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Static hydrothermal processing and fractionation for production of a collagen peptide with anti-oxidative and anti-aging properties

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ABSTRACT

Collagen, in a form suitable for human consumption, can be obtained from the large amounts of fish skin via protein hydrolysis to produce low-molecular-weight substances with enhanced bioavailability and function. To optimize this method, we investigated the synergistic effect of elevated temperature (150–250 °C) and pressure (350–3900 kPa) of a hydrothermal process on the hydrolytic ability and characteristics of collagen hydrolysates. Elevated temperature and pressure of the hydrothermal process increased the levels of free amino groups and lower-molecular-weight collagen hydrolysates, particularly at 210 °C and 2100 kPa. The resulting hydrolysates were fractionated by ultrafiltration membranes of different molecular weight cutoff and evaluated for their antioxidant (ABTS radical scavenging activity and reducing power) and anti-aging (tyrosinase and collagenase inhibition) activities. The < 1 kDa fraction had the highest antioxidant activities, whereas the 5–10 kDa fraction had the highest anti-aging activities. Therefore, fish skin could be successfully modified into biologically active collagen peptides by a hydrothermal process (hydrolysis) and ultrafiltration (separation), and the resulting bioactive peptides have potential for development as antioxidants and anti-aging ingredients in the food, nutraceutical, and cosmetic industries.

1. Introduction

Fish by-products of the food industry are very valuable protein and mineral sources; however, more than 60% of the by-product waste, including the skin, head, trimmings, liver, frames, bones, and roes, is ultimately discarded in the fish processing industry [1]. The rapid growth of aquaculture is predicted to result in the accumulation of large amounts of by-products that have potential for human consumption [2]. In particular, these fish by-products contain a significant amount of collagen, which plays an essential role in several organs of the body, particularly in the bone and cartilage, and has several health benefits as a supplement. However, collagen cannot readily be directly absorbed in the human body because of its insolubility and high molecular weight. Therefore, an efficient way to add value to collagen from fish by-products is through processing via protein hydrolysis to produce low-molecular-weight substances (i.e., collagen-derived hydrolysates and peptides) with enhanced bio-suitability and bio-availability [3,4].

Methods based on chemical or enzymatic hydrolysis are conventionally used to produce collagen-derived hydrolysates and peptides with a low molecular weight. Chemical hydrolysis, including acid or alkaline solvent extraction, is a simple and effective method for degrading collagen with a high molecular weight, but also has several disadvantages such as difficulty in control of the process and the generation of toxic chloride compounds (e.g., 3-monochloropropane-1,2-diol and 1,3-dichloropropan-2-ol) [5]. Although the enzymatic hydrolysis method avoids such side reactions and does not diminish the nutritional value of the collagen since the process is performed under relatively mild conditions [6], this method results lacks cost-effectiveness owing to the requirement of proteases, which also leads to an undesirable bitter taste of the resulting products [5,6]. An alternative to the hydrolysis method is to use a hydrothermal process system, which is usually performed in water at high temperature (> 150 °C) under pressure up to 25,000 kPa [7,8]. Hydrothermal processing is an environmentally friendly and effective technology for extracting bioactive components, with several general but significant advantages, including a simple and economic process owing to the ability to conduct the operation under self-generated pressure, and it is non-toxic and safe because of the use of purified water as a solvent [8,9]. For these

Abbreviations: CH, collagen hydrolysate; MWCO, molecular weight cut-off; GPC, gel permeation chromatography; ABTS, 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); CCP, commercial collagen peptide; CP, collagen peptide; L-DOPA, 3, 4-dihydroxyphenyl-l-alanine; NCAA, negatively charged amino acid

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reasons, the hydrothermal process can be considered a cost-effective and sustainable extraction technology. Recently, attention has been paid to application of the hydrothermal process for hydrolyzing or enhancing the solubility of high-molecular-weight substances for producing bioactive substances with specific functions. For example, Ramachandraiah, Koh, Davaatseren and Hong [5] investigated the effect of hydrothermal processing on the hydrolysis efficiency of soybean protein. Previous studies also reported that porcine skin could be hydrolyzed by hydrothermal processing to porcine collagen hydrolysates (CHs) with good antioxidant activities [3,8].

Bioactive peptides are specific protein fractions containing 3–20 amino acid residues with molecular masses below 6000 Da [10,11]. Their bioactivities and structural properties are dependent on the amino acid sequence and composition [11]. Bioactive peptides have several potential applications for functional food, cosmetics, and pharmaceuticals owing to their multi-functional bioactivities, including antimicrobial, antioxidant, emulsifying, antihypertensive, antidiabetic, and immunomodulatory effects [3,11–13]. In particular, collagen-derived hydrolysates and peptides generated from fish protein show improved functional and nutritional properties compared to the original proteins, and have been widely applied to the development of value-added products. They can also be easily absorbed and utilized for various metabolic activities [1].

The aim of the present study was (a) to determine the optimal hydrothermal hydrolysis temperature (150–250 °C) under self-generated pressure (350–3900 kPa) for the production of CHs from fish by-products with high hydrolysis capacity and quality characteristics; (b) to confirm the effect of ultrafiltration purification (1–10 kDa molecular weight cut-offs) of collagen peptides among CHs obtained by hydrothermal hydrolysis; (c) and to investigate their in vitro antioxidant activities and anti-aging activities.

