Effect of Sub- and Super-critical Water Treatment on Physicochemical Properties of Porcine Skin

Yeon-Ji Jo, Jae-Hyeong Kim, Kyung-Hun Jung, Sang-Gi Min*, and Ji-Yeon Chun*
Department of Bioindustrial Technologies, Konkuk University, Seoul 143-701, Korea

Abstract

Super- and sub-critical water treatments have been of interest as novel methods for protein hydrolysis. In the present study, we studied the effect of sub-critical water (Sub-H₂O, 300°C, 80 bar) treatment as well as super-critical water (Super-H₂O, 400°C, 280 bar) treatment on the physicochemical properties of porcine skin (PS), which has abundant collagen. Porcine skin was subjected to pre-thermal treatment by immersion in water at 70°C, and then treated with sub- or super-critical water. Physicochemical properties of the hydrolysates, such as molecular weight distribution, free amino acid content, amino acid profile, pH, color, and water content were determined. For the molecular weight distribution analysis, 1 kDa hydrolyzed porcine skin (H-PS) was produced by Super-H₂O or Sub-H₂O treatment. The free amino acid content was 57.18 mM and 30.13 mM after Sub-H₂O and Super-H₂O treatment, respectively. Determination of amino acid profile revealed that the content of Gly (28%) and Pro (30%) was higher after Super-H₂O treatment than after Sub-H₂O treatment, whereas the content of Cys (28%) and Ala (13.1%) was higher after Sub-H₂O treatment. Super-H₂O or Sub-H₂O treatment affected the pH of PS, which changed from 7.29 (Raw) to 9.22 (after Sub-H₂O treatment) and 9.49 (after Super-H₂O treatment). Taken together, these results showed that Sub-H₂O treatment was slightly more effective for hydrolysis than Super-H₂O. However, both Sub-H₂O and Super-H₂O treatments were effective processing methods for hydrolysis of PS collagen in a short time and can be regarded as a green chemistry technology.

Key words: sub-critical water, super-critical water, porcine skin, collagen, hydrolysates

Introduction

Collagen has been used in medical and pharmaceutical industry, for cosmetic surgery, reconstructive surgery, wound healing, etc. Nowadays, collagen is used in many beauty products, functional cosmetic products and food products because of the trends in well-being and increase in ageing population. Collagen is found in the byproducts of animals and marine life forms, such as skins, bones, and tissues. Collagen is a protein made up of amino acids and contains essential amino acids like glycine, proline, hydroxyproline, and arginine (Cho et al., 2006; Chun et al., 2014; Jung et al., 2014; Lee et al., 2013). However, most byproducts of animals and marine life-forms have high-molecular-weight (about 300 kDa) collagen that cannot be absorbed by the human body and that cannot penetrate human skin; animal byproducts such as porcine skin are rich in collagen; however, this collagen is not suitable for use in food or cosmetic products (Cho et al., 2006). Low-molecular-weight peptides are of interest in the food and cosmetic industry because of their anti-osteoarthritis, anti-osteoporosis, anti-hypertension, anti-wrinkle, and anti-oxidant activity (Mosquera et al., 2014; Shigemura et al., 2011; Zhang et al., 2006). High-molecular-weight proteins are usually hydrolyzed by thermal treatment prior to hydrolysis by acidic or alkaline enzymatic treatment (using trypsin, pepsin, chymotrypsin, or papain enzyme) (Chun et al., 2014; Jung et al., 2014). Such conventional methods of hydrolysis require long processing times, and the hydrolysates obtained are not safe for direct consumption without purification.

Super-critical water (Super-H₂O) and sub-critical water (Sub-H₂O) exhibit unique properties above the critical point 374°C and 221 MPa (Alargov et al., 2002; Lee et al., 2013). Super-H₂O and Sub-H₂O have been used for hydrolysis, extraction, separation, oxidation, and gasification. The advantages of Super-H₂O and Sub-H₂O treat-
ments as green chemistry processes are the use of water as a solvent rather than hazardous substances and their short processing time (Lee et al., 2013). In contrast, conventional hydrolysis or extraction methods use enzyme or acid. The effect of high-pressure/high-temperature treatment on porcine placenta has been reported (Lee et al., 2013). Treatment at a particular pressure and temperature converted collagen to gelatin; the hydrolysis was partial or complete depending on the pressure and temperature. Our previous study showed that Sub-H$_2$O treatment could hydrolyze collagen within an hour (Chun et al., 2014; Lee et al., 2013).

