Low molecular weight bioactive peptides derived from the enzymatic hydrolysis of collagen after isoelectric solubilization/precipitation process of turkey by-products

Zied Khiari, Maurice Ndagijimana, and Mirko Betti[1]

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5

ABSTRACT A process based on the isoelectric solubilization/precipitation (ISP) method was developed to recover collagen from low value poultry by-products. The application of the ISP process to turkey heads generated protein isolates and an insoluble biomass that was used to extract collagen. Isolated turkey head collagen was then enzymatically hydrolyzed for different time periods using alcalase, flavorzyme, and trypsin. The enzymatic hydrolysis approaches consisted of digesting collagen with each one of the 3 enzymes alone (alcalase, flavorzyme, or trypsin), or one of the 3 combinations of 2 enzymes (alcalase/flavorzyme, alcalase/trypsin, or flavorzyme/trypsin), or a cocktail of all 3 enzymes together (alcalase/flavorzyme/trypsin). The molecular weight distribution of turkey head collagen hydrolysates was determined using size exclusion chromatography and matrix-assisted laser desorption ionization-time of flight-mass spectrometry. The enzyme cocktail produced collagen hydrolysates with the greatest amount of low molecular weight peptides ranging from 555.26 to 2,093.74 Da. These collagen peptides showed excellent solubility over a wide pH range (2–8) and were able to bind cholic and deoxycholic acids and significantly ($P < 0.05$) inhibited plasma amine oxidase in a dose- and time-dependent manner. The ISP process combined with enzyme cocktail hydrolysis represents a potential new way to produce low molecular weight bioactive collagen peptides from low value poultry by-products.

Key words: turkey collagen, isoelectric solubilization/precipitation, enzyme cocktail, anti-inflammatory peptide, anticholesteremic peptide

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INTRODUCTION

The functional food and drink sector has been growing substantially over the last decade. According to Valls et al. (2013), the functional food and nutraceutical industry has a global market value over $40 billion. This continuous growth is associated with a consumer awareness of the role of functional foods in preventing disease and promoting health (Jackson and Paliyath, 2011). Food-derived bioactive peptides represent potential functional food ingredients. Many dietary peptides have been recognized to protect against cancer (Kobayashi et al., 2004), inflammation (Udenigwe et al., 2009), hypertension (Mizuno et al., 2004), and hypercholesterolaemia (Zhong et al., 2006). The most biologically potent peptides are commonly short in length, comprising 2 to 9 amino acids, and possessing molecular weights lower than 3,000 Da (Hayes, 2013).

Collagen is a fibrous insoluble protein mostly found in the skin, cartilage, and connective tissues. It constitutes about 30% of total body protein, making it the most abundant animal protein (Johnston-Banks, 1990). Recently, there has been a growing demand for collagen peptides due to their beneficial effects on skin. The oral ingestion of collagen-derived peptides (i.e., gelatin hydrolysates) promotes collagen synthesis in the skin and increases the size of collagen fibrils in the dermis (Minaguchi et al., 2005), which is believed to improve the hydration of skin and prevent wrinkle formation. Collagen peptides also possess antihypertensive and antioxidant activities that are associated with the presence of low molecular weight (**MW**) peptides (Khiari et al., 2014).

The industrial production of collagen peptides requires 2 separate operations. In the first step, gelatin is extracted and purified, and in the second step collagen peptides are enzymatically produced, sterilized, and finally dried (Moskowitz, 2000). The extraction of

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[1]Corresponding author: Mirko.Betti@ales.ualberta.ca

gelatin is time and energy consuming. For instance, the production of type A gelatin (i.e., acid pretreated by-products) requires up to 30 h of pretreatment, whereas type B gelatin (i.e., alkali pretreated by-products) needs a longer pretreatment period (Keenan, 2012). For both gelatin types, the extraction is performed in 4 to 5 successive batch operations, each lasting from 4 to 8 h using elevated temperatures from 55 to 100°C (Keenan, 2012). Hence, new time- and energy-efficient processes for collagen peptide preparation are still needed.

Recent studies showed that the isoelectric solubilization/precipitation technology is a useful way to produce functional protein isolates from meat processing by-products and underutilized poultry species (Hrynets et al., 2011; Wang et al., 2013). In this process, sarcoplasmic and myofibrillar proteins are solubilized at either acidic or alkaline pH values, then precipitated at their isoelectric point. In addition to the protein isolates, insoluble protein biomass and fat are also generated. The insoluble protein material represents a significant source of relatively pure collagen suitable for the extraction of gelatin and collagen peptides in a shorter time and with less pretreatment operations (Du et al., 2014).

Commercially available collagen peptides have broad MW distributions ranging from 2,000 to 20,000 Da (Schrieber and Gareis, 2007; Haug and Draget, 2011). According to a personal communication received by the authors from a gelatin manufacturer, the development of a process capable of producing collagen peptides with MW less than those readily available in the market (<2,000 Da) would be preferable in terms of solubility and potential marketability. Koopman et al. (2009) reported that the ingestion of low MW peptides increases their intestinal absorption resulting in a higher bioavailability. Babel et al. (2008) invented a method for the preparation of low MW (i.e., 100–2,000 Da) gelatin hydrolysates. The process is based on treating gelatin with 3 endopeptidases (bromelain, papain, and proteases from *Bacillus subtilis*) followed by 2 exopeptidases (proteases from *Aspergillus oryzae* and *Aspergillus sojae*). Although this process seems to be efficient in obtaining low MW peptides, the use of 5 highly purified and costly enzymes lowers the chance of any industrial implementation. Therefore, the main objective of the present study was to develop an efficient process, capable of producing collagen peptides lower than 2,000 Da. In this respect, a designed cocktail containing 3 commercially available enzymes (alcalase/flavorzyme/trypsin) was compared with a single-enzyme system comprised of one of the individual enzymes of the cocktail (alcalase, flavorzyme, or trypsin) or a dual-enzyme system containing 2 of the enzymes (alcalase/flavorzyme, alcalase/trypsin, or flavorzyme/trypsin). The anti-inflammatory capacities of the extracted collagen peptides were determined using the plasma amine oxidase (**PAO**) inhibition assay, and their anticholesteremic properties were estimated by the cholic and deoxycholic acid binding assay.

MATERIALS AND METHODS

Materials

Ground turkey heads were supplied by Rossdown Natural Foods and Rossdown Farms (Abbotsford, BC, Canada). Upon arrival to the laboratory in a frozen state, the material was divided into 3 main batches and stored at −20°C until used in less than 3 mo. Two different protein fractions, termed as protein isolates (mainly containing sarcoplasmic and myofibrillar proteins) and insoluble protein biomass (mainly composed of stromal proteins and bone debris), were separated using the acid-aided isoelectric solubilization/precipitation process (summarized in Figure 1) as described by Hrynets et al. (2011). All chemical reagents were either analytical or HPLC grade.

Enzymes

Pepsin (EC 3.4.23.1), prepared by MP Biomedicals, LLC (Solon, OH), was purchased from Fisher Scientific (Edmonton, Canada). Alcalase (EC 3.4.21.62), prepared by Novozyme Co. (Copenhagen, Denmark), flavorzyme (EC 3.4.11.1), prepared by DSM Nutritional Products Inc. (Kaiseraugst, Switzerland), and trypsin (EC 3.4.21.4), prepared by Sigma-Aldrich Co. (St. Louis, MO), were purchased from Sigma-Aldrich Co. (Edmonton, Canada). The sources of each enzyme and their declared activities are presented in Table 1.

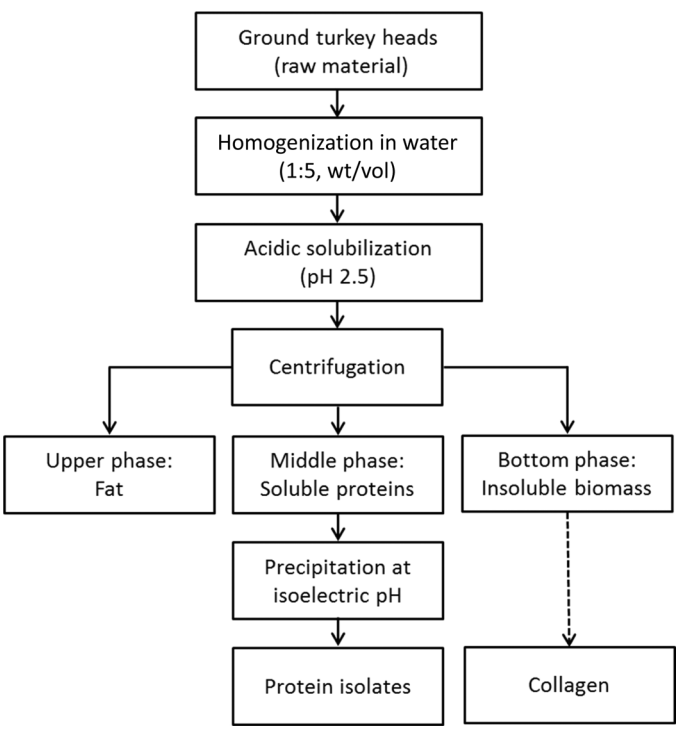

Figure 1. General scheme for the extraction of protein isolate and insoluble protein biomass from turkey heads through the isoelectric solubilization/precipitation process.