2. Materials and methods

2.1. Materials

Fish skin (from Bigeye tuna, *Thunnus obesus*) was supplied fresh on ice by Tuna Factory (Kyonggi-do, South Korea) and maintained at –60 °C until use. All reagents for the functionality test (antioxidant and anti-aging effects) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial fish skin peptide (Naticol 4000, four kDa collagen peptide) was supplied from CREMAR (Seoul, South Korea) and used as positive control of the functionality test. All other reagents were of the highest grade commercially available.

2.2. Fish skin pretreatment

The frozen fish skin was thawed at 4 °C overnight and pretreated according to the method of Min, Jo and Park [8]. After pretreatment, approximately 100 g of the sample (66.7% solid contents) was vacuum-packaged and frozen at −80 °C until use (within one month). For preparation for hydrothermal hydrolysis, the pretreated fish skin was mixed with water at a 1:9 (w/v) ratio at ambient temperature overnight, and a 100 mL aliquot of the sample solution (6.7% solid content) was placed into a high temperature- and high pressure-resistant glass bottle.

2.3. Static hydrothermal hydrolysis

The hydrothermal process system is a custom-designed, laboratory-scale, high-temperature, moderate-pressure processing reactor (1 L capacity, maximum temperature: 400 °C, maximum pressure: 30,000 kPa; R 101, Rexo Engineering, Seoul, South Korea). In this study, the high-temperature, moderate-pressure condition for protein hydrolysis was conducted according to the method of Min, Jo and Park [8]. From the hydrothermal process, the temperatures were set from 150 °C to 250 °C under the self-generated pressure of 350–3900 kPa. The target temperature was maintained for 10 min to ensure sufficient holding time for protein hydrolysis. Pressure transducer (P201, Allsensor, Seoul, Korea) was installed in the bottom of pressure chamber and it transmitted the pressure changes to digital pressure indicator (HI4200, Allsensor, Seoul, Korea) and data acquisition system (34970A, Agilent Technologies, USA). After the hydrothermal process, the samples were immediately removed from the pressure chamber and cooled to 0 °C using ice water, and CHs were finally collected after centrifugation at 4000 $\times g$ for 15 min. The CHs derived under different processing conditions were named CH-I (150 °C, 350 kPa), CH-II (170 °C, 660 kPa), CH-III (190 °C, 1100 kPa), CH-IV (210 °C, 2100 kPa), CH-V (230 °C, 2600 kPa), and CH-VI (250 °C, 3900 kPa).

2.4. Fractionation of CHs

CH was fractionated by ultrafiltration using an Amicon®Stirred Cells system (Catalog No. UFSC 20001, EMD Millipore Corporation, MA, USA) with four membranes with different molecular weight cutoff (MWCOs) of 10, 5, 3, and 1 kDa (Ultracel®Membrane, EMD Millipore Corporation, MA, USA) at 60 psi under nitrogen gas pressure under 20 °C. The permeate from each MWCO membrane was collected as the < 1 kDa (CP-I), 1–3 kDa (CP-II), 3–5 kDa (CP-III), 5–10 kDa (CP-IV), and > 10 kDa (CP-V) peptide fraction, respectively. The fractions were freeze-dried and kept in airtight containers at 25 °C until analysis. Production yields of powder type by the permeate from each MWCO membrane were calculated as the dry peptide weights against the wet peptide weights.

2.5. Protein and free amino group content determination

The soluble protein content of the samples (control and CH) was determined with a bicinchoninic acid protein (BSA) assay according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). Protein standard curves were constructed using samples of bovine serum albumin. The free amino group content was determined using a 2, 4, 6-trinitrobenzene sulfonic acid assay according to the manufacturer's instructions (Thermo Fisher Scientific, MA, USA). The free amino group content was then expressed in terms of the L-leucine level.

2.6. Empirical model fitting

The data on free amino group formation were empirically fitted as a function of elevated temperature (150, 170, 190, 210, 230, and 250 °C) and pressure (350, 660, 1100, 2100, 2600, and 3900 kPa) as suggested by our previous work [8]. The following empirical model (Eq. (1)) adopted the second-order multivariate polynomial regressions of SAS, 9.1.3, software (SAS Inst. Inc., Cary, NC, USA).

$$A_f = \beta_0 + \beta_1 \cdot T + \beta_2 \cdot P + \beta_3 \cdot T^2 + \beta_4 \cdot P^2 + \beta_5 \cdot T \cdot P \pm \varepsilon \quad (1)$$

where A_f (%) is the indicator of the degree of protein hydrolysis and conversion to free amino groups (%) in the empirical model fit, $\beta_{0\text{-}5}$ are the parameters in the empirical model, T and P are the temperature and pressure, respectively, and ε is the standard error of estimate in the empirical model.

2.7. Molecular weight distribution

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed to determine the molecular weight distribution (range of high molecular weight) of protein hydrolysates.

Samples were diluted with 8 M urea (final protein concentration, 4 mg/mL), and then mixed with one part of KTG 020 sample buffer (10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% β-mercaptoethanol, and 63 mM Tris-HCl, pH 6.8; KOMA Biotech Inc., Korea). Electrophoresis was performed on a EzCell Vertical Electrophoresis System (KOMA Biotech Inc., Korea) using a EzWay™ PAG 6% acrylamide gels according to the manufacturer's instructions. Then, samples were visualized by staining with Coomassie brilliant blue (0.025% (w/v) in 10% acetic acid) and destained in 40% methanol and 10% acetic acid. A molecular-weight standards kit (K18010 EzWay Protein PreBlue Ladder, 11 bands, 10–210 kDa) served as a standard.