In the present study, porcine skin was treated with Super-H$_2$O or Sub-H$_2$O and the hydrolyzing activity of Super-H$_2$O and Sub-H$_2$O was compared by determining the physicochemical properties of the PS hydrolysates, such as molecular weight distribution, free amino acid content, amino acid profile, pH, color, and water content.

**Materials and Methods**

**Pre-thermal treatment**

PS was purchased from a local butcher shop (Daeho Chooksan, Seoul). Fat and residual material in the PS were removed by immersion in water at 70°C, for 2 h. PS and DW (1:2 w/w) was prepared and the PS was then sliced (0.5 cm × 0.5 cm) and then blended with distilled water (DW) for 3 min using four-wing blade blender (CNHR-26, Bosch, Hong Kong). Finally, the blended PS was homogenized at high-speed (25,000 rpm) for 5 min by using Ultra Turrax® (T25, IKA Labotechnik, Germany).

**Super- or sub-critical water treatment**

A lab-scale super-critical fluid extraction system (SFE system, CS-1000) was collaboratively re-designed by the Laboratory of Food Engineering in Konkuk University and REXO Engineering (Korea). The system composed of a control box, vessel (250 mL, reactor), water bath, heater, temperature controller, and pressure controller (Fig. 1). A mixture of PS and DW (1:2 w/w) was prepared in the vessel. The vessel was heated to 300°C at 80 bar (for Sub-H$_2$O) or 400°C at 280 bar (for Super-H$_2$O) with shaking (the vessel was moved up and down during processing). After reaching the conditions required for Sub-H$_2$O or Super-H$_2$O, the vessel was directly cooled down to 40-45°C (pressure 0.1 bar) by placing in a water bath (4°C) with circulating coolant. The entire process was carried out to produce hydrolysates of porcine skin (H-PS) within 1 h.

**Gel permeation chromatography (GPC)**

The samples obtained after the Sub-H$_2$O or Super-H$_2$O treatment were centrifuged at 10,000 g for 5 min, and the molecular weight of peptides in the supernatant was determined by GPC based on a reported method (Gu et al., 2011). YL 9100 high performance liquid chromatog-
raphy (HPLC) system (Younglin Instrument Co. Ltd., Korea), equipped with three Ultrahydrogel™ 120 columns (7.8 × 30 mm, Waters, USA) was used for GPC analysis. The flow rate of mobile phase (deionized/distilled water) was 1 mL/min. The molecular weight (Mw) distribution of the peptides was monitored using YL 9100 refractive index detector (YL Instrument Co. Ltd., Korea) at 40°C and molecular weight standards kit (0.68–1,670 kDa, Polymer standards service, Germany) was applied as the standard peaks.

**Free amino acid content**

Free amino acid content was determined using the method of Benjakul and Morrissey (1997). The samples obtained after the Sub-H₂O or Super-H₂O treatment were centrifuged at 1,000 g for 15 min, and the supernatant was collected. Supernatant (125 mL) of the hydrolysates derived from PS (H-PS) was treated with 2 mL of sodium phosphate buffer (pH 8.2, 0.2152 M) and 1 mL of 0.01% (w/v) 2,4,6-trinitrobenzenesulfonic acid for 30 min at 50°C. The treated H-PS was cooled at ambient temperature and was further treated with 1 mL of 0.1 M sodium sulfite. Absorbance of the reactant (treated H-PS) was determined at 420 nm using a UV/VIS spectrophotometer. The free amino acid content was expressed in terms of L-leucine (Nagarajan et al., 2012).

**Amino acid profile and crude protein content**

The amino acid profile and crude protein content were determined by the Animal Resources Research Center in Konkuk University. Amino acid profiles (S4300 Amino Acid Reaction Module system & Komponenten analytischer Meßtechnik, Germany) and crude protein contents (Kjeltec 1035, Denmark) were determined by using the standard method released by the National Agricultural Products Quality Management Service (NAQS, Korea).

**Moisture content, pH, and color measurement**

The H-PS solution was filtered and the pH was determined using a pH meter (Model S220, Mettler Toledo GmbH, Switzerland). The moisture content of H-PS was determined by air drying at 102°C. The color of the H-PS solution was determined using a colorimeter (Minolta Chromameter CR-210, Japan) calibrated with a white standard (CIE L* = +97.83, CIE a* = -0.43, CIE b* = +1.96). The color measurement of H-PS was performed on the surface and measured five times. The color values, CIE L*, a*, and b* were determined as indicators of lightness, redness, and yellowness, respectively.

