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Volatile Compound, Physicochemical, and Antioxidant Properties of Beany Flavor-Removed Soy Protein Isolate Hydrolyzates Obtained from Combined High Temperature Pre-Treatment and Enzymatic Hydrolysis

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ABSTRACT: The present study investigated the volatile compound, physicochemical, and antioxidant properties of beany flavor-removed soy protein isolate (SPI) hydrolyzates produced by combined high temperature pre-treatment and enzymatic hydrolysis. Without remarkable changes in amino acid composition, reductions of residual lipoxygenase activity and beany flavor-causing volatile compounds such as hexanol, hexanal, and pentanol in SPI were observed after combined heating and enzymatic treatments. The degree of hydrolysis, emulsion capacity and stability, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, and superoxide radical scavenging activity of SPI were significantly increased, but the magnitudes of apparent viscosity, consistency index, and dynamic moduli (G', G'') of SPI were significantly decreased after the combined heating and enzymatic treatments. Based on these results, it was suggested that the enzymatic hydrolysis in combination with high temperature pre-treatment may allow for the production of beany flavor-removed SPI hydrolyzates with superior emulsifying and antioxidant functionalities.

Keywords: soy protein isolate, beany flavor, hydrolyzates

INTRODUCTION

Soy protein has been widely used as a functional ingredient in many different food products, because it is nutritious and has excellent functional properties. Soy protein isolates (SPI), soy protein concentrates, and soy protein flours are 3 types of widely used soy protein products (1-3). Among them, SPI is frequently preferred due to the higher protein content (about 90%). It is known that SPI provides a good balance in amino acid composition, since all the essential amino acids are present, and it contains physiologically beneficial components which are known to lower cholesterol and reduce the risk of hyperlipidemia and cardiovascular diseases (4,5). Furthermore, it has excellent functional properties such as gelling, emulsifying ability, and water- and oil-holding capacity (6,7).

One of the continuing obstacles to the acceptance of soy protein in the food industry is its beany flavor due to lipoxygenase-catalysed oxidation of unsaturated fatty acids, subsequently leading to the development of off-flavors and color degradation (8,9). Several studies have investigated the removal of the beany flavor by the inactivation of lipoxygenase present in soy foods using heat treatment, including steaming, roasting, and baking (8-11). According to Kong et al. (7), heat treatment effectively decreased the lipoxygenase activity in SPI. According to Kudre and Benjakul (11), the reduction of beany flavor in Barbara groundnut (*Vigna subterranean*) flour, an indigenous legume in the southern part of Thailand, was positively related to the decrease in the lipoxygenase activity.

Enzymatic hydrolysis has been widely used to enhance the functionalities of various proteins. The physicochemical properties of protein hydrolyzates generally rely on the degree of hydrolysis and types of protease employed (7,12,13). In particular, few researches noted that enzymatic hydrolysis of globular proteins may enhance their emulsifying properties, by improving overall protein solubility, exposing hidden hydrophobic residues, and reducing molecular size (2). In addition, protein hydrolysates have been shown to possess improved physiological ac-

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tivities such as antioxidant and antihypertensive activities, and hypocholesterolemic effects as compared to the intact protein (14-16).

Previous studies reported that soy proteins are not very accessible to enzymatic hydrolysis. A great deal of hydrolysis sites are shielded and difficult to be accessed by proteases, due to the compact quaternary and tertiary structures in soy protein (17,18). Heat treatment is frequently used in the food industry to modify the structures of proteins; thus, facilitating the enzymatic hydrolysis of soy proteins. Therefore, it was assumed in the present study that combined high temperature pre-treatment and enzymatic hydrolysis could be very efficient to improve the accessibility of soy proteins by protease and to enhance physicochemical properties and physiological activities.

In the present study, combined high temperature pretreatment and enzymatic hydrolysis was employed to remove beany flavor from SPI and produce beany flavor-removed SPI hydrolyzates. Firstly, the high temperature pre-treatment was used to inactivate beany flavor-producing enzymes in SPI and to facilitate the enzymatic hydrolysis of SPI. Secondly, the degree of hydrolysis, lipoxygenase activity, volatile compounds, sensory properties, amino acid composition, emulsifying activity, antioxidant activity, and rheological properties of beany flavor-removed SPI hydrolyzates were investigated.