Table 1. Source and activity of enzymes used for the extraction and hydrolysis of collagen

Enzyme	Source	Activity	Unit definition
Flavorzyme	*Aspergillus oryzae*	≥500 units/g	1 unit is the amount of enzyme that hydrolyzes 1 μ*M* of L-leucine-*p*-nitroanilide per minute (Chien et al., 2002).
Alcalase	*Bacillus licheniformis*	≥2.4 Anson units/g	1 Anson unit is the amount of enzyme that hydrolyzes urea-denatured hemoglobin at an initial rate such that there is liberated an amount of trichloroacetic acid soluble peptides per minute, which gives the same color with reagent as 1 mEq of tyrosine at 25°C and pH 7.50 (Anson, 1938).
Trypsin	Porcine pancreas	1,000 to 2,000 BAEE units/mg of solid	1 *N*-benzoyl-L-arginine ethyl ester (BAEE) unit produces a ΔA_{253} of 0.001 per min at pH 7.6 at 25°C using BAEE as substrate (Öste et al., 1986).
Pepsin	Porcine gastric mucosa	10,000 FCC units	1 Food Chemical Codex (FCC) unit digests 3,000 units coagulated egg albumin at 52°C, pH 2 to 3 (Food Chemical Codex, 2012).

Characterization of Protein Fractions Obtained Through the Isoelectric Solubilization/Precipitation Process

Chemical Composition. The chemical compositions of turkey heads, protein isolates, and the insoluble protein biomass obtained through the isoelectric solubilization/precipitation process were determined according to the standard methods of the Association of Official Analytical Chemists (AOAC International, 2000) following the method numbers 950.46, 920.153, and 960.39 for moisture, ash, and fat contents, respectively. The nitrogen contents were estimated by a TruSpec CN carbon/nitrogen determinator (Leco Corp., St. Joseph, MI), then converted to protein content using nitrogen conversion factors of 6.25 for turkey heads and turkey head protein isolates and 5.4 for turkey collagen biomass (Eastoe and Eastoe, 1952).

Hydroxyproline Content. The hydroxyproline contents of turkey heads, protein isolates, and the insoluble protein biomass obtained through the isoelectric solubilization/precipitation process were determined according to the method of Edwards and O'Brien (1980). Their respective collagen contents were calculated through multiplication of the hydroxyproline content by 7.14.

Extraction of Collagen

Turkey head collagen was purified from the insoluble protein biomass, obtained through the isoelectric solubilization/precipitation process (Figure 1), following the method described by Heu et al. (2010) with some modifications. To remove impurities and fat, the insoluble protein biomass was first homogenized with distilled water at a ratio of 1:5 (wt/vol) for 2 min using a homogenizer (Power Gen 1000 S1, Fisher Scientific, Schwerte, Germany) and then centrifuged at 10,000 × *g* for 10 min at 4°C. The sediment was collected and subjected to the above-mentioned pretreatment step 2 more times. Collagen was then extracted through a pepsin-aided solubilization in acetic acid as follows. The clean sediment was mixed with 0.5 *M* acetic acid (at a ratio of 1:100, wt/vol), then pepsin was added at an enzyme/substrate ratio of 1:20 (wt/wt). Collagen was solubilized for 48 h at 4°C under continuous stirring then collected by centrifugation at 10,000 × *g* for 10 min at 4°C. The NaCl was added to the supernatant (containing soluble collagen) to a final concentration of 2 *M* to precipitate the protein. The precipitated collagen was recovered by centrifugation at 20,000 × *g* for 10 min at 4°C (Avanti J-E High-performance centrifuge, Beckman Coulter Inc., Palto Alto, CA), dispersed in distilled water (in a ratio of 1:10, wt/vol) and dialyzed (MW cut-off of 12,400 Da) against distilled water for 48 h at 4°C with regular water changes. Dialyzed collagen solution was frozen at −20°C for 2 h in a Wood's freezer (W. C. Wood Corporation Inc., Ottawa, OH) then dried under vacuum (0.210 torr) at low temperature (−45°C) for 48 h (Labconco Corporation, Kansas City, MO).

Protein Profile of Extracted Proteins

The protein profiles of turkey heads, protein isolates, insoluble protein biomass, and the extracted collagen were determined by SDS-PAGE according to Khiari et al. (2013). Samples at a concentration of 5 mg/mL were first diluted 1:1 (vol/vol) with sample buffer (65.8 m*M* Tris-HCl at pH 6.8 containing 2.1% (wt/vol) SDS, 26.3% (wt/vol) glycerol, 0.01% (wt/vol) bromophenol blue, and 5% (vol/vol) β-mercaptoethanol) then heated to 85°C for 10 min. Aliquot of each sample (20 μL corresponding to 50 μg of proteins) and MW markers (20 μL) were loaded on a precast 4 to 20% ready gel. The precast gel and the MW markers were both purchased from Bio-Rad Laboratories Inc. (Hercules, CA). The analysis was performed on a PowerPack Basic electrophoresis apparatus (Bio-Rad Laboratories Inc.) at a constant voltage of 120 V. To visualize the protein bands, the gel was stained with Coomassie Brilliant Blue R250 and destained using a mixture composed of 50% (vol/vol) distilled water, 40% (vol/vol) methanol, and 10% (vol/vol) acetic acid. The gel was scanned using Image Scanner (Amersham Biosciences, Soeborg, Denmark) in transmission mode that was controlled with Magic Scan software (version 4.6, UMAX). The intensity of the bands was quantified using Image Quant TL software (version 2005, Amersham Biosciences).

Collagen Hydrolysis

One gram of freeze-dried collagen extracted from turkey heads was mixed with 90 mL of distilled water, then homogenized for 1 min at room temperature. The solution was then heated to 80°C for 10 min in a water bath (Isotemp 2320, Fisher Scientific, Marietta, OH) to inactivate the endogenous enzymes. After cooling to 50°C, the pH of the mixture was adjusted to 8 using 1 *M* NaOH and the volume was made up to 100 mL by adding water with a pH value previously adjusted to 8. Different hydrolysis approaches: single enzyme (alcalase, flavorzyme, or trypsin), a combination of 2 enzymes (alcalase/flavorzyme, alcalase/trypsin, or flavorzyme/trypsin), and a cocktail mixture containing all the 3 enzymes (alcalase/flavorzyme/trypsin) were performed separately. Both the dual enzyme mixtures and the enzyme cocktail were prepared by combining equal concentrations of each individual enzyme. For all the enzymatic approaches, the enzyme to substrate ratio was set to 1:50 (wt/wt) for each enzyme. Collagen was then hydrolyzed for 24 h under continuous shaking at 250 rpm in a jacketed glass beaker connected to a bath circulator heated to 50°C (Haake S7, Fisher Scientific, Newington, NH). The pH was kept constant, during the whole hydrolysis period, using a pH-stat titrator (Titrando 842, Metrohm, Herisau, Switzerland). After hydrolysis, collagen hydrolysates were heated to 80°C for 10 min to inactivate the enzymes, then cooled at room temperature for 60 min and centrifuged at 20,000 $\times$ *g* for 10 min at 4°C. The supernatant, containing soluble collagen peptides, was collected and split into small portions (~30 mL), then frozen at −20°C for 2 h. Extracted collagen peptides were dehydrated through water sublimation at −45°C for 48 h under vacuum (0.210 torr). The experimental hydrolysis procedures of turkey head collagen are summarized in Figure 2.

Determination of the Stability of the Enzyme Preparations

The stability of the enzyme cocktail, the combination of 2 enzymes, and each individual enzyme for 24 h of incubation at 50°C was determined through the evaluation of their protein patterns using the SDS-PAGE analysis (as described above) and through the quantification of free amino groups, using serine as a standard, according to the o-phthaldialdehyde method (Church et al., 1983) at time 0 and at the end of the hydrolysis reaction (i.e., 24 h).

Determination of the Degree of Hydrolysis

The degree of hydrolysis (**DH**), which is defined as the ratio of the number of peptide bonds cleaved with respect to the total number of bonds per unit weight (Khiari et al., 2014), was determined according to the pH-stat method described by Adler-Nissen (1986).

Determination of the MW Distribution

Size Exclusion Chromatography. The MW distribution of turkey head collagen peptides was estimated using size-exclusion chromatography on an AKTA fast protein liquid chromatography system (GE Healthcare Life Sciences, Baie-D'Urfe, QC, Canada). To observe the general MW profiles of collagen peptides, 100 μL of each collagen peptide samples (10 mg/mL in HPLC water) was loaded on a Superdex peptides 10/300 GL column (GE Healthcare Life Sciences) and separated using an isocratic elution at a flow rate of 0.5 mL/min with 50 m*M* sodium phosphate buffer containing 30 m*M* NaCl at pH 7.4. The detection of peptides was carried out at an absorbance of 214 nm. The column was equilibrated with buffer before injecting the samples and was calibrated using protein, peptide, and amino acid mixture containing cytochrome C (12,340 Da), aprotinin (6,511 Da), vitamin B12 (1,855 Da), angiotensin II (1,322 Da), leucine-enkaphalin (555 Da), and glycine-tyrosine (256 Da). A standard calibration curve was obtained by plotting the ratio of peak elution volume (V_e) to the void volume (V_0; i.e., V_e/V_0) against the logarithm of MW.

