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ARTICLE

Effects of High Pressure/High Temperature Processing on the Recovery and Characteristics of Porcine Placenta Hydrolysates

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Abstract

This study was performed to investigate the effects of high pressure/high temperature (HPHT) treatment on the recovery efficiency and characteristics of porcine placenta hydrolysates. The placenta hydrolysates were characterized by solubility, free amino acid contents, gel electrophoresis, gel permeation chromatography (GPC) and amino acid composition. Placenta was treated at 37.5 MPa of pressure combined with various temperatures (150, 170, and 200°C) or various holding times (0, 30, and 60 min at 170°C). Insoluble raw placenta collagen was partially solubilized (> 60% solubility) by the HPHT treatment. Free amino group content of placenta collagen was increased from 0.1 mM/g collagen to > 0.3 mM/g collagen after HPHT treatment, reflecting partial hydrolysis of collagen. The molecular weight (M_w) distribution showed evidence of collagen hydrolysis by shifting of M_w peaks toward low molecular weight when treated temperature or holding time was increased. Alanine (Ala), glycine (Gly), hydroxyproline (Hyp), and proline (Pro) contents increased after the HPHT treatments compared to a decrease in the others. In particular, the increase in Gly was obvious, followed by Hyp and Pro, reflecting that placenta hydrolysates were mainly composed of these amino acids. However, increasing temperature or holding time hardly affected the amino acid compositions. These results indicate that the HPHT treatment is advantageous to hydrolyze collagen derived from animal by-products.

Key words: high pressure, high temperature, subcritical water, placenta collagen, hydrolysis

Introduction

Collagen is a ubiquitous protein occurring in by-products such as tissues, bones, skins and organs of animals or marine organisms. Due to its low nutritional value and lack of essential amino acids, collagen has not been attracted much attention as an edible protein source. In contrast, collagen is an important source in medicine, pharmaceuticals, cosmetics as well as functional food industry because of its low biodegradability and less antigenicity (Kim *et al.*, 2010; Zhang *et al*., 2006). Collagen is a high molecular weight (M_w) protein (M_w = 300 kDa) and is composed of three sub-chains of α_1, α_2 (~100 kDa) and β (~200 kDa) associated through a triple-helix structure (Zhang *et al*., 2006). Although collagen is classified into several types based on chain composition, the primary structure of collagen is normally characterized by the presence of an uninterrupted glycine-X-Y repetition in which X and Y are mainly composed of Pro and Hyp (Miller, 1988).

Low M_w peptides derived from various proteins have been extensively investigated due to their physiological functions including anti-osteoarthritis, anti-osteoporosis, anti-oxidation and anti-hypertension properties (Denis *et al*., 2008). Commercial collagen hydrolysates are obtained by treatment with proteases such as trypsin, pepsin, chymotrypsin, alcalase, collagenase, and papain (Gómez-Guillén *et al*, 2011). Insoluble collagen must be converted to a soluble gelatin by mild thermal treatment prior to enzymatic hydrolysis, thereafter being partially hydrolyzed by acidic or alkaline treatment (Denis *et al*., 2008). However, this procedure requires too long processing time to achieve low M_w collagen peptides.

Hot pressurized water (namely subcritical water) treatment has been studied as a novel protein hydrolysis technology. The critical point of water is 374°C and 22 MPa at which the water ionization constant increases, hence, water ionizes readily to hydrogen and hydroxide ions (Watchararuji *et al*., 2008). Hydrogen ions disrupt peptide

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bonding thereby resulting in hydrolysis of peptides (Brunner, 2009). Information about the effects of subcritical water on the hydrolysis of soybean and rice bran protein is available (Sunphorka *et al*., 2012; Watchararuji *et al*., 2008). However, there was no study about collagen hydrolysis using subcritical water. Therefore, we investigated the effects of various high pressure/high temperature (HPHT) processing conditions on the recovery efficiency and the characteristics of porcine placenta hydrolysates.

Materials and Methods

Materials

Frozen porcine placenta was kindly provided by Samwoo Husbandry (Korea). The frozen placenta was thawed at 4°C overnight and washed in water several times to remove residual blood. All visible fat was trimmed off and the placenta was cut into 5 cm lengths. The moisture content of the placenta was estimated according to AOAC (1990). Crude protein of the porcine placenta was determined by Kjeldahl (%$N \times 6.25$). Approximately 100 g of placenta was vacuum-packaged and frozen at -50°C prior to use (within 2 wk). All chemicals used in this study were analytical grade and purchased from Sigma-Aldrich Corp. (USA).

