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ARTICLE

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

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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-H_2O, 300ºC, 80 bar) treatment as well as super-critical water (Super-H_2O, 400ºC, 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 70ºC, 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-H_2O or Sub-H_2O treatment. The free amino acid content was 57.18 mM and 30.13 mM after Sub-H_2O and Super-H_2O treatment, respectively. Determination of amino acid profile revealed that the content of Glu (22.5%) and Pro (30%) was higher after Super-H_2O treatment than after Sub-H_2O treatment, whereas the content of Gly (28%) and Ala (13.1%) was higher after Sub-H_2O treatment. Super-H_2O or Sub-H_2O treatment affected the pH of PS, which changed from 7.29 (Raw) to 9.22 (after Sub-H_2O treatment) and 9.49 (after Super-H_2O treatment). Taken together, these results showed that Sub-H_2O treatment was slightly more effective for hydrolysis than Super-H_2O was. However, both Sub-H_2O and Super-H_2O 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, hydroxylproline, 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-H_2O) and sub-critical water (Sub-H_2O) exhibit unique properties above the critical point 374°C and 221 MPa (Alargov *et al*., 2002; Lee *et al*., 2013). Super-H_2O and Sub-H_2O have been used for hydrolysis, extraction, separation, oxidation, and gasification. The advantages of Super-H_2O and Sub-H_2O treat-

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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_2O 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_2O or Sub-H_2O and the hydrolyzing activity of Super-H_2O and Sub-H_2O 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_2O) or 400°C at 280 bar (for Super-H_2O) with shaking (the vessel was moved up and down during processing). After reaching the conditions required for Sub-H_2O or Super-H_2O, 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_2O or Super-H_2O 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-

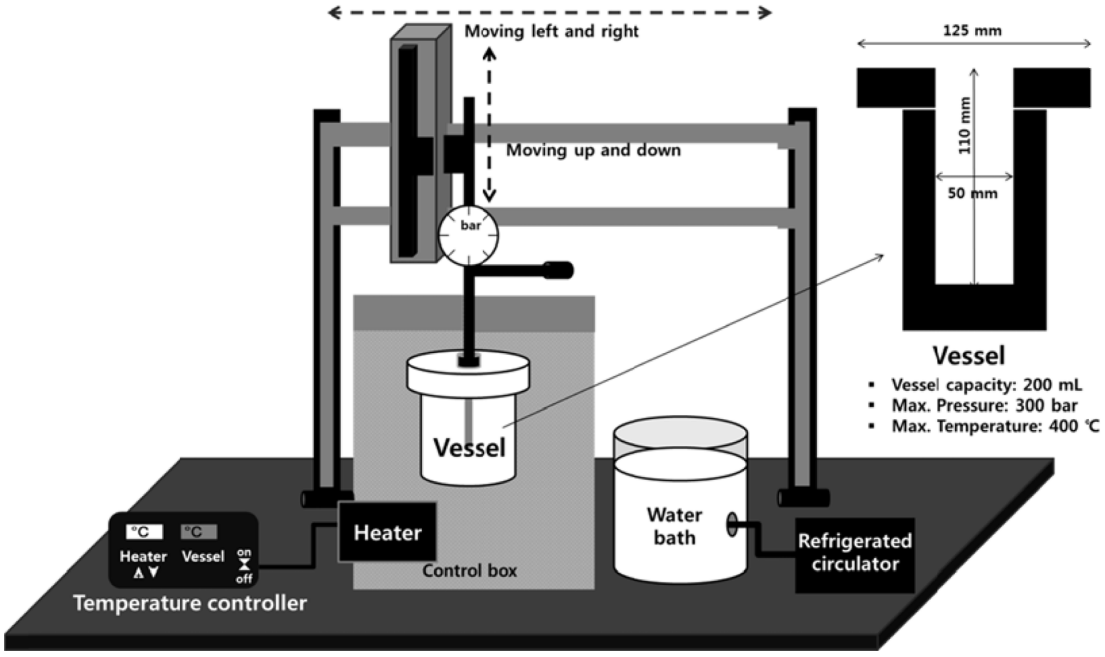

Fig. 1. Schematic diagram of Super-H_2O or Sub-H_2O system.

