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# Characterization of soy protein hydrolysates produced by varying subcritical water processing temperature

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ABSTRACT

The objective of this study was to investigate the effect of subcritical water processing (SWP) temperature on the hydrolysis pattern and quality characteristics of soybean protein hydrolysates. Two common cultivars of soybean were subjected to SWP at temperature ranging from 150 to 250 °C and pressure of 22 MPa. The highest free amino group content and yield were obtained at a processing temperature of 190 °C for both the cultivars ($p < 0.05$). Gel electrophoresis analysis exhibited low molecular mass hydrolysates at this processing temperature. Increasing processing temperature generated lower molecular mass peptides, as revealed by gel permeable chromatography. The color parameters L*, a* and b* were also highly influenced by the elevated processing temperatures, particularly at 190 °C. Increasing the processing temperatures of soybean hydrolysates also affected the viability of murine macrophage. The optimization of processing temperature could produce peptide fractions for food applications.

## 1. Introduction

Soybean (*Glycine* max *Merrill*) has been a major crop for human consumption due to its nutritional and physiological functions. Owing to soybean's rich protein content of approximately 40% (Belitz, Grosch, & Schieberle, 2009), this crop is a major source for dietary protein in several countries. Soy protein is recognized as a superior protein derived from plant resources, as it contains most essential amino acids, although it possesses less sulfur-containing amino acids (cysteine, methionine and tryptophan) compared to animal proteins (Belitz et al., 2009). Consequently, soy proteins are extensively used in various processed food formulations (Seo, Seo, & Yang, 2016).

Recently, protein hydrolysates have attracted considerable attention due to their physiological activities, including antihypertension, anti-obesity and hypocholesterolemic activities (Park & Nam, 2015; Wang & De Mejia, 2005). These bioactive peptides are normally composed of 3 to 20 amino acid residues, and their physiological activities are dependent on the composition and sequence of amino acids (Pihlanto-Leppälä, 2001). Furthermore, proteins and peptides with high molecular weight (Mw) are known to cause allergies and lack suitability for inclusion in special diets (Tello, Camacho, Jurado, Paez, & Guadix, 1994). A common method to produce physiologically active peptides has been enzymatic hydrolysis. However, the usage of proteases is associated with challenges such as lack of cost effectiveness and undesirable bitterness (Sarmadi & Ismail, 2010). Meanwhile, the chemical hydrolysis method (using HCl) is a simple and effective way to produce low Mw peptides, but it results in the generation of toxic chloride compounds such as 3-monochloropropane-1,2-diol and 1,3-dichloropropan-2-ol (Arisseto, Vicente, Furlani, & Toledo, 2013).

An alternative processing tool is the subcritical water process (SWP), which involves the hydrolysis of organic compounds, such as proteins and carbohydrates. With the exclusion of costly acids and enzymes and the use of benign solvent, SWP has been mostly employed in the hydrolysis of protein to generate amino acids and peptides (Powell, Bowra, & Cooper, 2016). Water has a critical point of 22.1 MPa and 374 °C, and SWP is conducted below the critical point of water (Brunner, 2009). Since water is used as a reactive medium, this process is recognized as a promising eco-friendly hydrolysis technology. Apart from being eco-friendly and energy efficient, this efficient process is not only less complicated but also has the potential to be scaled up. Due to the higher efficiency, SWP can be suitably scaled up for industrial application. By replacing larger extraction cells with lab-scale cell, it was shown that the extraction efficiency could be increased 30-fold and 167-fold (Islam, Jo, Jung, & Park, 2013).

Various researchers have attempted to investigate the effect of SWP on the hydrolysis efficiency of fish muscle (Yoshida, Terashima, & Takahashi, 1999), rice bran (Sereewatthanawut et al., 2008) and pork collagen (Jo, Kim, Jung, Min, & Chun, 2015; Lee, Choi,

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Chun, Min, & Hong, 2013). Although SWP can generate value added products and be scaled-up, studies pertaining to SWP of soy protein hydrolysates are limited. Watchararuji, Goto, Sasaki, and Shotipruk (2008) reported that hydrolyzed soybean meal using SWP (200–220 °C for 10–30 min) possessed antioxidant activities. Despite the broad range of applications of soy protein hydrolysates, a detailed relationship between the hydrolysis pattern of soy protein and processing temperature in SWP is not clearly understood. Apart from determination of Mw and free amino group content, investigations on color and pH could be beneficial in the development of novel products. These properties greatly impact on appearance and texture of food products and in turn influence its consumer acceptance. Cytotoxicity investigations could be used initially to extrapolate doses required for in-vivo animal models or clinical studies and finally in product development. Therefore, this study explores the effect of temperature (150–250 °C) in SWP on the protein hydrolysis pattern and quality characteristics of soybean hydrolysates.

## 2. Materials and methods

### 2.1. Materials

Two domestic soybean cultivars, Daewonkong (DWK; normal cultivar with crude protein content of 39.4%) and Saedanbaek (SDB; high protein cultivar with crude protein content of 48.7%), were kindly donated by the National Institute of Crop Science, Rural Development Administration (Miryang, Korea). The soybeans were finely ground by passing ten times through a commercial milling machine (S99-23, Shin Woo General Machinery Co., Incheon, Korea). The soybean powder was vacuum-packaged and kept at 4 °C prior to use (within 5 days). All chemicals used in this study were of analytical grade.

