ARTICLE

Effect of High Pressure on the Porcine Placenral Hydrolyzing Activity of Pepsin, Trypsin and Chymotrypsin

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Abstract

This study investigated the effects of protease treatments (trypsin, chymotrypsin, and pepsin) under various pressure levels (0.1-300 MPa) for the characteristics of porcine placenta hydrolysates. According to gel electrophoretic patterns, the trypsin showed the best placental hydrolyzing activity followed by chymotrypsin, regardless of the pressure levels. In particular, the peptide bands of tryptic-digested hydrolysate were not shown regardless of applied pressure levels. The peptide bands of hydrolysate treated chymotrypsin showed gradual decreases in molecular weights (M_w) with increasing pressure levels. However, the pepsin did not show any evidences of placental hydrolysis even though the pressure levels were increased to 300 MPa. The gel permeation chromatography (GPC) profiles showed that the trypsin and pepsin had better placental hydrolyzing activities under high pressure (particularly at 200 MPa), with lower M_w distributions of the hydrolysates. Pepsin also tend to lower the M_w of peptides, while the major bands of hydrolysates being treated at 300 MPa were observed at more than 7,000 Da. There were some differences in amino acid compositions of the hydrolysates, nevertheless, the peptides were mainly composed of glycine (Gly), alanine (Ala), hydroxyproline (Hyp) and proline (Pro). Consequently, the results indicate that high pressure could enhance the placental hydrolyzing activities of the selected proteases and the optimum pressure levels at which the maximum protease activity is around 200 MPa.

Key words: porcine placenta, high pressure, hydrolysates, protease, enzyme activity

Introduction

Although collagen is the most abundant protein source which can be obtained from various animal wastes or by-products, it has not been attracted much attention as a nutritional source due to a lack of some essential amino acids such as tryptophan (Szpak, 2011). Collagen has a molecular weight (M_w) of about 300 kDa and composed of double α-chains (~100 kDa) and β-chain (~200 kDa) structured by triple-helix (Furthmayr and Timpl, 1971). Recently, interests in collagen hydrolysates are greatly grown as a food, cosmetic or pharmaceutical ingredient since various bioactivities and functionalities of the hydrolysateshave been identified (Giménez *et al.*, 2009; Iwai *et al.*, 2005). It has been reported that collagen hydrolysates show different bioactivity depending on the molecular weights and amino acid sequence. For instant, antioxidant activity was identified in the collagen hydrolysates which

were composed of 10-15 amino acids while antihypertensive (ACE inhibitory) activity were shown in the peptides consisted of 2-4 amino acids (Gómez-Guillén *et al.*, 2011).

Commercial collagen hydrolysatesare produced mainly by acid treatments or by enzymatic digestion. In particular, acid treatment (normally HCl) a thigh temperature has been a standard way to produce collagen hydrolysates within a few hours (Tsugita and Sheffler, 1982), however, it also implies a generation of waste water and a requirement of neutralization/desalting processes. In more recent, subcritical water processing has been introduced as a novel collagen hydrolysis technology (Lee *et al.*, 2013). Upon elaborated pressure and temperature (subcritical region), collagen converts into gelatin and undergoes partial or complete hydrolysis depending on the subcritical conditions such as temperature. Our previous study indicated that subcritical water processing (37.5 MPa and 170ºC) had a potential advantage to hydrolyze collagen within a few minutes (Lee *et al.*, 2013). Disadvantage of subcritical hydrolysis of collagen was relatively high M_w (>5 kDa) of collagen hydrolysates.

The usage of proteases is an alternative of the second hydrolyze collagen. In spite of high cost of final products,

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enzymatic hydrolysis is a common method to produce high quality collagen peptides (Clemente, 2000). Various enzymes including trypsin, pepsin, chymotrypsin, alcalase as well as collagenase are involved in collagen hydrolysis. Intriguingly, hydrolysis activity of proteases has been reported to be enhanced under and/or after high pressure treatment (Northrop, 2002). It is likely that enhanced proteolysis activity of the enzymes provides various advantages in reducing the M_w of final product with reduced cost. Therefore, this study was conducted to evaluate the effects of high pressure (0.1-300 MPa) on the collagen-hydrolyzing activity of various proteases (pepsin, trypsin, and chymotrypsin).