Treatments

A lab-scale high pressure system equipped with an ohmic heater was used to extract and hydrolyze the placenta. In brief, the system was composed of a S-40 pressure generator (Seowon Compressor Co., Korea), a HSF-300 pressure intensifier (Haskel International Inc., USA), a pressure vessel with working volume of 1 L, and an ohmic heater connected with a SDU-990 temperature controller (TC Sensor Manufacturing Ltd., Korea) as depicted in Fig. 1. Water was used as the pressure-transmitting medium. Vacuum-packaged frozen placenta was thawed overnight and blended using a HR-2084 food processor (Philips Electronics, Korea). Aliquots 30 g of placenta homogenates were filled into a polyimide film and sealed with precursor using a MH-7 heating press (Masada Seisakusho Co. Ltd., Japan). Samples were divided into two groups for two different experiments to evaluate the temperature and holding time effects. Samples in the first group were inserted into the vessel and heated from 20°C to the target temperature (150, 170, or 200°C) under 37.5 MPa of pressure. When the inner vessel temperature reached the target, the vessel was cooled down to 40°C using ice. The remaining samples were heated under 37.5 MPa of pressure to evaluate the effects of holding time. When vessel temperature was 170°C, the samples were held for 0, 30, and 60 min under the pressure/temperature-controlled conditions and cooled down to 40°C using ice. After cooling, the samples were removed from the vessel and analyzed without further storage. A non-treated sample was used as a control.

Gel electrophoresis

After treatment, samples (control and treatment at 170°C for 30 min) were transferred to test tube and vigorously vortexed. Aliquots 100 μL of sample were diluted with 400 μL of 8 M urea (final protein concentration of 4 mg/mL). Peptide profiles of the sample were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using EzWay™ PAG 6% acrylamide gels (KOMA Biotech Inc., Korea) based on the method of Laemmli (1970). The sample was mixed with one part KTG 020 sample buffer (KOMA Biotech Inc., Korea), consist-

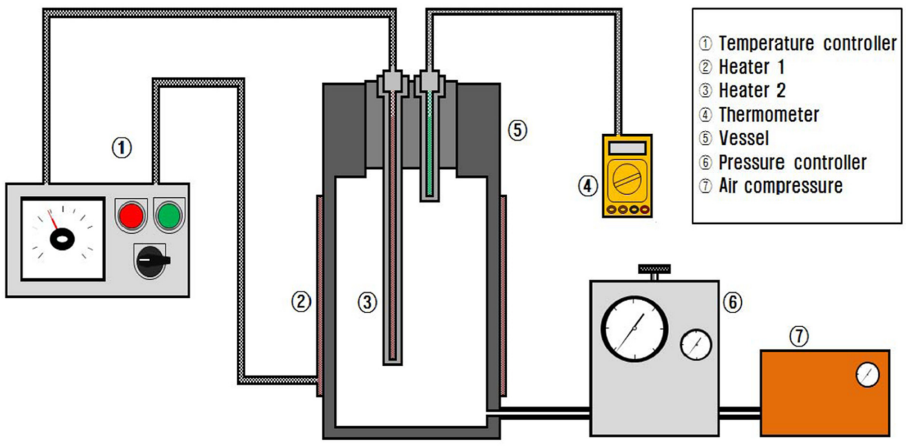

Fig. 1. Schematic diagram of the high pressure/high temperature processing apparatus.

ing of 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% β-mercaptoethanol and 63 mM Tris, pH 6.8). The sample was boiled for 2 min and 20 μL of sample mixture was loaded into the gel wells. Peptide separation was performed at a constant voltage of 140 V (~1 h).

Solubility

All treatments were centrifuged at 1,000 *g* for 15 min, and the supernatant was collected. The protein content of the supernatant was determined by the method of Kjeldahl (%$N \times 6.25$) and solubility was expressed as percent protein recovery in the supernatant over the initial protein content in the sample.