Matrix-Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry. The determination of the MW of turkey head collagen peptides, obtained through the cocktail enzymes, was determined by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (**MALDI-TOF-MS**) according to the method described by Hong et al. (2014). Briefly, the freeze-dried sample was solubilized in HPLC water at a concentration of 10 mg/mL, then diluted in 50% acetonitrile/water mixture containing 0.1% trifluoroacetic acid. An aliquot of sample (1 μL) was then diluted with α-cyano-4-hydroxycinnamic acid (10 mg/mL in 50% acetonitrile/water + 0.1% trifluoroacetic acid) at a ratio of 1:1 (vol/vol). One microliter of the sample/matrix solution was transferred to a stainless steel target plate and dried at room temperature. The mass spectra were obtained in positive ion mode after acceleration from the ion source by 25 kV using a Bruker Ultraflex MALDI-TOF/TOF (Bruker Daltonic, GmbH, Rheinstetten, Germany). In this study, only peptides greater than 500 Da were taken into consideration due to the interference of the matrix components with the sample in the low MW range (i.e., 1–500 Da).

Collagen Peptide Solubility

The solubility of collagen peptides, as a function of pH, was determined according to the method described by Khiari et al. (2013). Collagen peptide solution was made by homogenizing 1.5 g of freeze-dried sample in 500 mL of deionized water. Twenty milliliters of sample was transferred to a glass test tube and the pH was adjusted to 2, 4, 6, and 8 with either 1 *M* HCl or 1 *M* NaOH using a calibrated pH meter (UB-10, Ultra Basic pH meter, Denver Instrument, Bohemia, NY). The fi-

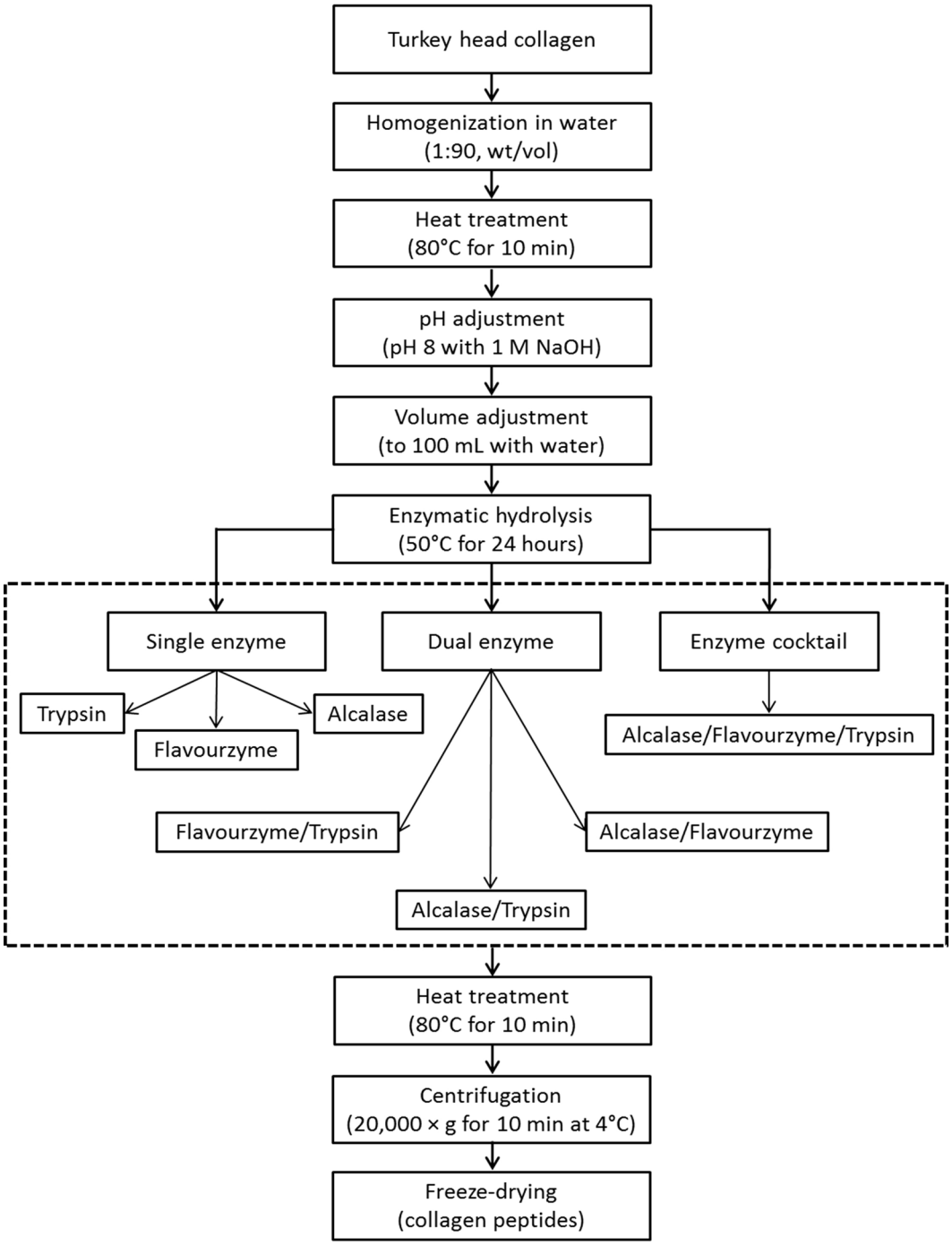

Figure 2. General scheme for the enzymatic hydrolysis of turkey head collagen.

nal volume was then adjusted to 25 mL using deionized water having the same pH as the sample. Each sample was centrifuged at 10,000 × *g* for 15 min at 5°C, and the protein content of the supernatant was determined using the Biuret assay (Gornall et al., 1949) and BSA (Sigma-Aldrich Inc.) as a protein standard. The solubility was calculated as the ratio of the protein content in the sample before and after centrifugation as follows:

$$\text{solubility } (\%) = \frac{\text{protein content in the supernatant}}{\text{protein content in the sample}} \times 100.$$

Screening for Bioactivity of Collagen Peptides

Bile Acid Binding Capacity. The in vitro bile acid binding capacity of collagen peptides, obtained by the enzyme cocktail, was assayed using sodium cholate and sodium deoxycholate according to the method described by Yoshie-Stark and Wäsche (2004) with some modifications. Both bile acids were separately dissolved in 50 m*M* sodium phosphate buffer at pH 6.5 to a final concentration of 2 m*M*. Different amounts of freeze-dried collagen peptides (5, 10, or 20 mg) were added to 1 mL

of each bile acid and vortexed for 30 s. Individual bile acid solutions, without samples, were used as blanks. Cholestyramine, a known bile acid binding resin, was used as a positive control. All samples, blanks, and controls were incubated at 37°C for 2 h under continuous shaking (250 rpm) and then centrifuged at 10,000 × *g* for 30 min at 25°C.

The supernatant was collected and analyzed on a prominence ultra-fast liquid chromatography UFLC-CXR (Shimadzu, Tokyo, Japan) system equipped with a CBM-20A communication bus module, 2 LC-20AD XR pumps, a DGU-20A3 vacuum degasser, an SIL-20AC XR auto-sampler, a CTO-20AC column oven, and a SPD-M20A diode array detector. The chromatographic separations were performed on a BEH C_{18} (2.1 × 150 mm, 1.7 μm) column (Waters, Milford, MA) at 30°C. The temperature of the auto-sampler was set to 4°C. The elution of samples (10 μL) was carried out in an isocratic mode with 40% acetonitrile containing 0.05% phosphoric acid (pH 6.5) using a flow rate of 0.3 mL/min for 40 min. The UV spectra were collected at 210 nm. Eluted bile acid peaks were quantitated using standard calibration curves generated from the peak area responses of the standard solutions (0.1–4 m*M*). The bile acid binding capacity (%) was calculated as follows:

$$\text{bile acid binding capacity } (\%) = \left[\frac{CB - CS}{CB}\right] \times 100,$$

where *CB* is the bile acid concentration in the blank and *CS* is bile acid concentration in the sample.

PAO Inhibitory Activity. The in vitro PAO inhibitory activity of collagen peptides was determined according to the method described by Holt and Palcic (2006). Freeze-dried collagen peptides were dissolved in HPLC grade water to a final concentration of 40 mg/mL, then centrifuged at 2,000 × *g* for 5 min at room temperature and filtered through a 0.2 μm nylon syringe filter. Different concentrations: 40, 20, 10, and 5 mg/mL (respectively corresponding to 10, 5, 2.5, and 1.25 mg/mL in the final enzymatic assay) were prepared through serial dilutions. The assay was performed in the presence of 5 m*M* benzylamine and 2.5 mg/mL of PAO (19.5 units/mg of dry weight, Worthington Biochemical Corp., Lakewood, NJ). The assay buffer was 100 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (**HEPES**) at pH 7.4. Calcium chloride ($CaCl_2$), potassium chloride (KCl), magnesium chloride ($MgCl_2$), and sodium chloride (NaCl) were added to the HEPES buffer to final concentrations of 4, 10, 2.8, and 280 m*M*, respectively. The PAO activity was detected using a chromogenic solution prepared by mixing horseradish peroxidase (4 units/mL) to 4-aminoantipyrine (500 μ*M*) and vanillic acid (1 m*M*) in physiological HEPES buffer.