Materials and Methods

Materials

Frozen porcine placenta was kindly donated by Samwoo Husbandry Co. (Korea). The frozen placenta was thawed in running water for 4 h and washed with water containing detergent to remove residual blood. The placenta was further washed with water several times and dewatering process was done by centrifugation at ×1,000 g for 15 min. Visual fat was completely trimmed off and the placenta was cut into about 5 cm in length. Aliquots of 1 kg placenta were vacuum-packaged using polyethylene pouch and frozen at -50°C prior to use (within 2 mon). Pepsin (E.C. 3.4.23.1, from porcine gastric mucosa, 250 units/mg protein), trypsin (E.C. 3.4.21.4, from bovine prancrease, 10,000 BAEE (N-benzoyl-L-arginine ethyl ester) units/mg protein) and α-chymotrypsin (E.C. 3.4. 21.1, from bovine prancrease type-II, 40 units/ mg protein) were purchased from Sigma-Aldrich Co. (USA). All che- micals used in this study were analytical grade.

Treatment

Frozen placenta was thawed at 4°C overnight. Moisture and crude protein contents of the placenta were determined respectively by 102°C air drying and by Kjeldahl (%N×6.25). The placenta was blended using a SMT homogenizer (SMT Co. Ltd., Japan) for 5 min and aliquot 50 mL was mixed with each protease (40 unit/mg of pepsin and chymotrypsin, 25.0 BAEE unit/mg of trypsin). High pressure was applied using a lab-assembled barostatic device. In brief, the device was composed of pressure generator, intensifier and pressure controller, and water was used for pressure-transmitting medium. Samples were pressurized at 100, 200, and 300 MPa for 5 min

at ambient temperature. After depression, all samples were further incubated at 4°C for 2 h and the enzymes were inactivated by immersing the samples in 75°C water bath for 15 min.

Gel electrophoresis

Samples were vigorously voltexed and diluted to final 4 mg/mL protein concentration by adding adequate amount of distilled water. Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) was conducted using Ez-Way™ PAG 12% acrylamide gels (KOMA Biotech Inc., Korea) based on the method of Laemmli (1970). The sample dilutes were mixed with the same amount of KTG20 sample buffer (KOMA Biotech Inc., Korea) consisting of 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% β-mercaptoethanol and 63 mM Tris (pH 6.8). The mixture 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).

Gel permeation chromatography (GPC)

Samples were centrifuged at ×10,000 g for 5 min and the molecular weight (M_w) of peptides in the supernatant was determined by GPC based on the method of Gu *et al*. (2011). 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) was used for GPC analysis. The flow rate of mobile phase was 1 mL/min using deionized/distilled water. The M_w distributions of the peptides were monitored using a YL 9100 refractive index detector (YL Instrument Co. Ltd., Korea) at 40°C. A M_w 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 DIS-MIC-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 sam-

ples 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

Completely randomized design was adopted to evaluate the effect of pressure levels or types of enzyme. One-way analysis of variance (ANOVA) was conducted using a SAS statistical program (ver. 9.1) and the means were separated by Duncan's multiple range test when the main effect (pressure levels) was significant ($p < 0.05$).

Results and Discussion

Gel electrophoresis

The three selected proteases exhibited different hydrolyzing features based on peptide band patterns of placenta (Fig. 1). For pepsin treatments, high M_w bands were shown in the M_w range of >55 kDa, reflecting that pepsin had a poor placental hydrolysis activity. Contrary to literature which postulates proteolysis activity of pepsin (Skierka and Safdowska, 2007), poor hydrolysis in the present study would be due to type of substrate (mainly collagen) and environmental conditions (pH). Pepsin is reported to cleave peptide bonds between hydrophobic and aromatic amino acids (Dunn, 2001). Because collagen rarely contains the aromatic amino acids such as tyrosine, tryptophan and phenylalanine (Dunn, 2001; Nelson and Cox, 2008), placenta suspension is not a good substrate for the peptic-digestion. In addition, the optimum reaction of

Fig. 1. SDS-PAGE pattern of porcine placenta treated with proteases and pressurized for 5 min after incubation at 4°C for 2 h.

pepsin is occurred at around pH 2. At higher than neutral region, pepsin is inactivatedand irreversibly denatured (Johnston *et al*., 2001). The placenta suspension had a pH about 6.3 which was too high for pepsin to hydrolyze the substrates as reported by Lee *et al*. (2013). These factors would also affect the activity of pepsin under high pressure, being shown no visual changes in peptide band patterns.