MATERIALS AND METHODS

Materials

SPI was purchased from Vegemom Co. (Seoul, Korea). SPI had 92% protein, 3.2% carbohydrate, 0.8% lipid, and 4% ash, on a dry basis. Protamex from *Bacillus amyloliquefaciens* and *Bacillus licheniformis* (1.5 AU-NH/g) was obtained from Novozymes (Bagsværd, Denmark). Standards for amino acids (glycine, alanine, valine, isoleucine, leucine, aspartic acid, glutamic acid, serine, threonine, cysteine, methionine, lysine, arginine, histidine, phenylalanine, tyrosine, and proline) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of reagent grade unless otherwise specified.

Combined temperature pre-treatment and enzymatic hydrolysis

To produce beany flavor removed-SPI hydrolysates, combined high temperature pre-treatment and enzymatic hydrolysis were used. SPI (200 g) was transferred to borosilicate glass beakers (600 mL) and sealed with vinyl wrap and aluminum foil. The sample was transferred to an autoclave (LAB-2AC5100S, LabTech, Daegu, Korea) when the autoclave temperature was 30°C, and the temperature was increased to 121°C at heating rate of 17°C/min. After heating, the temperature was held for 3 min at 121 °C, cooled for 2 h at room temperature, and dried in a drying oven (DMC-122SP, Daeil Engineering Co., Seoul, Korea) at 50°C for 2 h to produce the beany flavor-removed SPI.

After the high temperature pre-treatment, the beany flavor-removed SPI was suspended in distilled water (10%, w/v) and homogenised at a speed of 10,000 rpm for 1 min using a homogeniser (T-10 basic, IKA Werke GmbH & Co. KG, Staufen, Germany). The homogenates were hydrolysed by Protamex (100 mg) in a shaking water bath (VS-1205SW1, Vision Scientific, Bucheon, Korea) under optimal enzyme conditions (pH 6.0 and 45°C) for different hydrolysis time of 0, 10, 20, 30, and 40 min. Once the desired hydrolysis time reached, the solution was heated at 95°C for 10 min to inactivate the protease and was then cooled in ice water. The hydrolyzates were centrifuged at 3,000 *g* at 25°C for 30 min (VS-12SMT, Vision Scientific) to separate insoluble and soluble fractions. Finally, the supernatants were freeze-dried to produce beany flavor-removed SPI hydrolyzates. The samples were stored at −20°C until needed.

Degree of hydrolysis

The degree of hydrolysis for the control (no treatment), beany flavor-removed SPI (only hot temperature pretreatment without enzymatic hydrolysis), and beany flavor-removed SPI hydrolyzates (combined hot temperature pre-treatment and enzymatic hydrolysis using different hydrolysis time) was evaluated according to the procedure of Sathivel et al. (19) with slight modifications. The degree of hydrolysis (%) was defined as the percentage of soluble protein in trichloroacetic acid (TCA). A sample aliquot (10 mL) was mixed with 10 mL of 20% TCA to obtain 10% TCA-soluble nitrogen and the mixture was centrifuged at 5,000 *g* for 15 min at 25°C. The soluble nitrogen in the supernatant and the total nitrogen were determined using the AACC International Approved Methods 46-13 (20). The degree of hydrolysis was calculated using the following equation:

$$\text{Degree of hydrolysis (\%)} = \frac{\text{10\% TCA soluble nitrogen in the sample}}{\text{Total nitrogen in the sample}} \times 100$$

Lipoxygenase activity

The lipoxygenase activity in all the samples was measured by the procedure of Kong et al. (7).

Sample preparation for identifying volatile compounds

The extract samples were prepared by the high volume headspace vial method. In brief, 2 g of each sample was taken in a 250 mL amber vial (Supelco Inc., Bellefonte, PA, USA). The vial was capped with a polytetrafluoroeth-

ylene septum (QMX Laboratories Ltd., Great Dunmow, Essex, UK) and placed in an oven (50°C for 10 min) to volatilize compounds. The sample was equilibrated for 1 h at the required temperature and the headspace gases were adsorbed onto a triple-bed adsorption tube using a vacuum pump and mass flow controller. The triple-bed adsorbent tube consisted of a three-bed packed Tenax-TA (Supleco Inc.) with a small amount of Carbopack B and Carbosieve S-III absorbent (Supleco Inc.). The compounds trapped in the tube were desorbed by a thermal desorber and injected into a gas chromatography-mass spectrometry (GC-MS) instrument.