Gel permeation chromatography (GPC) was used to determine the molecular weight distribution (range of low molecular weight). GPC was performed using a high-performance liquid chromatography (HPLC) system (YL 9100, Younglin Instrument Co., Ltd., Korea) equipped with three Ultrahydrogel™ 120 columns (7.8 × 3000 mm, Waters, USA). The mobile phase was distilled/deionized water at the flow rate of 1.0 mL/min, and the molecular weight distributions of the collagen peptides were monitored using refractive index detector (YL 9100, YL Instrument Co., Ltd., Korea) at 40 °C. A molecular-weight standards kit (106–67,500 Da, Polymer Standards Service, Germany) served as a standard.

2.8. Amino acid composition

The amino acid composition of the samples was determined using an Agilent HPLC 1200 system (Agilent Technologies Inc., CA, USA) equipped with a C18 column (4.6 × 150 mm, 5 μm in diameter). Two mobile phases were used: a 20 mM sodium phosphate buffer (pH 7.8) and a 45% (v/v) acetonitrile/45% (v/v) methanol solution. The samples were monitored for o-phthalaldehyde derivatives and 9-fluorenylmethyl chloroformate derivatives at 40 °C for two detectors [a fluorescence detector and an ultraviolet (UV) detector] (Agilent Technologies Inc., CA, USA). The content of each amino acid was calculated using the following equation (Eq. (2)):

$$\textit{Amino acid content}\ (\%) = \frac{A_{\textit{total amino acid}} - A_{\textit{free amino acid}}}{\textit{Sum of amino acid cotent}} \times 100 \quad (2)$$

where $A_{\text{total amino acid}}$ is the amino acid content after hydrolysis using 6 M HCl (at 130 °C for 24 h), and $A_{\text{free amino acid}}$ is the amino acid content after solubilizing in distilled water.

2.9. Antioxidant and anti-aging activity

2.9.1. 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging activity

The ABTS radical-scavenging activity of samples was carried out according to a previously described method [3]. The ABTS radical cation ($ABTS^{\bullet+}$) stock solution (7.0 mM ABTS and 2.45 mM potassium persulfate) was diluted with 5.0 mM phosphate-buffered saline, pH 7.4 to obtain an absorbance of 0.70 ± 0.02 at 734 nm. The diluted $ABTS^{\bullet+}$ solution (1 mL) and 1 mL of each sample were mixed, and the absorbance was recorded at 734 nm after 10 min (completion of the reaction time). Ascorbic acid and commercial collagen peptide (CCP) served as positive controls and were used for comparison. Ten minutes later, the absorbance of the sample (A_{sample}, with sample) and control (A_{control}, without sample) was measured at 734 nm using a microplate reader. The ABTS radical-scavenging activity (%) was then calculated as follows (Eq. (3)):

$$\textit{ABTS scavenging}\ (\%) = \frac{A_{\textit{control}} - A_{\textit{sample}}}{A_{\textit{control}}} \times 100 \quad (3)$$

2.9.2. Reducing power

The reducing power of the samples was then determined according to a previously described method [3]. In brief, 1 mL of each sample was mixed with 1 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% potassium ferricyanide. The reaction mixture was then incubated at 50 °C for 20 min followed by addition of 1 mL of 10% trichloroacetic. After centrifugation at 3000 *g* for 10 min, two milliliters of supernatant were added to distilled water (2 mL) and 0.1% ferric chloride solution (0.4 mL). Ten minutes later, the absorbance of the resultant solutions was recorded at 700 nm using a microplate reader. Ascorbic acid and CCP served as positive controls and were used for comparison. Higher absorbance of the reaction mixtures indicated a higher reducing power.

2.9.3. Inhibition of tyrosinase activity

Tyrosinase inhibition was determined according to a previously described method [3]. The sample (20 μL) was mixed with 30 μL of mushroom tyrosinase (enzyme: 167 U/mL) and 70 μL of 0.1 M phosphate buffer (pH 6.8) and incubated for 5 min at 30 °C. To initiate the enzymatic reaction, 100 μL of 3, 4-dihydroxyphenyl-l-alanine (substrate) was then added, and the absorbance was recorded at 492 nm for 20 min using a microplate reader. Ascorbic acid and CCP served as positive controls and were used for comparison. The percent inhibition was calculated as follows (Eq. (4)):

$$\textit{Tyrosinase inhibition}\ (\%) = \frac{[(A - B) - (C - D)]}{(A - B)} \times 100 \quad (4)$$

where A is a mixture with tyrosinase without samples, B is a mixture without the sample and tyrosinase, C is a mixture with the sample and tyrosinase, and D is a mixture with the sample but without tyrosinase.