**Results and Discussion**

**Molecular weight distribution**

In order to select low-molecular weight H-PS, the PS was hydrolysed at various temperatures, 200°C, 250°C, 300°C, 350°C, and 400°C, and pressures 40, 40, 80, 80, and 280 bar, respectively. The average molecular weight distribution of H-PS after Sub-H₂O or Super-H₂O treatment is shown in Fig. 2. Each sample showed peaks near 6950 Da (200°C), 2800 Da (250°C), 500 Da (250°C and 300°C), and 222 Da (350°C and 400°C) (data not shown). The lowest molecular weight H-PS (222 Da) were located at 350°C and 400°C and the second lowest molecular weight (500 Da) H-PS were seen at 300°C and 250°C. At increasing temperatures, peaks for low-molecular-weight H-PS appeared and peaks for high-molecular-weight H-PS disappeared. The molecular weight distribution results show that the 1-kDa H-PS was produced by treatments over 250°C.

In the present study, Sub-H₂O or Super-H₂O treatments were completed in a short time (within 1 h), and low-molecular-weight H-PS was produced using green chemistry processing and without any chemical treatments by using acid, alkali, or enzyme. Cho et al. (2006) carried out hydrolysis of porcine skin using irradiation; gel permeation chromatography of the hydrolysates of porcine skin irradiated at 300 kGy showed major peaks at 9,000, 8,200, 860, and 170 Da. The lowest molecular weight hydrolysate (170 Da) of porcine skin had slightly lower molecular weight than the hydrolysate (220 Da) produced by our method. Radiation technology can also be used to produce oligopeptides from PS collagen; this processing method causes less environmental pollution and has simple processing steps. In the study by Lee et al. (2013), low-molecular-weight hydrolysates of porcine placenta (below 106 Da) were produced by treatment at 170°C.
37.5 MPa (60 min processing time). Their study also showed that production of small peptides derived from animal by-products is possible by using high temperature and high pressure without chemical treatment.

**Free amino acid content**

Protein decomposition was determined by measuring free amino acid content in the H-PS. Lee et al. (2013) have stated that it is difficult to find the extent of hydrolyzation of the porcine placenta by high-pressure/high-temperature processing, because the amounts of soluble gelatin and collagen hydrolysates cannot be quantified. In this study, the free amino acid content was expressed in terms of \( \text{L-leucine} \) (Table 1). However, it could not be determined in raw PS and thermally pre-treated PS (70°C) because of their tough texture. Free amino acid content was 57.18 mM and 30.13 mM in H-PS obtained by Sub-H\(_2\)O and Super-H\(_2\)O treatments, respectively. Sub-H\(_2\)O treatment was more effective in decomposing PS protein according these results.

Lee et al. (2013) showed that raw porcine placenta was hydrolyzed when treated at high temperature of 150°C, 170°C, and 200°C, for 0, 30, and 60 min respectively, at a pressure of 37.5 MPa or 100 MPa. They found that the main influencing factor for hydrolysis of porcine placenta was not pressure, but temperature. The optimum conditions of treatment were 170°C and 30 min. The hydrolysis of squid skin at various extraction temperatures (50-80°C) showed increase in the free amino acid content with increase in temperature (Nagarajan et al., 2012). Therefore, the two earlier studies and the present study shows that the free amino acid content (as a measure of protein decomposition) was mainly influenced by temperature increase. Alargov et al., (2002) also observed the effect of temperature (250-400°C) or pressure (15-40 MPa) on oligomerization and decomposition of glycine. The diglycine and diketopiperazine contents of the reaction mixtures were high at temperature of 350°C and low pressures of 15 and 20 MPa. In other words, decrease in pressure led to the formation of diglycine and diketopiperazine in high concentrations (3.51 and 8.47 mM at 15 and 20 MPa, respectively). Increasing the pressure to 25 MPa resulted in reduction in the extent of glycine decomposition. Therefore, from the results of various studies, it can be concluded that high pressure and high temperature alone cannot be correlated with the hydrolysis activity; and optimum treatment depending on the type of material should be selected to improve the hydrolysis activity.

The traditional methods to hydrolyze animal protein are acid, alkali, or enzyme treatment. Moreover, many researchers have recently combined high-pressure processing with the conventional methods (Chun et al., 2014; Dong et al., 2014; Jung et al., 2014). In future studies, it is necessary to combine Sub-H\(_2\)O and Super-H\(_2\)O treatment with conventional methods such as enzyme treatment for improving PS hydrolysis.