Thermal desorber and GC-MS analysis

Volatile compounds in each sample were investigated by the method of US EPA TO-17 (21) using an automatics thermal desorber (ATD 400, PerkinElmer, Boston, MA, USA) integrated with a GC-MS (QP 5050A, Shimadzu Co., Kyoto, Japan) equipped with an AT-1 column (60 m×0.32 mm×1.0 μm; W. R. Grace & Co., Deerfield, IL, USA). The operating conditions of GC-MS allowed detection of highly volatile and low molecular weight compounds at a mass range of 20∼350 m/z using helium carrier gas with less than 1 ppm of impurities. Tentative identifications were performed based on comparison of the experimental mass spectrum with entries in the Wiley 221 mass spectral library (John Wiley & Sons, Inc., Hoboken, NJ, USA). The main volatile compounds (*n*-hexanal, *n*-pentanol, and *n*-hexanol) were compared by peak areas.

Sensory evaluation

Sensory characteristics of each sample were studied using a quantitative descriptive analysis carried out by a panel of 10 selected and trained assessors. Panel was consisted of experienced 10 panelists (5 females and 5 males) from the academic staff working in the Department of Food and Nutrition, Kyung Hee University. Panel evaluated each sample in terms of beany flavor, bitterness, solubility, and yellowness. The panel was trained 1 h before each evaluation session. Some sensory terms for taste, aroma and texture of the samples were introduced to panelists. Samples presented in 3 digit-coded glass plates were served with a glass of water and an unsalted cracker to the panelists. All samples were presented at the same time in each session. Sensory evaluation of samples in each analysis was carried out two times.

Amino acid composition

To evaluate the amino acid composition, each sample was applied into hydrolysis tube and hydrolysed with 6 M HCl at 110°C for 24 h. The hydrolyzed sample was dried, dissolved in 0.02 N HCl, and centrifuged at 3,000 *g* for 15 min. The amino acid composition was then obtained by automatic analysis algorithm of the amino acid automatic analyzer. The amino acid analyzer (HITACHI L-8900, Hitachi High-Technologies Corporation, Tokyo, Japan) attached HITACHI high performance liquid chromatography packed column with ion exchange resin No. 2622 PF (4.6 mm×60 mm; Hitachi High-Technologies Corporation) and UV detector (VIS1: 570 nm, VIS2: 440 nm) was used for analysis of amino acids. MCI Buffer L-8900 PFS-1, -2, -3, -4, and -RG (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used in this study. About 20 μL of each sample was injected. Amino acid determination was performed using ninhydrin reagent set (Wako Pure Chemical Industries, Ltd.).

Emulsion capacity and stability

The emulsion capacity and emulsion stability of the control, beany flavor-removed SPI, and beany flavor-removed SPI hydrolysates were investigated according to Yasumatsu et al. (22) with some modifications. The sample (0.01 g) was suspended in 10 mL of distilled water before mixing with 10 mL of vegetable oil. The water/oil mixture was emulsified using a homogenizer at 10,000 rpm for 1 min, and then the mixture was centrifuged at 1,300 *g* for 5 min. Emulsion capacity (%) calculated as follows:

$$\text{Emulsion capacity (\%)} = \frac{\text{Height of emulsion layer}}{\text{Total height of fluid}} \times 100$$

Emulsion stability was determined by heating the emulsion at 80°C for 30 min, cooling with tap water for 15 min, and centrifuging at 1,300 *g* for 5 min. Emulsion stability (%) was calculated as follows:

$$\text{Emulsion stability (\%)} = \frac{\text{Height of remaining emulsion layer}}{\text{Total height of fluid}} \times 100$$

DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of all the samples was investigated by the modified method of Zhu et al. (23). A series of solutions with different concentrations (0∼10 mg/mL) of samples was previously prepared for determining the DPPH radical scavenging activity. Each sample (1.0 mL) was added to 6.0 mL of DPPH solution (0.2 mM/L in 99% ethanol). The solution was then mixed, kept at room temperature in the dark for 30 min, and centrifuged for 10 min at 1,000 *g*. The supernatant was used for investigating DPPH radical scavenging activity. The reduction of DPPH free radicals was determined by measuring the absorbance at 517 nm with a UV-Vis spectrophotometer (UVmini-1240, Shimadzu Co.). A low absorbance of the reaction mixture indicates a high free radical scavenging

activity. The percentage of DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = 1 - \frac{A_{517sample} - A_{517control}}{A_{517blank}} \times 100$$

where $A_{517sample}$ is the value of 1.0 mL of sample solution mixed with 6.0 mL of DPPH solution, the $A_{517control}$ is the value of 1.0 mL of sample solution mixed with 6.0 mL of 99% ethanol, and the $A_{517blank}$ is the value of 1.0 mL of 99% ethanol mixed with 6.0 mL of DPPH solution.

Superoxide radical scavenging activity

The superoxide radical scavenging activity of all the samples was determined by the method of Marklund and Marklund (24) with slight modifications. Briefly, 0.2 mL solution of each sample at different concentrations (0~10 mg/mL) was mixed with 3 mL of 50 mM Tris-HCl buffer (pH 8.5) and 0.2 mL of 7.2 mM pyrogallol solution. The reacting mixture was shaken, kept at room temperature for 10 min, and centrifuged at 1,000 *g* for 10 min. The supernatant was used for investigating superoxide radical scavenging activity. The absorbance was measured at 420 nm using a UV-Vis spectrophotometer (UVmini-1240, Shimadzu Co.). The superoxide radical scavenging activity was determined as the percentage of inhibiting pyrogallol autoxidation, which was calculated from absorbance in the presence or absence of pyrogallol and samples.

$$\text{Superoxide radical scavenging activity (\%)} = 1 - \frac{A_{420sample} - A_{420control}}{A_{420blank}} \times 100$$

where $A_{420sample}$ is the value of 0.2 mL sample solution mixed with 3 mL of 50 mM Tris-HCl buffer (pH 8.5) and 0.2 mL of 7.2 mM pyrogallol solution, the $A_{420control}$ is the value of 0.2 mL sample solution mixed with 3.2 mL of 50 mM Tris-HCl buffer (pH 8.5), and the $A_{420blank}$ is the value of 0.2 mL distilled water mixed with 3 mL of 50 mM Tris-HCl buffer (pH 8.5) and 0.2 mL of 7.2 mM pyrogallol solution.

Steady and dynamic shear rheological properties

To measure the steady and dynamic shear rheological properties, 2 g of each sample were mixed with 10 g of 0.1 M phosphate buffered saline (pH 7.0, 1% NaCl and 0.02% KCl) in a screw-cap Erlenmeyer flask. Each mixture was moderately stirred for 1 h at room temperature to hydrate, and then heated at 95°C for 30 min in a shaking water bath with mild agitation. During heating, a screw-cap Erlenmeyer flask was used to prevent water evaporation. At the end of the heating period, the hot sample mixture was immediately placed on the rheometer plate to measure its rheological properties.

Steady shear rheological properties of each sample were determined on a strain controlled Physica MCR 102 Rheometer (Anton Paar, Graz, Austria). To obtain steady shear (shear stress and shear rate) data, a plate/plate geometry (50 mm diameter, 0.5 mm gap) was used at 25°C with shear rate from 0.1 to 1,000 s^{-1}. To describe the steady shear rheological properties of the samples, the data were fitted to the well-known power law (Eq. 1) and Casson (Eq. 2) models:

$$\sigma = K\dot{\gamma}^{n} \quad \text{(Eq. 1)}$$

$$\sigma^{0.5} = K_{oc} + K_c\dot{\gamma}^{0.5} \quad \text{(Eq. 2)}$$

where σ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), K is the consistency index ($Pa \cdot s^{n}$), *n* is the flow behaviour index (dimensionless), and $(K_c)^2$ is the Casson plastic viscosity (η_c). Casson yield stress (σ_{oc}) according to the Casson model (Eq. 2) was determined as the square of the intercept (K_{oc}) that was obtained from the linear regression of the square roots of the shear rate-shear stress data. Using the magnitudes of K and *n*, the apparent viscosity ($\eta_{a,100}$) at 100 s^{-1} was calculated.