Free amino group content

Free amino group content was determined by the method of Benjakul and Morrissey (1997). The sample supernatant (125 μL) was mixed with 2 mL 0.2125 M sodium phosphate buffer (pH 8.2), and 1 mL 0.01% 2,4,6-trinitrobenzenesulfonic acid was added into the sample mixture. The mixture was thermal-treated at 50°C for 30 min, and 2 mL of 0.1 M sodium sulfite was added to the sample to terminate the reaction. The mixture was cooled at ambient temperature for 15 min and absorbance was measured at 420 nm. The free amino group content was expressed in terms of L-leucine (Nagarajan *et al*., 2012).

Molecular weight distribution

The molecular weight (M_w) distribution of collagen peptides in the supernatant was determined by the method of Gu *et al*. (2011) with minor modifications. Gel permeation chromatography was performed using a YL 9100 high performance liquid chromatography (HPLC) system (Younglin Instrument Co. Ltd., Korea) equipped with three Ultrahydrogel™ 120 columns (7.8 × 3,000 mm, Waters, USA). The mobile phase was distilled/deionized water at a flow rate of 1 mL/min, and the M_w distributions of the collagen peptides were monitored using a YL 9100 refractive index detector (YL Instrument Co. Ltd., Korea) at 40°C. A molecular weight standards kit (0.68-1,670 kDa, Polymer standards service, Germany) was used as standards.

Amino acid composition

Amino acid composition of the sample supernatant was determined using an Agilent HPLC 1200 system (Agilent Technologies Inc., USA) equipped with two detectors (a fluorescence detector and a UV detector) and a C_{18} column (4.6 × 150 mm, 5 μm in diameter). The supernatant containing 3 mg of total protein was added to 30 mL of 6 M HCl and hydrolyzed at 130°C for 24 h. After hydrolysis, the sample was diluted with distilled/deionized water up to total volume of 100 mL, and then filtered using a DISMIC-13CP 0.45 μm syringe filter (Advantec Co., Japan). Two mobile phases were applied, i.e., one was 20 mM sodium phosphate buffer (pH 7.8) and the other was 45% (v/v) acetonitrile/45% (v/v) methanol solution. The samples were monitored at an emission wavelength of 450 nm and an excitation wavelength of 340 nm for the *o*-phthalaldehyde derivatives, and an emission wavelength of 305 nm and an excitation wavelength of 266 nm for the 9-fluorenylmethyl chloroformate derivatives at 40°C in the case of fluorescence detecting and an absorbance at 338 nm at 40°C in the case of UV detecting. An Agilent 5061-3330 (Agilent Technologies Inc., USA) was used as the standard.

Statistical analysis

A completely randomized design was adopted. Data collected from the triplicate determinations of each sample repeated twice are presented as mean±standard deviation. Analysis of variance was performed, and the mean differences were identified by Duncan's multiple range test using a SAS statistical software ver. 9.1 (SAS Institute, USA).

Results and Discussion

Solubility

The ground placenta was insoluble in water and exhibited 2.9% protein recovery in the supernatant (Fig. 2). A small portion of soluble protein would account for the minor contaminant possibly originating from blood. HPHT processing increased the solubility of the porcine placenta. The solubility of placenta was 58% when HPHT processing was applied at 150°C (Fig. 2A). More protein (71%) was recovered in the supernatant by increasing processing temperature (170°C) ($p<0.05$). Insoluble collagen is converted into soluble gelatin in water following mild thermal treatment (Montero and Gómez-Guillén, 2000). In this study, the applied temperatures (150-170°C) were high enough for placenta collagen to convert to gelatin, which could increase solubility of the samples in the supernatant. In addition, ionization of water under the HPHT conditions caused a decrease in water pH, hence, placenta collagen would be partially hydrolyzed to soluble peptides (Zhu *et al*., 2011). In contrast, the HPHT processing at 200°C caused a drastic decrease in solubility (55%) of placenta ($p<0.05$).

A parabolic change in placental solubility by the effect

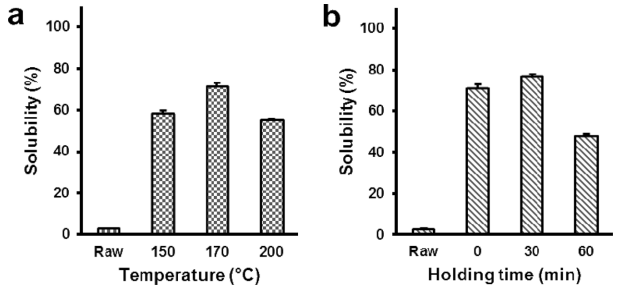

Fig. 2. Solubility profiles of raw (control) and high pressure/high temperature (HPHT) treated porcine placenta (A) at various temperatures for 0 min and (B) at 170°C for various holding times. All HPHT treated placenta suspensions were treated at 37.5 MPa of pressure. Vertical bars indicate standard deviations.