2.9.4. Inhibition of collagenase activity

Collagenase inhibition was conducted by a previously described method [3]. A N-[3-(2-furyl) acryloyl]-Leu–Gly–Pro–Ala (substrate; FALGPA) and collagenase (enzyme; from *Clostridium histolyticum*, Type IA, 0.5–50 FALGPA U/mg solid) were dissolved in 50 mM tricine buffer (pH 7.5) containing 10 mM calcium chloride and 400 mM sodium chloride. The sample (0.1 mL) was mixed with 0.25 mL FALGPA solution (1.0 mM) and 0.15 mL collagenase solution (0.2 mg/mL), and incubated for 20 min at room temperature. The reaction was terminated by addition of 0.5 mL citric acid (6%, w/v). Next, the ethyl acetate (1.5 mL) was added and centrifuged at 4000 x *g* for 5 min at 4 °C. The absorbance of the supernatant was measured at 345 nm using a microplate reader. Ascorbic acid and CCP served as positive controls and were used for comparison. The percent inhibition was calculated as follows (Eq. (5)):

$$\textit{Collagenase inhibition}\ (\%) = \frac{[(A - B) - (C - D)]}{(A - B)} \times 100 \quad (5)$$

where A is a mixture with collagenase without the sample, B is a mixture without the sample and collagenase, C is a mixture with the sample and collagenase, and D is a mixture with the sample but without collagenase.

2.10. Statistical analysis

Data are presented as the mean ± standard deviation. The significance of differences between groups was assessed using multiple comparisons and analysis of variance followed by the Tukey honest significant difference tests. Differences with p values less than 0.05 were considered statistically significant.

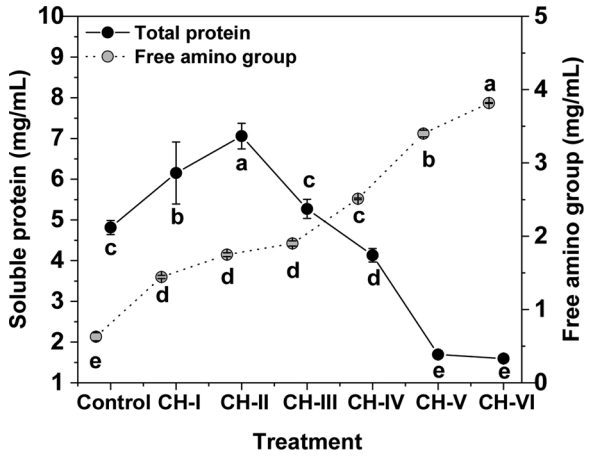

Fig. 1. Changes in soluble protein and free amino group contents as a function of temperature and pressure during the hydrothermal process. The control is a collagen suspension. CH-I, CH-II, CH-III, CH-IV, CH-V, and CH-VI were hydrolyzed at 150 °C & 350 kPa, 170 °C & 660 kPa, 190 °C & 1100 kPa, 210 °C & 2100 kPa, 230 °C & 2600 kPa, and 250 °C & 3900 kPa, respectively. Different letters (a–e) indicate statistically significant differences ($p < 0.05$).

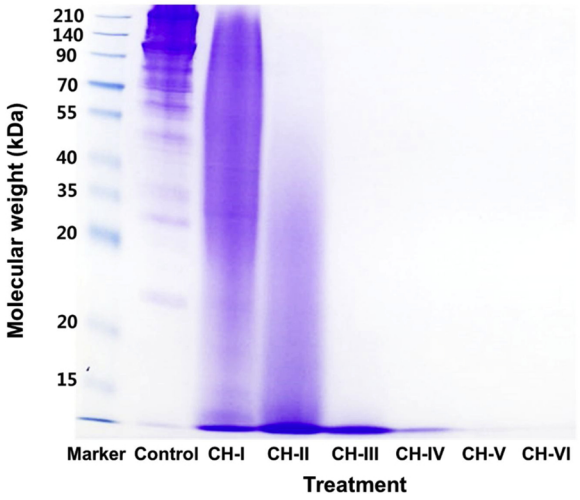

Fig. 2. Effects of elevated temperature and pressure on the results of SDS-PAGE of collagen hydrolysates (CHs) after the hydrothermal process. The control is a collagen suspension. CH-I, CH-II, CH-III, CH-IV, CH-V, and CH-VI were hydrolyzed at 150 °C & 350 kPa, 170 °C & 660 kPa, 190 °C & 1100 kPa, 210 °C & 2100 kPa, 230 °C & 2600 kPa, and 250 °C & 3900 kPa, respectively.

3. Results and discussion

3.1. Effect of temperature and pressure of the static hydrothermal process on CH production

Fig. 1 depicts total protein and free amino group content, typical parameters used for determining the efficiency of the hydrolysis of proteins, of CHs according to the temperature and the pressure of hydrothermal processing. The collagen suspension was 4.82 mg/mL of the total protein content. The protein content of CH-II (170 °C, 660 kPa) was significantly increased up to 7.06 mg/mL, indicating an increase in protein solubility. However, further increasing the temperature caused a significant decrease in the protein content. The protein content of CH-VI (250 °C, 3900 kPa) decreased to 1.60 mg/mL. This decrease in protein content over 190 °C and 1100 kPa in the hydrothermal process could indicate enhanced protein hydrolysis under this condition. The protein hydrolysis could have resulted from producing single amino acids (cysteine or cystine, tyrosine and tryptophan) measured by BCA assay [8]. During protein breakdown, proteins are hydrolyzed into shorter peptide or amino acid materials; thus, the higher free amino group content of the CH is indicative of increased protein breakdown. As shown in Fig. 1, the free amino group content of the collagen suspension was 0.63 mg/mL, which gradually and significantly increased from 1.44 mg/mL to 3.82 mg/mL with increasing temperature. A higher temperature can rapidly promote the hydrolysis of proteins into smaller molecules (peptides and amino acids) because water is a potent source of hydronium (H_3O^+) and hydroxide (OH^-) ions [8,9]. Thus, the higher temperature of hydrothermal processing can induce the thermal degradation of proteins to promote the conversion to amino acids. Therefore, the free amino group content will be increased simply by increasing the temperature. One study suggests that protein undergoes thermal hydrolysis in the temperature range of 150–190 °C, as indicated by the increase in free amino group content [5]. Moreover, protein hydrolysis dominated at temperatures of 240–260 °C, and protein was degraded into small MW peptides and free amino acids [7].