The assay was performed in a 96-well, clear, flat-bottomed Costar polystyrene plate (Corning Inc., Corning, NY). Seventy-five microliters of PAO solution together with 75 μL of samples (at different concentrations) and 75 μL of chromogenic solution were placed in the well at room temperature and incubated for 0, 1, or 2 h without the substrate (5 m*M* benzylamine). Using an 8-channel pipette, 75 μL of preheated substrate solution (37°C, 15 min) was quickly added to each well to start the enzymatic reaction. The microtiter plate was immediately transferred to a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA) and the absorbance was recorded at 490 nm every 3 min for 33 min at 37°C.

The control contained all reaction components except the sample (substituted with buffer). Blanks with no enzyme (replaced by HEPES buffer) or with no substrate (replaced by water) were also used. Two millimolar of propargylamine (Sigma-Aldrich Inc.), a known potent PAO inhibitor, was used as a positive control. The PAO activity was expressed as the slope of the increase in absorbance at 490 nm (ρA). The PAO inhibition (%) was calculated from the ratio of the slope in the presence of sample to the slope obtained with the control, according to the following formula:

$$\text{PAO inhibition } (\%) = \left[1 - \left(\frac{\rho A \text{ inhibitor}}{\rho A \text{ control}}\right)\right] \times 100.$$

The IC_{50}, the concentration required to achieve 50% inhibition, was determined from plots of PAO inhibition (%) versus the sample concentration.

Statistical Analysis

The extraction and the hydrolysis of collagen from turkey heads were carried out in triplicate at 3 different time periods (i.e., 3 independent trials) using 3 different poultry head batches. All the analyses were repeated 3 times for each trial, except for the PAO assay, which was replicated 4 times. All analyses were subjected to ANOVA using the PROC MIXED procedure of SAS (v. 9.1.3, SAS Institute Inc., Cary, NC). For all analyses, except DH, bile acid binding capacity, and PAO inhibitory activity, one-way ANOVA was performed. The model, in this case, tested the hydrolysis approach (single, dual, and enzyme cocktail) as a fixed effect. The DH, bile acid binding capacity, and PAO inhibitory activity, on the other hand, were analyzed using 2-way ANOVA. For DH, the hydrolysis approach and the hydrolysis period (4, 8, 12, 16, 20, and 24 h) were considered as fixed effects. For bile acid binding capacity, the sample (collagen peptides and cholestyramine) and the concentration (5, 10, and 20 mg/mL) were examined as fixed effects. For PAO inhibitory activity, the incubation time (0, 1, and 2 h) and the concentration (1.25, 2.5, 5, and 10 mg/mL) were analyzed as fixed effects. For both models (1- and 2-way ANOVA), the experimental replication (trial 1, 2, and 3) was considered as the random factor. Multiple-range

test Tukey's honestly significant difference ($P < 0.05$) was conducted to determine differences between treatment means, and letter groupings were obtained using the SAS PDMIX800 macro (Saxton, 1998).

RESULTS AND DISCUSSION

Characterization of Protein Fractions Obtained Through the Isoelectric Solubilization/Precipitation Process

The proximate analysis and the hydroxyproline content of turkey heads and the 2 protein fractions (the protein isolate and insoluble protein biomass), obtained through the isoelectric solubilization/precipitation process, are shown in Table 2. Moisture was the main component of all these materials with contents greater than 82%. Turkey heads had greater ($P < 0.05$) ash and fat contents compared with the protein isolate and the insoluble protein biomass. The presence of high ash level in turkey heads might be associated with the bone minerals. The protein isolate had significantly greater protein content (15.13%) compared with turkey heads and the insoluble protein biomass, which may confirm the effectiveness of the isoelectric solubilization/precipitation process in recovering protein rich fractions, mainly sarcoplasmic and myofibrillar proteins, and in eliminating fat as reported by Hultin and Kelleher (1999). The insoluble biomass had significantly the greatest Hyp level (4.95%, on a dry weight basis) compared with turkey heads (2.37%) and the protein isolates (0.05%). This finding may indicate that the insoluble biomass was predominantly a collagen-rich material with a collagen content of 35.40%. Results from this study regarding the Hyp content and the chemical composition of turkey heads were in full agreement with those reported by Du et al. (2013).

Protein Profile of Extracted Proteins

Figure 3 shows the SDS-PAGE pattern of turkey heads, protein isolate, insoluble protein biomass, and the extracted collagen. This analysis was conducted to evaluate the protein profiles of each fraction. Turkey heads, as a starting material (Figure 3, lane 2), contained all the 3 categories of meat proteins: sarcoplasmic, myofibrillar, and stromal proteins. The application of the isoelectric solubilization/precipitation process to turkey heads resulted in 2 main fractions, protein isolates (Figure 3, lane 3) and insoluble protein biomass (Figure 3, lane 4). According to Hultin and Kelleher (1999), the isoelectric solubilization/precipitation process is very effective in concentrating sarcoplasmic and myofibrillar proteins. This was evident by the increased concentration of these proteins in the SDS-polyacrylamide gels. For instance, the average band intensities in Figure 3, lane 3, corresponding to myosin heavy chain (~205 kDa), C-protein (~137 kDa), actin (~43 kDa), tropomyosin (~36 kDa), and myosin light chain (~24 kDa), were respectively, 9, 38, 3, 17, and 10% more intense than their corresponding bands in turkey heads (Figure 3, lane 2). The insoluble biomass (Figure 3, lane 4) showed clearly less protein bands compared with turkey heads and the protein isolates. The presence of bands characteristic of collagen, the monomeric α_1 and α_2 chains as well as the dimeric β-chains, were observed (Figure 3, lane 4). Minor contamination with myosin heavy chain (~205 kDa), C-protein (~137 kDa), and actin (~43 kDa) were also observed. The protein profile of the extracted collagen (Figure 3, lane 5) contained 30 and 36% more intense α_1 (upper band) and α_2 (lower band) collagen bands, respectively, compared with the insoluble biomass (Figure 3, lane 4). No other protein contamination was observed in the SDS-PAGE profile of the extracted collagen (Figure 3, lane 5). The presence of α_1 and α_2 bands indicated that the turkey head collagen was type I.

Determination of the DH

The DH was affected by the enzymatic approach (single, dual, or enzyme cocktail) and the hydrolysis period, but the interaction (enzymatic approaches × hydrolysis period) was not significant (Table 3). It seems that the addition of more than one enzyme to collagen solution has a positive additive effect on the hydrolysis of collagen. For instance and regardless of the hydrolysis period, the DH of each single enzyme (alcalase, flavorzyme, and trypsin) was significantly less than those obtained with dual-enzyme mixture (alcalase/flavorzyme, alcalase/trypsin, or flavorzyme/trypsin). The enzyme cocktail, which comprised all the 3 enzymes,

Table 2. Chemical composition, hydroxyproline content, and collagen level of turkey heads and the protein fractions obtained through the isoelectric solubilization/precipitation process[1]

Item	Moisture (%)	Ash (%)	Protein (%)	Fat (%)	Hyp[2] (%)	Collagen (%)
Material						
Turkey heads	81.96 ± 1.35^{b}	5.58 ± 0.47^{a}	9.67 ± 1.60^{b}	5.51 ± 1.10^{a}	2.37 ± 0.27^{b}	19.95 ± 1.92^{b}
Protein isolates	82.19 ± 0.55^{b}	0.56 ± 0.04^{c}	15.13 ± 0.42^{a}	1.18 ± 0.13^{b}	0.05 ± 0.03^{c}	0.35 ± 0.20^{c}
Insoluble biomass	86.96 ± 0.37^{a}	2.44 ± 0.60^{b}	8.05 ± 0.44^{b}	2.25 ± 0.30^{b}	4.95 ± 0.14^{a}	35.40 ± 0.99^{a}
P-value	<0.0001	<0.0001	0.0004	0.0005	<0.0001	<0.0001

$^{a–c}$Means within the same column sharing a common letter are not significantly different from each other.

[1]Results are presented as mean ± SD (n = 3).

[2]Hyp: hydroxyproline.