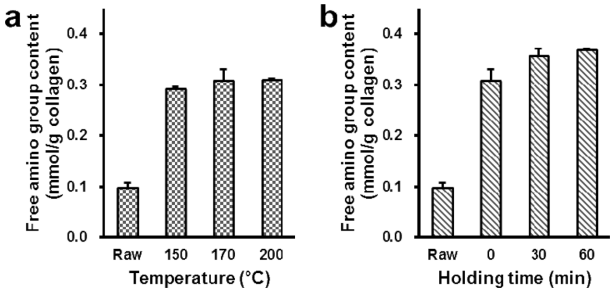

Fig. 3. Free amino group contents of raw (control) and high pressure/high temperature (HPHT) treated porcine placenta (A) at various temperatures for 0 min and (B) at 170°C for various holding time. All HPHT treated placenta suspensions were treated at 37.5 MPa of pressure. Vertical bars indicate standard deviations.

temperature was observed when the placenta was subjected to HPHT processing at 170°C for various holding times (Fig. 2B). The solubility of placenta was increased to 77% until 30 min of treatment at 170°C. Thereafter solubility was decreased down to 48% ($p<0.05$). These phenomena could be explained by decomposition of collagen peptides. Sunphorka *et al*. (2012) indicated that protein content of subcritical-processed rice bran decreases with increasing holding time, whereas amino acid content increased. Similarly, decomposition of amino acids occurs following subcritical (190-260°C) water treatment (Amashukeli *et al*., 2007; Dunn and Brophy, 1932). Consequently, the decomposition of peptides and amino acids would account for a decrease in solubility of the porcine placenta suspension.

Free amino group content

It was unclear how much placenta was hydrolyzed by the HPHT processing, because the amounts of soluble gelatin and collagen hydrolysates could not be quantified. Protein decomposition was determined in terms of free amino group content to evaluate the amount of hydrolyzed placenta (Nagarajan *et al*., 2012) and the results are depicted in Fig. 3. The free amino group content of raw placenta was 0.10 mM/g and increased up to 0.26 mM/g after HPHT treatment at 150°C ($p<0.05$) (Fig. 3A). The free amino group content was further increased with increasing temperature and was 0.31 mM/g at 170°C ($p<0.05$), confirming hydrolysis of placenta collagen. No difference in free amino group content occurred between the 170°C and 200°C treatments. The results indicate that HPHT processing at 170°C was the best condition from an energy efficiency perspective. Placenta hydrolysis may have occurred at > 170°C, whereas decomposition of free amino acids would also occur (Amashukeli *et al*., 2007), resulting in no significant increase in free amino group content of porcine placenta.

Increasing the holding time at 170°C was also attributed to higher free amino group content (Fig. 3B). The free amino group content increased from 0.31 to 0.36 mM/g by increasing the holding time from 0 to 30 min ($p < 0.05$). Alternately, further increasing holding time (60 min) resulted in a gradual increase in free amino group content (0.37 mM/g) compared to that at 30 min. Nevertheless the impact of the 60 min treatment was not significant compared to that at 30 min. Higher pressure level (100 MPa, 170°C, 30 min) was also compared with the corresponding counterpart of 37.5 MPa treatment (170°C, 30 min), but no difference in protein recovery or free amino group content was observed (data not shown). These results indicate that the main factor affecting placenta hydrolysis during HPHT processing was not pressure but temperature. Based on these results, the optimum condition of HPHT processing for placenta hydrolysis was 170°C for 30 min, where product yield was maximum.