We further evaluated the efficiency of protein hydrolysis with SDS-PAGE. Fig. 2 depicts the electrophoresis patterns of CHs dependent on the temperature of hydrothermal processing. The collagen suspension strongly displayed a band greater than 70,000 Da, indicating the partial unfolding of the polypeptide chains of the triple helix. The high molecular hydrolysates (> 70,000 Da) was also detected after CH-I (150 °C, 350 kPa) although the molecular weight distribution was extensive from low to high level. However, rapid hydrolysis was observed as the temperature of hydrothermal processing increased. At 170 °C and 660 kPa, there was a wide distribution and lower molecular weights detected for the CHs. With a further increase in the temperature and pressure beyond 190 °C and 1100 kPa, peptides of low molecular weight completely disappeared from the SDS-PAGE gel and accumulated at the < 10,000 Da marker. CHs are hydrolyzed by attacking the non-triple helical domain of native collagen above its denaturation temperature [14], which has greater energy to destroy most of the triple helices of the gelatin or collagen hydrolysate and cleave their peptide bonds [8,14]. Thus, since the higher temperature and pressure (> 190 °C, 1100 kPa) during hydrothermal processing could disintegrate peptides with low molecular weight, we suggest that the best temperature and pressure of hydrothermal processing to produce low-molecular-weight CHs is above 190 °C and 1100 kPa.

Since the SDS-PAGE analysis was not sufficient to detect the specific profile of CHs below 10 kDa, GPC analysis was further adopted to evaluate the molecular weight distribution of CHs with low molecular weights for further verification. Fig. 3 shows the relative molecular weight distribution of CHs dependent on the temperature of hydrothermal processing. CH-I (150 °C, 350 kPa) has no specific molecular weight peak, indicating that mild hydrolysis was not sufficient to disintegrate high-molecular-weight collagen into smaller molecules according to the detection limit of the index detector system used in this

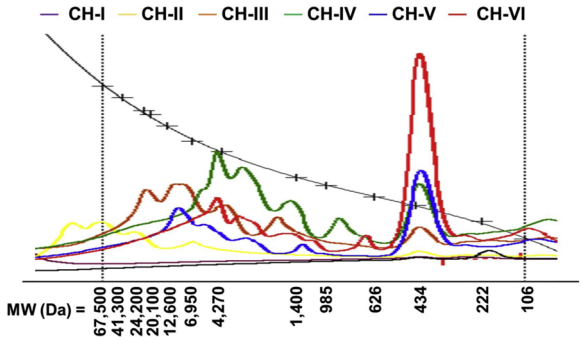

Fig. 3. Relative-molecular-weight (MW) distribution of collagen hydrolysates (CHs) after the hydrothermal process. CH-I, CH-II, CH-III, CH-IV, CH-V, and CH-VI were hydrolyzed at 150 °C & 350 kPa, 170 °C & 660 kPa, 190 °C & 1100 kPa, 210 °C & 2100 kPa, 230 °C & 2600 kPa, and 250 °C & 3900 kPa, respectively.

Table 1
Estimated coefficients and probability testing of fitted polynomial parameters to predict amino group formation (A_f %) as a function of temperature and pressure during hydrothermal processing ($A_f = \beta_0 + \beta_1 \cdot T + \beta_2 \cdot P + \beta_3 \cdot T^2 + \beta_4 \cdot P^2 + \beta_5 \cdot T \cdot P \pm \varepsilon$).

Coefficients	β_0 0.61997262	β_1 0.01658195	β_2 −0.00424863	β_3 −0.00007104	β_4 −0.00000056	β_5 0.00002934
Pr > |t|	0.0002	0.0140	< 0.0001	0.0682	< 0.0001	< 0.0001
R^2 values	0.99					
SEE (ε)*	0.08					

* Standard error of the estimate.

study. By contrast, the higher temperature (> 170 °C, 660 kPa) during hydrothermal processing showed detectable values in the higher range of the relative molecular weight distribution. CHs with a temperature and pressure above 170 °C and 660 kPa (CH-II–VI) exhibited two distinct distributions in molecular weight: one with a relatively high molecular weight (626–67,500 Da) and a wide distribution, and the other with a low molecular weight (434 Da) and narrow distribution. In the former distribution, CH-IV (210 °C, 2100 kPa) exhibited a maximum peak at 4270 Da, and had a similar molecular weight to that of the commercial bioactive peptide (3–4 kDa). Based on a report by Ramachandraiah, Koh, Davaatseren and Hong [5], the best condition of subcritical water processing to obtain protein hydrolysates with maximum yield in low MW peptide is between 190 and 210 °C. In addition, the peak of 434 Da with a narrow distribution had a large area in the order CH-VI > CH-V > CH-IV > CH-III > CH-II > CH-I; thus, a higher temperature and pressure during hydrothermal processing would generate more free amino acids.