For trypsin treatments, no bands were detected at atmospheric condition, indicating that trypsin had a potential to hydrolyze collagen. High M_w peptides were completely hydrolyzed which attributed no visual bands in SDS-PAGE gel, and the tryptic-digested hydrolysates had M_w below 20 kDa. Therefore, the effect of high pressure on the tryptic-digestion of placenta was not identical in SDS-PAGE.

The chymotrypsin-treated placenta showed different band intensities depending on applied pressure levels. All bands of placental hydrolysates were in the range of <40 kDa with more than 6 peptide bands. With increasing pressure, the bands of 30-40 kDa lost their intensities probably as a result of further hydrolysis, hencehigh pressure seemed to enhance the enzyme activity for placental hydrolysis. Meanwhile, the bands below 29 kDa were not affected by pressure levels.

Based on the results from gel electrophoresis, it could be concluded that trypsin had the best placental hydrolysis ability followed by chymotrypsin and pepsin. However, it should be noted that the trypsin concentration (BAEE units/mg protein) applied in this study could not compensate for that of chymotrypsin (unit/mg). On the other hand, trypsin in the present study was used after high dilution (×400). Therefore the usage of trypsin for collagen hydrolysis had advantages in product cost and hydrolyzing efficiency comparing to chymotrypsin and pepsin.

M_w distribution

Because gel electrophoresis did not provide information regarding the M_w distributions of placenta hydrolysates, supernatant of the enzyme-treated samples were analyzed by GPC (Fig. 2). Despite that pepsin exhibited poor placental hydrolysis ability in gel electrophoresis, the limited amounts of peptides produced by this enzymatic digestion were mainly separated by two groups, one group had a M_w higher than 20 kDa and the other group of which M_w was lower than 434 Da. Considering a repetitive primary structure of collagen, the former would be limited hydrolysates from collagen polymer while the latter was thought to be hydrolysates cleaved from C and/
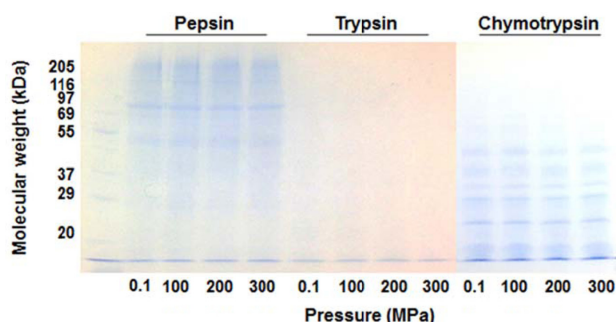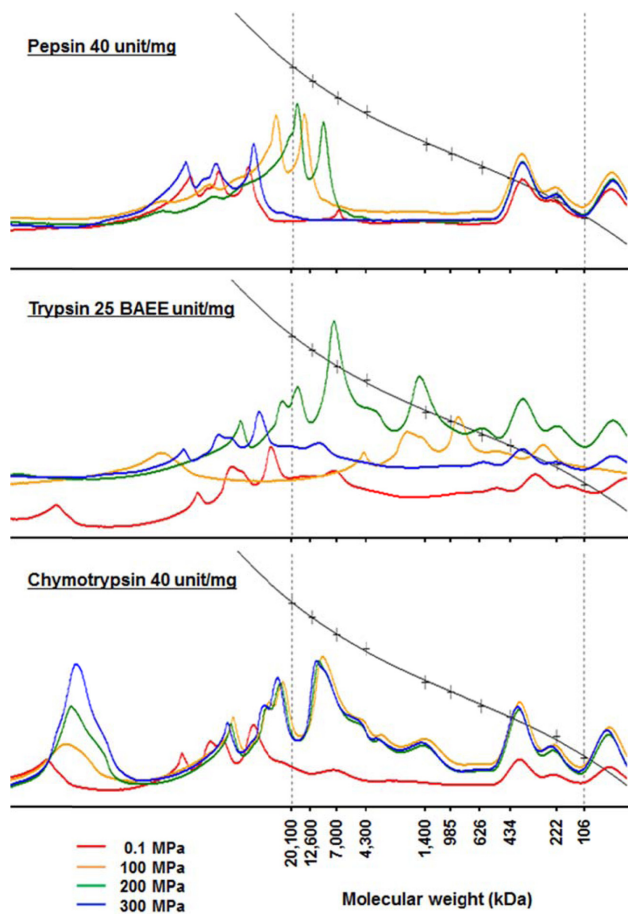
Fig. 2. Gel permeation chromatograph of porcine placenta treated with proteases and pressurized for 5 min after incubation at 4°C for 2 h.