Dynamic shear rheological properties [storage modulus (G'), loss modulus (G''), and complex viscosity (η^*)] of each sample were determined on a strain controlled Physica MCR 102 Rheometer (Anton Paar). Prior to the dynamic shear rheological measurements, a strain sweep test at a constant frequency of 6.3 rad/s determined the linear viscoelastic region. The dynamic measurements were performed at a strain value of 0.02 (2%) (within the linear viscoelastic region). Frequency sweep tests of all the samples were performed using a plate/plate geometry (50 mm diameter, 0.5 mm gap) at 25°C and frequency (ω) from 0.63 to 63.8 rad/s.

Statistical analysis

All statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was performed using the general linear models procedure to determine significant differences among the samples. Means were compared by using Fisher's least significant difference procedure. Significance was defined at the 5% level.

RESULTS AND DISCUSSION

Degree of hydrolysis

The degree of hydrolysis for SPI was significantly increased after combined high temperature pre-treatment and enzymatic hydrolysis (Table 1). Furthermore, the

Table 1. Degree of hydrolysis for beany flavor-removed soy protein isolate hydrolysates produced by combined high temperature pre-treatment and enzymatic hydrolysis using different hydrolysis time

Hydrolysis time (min)	Degree of hydrolysis (%)
Control[1)]	2.21±0.32[e]
0[2)]	3.16±0.27[e]
10	45.26±1.23[d]
20	51.38±2.12[c]
30	59.41±2.28[b]
40	63.60±2.04[a]

Data are presented as the mean±SD of three separate experiments.
Values with different letters (a-e) differ significantly (P<0.05).
[1)]No treatment.
[2)]Only high temperature pre-treatment without enzymatic hydrolysis.

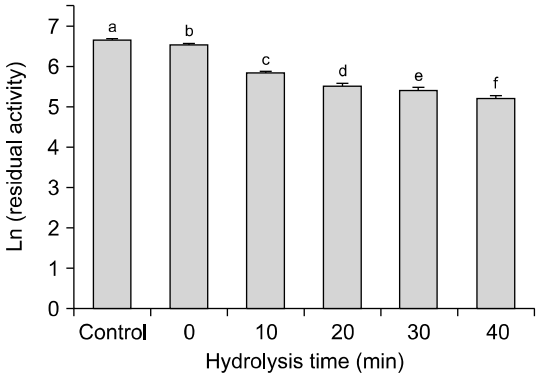

Fig. 1. Lipoxygenase activity for beany flavor-removed soy protein isolate hydrolysates produced by combined high temperature pre-treatment and enzymatic hydrolysis using different hydrolysis times. Control, no treatment; 0 min, only high temperature pre-treatment without enzymatic hydrolysis. Different letters (a-f) above the bars are significantly different at P<0.05.

degree of hydrolysis was significantly influenced by increasing the enzymatic hydrolysis time from 0 to 40 min. Surówka and Żmudziński (25) also found that the degree of hydrolysis for soy protein was significantly improved by enzymatic hydrolysis with Neutrase after extrusion pre-treatment. Proteins are known to form amorphous three and/or four-dimensional structures which are stabilized by non-covalent interactions, hydrogen bonds, disulfide bonds, and so on. Therefore, it is suggested in the present study that the enzymatic hydrolysis in combination with high temperature pre-treatment can enhance the susceptibility to protein hydrolysis due to the unfolding of proteins and the cleavage of disulfide bonds (1).

Lipoxygenase activity

The off-flavor in soy foods, such as beany or green flavor, is highly related to lipoxygenase activity and could be eliminated by the inactivation of lipoxygenase (7). Otherwise, the stronger the activity of lipoxygenase in soy foods, the stronger the beany flavor in soy foods. The combined effects of high temperature pre-treatment and enzymatic hydrolysis on the inactivation of lipoxygenase in SPI are shown in Fig. 1. The combined high temperature pre-treatment and enzymatic hydrolysis resulted in a significant reduction of lipoxygenase activity in SPI. This finding was in accordance with Kong et al. (7) and Kudre and Benjakul (11). According to Kudre and Benjakul (11), the lipoxygenase activity of Barbara groundnut (*Vigna subterranean*) flour, an indigenous legume in the southern part of Thailand, was remarkably declined after heating at the temperature of 70°C due to the denaturation of lipoxygenase at the temperature. Kong et al. (7) investigated the effects of heating treatment on inactivation of lipoxygenase in defatted soybean flour, and they stated that with heating treatment at boiling temperature, the residual lipoxygenase activity was considerably decreased.