3.2. Empirical model fitting of estimated free amino acid formation (Af %)

Table 1 shows the estimated coefficients and probability test of the empirical model fit to the amino acid formation data as a function of elevated temperature and pressure during hydrothermal processing. Both temperature and pressure of hydrothermal processing are key factors which influence on the hydrolysis efficacy of high molecular substances since temperature affects pressure, while saturated pressure affecting heating process in turn [8,15]. The model fit showed a positive linear coefficient for temperature ($\beta_1 = 0.01658195$, $p < 0.05$). The model implied that elevated temperature would result in protein hydrolysis and subsequent formation of free amino acids. Protein hydrolysis was 1.2–1.6 times higher at high temperatures (160–180 °C) as compared to that reported at 110 °C [16]. In contrast to temperature, a negative linear coefficient of pressure ($\beta_2 = -0.00424863$, $p < 0.05$) was observed. It is expected that pressure would not be able to hydrolyze high-molecular-weight proteins. Indeed, the use of only pressure does not have a significant effect on protein hydrolysis in hydrothermal processing, or on the yield of amino acids in a hydrolysate as compared with the effects of temperature and time [8,17]. The quadratic function of the temperature increase showed a negative coefficient ($\beta_3 = -0.00007104$); however, this was not statistically significant ($p > 0.05$). The quadratic function of the pressure increment was also negative ($\beta_4 = -0.00000056$) with statistical significance ($p < 0.05$); however, its magnitude was quite minor, indicating that a quadratic function for elevated pressure would not be meaningful. The combination of elevated temperature and pressure showed a positive relationship ($\beta_5 = 0.00002934$, $p < 0.05$). Thus, in our study, the synergy of elevated temperature and pressure resulted in more efficient protein hydrolysis and subsequent production of amino acids. Empirical model also appropriately explained the efficacy of parameters (temperature, pressure) for hydrothermal processing.

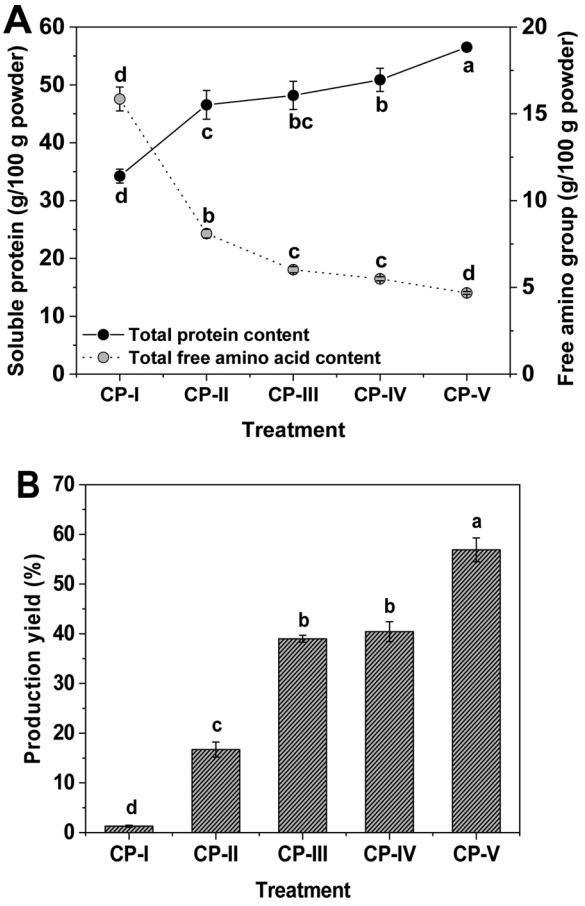

Fig. 4. Soluble protein and free amino group content (A), and production yield (B) of collagen peptides (CP) obtained by ultrafiltration. Samples were fractionated with ultrafiltration membranes of different molecular weight cutoffs (MWCOs), and the resulting fractions were named CP-I (MWCO < 1 kDa), CP-II (MWCO 1–3 kDa), CP -III (MWCO 3–5 kDa), CP-IV (MWCO 5–10 kDa), and CP -V (MWCO > 10 kDa). Different letters (a–e) indicate statistically significant differences ($p < 0.05$).

3.3. Ultrafiltration effect for collagen peptides with various MWCOs

The soluble CHs generated from the optimal condition (CH-IV: 210 °C, 2100 kPa) of the hydrothermal process were sequentially fractionated on an ultrafiltration membrane using different MWCOs of 10, 5, 3, and 1 kDa for separation into five different CH fractions. Ultrafiltration is a conventional process used to separate protein hydrolysates with some advantages: requires minimal protein denaturation compared to precipitation, is faster than dialysis, operates at a lower temperature than evaporation or spray-drying, is cost-effective, and can be readily scaled-up [18,19]. Fig. 4 shows the protein content, free amino group content, and yield of the different collagen peptide

Table 2
Amino acid composition of collagen peptides (CPs) obtained by ultrafiltration.