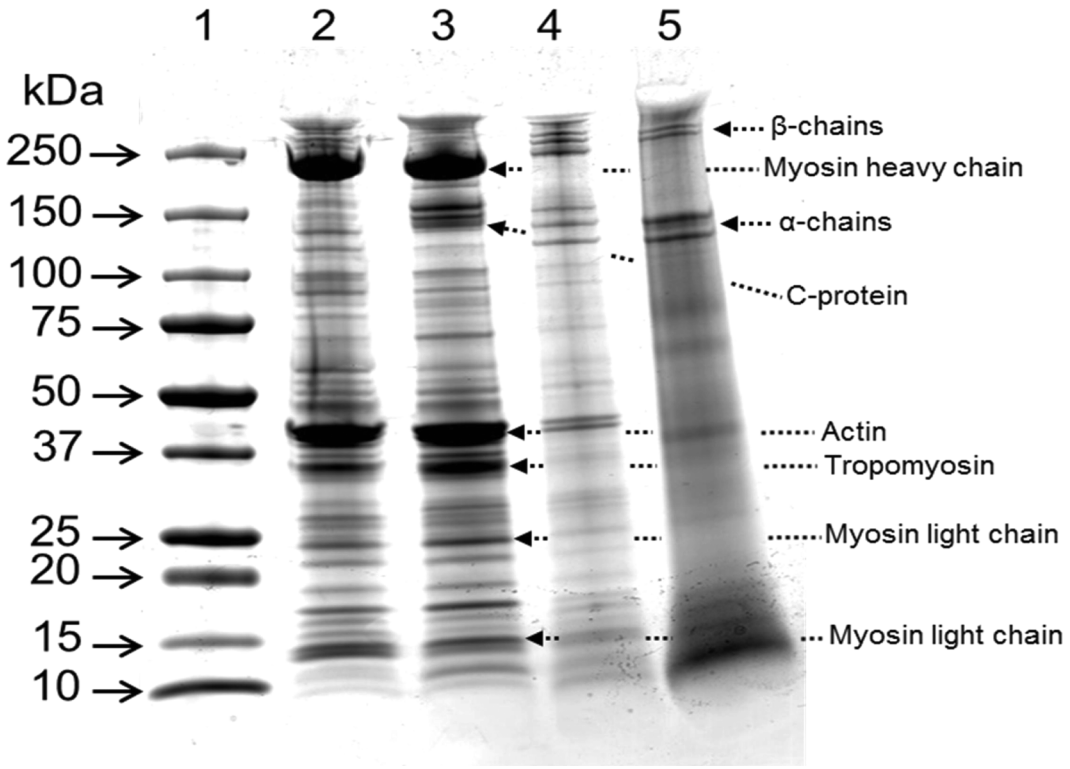

Figure 3. The SDS-PAGE profile of turkey heads and different protein fractions obtained through the isoelectric solubilization/precipitation process. Lane 1: molecular weight markers, lane 2: total proteins in turkey heads, lane 3: protein isolates, lane 4: insoluble protein biomass, and lane 5: collagen.

showed greater ($P < 0.05$) DH compared with the rest of the other enzymatic approaches (i.e., single and dual). Regardless of the hydrolysis approach, the DH significantly increased during 16 h of hydrolysis reaching an average value of 5.79%. Increasing the hydrolysis period more than 16 h did not ($P > 0.05$) increase the DH (Table 3). Therefore, to achieve the greatest DH, it is advisable to use the enzyme cocktail for 16 h.

So far, no previous study investigated the preparation and characterization of turkey collagen peptides. Chicken leg collagen peptides, on the other hand, were prepared by flavorzyme (i.e., proteases from *Aspergillus oryzae*) and screened for bioactivity (Iwai et al., 2005; Saiga et al., 2008). However, no information about the DH was provided in both studies.

Stability of the Enzyme Preparations

To study the stability of the enzymes and the enzyme mixtures against digestion or self-digestion, the protein profiles and the amount of free α-amino groups of all the individual enzymes and the enzyme mixtures at time zero and after incubation for 24 h were evaluated. The electrophoretic band patterns of each single enzyme, the dual enzyme mixture and the enzyme cocktail at time zero (Figure 4, I) and after 24 h of incubation (Figure 4, II) were in general similar with minor differences in band intensities. In addition, at 24 h, the free α-amino groups in all enzyme preparations were slightly greater (but not significant) than those at time zero (Figure 4, III), which indicated minor degradation of the enzymes. These results may reveal that all the enzyme preparations were stable under the experimental conditions of the present study.

Table 3. Degree of hydrolysis (DH) of turkey head collagen using several enzymatic approaches at different hydrolysis periods[1]

Item	DH (%)
Enzymatic approach	
Alcalase	3.35 ± 0.31^{d}
Flavorzyme	3.73 ± 1.33^{d}
Trypsin	1.85 ± 0.26^{e}
Alcalase/flavorzyme	6.63 ± 1.42^{b}
Alcalase/trypsin	5.07 ± 1.93^{c}
Flavorzyme/trypsin	6.48 ± 0.60^{b}
Alcalase/flavorzyme/trypsin	10.42 ± 1.10^{a}
Hydrolysis period (h)	
0	—
4	4.25 ± 2.61^{c}
8	4.69 ± 2.84^{c}
12	5.14 ± 2.93^{bc}
16	5.79 ± 2.84^{ab}
20	6.06 ± 2.88^{ab}
24	6.24 ± 2.91^{a}
Sources of variation (P-value)	
Enzymatic approach	<0.0001
Hydrolysis period	<0.0001
Interaction	0.9813

[a–e]Means within the same column and within the same group sharing a common letter are not significantly different from each other.

[1]Results are presented as mean ± SD (n = 3).

Determination of MW Distribution of Collagen Peptides

Figure 5 illustrates the size exclusion chromatographic (**SEC**) profiles of collagen peptides obtained with different enzymatic hydrolyses after 24 h of hydrolysis. Similarly to the DH analysis, it seems that there is a relation between the enzymatic approach and the molecular size of peptides. A decrease in MW masses and a reduction in high MW fractions were clearly observed when more than one enzyme was used (Figure 5). The MW distribution of collagen peptides varied depending on the enzymatic approach used. For instance, collagen hydrolysis with single enzyme approach produced great proportions (63.5–70.8%) of peptides with MW higher than 2,000 Da (Table 4). The dual enzyme mixture reduced the proportion of peptides with MW higher than 2,000 Da to 48.2 to 51.9%. The majorities of collagen peptides (85.3%) produced with the enzyme cocktail had MW lower than 2,000 Da. About one-half of the low MW peptides produced with the enzyme cocktail (45.6%) were less than 1,000 Da in size (Table 4).

Trypsin and alcalase are serine endopeptidase and endoproteinase, respectively, whereas flavorzyme is a crude endo- and exopeptidase mixture. In this study, the use of 3 enzymes, with a broad endo- and exo-specificity produced shorter chain collagen peptides compared with those obtained with either single enzyme or the mixture of 2 enzymes. Cocktail enzymes, containing several individual enzymes with single or different catalytic sites, are widely used for degrading cellulose (Resch et al., 2013; Su et al., 2013). It is known that alcalase and flavorzyme have wide catalytic function, whereas trypsin preferentially hydrolyzes peptides on the C-terminal of lysine and arginine amino acid residues (Keil-Dlouhá et al., 1971). The presence of 3 different individual enzymes in the same mixture may have resulted in different substrate specificities. In this regard, the 3 enzymes possessing various exo- and endo- activities may have worked in an additive manner through the exposure of new reactive sites for the other enzymes.

The MALDI-TOF-MS analyses (Figure 6) were performed to complement the SEC analyses and to accurately determine the MW profile of turkey head collagen peptides. The MS spectrum of the collagen peptides obtained through the cocktail enzymes confirmed the result obtained by the size exclusion chromatograms showing that the smallest and the largest peptides had MW of 555.26 and 2,093.74 Da, respectively. The molecular size is one of the factors that control the peptide bioavailability. It has been reported that the peptide bioavailability strongly decreases beyond 700 Da (Antosova et al., 2009). Based on the SEC and MALDI/TOF/MS analyses, it is suggested that the enzyme cocktail, developed in this study, is more efficient in degrading collagen compared with both single and dual enzyme hydrolysis strategies. Because the enzyme cocktail generated significantly higher proportions of very low MW peptides; therefore, the hydrolysis of collagen with the prepared enzyme cocktail may be an efficient practical way to improve the absorption of collagen peptides in the small intestine (i.e., bioavailability). According to the laboratory analyses, the preparation of collagen peptides using the ISP process combined with the enzyme cocktail hydrolysis could reduce the processing time by at least 16 h compared with the conventional method. In addition, the application of the ISP method recovers valuable meat proteins (in the form of protein isolates), which are usually discarded during the pretreatment step of the conventional gelatin preparation process.

Solubility of Collagen Peptides

The solubility of turkey head collagen peptides obtained with different hydrolysis approaches (single, dual, and enzyme cocktail) is shown in Figure 7. At pH 2 and 4 (Figure 7 I and II, respectively), collagen peptides obtained with alcalase and trypsin had the least ($P < 0.05$) solubility values compared with the rest of the samples. No significant difference in solubility among all the collagen peptides, regardless of the hydrolysis approach, was observed when changing the pH to either 6 or 8 (Figure 7, III and IV). High solubility values are usually associated with the presence of low molecular mass peptides. According to Dong et al. (2008), the solubility increases with the reduction of the molecular size of the protein or peptide fractions. This can be due to the exposure of polar amino acid residues capable of binding water through hydrogen bonds (Gbogouri et al., 2004). The least solubility value observed for alcalase and trypsin derived peptides at pH 2 and 4 could be due to the insufficient hydrolysis of collagen. Despite being produced with a single enzyme, flavorzyme-derived peptides had greater solubility compared with those obtained with alcalase and trypsin. This could be due to the endo-exo activity of flavorzyme, which may have resulted in better hydrolysis of collagen. The use of flavorzyme alone, dual-enzyme mixture, or enzyme cocktail is a more efficient way to produce highly soluble peptides at low pH values compared with single enzyme hydrolysis with either alcalase or trypsin alone. Regardless of the pH value and the hydrolysis approach, all collagen peptides had excellent solubility, which varied from 54.4 to 93.3%.

