposing the strong linkage between collagen and hydroxyapatite, which therefore facilitates the extraction of collagen by hot water treatment. All extracts were identified as type-I collagen, and possessed high physicochemical functionalities. These collagens could be potentially useful in a variety of commercial applications.

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