### 2.2. SWP treatment

The soybean powder from each cultivar (125 g) was individually suspended into 1.7 L of distilled water, and the final volume was adjusted to 2 L. The suspensions were gently stirred in a 4 °C refrigerator prior to SWP treatment. The SWP was conducted using a lab-assembled supercritical water processor as described in our previous study (Lee et al., 2013). In brief, the device consisted of a reactor with a working volume of 300 mL, a cooling system and a pressure regulator. An aliquot of suspension (240 mL) was transferred to the reactor and enclosed tightly. The reactor was subsequently heated at 6 °C/min using an ohmic heater. To avoid localized overheating, the sample in the reactor was vertically agitated by the heater through mechanical movement. Pressure inside the reactor by nitrogen gas injection was adjusted to 22 MPa throughout the entire processing. Once the reactor (inside temperature) reached the target temperature (150–250 °C), the reactor was immediately cooled to 40 °C in a 4 °C water without holding. During cooling, when the reactor temperature reached 40 °C (~30 min) the reactor was opened to collect the sample suspension into a glass bottle. A small portion of the sample (1 mL) was taken for gel electrophoresis, and the remaining suspension was centrifuged at 5000 × *g* for 10 min at ambient temperature. The suspension was allowed to phase separate to form cream (lipids), skim (soluble fraction) and precipitates (insoluble fraction). The skimmed layer was collected and separated into two groups. One group was transferred to a wide plastic dish and frozen at −50 °C overnight. The following day, the frozen samples were lyophilized for cell toxicity evaluation. The other group was kept at ambient temperature (~18 °C) prior to analyses (within 2 h).

### 2.3. Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the suspension was conducted based on the method of Laemmli (1970) using an EzCell Electrophoresis system (Komabiotech Inc., Seoul, Korea). Protein concentration of the suspension was adjusted to 4.0 mg/mL by the addition of distilled water and mixing with the same volume of sample buffer (Komabiotech Inc., Seoul, Korea) consisting of 126 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.005% bromophenol blue and 10% 2-merchaptoethanol. The mixture was then heated at 85 °C for 2 min and centrifuged at 10,000 × *g* for 2 min to remove any precipitates. Peptide separation was conducted using a 12% acrylamide gel under a constant voltage of 140 V (~1 h).

### 2.4. Color and pH

Aliquots of 40 mL (suspension) were transferred to three transparent plastic cylinders and placed on the head of a color reader (SC80, SADT Ltd., Beijing, China) that was calibrated with white and black standard plates. Colorimetric parameters, CIE L* (lightness), a* (redness) and b* (yellowness), of the samples were recorded. pH of the suspension in each cylinder was determined using a pH meter (pH 900, Precisa Co., Dietikon, Switzerland).

### 2.5. Yield and free amino group content

Suspensions of each treatment were again centrifuged at 10,000 × *g* for 15 min at 20 °C. The supernatant was filtered through a 0.45 μm syringe filter (MIC-13CP, Advantec Co., Tokyo, Japan), and protein concentrations of the filtrates were measured in duplicates by the Kjeldahl (%N × 6.25) method. Yield was calculated as percent soluble nitrogen in the supernatant over total nitrogen in the raw suspension.

Measurement of free amino group content was performed in duplicate as described by Benjakul and Morrissey (1997). The filtrate (125 μL) was mixed with 2 mL (0.2125 M) sodium phosphate buffer (pH 8.2) and 1 mL of 0.01% 2,4,6-trinitrobenzenesulfonic acid. The mixture was incubated in a water bath at 50 °C for 30 min and the chemical reaction was terminated by the addition of 2 mL of (0.1 M) sodium sulfide. Next, the mixture was kept at ambient temperature for 15 min, and the absorbance at 412 nm was measured. The free amino group content was calculated using L-leucine (131.17 g/mol) as a standard (Nagarajan, Benjakul, Prodpran, Songtipya, & Kishimura, 2012).

### 2.6. Molecular weight distribution

Following SWP, the solubilized portion of centrifuged samples were filtered prior to analysis. The Mw distribution of soybean hydrolysates in the filtrates was determined as described by Gu, Li, Liu, Yi, and Cai (2011) with minor modification (Lee et al., 2013). Gel permeable chromatography (GPC) was applied using a high-performance liquid chromatography (HPLC) system (YL 9100, Younglin Instrument Co. Ltd., Seoul, Korea) equipped with an Ultrahydrogel™ 120 column (7.8 × 3000 mm, Waters, Milford, MA, USA). Distilled pure water (HPLC grade, Samchun Chemical Co., Seoul, Korea) was used for the mobile phase, and the flow rate was adjusted to 1 mL/min. The Mw distribution was monitored using a refractive index detector (YL 9100, YL Instrument Co. Ltd., Seoul, Korea) equilibrated at 40 °C, and a Mw standard kit (680–1,670,000 Da, Polymer standards service, Mainz, Germany) was used as the standard.

### 2.7. Cell viability

Murine macrophage (ATCC® TIB-71™, American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's mediums (DMEM; LM 001-11, WelGene Inc., Daegu, Korea) with 10% fetal bovine serum (FBS; S001-07, WelGene Inc., Daegu, Korea). The cells ($4 \times 10^4$/well) were incubated for 24 h in a 96-well plate under 95% air with 5% $CO_2$ at 37 °C and starved in DMEM with 1% FBS overnight. The MTT assay was conducted based on the method of Twentyman and Luscombe (1987). The samples were treated with 3-

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