Screening for Bioactivity of Collagen Peptides

Peptide bioactivities, in terms of plasma amine oxidase inhibition and bile acid binding properties, are usually associated with the presence of low MW fractions (Udenigwe et al., 2009; Kongo-Dia-Moukala et al., 2011). Results from SEC (Table 4) and MALDI-TOF-MS (Figure 6) analyses indicated that the enzyme cock-

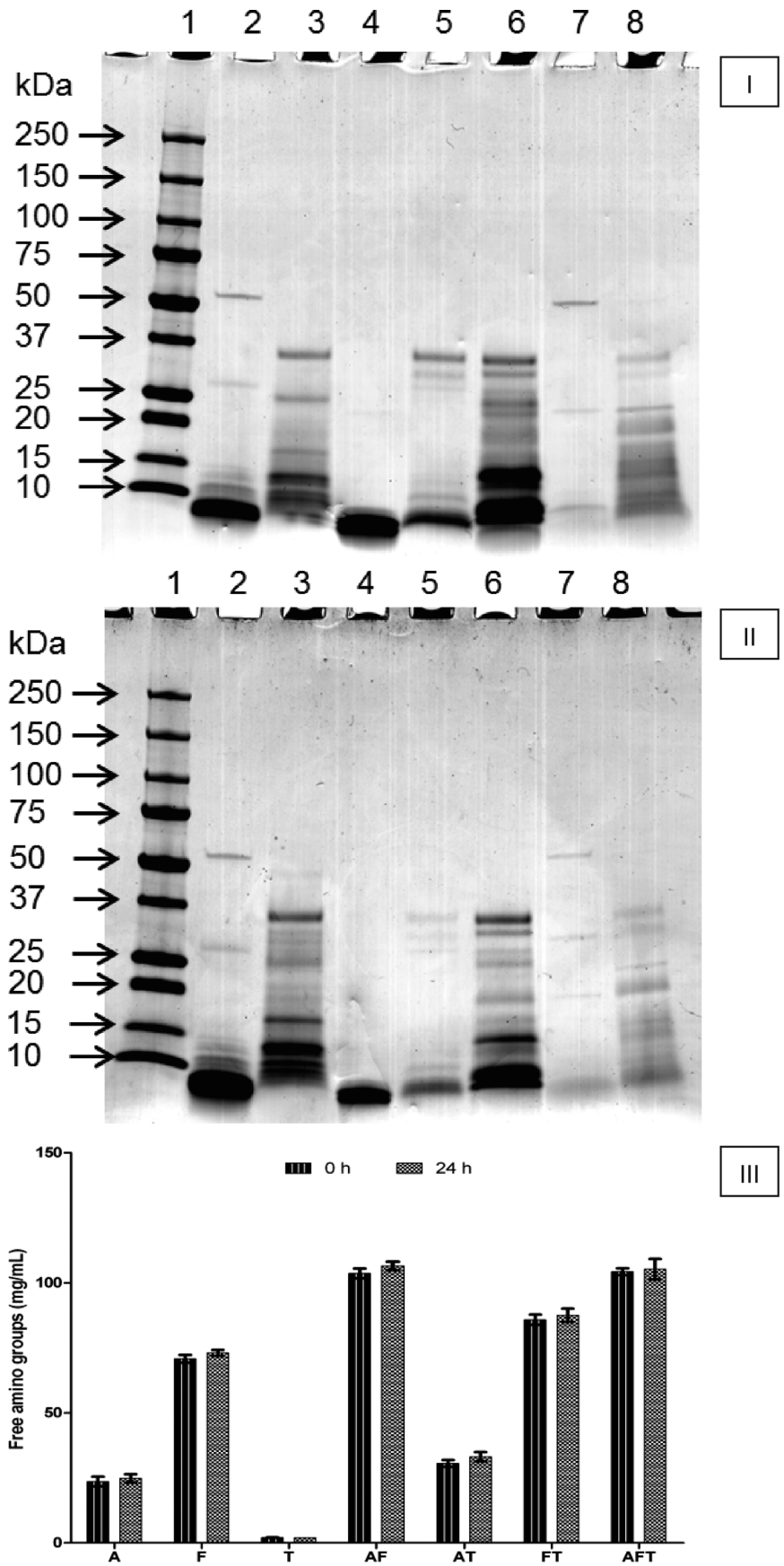

Figure 4. Stability of the enzyme mixtures. I and II: SDS-PAGE profiles of different enzyme preparations at time 0 and after 24 h of hydrolysis, respectively. Lane 1: molecular weight markers; lane 2: alcalase; lane 3: flavorzyme; lane 4: trypsin; lane 5: alcalase/flavorzyme; lane 6: alcalase/trypsin; lane 7: flavorzyme/trypsin, and lane 8: alcalase/flavorzyme/trypsin. III: The amount of free amino groups at time 0 and after 24 h of hydrolysis. A: alcalase, F: flavorzyme, T: trypsin, AF: alcalase/flavorzyme, AT: alcalase/trypsin, FT: flavorzyme/trypsin, and AFT: alcalase/flavorzyme/trypsin.

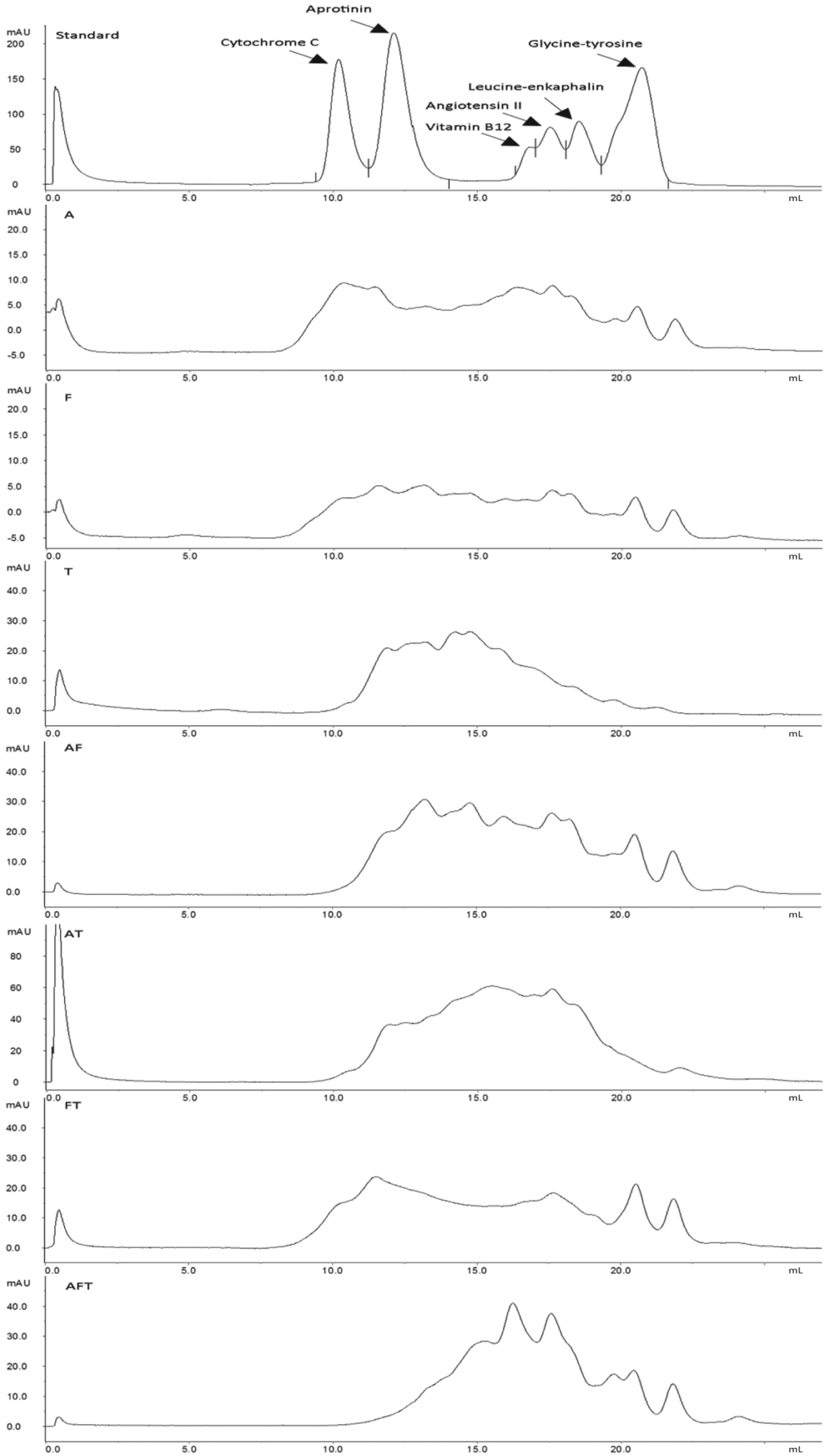

Figure 5. Representative size exclusion chromatograms of turkey head collagen peptides obtained with different enzymatic approaches. A: alcalase, F: flavorzyme, T: trypsin, AF: alcalase/flavorzyme, AT: alcalase/trypsin, FT: flavorzyme/trypsin, and AFT: alcalase/flavorzyme/trypsin.