**Amino acid profiles**

The amino acid profile of PS treated with Sub-H\(_2\)O or Super-H\(_2\)O were determined (Fig. 3). The content of Glu (22.5%) and Pro (30%) was higher with Super-H\(_2\)O treatment than Sub-H\(_2\)O treatment whereas the content of Gly (28%) and Ala (13.1%) were higher with Sub-H\(_2\)O treatment. The contents of Glu and Pro with both the treatments were more than 20% of total amino acid content. The Arg content with Super-H\(_2\)O treatment was 15% of the total amino acid content. Contents of Asp, Thr, Ser, and Cys were not determined after Sub-H\(_2\)O and Super-H\(_2\)O treatment. In spite of different source materials, the amino acid profile of PS was similar to that reported by Lee et al. (2013) (Thermal processing of porcine placenta at high pressure) and Nagarajan et al. (2012) (Thermal processing of splendid squid). Composition of total amino acids showed that Gly content was the highest, followed by Ala, Pro and Glu; Cys peak was not observed. For the Gly function, it serves to release the energy required for

---

**Table 1. Effect of sub- and super-critical water treatment on free amino acid contents of hydrolyzed porcine skin**

| Treatment | Free amino acid (mM) |
|-----------|----------------------|
| Sub-H\(_2\)O | 57.18±0.69 |
| Super-H\(_2\)O | 30.13±0.70 |

---

**Fig. 3. Amino acid profile of porcine skin hydrolyzed by Sub-H\(_2\)O and Super-H\(_2\)O treatment.**
muscle function by breaking the glycogen and control protein self-organization into elastomeric or amyloid fibrils. Moreover, Gly helps the immune system, supports the non-essential amino acid synthesis, and reduces blood cholesterol (Rauscher et al., 2006).

Moisture content, crude protein, pH, and color

Table 2 shows the physical properties of PS treated by various treatments. Initial moisture content was approximately 59% and there was no significant difference in this value after the various treatments. The crude protein contents were 3.10% and 3.82% with Super-H$_2$O and Sub-H$_2$O treatments, respectively. Super-H$_2$O or Sub-H$_2$O treatments affected the pH of PS, which changed from 7.29 (Raw) to 9.22 (Sub-H$_2$O) and 9.49 (Super-H$_2$O).

There are two explanations for the pH change. First, H-PS was alkaline (pH>9.0), which indicates that self-ionization of water might have increased by Super-H$_2$O. Second, it is well known that self-ionization of water is increased under Super-H$_2$O conditions (300-400°C and 22.2-40 MPa). In that study, hydrolyzed glycine at 40 MPa showed a pH change from 6.4 to 8.0 by temperature increase. This suggested that when glycine decomposes at high temperature (e.g., melting point 233°C), the pH of the reaction mixture increases because of formation of methylamine and other amines (Alargov et al., 2002; Sato et al., 2002).

In order to evaluate how pre-thermal, Super-H$_2$O and Sub-H$_2$O treatments influence the color of the hydrolysates, the lightness, redness and yellowness of PS hydrolysates were measured. The color of the hydrolyzed porcine skin changed from pink to brown (image not shown). Lightness decreased by pre-thermal, Super-H$_2$O and Sub-H$_2$O treatments to 47.5, 33.6, and 33.7, respectively. Redness also showed a decreasing trend similar to lightness.

Although yellowness decreased slightly, the values for the hydrolyzed samples were not significantly different from those before treatment.

Conclusion

Traditionally, acid and alkali treatments have been used for the hydrolysis of animal protein or fish protein; however, these methods are time consuming and involve chemical processing, which could affect the properties of the source materials. The present study demonstrated the effect of sub- and super-critical water treatment on the physicochemical properties of PS. The overall results indicate that sub-critical water is slightly more effective for hydrolysis of PS than super-critical water. However, both sub- and super-critical water treatments are green chemistry processes and effectively hydrolyzed PS collagen to low-molecular-weight hydrolysate (less than 1 kDa) in a short time (within 1 h) without any chemical treatment. This hydrolysis by using sub- or super-critical water which is short time and safe processing without any chemical reaction may applied into other byproducts derived from other animal protein such as fish and beef.

Acknowledgements

Financial support for this study was obtained from the Korean Institute of Planning and Evaluation for Technology in Food, Agriculture, Forest, and Fisheries, Korea (iPET Project No. 311029-3). This paper was supported by the KU Research Professor Program of Konkuk University.