Unit: %

Amino acids	CP-I[1)]	CP-II	CP-III	CP-IV	CP-V
Aspartic acid	6.5	5.3	4.7	4.7	5.0
Glutamic acid	18.6	14.0	12.7	12.5	12.3
Asparagine	N.D.	N.D.	N.D.	N.D.	N.D.
Serine	4.7	3.9	3.6	3.7	3.9
Glutamine	N.D.*	N.D.	N.D.	N.D.	N.D.
Histidine	1.2	1.0	1.0	1.0	1.2
Glycine	22.6	23.8	23.5	23.3	21.6
Threonine	2.3	3.0	3.1	3.1	3.8
Arginine	6.7	8.5	9.7	9.9	10.0
Alanine	10.6	11.2	10.9	10.7	10.0
Taurine	N.D.	N.D.	N.D.	N.D.	N.D.
GABA	N.D.	N.D.	N.D.	N.D.	N.D.
Tyrosine	1.2	0.8	0.9	1.0	1.6
Valine	2.5	3.0	3.0	3.1	3.2
Methionine	1.7	1.9	2.1	2.2	1.8
Phenylalanine	1.8	2.2	2.6	2.7	3.1
Isoleucine	1.3	1.6	1.8	1.9	2.3
Leucine	2.7	3.4	3.6	3.8	4.5
Lysine	3.7	3.8	3.8	3.5	3.6
Proline	11.9	12.5	13.2	13.0	12.1
HAA[2)]	53.5	57.7	58.5	58.5	56.8
NCAA[3)]	25.1	19.3	17.4	17.2	17.2

[1] Samples were fractionated by means of ultrafiltration membranes with a different molecular weight cutoffs (MWCO) and the resulting fractions were named CP-I (MWCO < 1 kDa), CP-II (MWCO 1–3 kDa), CP -III (MWCO 3–5 kDa), CP-IV (MWCO 5–10 kDa), and CP -V (MWCO > 10 kDa).
[2] HAA = hydrophobic amino acids (Glycine, Alanine, Valine, Phenylalanine, Isoleucine, Leucine, Proline).
[3] NCAA = negatively charged amino acids (Aspartic acid, Asparagine, Glutamic acid, Glutamine).
* N.D.: Not detected; GABA: gamma-aminobutyric acid.

fractions. The protein content of the collagen peptides significantly increased from 34.2%–56.5%, whereas the free amino groups decreased from 15.8% to 4.7% with increasing MWCO of the ultrafiltration membrane ($p < 0.05$). The lowest and highest production yield was obtained by CP-I (1.3%) and CP-V (56.9%), respectively. A similar trend was obtained by Choi, Min and Jo [3]. Therefore, ultrafiltration is useful for the concentration, separation, and purification of low-molecular-weight products but results in a decreased yield.

Table 2 shows the amino acid compositions of the collagen peptide fractions. The most abundant amino acids included glycine, glutamic acid, and proline for all fractions. Various functionalities of protein hydrolysates/peptides are based on the molecular size or amino acid composition. In particular, peptide sequences with a greater composition of hydrophobic amino acids are considered to contribute to superior antioxidant activities [20]. Hydrophobic amino acids (HAAs) such as alanine, isoleucine, leucine, phenylalanine, valine, proline, and glycine, could be enhanced by radical scavenging due to increased solubility in lipids; the proportion of HAA groups of CP-I, CP-II, CP-III, CP-IV, and CP-V was found to be 53.5%, 57.7%, 58.5%, 58.5%, and 56.8%, respectively. In addition, negatively charged amino acids (NCAAs) such as aspartic acid, glutamic acid, asparagine, and glutamine can donate their excess electrons via free radical reaction to improve antioxidant properties [21]. The NCAA content of the CHs decreased from 25.1% (CH-I) to 17.2% (CH-V) with increasing MWCO of the ultrafiltration membrane, indicating that the collagen peptide fraction with a higher NCAA content would have stronger antioxidant effects.

3.4. In vitro antioxidant and anti-aging activities of collagen peptides

ABTS radical scavenging activity and reducing power assays were carried out on the CP fractions to verify their in vitro antioxidant activity (Table 3). ABTS radical scavenging activity is exclusively measured by the ability of antioxidant peptides to be participate in hydrogen atom transfer, which neutralizes generated $ABTS^{\cdot+}$ [22]. The CP fractions showed a range of ABTS radical scavenging activity from 44.64% to 56.22%, which were all significantly higher than that of the positive control CCP (24.48%) but lower than vitamin C (50 μg/mL), with the most potent scavenging activity (63.69%) overall. Of the five CP fractions, CP-I exhibited the highest ABTS radical scavenging activity (56.22%); however, there was no difference among the other CP fractions.

The reducing power assay is commonly used to evaluate the ability of an antioxidant to donate a hydrogen or an electron; the presence of these antioxidant reducers converts or reduces the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) [23]. The CP fractions exhibited a significant ($p < 0.05$) difference in their reducing power, which increased from Abs 0.231 to Abs 0.499 with a decrease in the MWCO of the ultrafiltration membrane. This result indicates that the reducing power of collagen peptides was mainly influenced by the molecular weight; hence, CP with a lower molecular weight (MWCO < 1 kDa fraction; CP-I) had high antioxidant potential. Vitamin C (50 μg/mL) had the highest (Abs 0.521) reducing power, whereas that of CPP was not detectable (Abs 0.007). In general, the molecular size of protein hydrolysates is considered to be an important factor determines their biological properties. In particular, a low-molecular weight product obtained after ultrafiltration could be a more potent antioxidant. Moreover, some researchers suggested that the desired molecular size and higher bioactivities are largely dependent on the composition of the protein hydrolysates and the activity [24,25].