Table 4. Molecular weight distribution of turkey head collagen peptides using several enzymatic approaches[1]

	Amount (% of integration area[2])		
Item	>2 kDa	1 to 2 kDa	<1 kDa
Enzymatic approach			
Alcalase	63.5 ± 5.4^{c}	14.2 ± 2.5^{d}	22.4 ± 2.9^{cd}
Flavorzyme	66.7 ± 0.5^{c}	6.6 ± 1.0^{e}	26.6 ± 1.5^{c}
Trypsin	70.8 ± 1.8^{c}	21.3 ± 2.2^{c}	7.8 ± 0.4^{e}
Alcalase/flavorzyme	48.2 ± 1.1^{b}	17.3 ± 0.3^{cd}	34.5 ± 1.5^{b}
Alcalase/trypsin	51.9 ± 0.6^{b}	28.0 ± 1.2^{b}	20.0 ± 0.5^{d}
Flavorzyme/trypsin	50.9 ± 2.0^{b}	14.6 ± 1.2^{d}	34.4 ± 0.8^{b}
Alcalase/flavorzyme/trypsin	14.7 ± 0.3^{a}	39.7 ± 1.1^{a}	45.6 ± 1.5^{a}
P-value	<0.0001	<0.0001	<0.0001

[a–e]Means within the same column sharing a common letter are not significantly different from each other.

[1]Results are presented as mean ± SD (n = 3).

[2]The amount of each molecular weight fraction was calculated as a percentage of the integration area with respect to the total peak area under the chromatogram.

tail produced significantly higher fractions of peptides having low and very low MW (less than 2,000 and 1,000 Da, respectively) compared with the rest of the hydrolysis approaches. Therefore, only hydrolysates obtained from turkey head collagen using the enzyme cocktail were screened for bioactivity.

Bile Acid Binding Capacity. The in vitro bile acid binding assay was originally developed to test the anticholesteremic properties of plant fibers. The method was then adapted to assess the anticholesteremic properties of food-derived peptides. Peptides with high bile acid binding capacity are believed to block the resorption of bile acid and therefore decrease the blood cholesterol level (Iwami et al., 1986). Unlike whey, egg, and soybean proteins, very few hypocholesterolemic peptides from meat proteins have been identified (Nagaoka, 2005). In this study, cholic and deoxycholic acids were used to assay the in vitro binding capacity of collagen peptides produced after 24 of hydrolysis with the enzyme cocktail, and results are presented in Figure 8A and B. For both bile acids, increasing collagen peptide concentrations significantly increased the percentage of bound bile acid. The binding of cholestyramine to cholic and deoxycholic acids showed a different trend. Increasing the cholestyramine levels resulted in a higher binding capacity toward sodium cholate

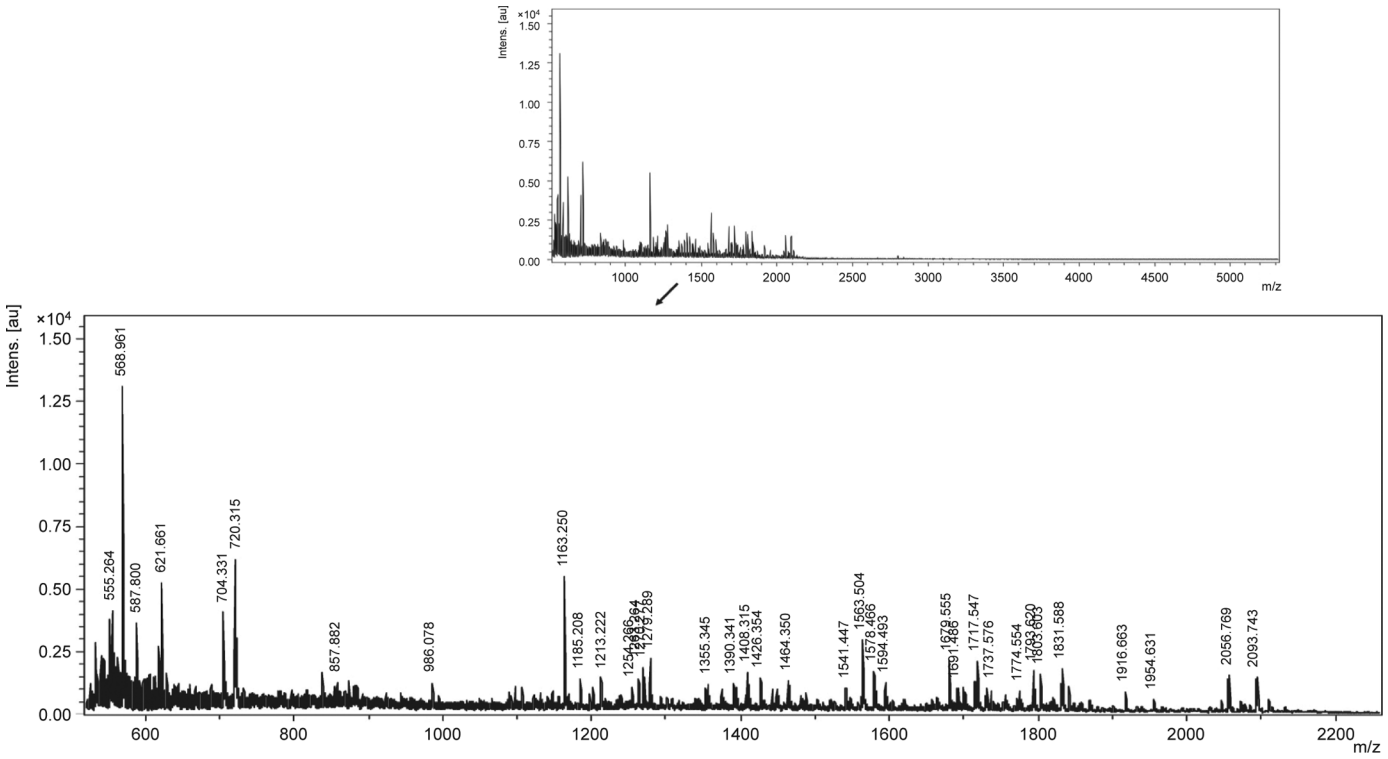

Figure 6. Matrix-assisted laser desorption ionization-time of flight-mass spectrometry spectrum of turkey head collagen peptides obtained using the enzyme cocktail after 24 h of hydrolysis. Intens: peak intensity; au: arbitrary unit.

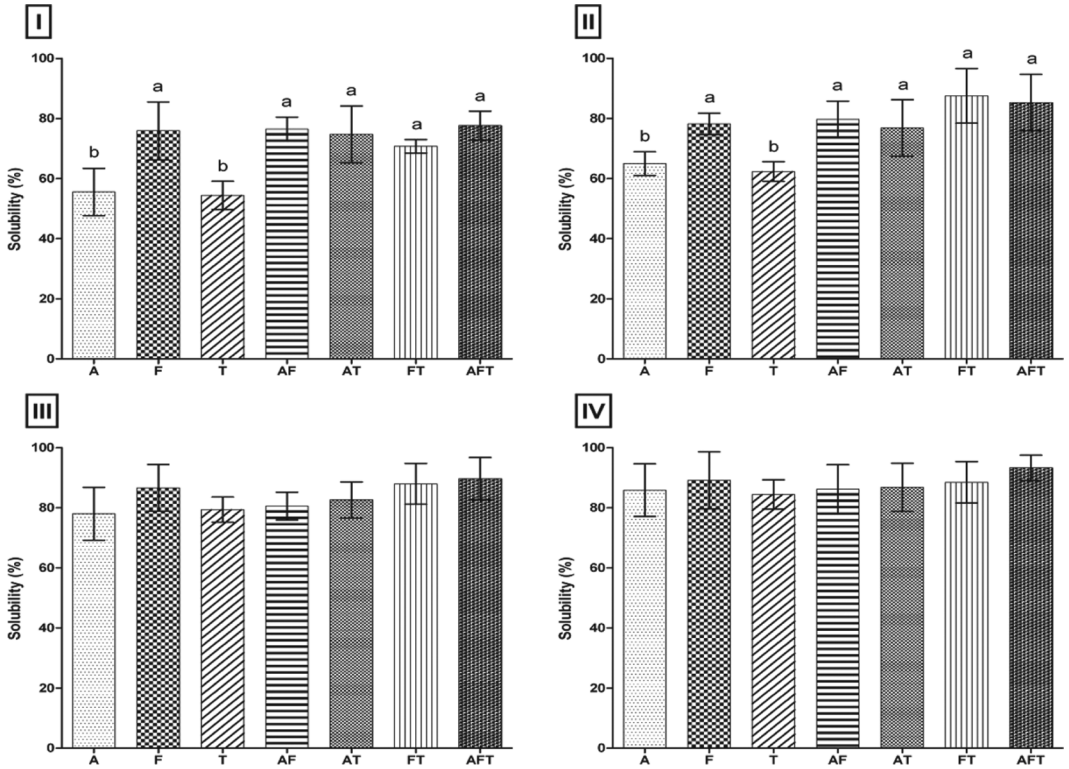

Figure 7. Solubility (%) of turkey head collagen peptides (n = 3). I: pH 2; II: pH 4; III: pH 6; IV: pH 8. A: alcalase, F: flavorzyme, T: trypsin, AF: alcalase/flavorzyme, AT: alcalase/trypsin, FT: flavorzyme/trypsin, and AFT: alcalase/flavorzyme/trypsin. Means sharing a common letter (a,b) are not significantly different from each other.