Gel electrophoresis

Porcine placenta peptides exhibited four major bands near 200 and 100 kDa (Fig. 4). The former indicates α-chains (namely α1 and α2) which have M_w of 116 kDa and the latter were β-chains (205 kDa) as revealed in other studies (Ahmad *et al*., 2010; Klomklao *et al*., 2006; Liu *et al*., 2012). These results indicate that the composition of protein in porcine placenta was mainly collagen. There was no difference in SDS-PAGE profiles between reducing and non-reducing conditions reflecting no disulfide bond in placenta peptides. Most bands lost their intensity following the HPHT processing (170°C for 30 min) and the bands were shifted to low M_w ranges (< 55 kDa). These changes in collagen band profiles were in agreement with

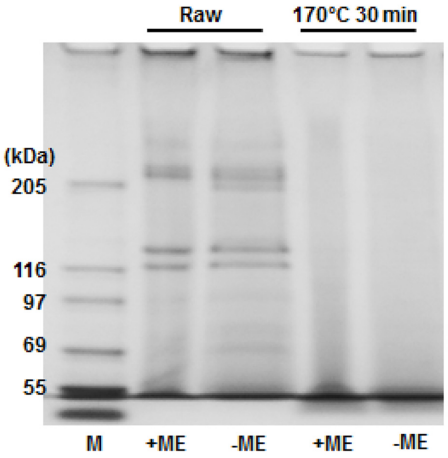

Fig. 4. SDS-PAGE profiles of non-reduced (-ME) and reduced (+ME) raw (control) and high pressure/high temperature (HPHT) treated porcine placenta. The HPHT treatment was conducted at 170°C for 30 min under 37.5 MPa. Lane M indicates molecular weight standard.

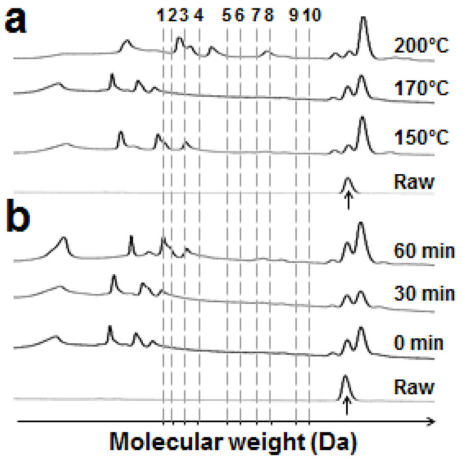

Fig. 5. Relative molecular weight distributions of raw (control) and high pressure/high temperature (HPHT) treated porcine placenta (A) at various temperatures for 0 min and (B) at 170°C for various holding time. All HPHT treated placenta suspensions were treated at 37.5 MPa of pressure. Molecular weights presented as Arabic numbers are (1) 20,100 Da, (2) 12,600 Da, (3) 6,950 Da, (4) 4,270 Da, (5) 1,400 Da, (6) 985 Da, (7) 626 Da, (8) 434 Da, (9) 222 Da and (10) 106 Da. Upper arrows indicate system peaks.

Zhang *et al*. (2006) who reported that gelatin and/or collagen hydrolysate exhibit a broad distribution with lower M_w than collagen. In contrast to enzymatic hydrolysis, which has several steps in hydrolysis from long chains to short chains (or amino acid), thermal hydrolysis of collagen occurred at relatively high temperature (> 150°C), hence SDS-PAGE profiles of HPHT treatments in the present study exhibited same results regardless of temperature and holding time (data not shown).

Molecular weight distribution

Due to the insoluble properties of raw placenta, no M_w peak was observed in the control supernatant (Fig. 5). The HPHT treatment at 150°C exhibited two distinctive peak groups out of the range of the M_w standards (Fig. 5A). The first group consisted of four high M_w peaks (> 20.1 kDa) and the second group was two low M_w peaks (< 106 Da). The former would be solubilized gelatin and/or partially hydrolyzed high M_w peptides, and the latter possibly indicated small units of peptide hydrolysates. Increasing temperature shifted the high M_w peaks toward the lower M_w region. Moreover, the highest M_w peak mostly disappeared at 200°C, whereas new peaks were generated in the average M_w range of 1,400-4,270 Da and 424 Da, indicating that hydrolysis of placenta progressed with increasing temperature.

Similar results were also obtained when the placenta was treated by HPHT processing at constant temperature (170 °C) and increasing holding time (Fig. 5B). Movements of peaks toward the low M_w region was not considerable between the 0 and 30 min treatments, whereas the 60 min HPHT treatment resulted in relatively low M_w peaks and a new peak. Nevertheless, the overall peak M_w from the HPHT treatment at 170°C for 60 min was higher than those treated at 200°C for 0 min. The results demonstrate that HPHT processing has a potential advantage to produce small peptides derived from animal by-products. Although, there is no comparative literature, HPHT processing enabled to produce collagen peptides without chemical treatment within 1 h.