To assess the in vitro anti-aging activity of the CP fractions, their tyrosinase and collagenase inhibition abilities were assessed (Table 3). Tyrosinase is a key enzyme involved in melanogenesis. Melanin is a unique pigment with the ability to protect the human skin from UV radiation-induced damage. However, increased melanin levels can lead to pigmentation disorders such as the formation of melisma, spots, and freckles in the human skin [26]. Therefore, substantial attention has been paid to the development of tyrosinase inhibitors as an effective strategy to promote skin whitening for cosmetic purposes [27]. In this study, vitamin C (500 mg/mL, positive control) strongly inhibited tyrosinase activity (98.08%), indicating a better skin whitening effect. CPP inhibited tyrosinase activity by 60.09%, with a statistically similar effect to that of CP-II (59.90%), CP-III (59.96%), and CP-IV (63.10%). Of the five CP fractions, CP-V exhibited the lowest level of inhibitory activity (26.05%) toward tyrosinase.

Collagenase hydrolyzes collagen, resulting in loss of hydration and elasticity in the skin, and consequent dehydration and wrinkle formation on the skin surface [28]. Vitamin C (500 mg/mL) and CCP inhibited collagenase activity by 58.10% and 60.77%, respectively. Among the five CP fractions, CP-IV exhibited the highest level of inhibitory activity (84.94%) toward collagenase, whereas CP-I showed the lowest level (32.37%). Therefore, CP-IV appears to offer the most effective protection against wrinkle formation. Taken together, these in vitro studies indicated that the MWCO 5–10-kDa fraction (CP-IV) of the CH obtained using our optimized hydrothermal method would be a good tyrosinase and collagenase inhibitor with the best potential anti-aging activity.

4. Conclusion

CHs were produced by a hydrothermal process using the synergistic effect of elevated temperature (150–250 °C) and pressure (350–3900 kPa). Empirical model fitting suggested that both elevated temperature and pressure are required for enhanced protein hydrolysis. The formation of collagen hydrolysates with low molecular weight was

Table 3
Antioxidant and anti-aging activities of collagen peptides (CPs).

Treatment[1)]	Antioxidant activity assay		Anti-aging activity assay	
	ABTS radical scavenging activity (%)	Reducing power (Abs at 700 nm)	Tyrosinase inhibition (%)	Collagenase inhibition (%)
Ascorbic acid[2)]	63.69 ± 2.174[a*]	0.521 ± 0.041[a]	98.08 ± 0.124[a]	58.10 ± 11.73[bc]
CCP[3)]	24.48 ± 3.660[e]	0.007 ± 0.001[d]	60.09 ± 0.860[b]	60.77 ± 7.218[b]
CP-I	56.22 ± 1.215[a]	0.499 ± 0.067[a]	56.10 ± 1.060[c]	32.37 ± 1.340[d]
CP-II	44.64 ± 3.010[d]	0.309 ± 0.028[b]	59.90 ± 1.653[b]	42.25 ± 1.604[cd]
CP-III	45.35 ± 4.402[cd]	0.249 ± 0.025[c]	59.96 ± 1.934[b]	53.05 ± 0.100[bc]
CP-IV	49.79 ± 2.900[bcd]	0.246 ± 0.018[c]	63.10 ± 1.341[b]	84.94 ± 5.882[a]
CP-V	53.47 ± 1.294[bc]	0.231 ± 0.014[c]	26.05 ± 0.752[d]	62.71 ± 1.404[b]

[1] Samples were fractionated by means of ultrafiltration membranes with different molecular weight cutoffs (MWCO) and the resulting fractions were named CP-I (MWCO < 1 kDa), CP-II (MWCO 1–3 kDa), CP -III (MWCO 3–5 kDa), CP-IV (MWCO 5–10 kDa), and CP-V (MWCO > 10 kDa). CP samples were dissolved at 1 mg/mL (ABTS radical scavenging activity), 10 mg/mL (reducing power), and 5 mg/mL (tyrosinase inhibition and collagenase inhibition activity).
[2] Ascorbic acid (ABTS radical scavenging activity and reducing power: 50 μg/mL, tyrosinase inhibition and collagenase inhibition activity: 500 μg/mL).
[3] Commercial collagen peptide with a 4 kDa molecular weight.
* Different letters (a–e) indicate statistically significant differences ($p < 0.05$).

dependent on processing at elevated temperature and pressure, as shown by molecular size distribution (SDS-PAGE and GPC). These findings suggest that the optimal hydrothermal processing condition for production of useful protein hydrolysates from fish by-products is 210 °C and 2100 kPa (CH-IV). Furthermore, CHs were successfully modified into biologically active collagen peptides using ultrafiltration purification with different MWCOs. The MWCO 5–10 kDa fraction (CP-IV) peptides exhibited the most potent antioxidant activity and the MWCO < 1 kDa fraction (CP-I) peptides had enhanced anti-aging activity. These active peptides show excellent potential for application as antioxidant and anti-aging ingredients in the development of value-added foods, cosmetics, and pharmaceutical products.

Declarations of interest

None.

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