(Figure 8A); however, deoxycholic acid was bound to cholestyramine by 93.5% at all concentrations (Figure 8B). In this study, the binding capacity of cholestyramine to cholic and deoxycholic acids was similar to that reported by Camire and Dougherty (2003) and Story and Kritchevsky (1976), respectively.

At 20 mg/mL, both the cholate and deoxycholate binding capacities of collagen peptides (36.7 and 37.5%, respectively) were about 2.5-fold lower than that of cholestyramine (∼93%). The in vitro binding assay showed that, at 5 mg/mL, collagen peptides bind deoxycholic acid at higher percentages ($P < 0.05$) compared with cholic acid. Cholic acid is a primary bile acid with 3 hydroxyl groups, whereas deoxycholic acid is a secondary bile acid with 2 hydroxyl groups (Kongo-Dia-Moukala et al., 2011). The binding of bile acid is mainly due to hydrophobic interactions (Kern et al., 1978). Various hydrophobic amino acid residues (Ala, Iso, Leu, Phe, Val, Pro, Met, and Tyr) are present in gelatin. Although the amino acid sequences of the bile acid binding peptides were not determined in the present study, previous investigations have identified peptides containing Pro (Kwon et al., 2002), Ala (Nagaoka et al., 2001), or both Ala and Pro (Zhong et al., 2007) as potent hypocholesterolemic peptides. According to Du et al. (2013), gelatin extracted from turkey heads comprised 33% hydrophobic amino acid residues in which Ala and Pro alone represented more than 75% of the total hydrophobic amino acid residues. The ability of turkey collagen peptides to bind bile acid may therefore be linked to the presence of Ala and Pro. The structure elucidation of the possible hypocholesterolemic peptides and their mechanisms of interaction with bile acids need to be further investigated.

PAO Inhibitory Activity. Plasma amine oxidase is a group of copper-containing amine oxidases, including semicarbazide-sensitive amine oxidase, that convert primary amines to corresponding aldehydes with the generation of the reactive oxygen species hydrogen peroxide and ammonia (Olivieri et al., 2007). Hydrogen peroxide and aldehydes play major roles in generating oxidative stress and have been implicated in various inflammatory pathologies (Yadav and Ramana, 2013). Therefore, the ability to inhibit PAO represents a useful in vitro indicator to screen for an ingredient's ability to control inflammation.

The PAO inhibitory activity of collagen peptides produced after 24 h of hydrolysis by the enzyme cocktail is presented in Figure 9. Regardless of the preincubation time, it was observed that increasing the peptide concentration increased the PAO inhibition. For all peptide concentrations, the preincubation of PAO for 1 and 2 h resulted in higher inhibitions compared with the nonincubated samples. However, the preincubation of sample with PAO for 2 h did not improve the enzyme inhibition ($P > 0.05$) compared with those preincubated for 1 h. Significant differences among the PAO inhibitory activities of collagen peptides were only observed when using peptide concentrations higher than 5 mg/mL and preincubation periods longer than 1 h. This indicates

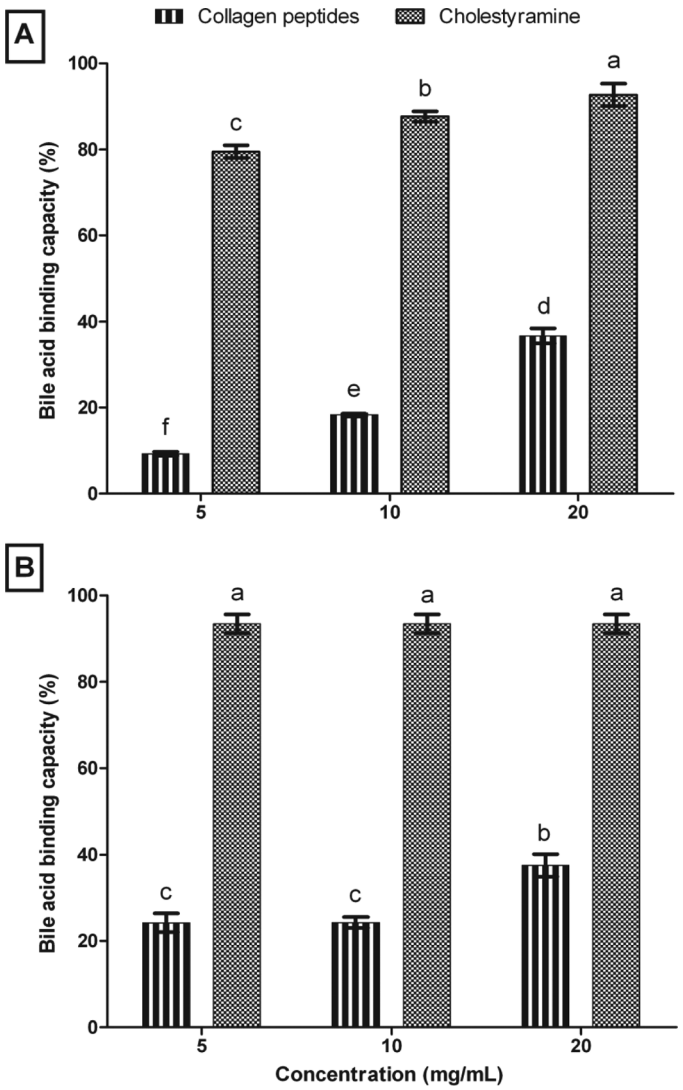

Figure 8. Bile acid binding capacity (%) of turkey head collagen peptides at different concentrations (n = 3). A: In vitro binding capacity tested with cholic acid. B: In vitro binding capacity tested with deoxycholic acid. Means sharing a common letter (a–f) are not significantly different from each other.

that the extracted collagen peptide inhibits PAO in a time- and dose-dependent manner. This type of PAO inhibition could be due to the presence of lysine-containing collagen peptides. Olivieri et al. (2007) reported that L-lysine inhibited semicarbazide-sensitive amine oxidase activity. It has also been shown that peptides with a lysine residue in the central position represent a potent inhibitor of PAO (Yegutkin et al., 2004). Both these studies reported an inhibition of PAO in a time- and dose-dependent manner.

Amino-sugars, such as galactosamine, can inhibit PAO (O'Sullivan et al., 2003). Although the IC_{50} (4.06 ± 0.1 mg/mL) of the extracted crude collagen peptides was about 6-fold higher than the inhibitory concentration (4 m*M* equivalent to 0.7 mg/mL) of galactosamine (Yegutkin et al., 2004), the use of collagen peptides as anti-inflammatory ingredients would probably be more therapeutically feasible because the use of amino sugars may increase blood sugar and have negative health consequences (Yegutkin et al., 2004).

Although the in vitro screening for bioactivity indicated that turkey head collagen peptides bound to bile acid and inhibited PAO, it was important to mention that the in vitro bioactive assays may not necessarily correlate with the in vivo anticholesteremic and the anti-inflammatory activities. It has been reported that the bioavailability, reactivity, and stability of the bioactive peptides are the main factors responsible for such lack of correlation between the in vitro and the in vivo effects (Sarmadi and Ismail, 2010). Therefore, the efficacy of turkey head collagen peptides in blocking inflammatory responses and lowering blood cholesterol levels still needs to be clinically verified.

Conclusion

A process was developed for the extraction and the hydrolysis of poultry collagen using an enzyme cocktail, prepared with alcalase, flavorzyme, and trypsin. The enzyme cocktail was stable against self-digestion and efficiently produced high proportions of very low MW peptides. These extracted collagen peptides were highly soluble in the pH range of 2 to 8, and were able to bind both cholic and deoxycholic acids and to inhibit PAO in a time- and dose-dependent manner, thus indicating both hypocholesterolemic and anti-inflammatory activities. The combination of the isoelectric solubilization/precipitation process and the enzyme cocktail hydrolysis represents a potential new way to produce high value functional food ingredients from a low value poultry by-product. The resulting soluble collagen peptides possessed excellent functional and bioactive properties and may have potential industrial applications.

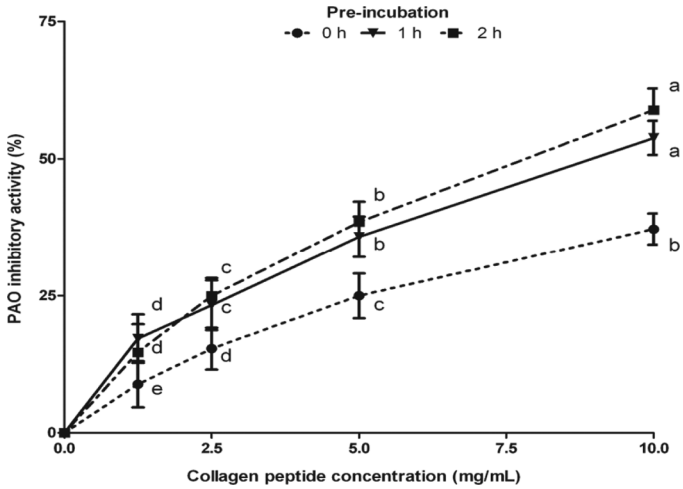

Figure 9. Plasma amine oxidase (PAO) inhibitory activity (%) of turkey head collagen peptides at different concentrations and preincubation periods (n = 3). Means sharing a common letter (a–e) are not significantly different from each other.

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