Volatile compounds

As mentioned earlier, lipoxygenase acts as a catalyst in the oxidation of polyunsaturated fatty acid, such as linoleic and linoleic acid, and the hydroperoxides formed during oxidation are very unstable and they can easily be transformed to beany flavor-causing compounds (26). In particular, it is commonly noted that typical beany flavor-causing compounds in soy products are pentanol, hexanol, and hexanal (8,26,27). Accordingly, in the present study, we evaluated the quantities of pentanol, hexanol, and hexanal by comparing their peak areas in order to elucidate the effects of combined high temperature pre-treatment and enzymatic hydrolysis on the removal of beany flavor from SPI. The most abundant beany flavor-causing compound in the control was hexanol (Table 2). The result was consistent with Wang et al. (27), who found that the amounts of hexanol in soy milk were the higher than hexanal and pentanol. In the present study, the peak areas of hexanal, pentanol, and hexanol in the control were significantly decreased by 69.6, 71.3, and 99.8%, respectively, during high temperature pre-treatment. The reduction of beany flavor in SPI during heat treatment can be probably correlated with the decrease in the lipoxygenase activity, as noted by Kudre and Benjakul (11). Furthermore, enzymatic hydrolysis at all the hydrolysis times examined led to a significant decrease in the peak areas of hexanal, pentanol, and hexanol. In particular, hexanol was not detected after combined high temperature pre-treatment and enzymatic hydrolysis. Based on the findings obtained from the present study on lipoxygenase activity and volatile compound analysis, it was suggested that the combined high temperature pre-treatment and enzymatic hydrolysis could be the efficient technology to inactivate residual lipoxygenase and remove the beany flavor from SPI.

Table 2. Main volatile compounds of beany flavor-removed soy protein isolate hydrolysates produced by combined high temperature pre-treatment and enzymatic hydrolysis using different hydrolysis times (unit: peak area)

Compound	Control[1)]	Hydrolysis time (min)				
		0[2)]	10	20	30	40
n-Hexanal	2,145,996[a]	651,851[b]	214,030[c]	191,187[c]	206,061[c]	209,419[c]
n-Pentanol	3,575,716[a]	1,025,832[b]	375,823[c]	342,134[c]	362,963[c]	349,069[c]
n-Hexanol	5,192,113[a]	962,113[b]	ND[3)]	ND	ND	ND

Values with different letters (a-c) within the same row differ significantly ($P<0.05$).
[1)]No treatment.
[2)]Only high temperature pre-treatment without enzymatic hydrolysis.
[3)]ND: not detected.

Table 3. Sensory properties for beany flavor-removed soy protein isolate hydrolysates produced by combined high temperature pre-treatment and enzymatic hydrolysis using different hydrolysis times

Sensory property	Control[1)]	Hydrolysis time (min)				
		0[2)]	10	20	30	40
Beany flavor	7.25[a]	4.15±0.63[b]	3.33±0.81[c]	2.50±0.54[d]	2.50±0.54[d]	2.16±0.40[d]
Bitterness	3.20[ab]	2.87±0.44[bc]	2.50±0.54[c]	3.00±0.63[b]	3.00±0.63[b]	3.50±0.83[a]
Solubility	4.01[b]	4.05±0.41[b]	8.33±0.40[a]	8.50±0.54[a]	8.50±0.54[a]	8.83±0.40[a]
Yellowness	3.12[b]	4.30±0.84[a]	1.66±0.49[d]	1.83±0.40[c]	1.66±0.40[d]	1.83±0.40[c]

Data are presented as the mean±SD of three separate experiments.
Values with different letters (a-d) within the same row differ significantly ($P<0.05$).
[1)]No treatment.
[2)]Only high temperature pre-treatment without enzymatic hydrolysis.