Amino acid profiles

The amino acid compositions of the HPHT treated placenta were determined (Fig. 6). Raw placenta exhibited various amino acid compositions. In particular, Asp, Glu, and Gly exhibited > 10% of total amino acid profiles. These amino acid profiles were similar to those obtained by Gu *et al*. (2011). Nalinamon *et al*. (2011) reported that the major amino acids of type I collagen extracted from ornate threadfin bream were Ala (13.4%), Gly (32.1%), and Pro (10.4%). Different compositions of collagen are due to different sources (animals versus marine organisms) and different experimental conditions (supernatant versus whole collagen). It is reported that placental membranes contain type I and VII collagens compared to type IV and VI collagens in placental villi (Miller, 1988). Although collagen type is classified by sub-chain composition, each sub-chain exhibits similar amino acid composition (Nimni and Hark-

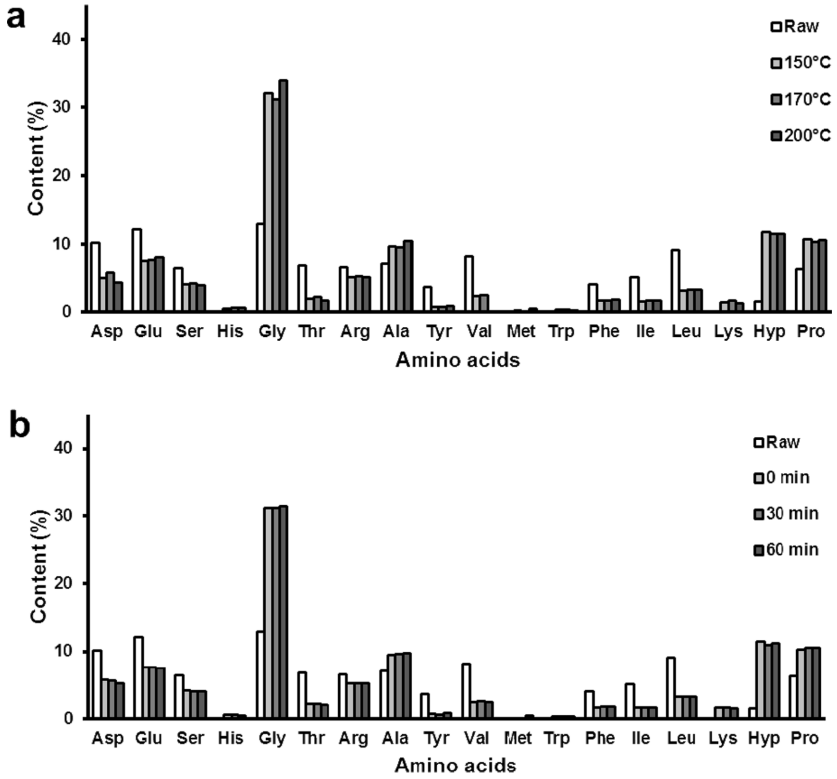

Fig. 6. Amino acid compositions of raw (control) and high pressure/high temperature (HPHT) treated porcine placenta (A) at various temperatures for 0 min and (B) at 170°C for various holding times. All HPHT treated placenta suspensions were treated at 37.5 MPa of pressure.

ness, 1988). It should be noted that the amino acids of raw placenta in this study were based on the supernatant (solubilized proteins). Regardless of treatment conditions, the amino acid contents of most placenta supernatants decreased greatly after HPHT treatment with the exception of Gly, Ala, Hyp and Pro which increases in content over the total amino acid compositions (p<0.05). These patterns exhibited typical amino acid profiles of collagen (Nalinamon *et al*., 2011; Nimni and Harkness, 1988). However, no differences in amino acid contents were observed among treatments, indicating that the collagen extraction and hydrolysis could be achieved by HPHT processing.

Conclusion

This study applied HPHT processing to extract and hydrolyze porcine placenta collagen. Among the physical factors, increasing temperature produced low M_w collagen peptides rather than holding time. Based on the results, HPHT processing at 170°C for 30 min was favorable to produce low Mw collagen peptide. Although the main mechanisms involved in collagen hydrolysis under high pressure and high temperature domains are still obscure, HPHT processing exhibited the best way to achieve value-added collagen hydrolysates from less valuable animal wastes or by-products.

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