or N-terminal of collagen which was composed of 2-3 amino acids (Lee *et al*., 2013). With increasing pressure levels, the low M_w group was not affected irrespective of pressure levels while the high M_w peaks were shifted toward lower M_w range, indicating that hydrolysis activity of pepsin was enhanced after pressurization. The shift of high M_w peaks was preceded when the enzyme/placenta mixture was pressurized at 200 MPa. In particular, all peptides belonging to high M_w group were observed within 20 kDa region, indicating the optimum pressure level for the maximum enhancement of peptic-digestion. The pressure-induced activity enhancement of pepsin was also reported when the substrate was soybean (Peñas *et al*., 2006) or milk proteins (Chicón *et al*., 2008). Alternately, further increase in pressure (300 MPa) resulted in higher M_w peaks than control (0.1 MPa). The result was not in agreement with Chicón *et al*. (2008) where the authors demonstrated that pepsin-catalyzed β-lactoglobulin hydrolysis was intense with increasing pressure levels up to 400 MPa. Different substrate pH would result in different structural

tolerance of pepsin over pressure.

The patterns in tryptic-digestion of placenta were also similar to those of pepsin. At atmospheric condition, hydrolysates of trypsin treatment had broad ranges of M_w peaks. With increasing pressure (100 MPa), all M_w peaks shifted greatly to lower M_w region, and the main peaks were detected at 1-5 kDa. At 200 MPa, the peaks were detected at lower M_w ranges comparing to those of control (0.1 MPa), however, the placental hydrolysis activity was lesser than that treated at 100 MPa. The decrease in tryptic-digestion activity was also shown when the hydrolyzing reactant was subjected at 300 MPa at which the M_w peaks were displayed at slightly higher ranges than those of control. Consequently, the optimum pressure level for tryptic-digestion was found at 100 MPa. Peñas *et al*. (2004) also noted similar results using soybean protein and concluded that pressure-induced aggregation of substrate diminished the hydrolysis activity of trypsin. In the current study, however, it was hardly expected that high pressure affected the placental collagen, because high pressure was reported not to attribute the collagen structure (Suzuki *et al*., 1993). It was recognized that transient pressure of trypsin between native and denatured states was 6.5 kbar (650 MPa), while it depending on the concentration of proteins, ratio of enzyme to substrate and temperature (Ruan *et al*., 1997). In the mixed conditions with substrate, there would be an interaction between hydrolysates and enzyme under high pressure and resulted less activity at higher than 200 MPa (Peñas *et al*., 2004).

The placental hydrolysates obtained by chymotrypsin showed some unique pattern under high pressure. Increasing pressure up to 100 MPa resulted better hydrolysis of placenta as shown in lower M_w peaks than those obtained by control. However, no peak changes were shown with increasing pressure (>200 MPa). The only attribute of chymotryptic digestion was an increase of the highet M_w peak dimension. Partial collagen peptides appeared to be fractionated and likely accumulated in the suspension.