References

1. Alargov, D., Deguchi, S., Tsujii, K., and Horikoshi, K. (2002) Reaction behaviors of glycine under super- and subcritical water conditions. *Org Life Evol Biosph.* 32, 1-12.
2. Benjakul, S. and Morrissey, M. T. (1997) Protein hydrolysates from pacific whiting solid wastes. *J. Agric. Food Chem.* 45, 3423-3430.
3. Brunner, G. (2014) Supercritical fluid science and technology. Chapter 2. Properties of pure water. Brunner, G. (ed),
4. Brunner, G. (2009) Near critical and supercritical water. Part I. Hydrolytic and hydrothermal processes. *J. Supercritical Fluid.* 47, 373-381.

5. Cho, Y. J., Seo, J. E., Kim, Y. J., Lee, N. H., Hong, S. P., and Kim, Y. H. (2006) Study on the degradation of pig skin collagen using irradiation technique. *J. Korean. Soc. Food. Sci. Nutr.* 35, 588-593.

6. Chun, J. Y., Jo, Y. J., Min, S. G., and Hong, G. P. (2014) Effect of high pressure on the porcine placental hydrolyzing activity of pepsin, trypsin and chymotrypsin. *Korean J. Food Sci. An.* 34, 14-19.

7. Dong, X. B., Li, X., Zhang, C. H., Wang, J. Z., Tang, C. H., Sun, H. M., Jia, W., Li, Y., and Chen, L. L. (2014) Development of a novel method for hot-pressure extraction of protein from chicken bone and the effect of enzymatic hydrolysis on the extracts. *Food Chem.* 157, 339-346.

8. Gu, R. Z., Li, C. Y., Liu, W. Y., Yi, W. X., and Cai, M. Y. (2011) Angiotensin I-converting enzyme inhibitory activity of low-molecular-weight peptides from Atlantic salmon (*Salmo salar* L.) skin. *Food Res. Int.* 44, 1536-1540.

9. Jung K. H., Choi, Y. C., Chun, J. Y., Min, S. G., and Hong, G. P. H. (2014) Effects of concentration and reaction time of trypsin, pepsin, and chymotrypsin on the hydrolysis efficiency of porcine placenta. *Korean J. Food Sci. An.* 34, 151-157.

10. Lee, M. Y., Choi, Y. C., Chun, J. Y., Min, S. G., and Hong, G. P. (2013) Effects of high pressure/high temperature processing on the recovery and characteristics of porcine placenta hydrolysates. *Korean J. Food Sci. An.* 33, 474-480.

11. Mosquera, M., Giménez, B., da Silva, I. M., Boelter, J. F., Montero, P., Gómez-Guillén, M. C., and Brandelli, A. (2014) Nanoencapsulation of an active peptidic fraction from sea bream scales collagen. *Food Chem.* 156, 144-150.

12. Nagarajan, M., Benjakul, S., Prodpran, T., Songtipya, P., and Kishimura, H. (2012) Characteristics and functional properties of gelatin from splendid squid (*loligo formosana*) skin as affected by extraction temperatures. *Food Hydrocolloid.* 29, 389-397.

13. Penninger, J. M. L., Kersten, R. J. A., and Baur, H. C. L. (2000) Hydrolysis of diphenylether in supercritical water: Effects of dissolved nac. *J. Supercrit Fluids.* 17, 215-226.

14. Rauscher S., Baud S., Miao M., Keeley F. W., and Pomès R. (2006) Proline and glycine control protein self-organization into elastomeric or amyloid fibrils. *Structure* 14, 1667-1676.

15. Ravber, M., Knez, Ž., and Škerget, M. (2015) Simultaneous extraction of oil- and water-soluble phase from sunflower seeds with subcritical water. *Food Chem.* 166, 316-323.

16. Sato, N., Daimon, H., and Fujie, K. (2002) Decomposition of glycine in high temperature and high pressure water. *Kag. Kog. Ronbunshu.* 28, 113-117.

17. Shigemura, Y., Akaba, S., Kawashima, E., Park, E. Y., Nakamura, Y., and Sato, K. (2011) Identification of a novel food-derived collagen peptide, hydroxyproplyl-glycine, in human peripheral blood by pre-column derivatisation with phenyl isothiocyanate. *Food Chem.* 129, 1019-1024.

18. Watchararuji, K., Goto, M., Sasaki, M., and Shotiprunk, A. (2008) Value-added subcritical water hydrolysate from rice bran and soybean meal. *Bioresour. Technol.* 99, 6207-6213.

19. Zhang, Z., Li, G., and Shi, B. (2006) Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. *J. Society Leather Technol. Chem.* 90, 23-28.

(Received 2014.9.24/Revised 2014.10.17/Accepted 2014.10.23)