raphy (HPLC) system (Younglin Instrument Co. Ltd., Korea), equipped with three Ultrahydrogel™ 120 columns (7.8 × 300 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-H_2O or Super-H_2O 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-H_2O or Super-H_2O 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-H_2O or Super-H_2O 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,

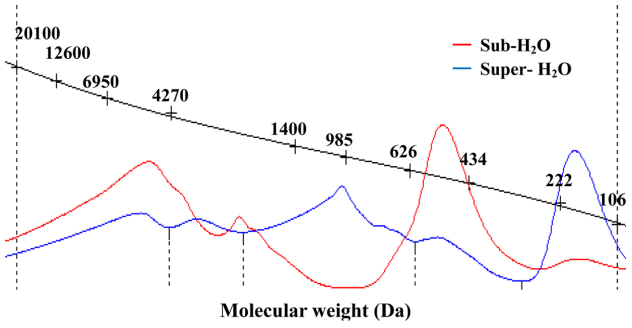

Fig. 2. Gel permeation chromatograph of porcine skin treated with Sub-H_2O (red line) or Super-H_2O (blue line).

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 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_2O and Super-H_2O treatments, respectively. Sub-H_2O 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 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.

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_2O	57.18±0.69
Super-H_2O	30.13±0.70

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_2O and Super-H_2O 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_2O or Super-H_2O were determined (Fig. 3). The content of Glu (22.5%) and Pro (30%) was higher with Super-H_2O treatment than Sub-H_2O treatment whereas the content of Gly (28%) and Ala (13.1%) were higher with Sub-H_2O 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_2O treatment was 15% of the total amino acid content. Contents of Asp, Thr, Ser, and Cys were not determined after Sub-H_2O and Super-H_2O 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

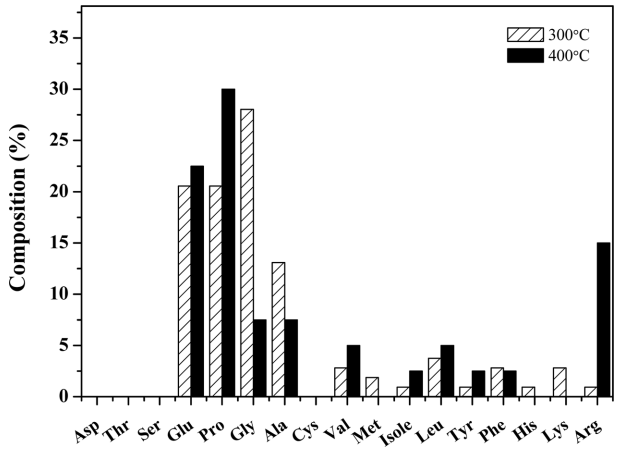

Fig. 3. Amino acid profile of porcine skin hydrolyzed by Sub-H_2O and Super-H_2O treatment.

Table 2. Physicochemical properties of porcine skin before and after various treatments

Treatment	Moisture content	pH	*L**-value	*a**-value	*b**-value
Raw (10°C)	59.22±1.89	7.29±0.01	69.5±1.28	6.63±0.82	12.4±1.58
Pre-thermal treatment (70°C)	60.97±6.67	7.98±0.00	47.5±1.77	3.7±0.54	13.7±1.01
Sub-H_2O (300°C)	57.46±3.89	9.22±0.01	33.6±0.05	0.59±0.06	11.4±0.06
Super-H_2O (400°C)	59.23±0.84	9.49±0.01	33.7±0.12	1.47±0.02	10.8±0.11

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_2O and Sub-H_2O treatments, respectively. Super-H_2O or Sub-H_2O treatments affected the pH of PS, which changed from 7.29 (Raw) to 9.22 (Sub-H_2O) and 9.49 (Super-H_2O).

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_2O or Sub-H_2O treatments. If $[H_3O^+]$ is higher than $[OH^-]$ then pH is below 7 and if $[H_3O^+]$ is lower than $[OH^-]$, then the pH is above 7 in pure water (Brunner, 2009, 2014; Penninger *et al*., 2000; Ravber *et al*., 2015; Watchararuji *et al*., 2008). In this study, $[OH^-]$ increased with temperature and pressure, and this caused self-ionization of water. The second explanation is consistent with the reasoning of Alargov *et al*. (2002), who observed the reaction behavior of glycine under Super- and Sub-H_2O 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 increae. 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_2O and Sub-H_2O 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_2O and Sub-H_2O 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.

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