Amino acid compositions

Comparing the amino acid compositions of raw placenta, all digested hydrolysates showed similar pattern of changes after enzymatic hydrolysis (Fig. 3). The enzymatic hydrolysis did not affect the contents of His and Met. Meanwhile significant increases in Gly, Ala, Lys, Hyp and Pro were observed with decreasing the other amino acid compositions ($p < 0.05$). The amino acids of placental hydrolysates were also in agreement with our previous study (Lee *et al*., 2013). For peptic and chymotryptic-digestions,
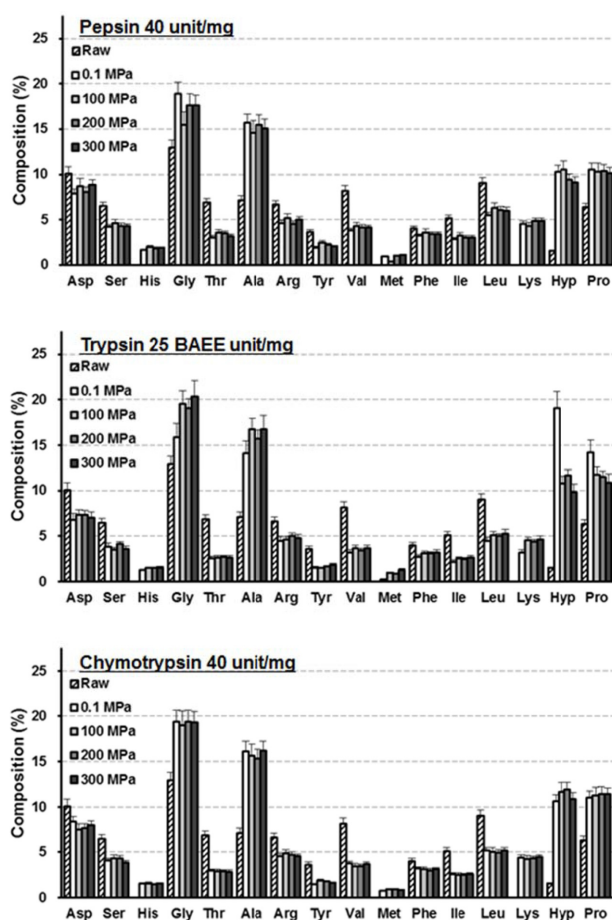
Fig. 3. Amino acid composition of porcine placenta treated with proteases and pressurized for 5 min after incubation at 4°C for 2 h. Vertical bars represent standard deviations (n=3).

changes in amino acid compositions with pressure level were not significant, though Gly in peptic-digested placenta hydrolysate was lowest at 100 MPa treatment ($p<0.05$). Meanwhile, tryptic-digested placenta hydrolysates showed clear changes in amino acid contents by pressurization. In particular, Gly and Ala contents of tryptic-digested hydrolystae increased at 100 MPa. The Ala content did not differ among pressure treatments while Gly content was further increased with increasing pressure up to 200 MPa ($p<0.05$) thereafter no differences in Gly contents were observed. In addition, Hyp and Pro contents decreased at 100 MPa ($p<0.05$) and tended to decrease with increasing pressure levels, reflecting pressure-dependent peptide clavage of trypsin. It is reported that trypsin cleaves the carboxyl residues of Lys or Arg. However, this enzyme does not attack the same residues when it is followed by Pro (Rodriguez *et al.*, 2008). Considering that the low M_w peptides of tryptic-digestion was found at 100 MPa treatment based on GPC, the relationship between

pressure levels and amino acid compositions of tryptic-digested placenta was not clear in the present study, which warranted further exploration.

Conclusion

This study demonstrated the enhancement of hydrolyzing activities of three selected proteases under high pressure. Trypsin revealed a potential protease to hydrolyze animal by-product collagen effectively. Although, it was obscure whether an interaction between enzyme and substrate was occurred under pressure or structural modification of the enzymes caused the enhancement of placental hydrolyzing activity, 100-200 MPa of pressurization was favorable to improve the proteolytic activity. Consequently, the results indicated that pressurization had a potential advantage in enzymatic-collagen hydrolysis.

Acknowledgements

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