Sensory profiles

The beany flavor score of the control was significantly higher than those of all the beany flavor-removed SPI hydrolysates (Table 3). It was well correlated with our previous results obtained from volatile compound analysis, which revealed the decreased hexanal, pentanol, and hexanol levels after combined high temperature pre-treatment and enzymatic hydrolysis. The bitter taste score was not significantly different after 10 min of enzymatic hydrolysis, but significantly increased after 20 min of enzymatic hydrolysis. The solubility score of the control was significantly increased from 4.01 and 8.83, but the yellowness score was significantly reduced from 3.12 to 1.83 after combined high temperature pre-treatment and enzymatic hydrolysis time of 40 min.

Amino acid compositions

The most dominant amino acid in the control and beany flavor removed-SPI hydrolyzates was glutamic acid, followed by aspartic acid, leucine, and lysine (Table 4). Wu and Ding (14) also found that the major two amino acids in soy protein are glutamic acid and aspartic acid. In the present study, the amino acid composition of all the beany flavor-removed SPI hydrolysates is the same as that of the control. Moreover, the quantities of glutamic acid, leucine, and lysine in all the hydrolyzates were not significantly different from those in the control, but the quantities of aspartic acid were slightly decreased from 97.88 to 94.48 mg/g at the enzymatic hydrolysis time of 40 min. Thus, it was found that combined high temperature pre-treatment and enzymatic hydrolysis can have the advantage of preserving the nutritive quality of SPI without considerably destruction of amino acids.

Emulsion capacity and stability

Both of emulsion capacity and stability were significantly increased after combined heating and enzymatic treatments (Table 5). Heating can induce the denaturation of proteins and weaken the forces that stabilize the tertiary and quaternary structures of proteins. The result is the formation of a new molecular aggregate structures (2,8). Bueno et al. (28) studied the effect of high temperature extrusion cooking on the emulsifying properties of soybean proteins and pectin mixtures, and found that the emulsifying capacity of the mixture increased 41% after extrusion processing. They reported that high temperature extrusion cooking of protein can lead to changes in structure and functional properties, such as unfolding of the molecules and exposing functional groups, including hydrophobic groups, from the inside, consequently improving the emulsion properties of proteins. Chen et al. (2) reported that reduced molecular weight and elevated protein solubility can be the major reasons for the remarkably enhanced emulsifying activities of soy protein hydrolyzates which produced after enzymatic hydrolysis with a mixture of trypsin (EC 3. 4. 21. 4), chymotrypsin (EC 3. 4. 21. 1), elastase (EC 3. 4. 4. 7), and carboxypeptidase A (EC 3. 4. 17. 1). Therefore, it is indicated in

Table 4. Amino acid composition of beany flavor-removed soy protein isolate hydrolysates produced by combined high temperature pre-treatment and enzymatic hydrolysis using different hydrolysis times (unit: mg/g)

	Control[1]	Hydrolysis time (min)				
		0[2]	10	20	30	40
Glycine	35.26±0.21^{ns}	35.27±0.17	34.03±0.18	34.11±0.11	34.06±0.22	34.03±0.22
Alanine	37.08±0.18^{ns}	37.10±0.51	35.79±0.22	35.88±0.23	35.82±0.31	35.79±0.32
Valine	40.93±0.15^{ns}	40.95±0.17	39.50±0.22	39.60±0.22	39.54±0.21	39.51±0.26
Isoleucine	39.10±0.11^{a}	39.11±0.22^{a}	37.73±0.32^{b}	37.83±0.31^{b}	37.77±0.25^{b}	37.74±0.15^{b}
Leucine	68.30±0.20^{ns}	68.32±0.31	65.91±0.53	66.08±0.36	65.98±0.16	65.92±0.19
Aspartic acid	97.88±0.16^{a}	97.92±0.19^{a}	94.47±0.26^{b}	94.71±0.32^{b}	94.56±0.32^{b}	94.48±0.21^{b}
Glutamic acid	162.77±0.73^{ns}	162.82±0.48	157.09±1.18	157.48±1.32	157.24±0.15	157.11±0.61
Serine	44.20±0.21^{ns}	44.22±0.21	42.66±0.18	42.77±0.22	42.70±0.19	42.67±0.22
Threonine	33.91±0.32^{ns}	33.93±0.33	32.73±0.19	32.81±0.18	32.76±0.21	32.74±0.19
Cysteine	9.67±0.15^{ns}	9.68±0.15	9.34±0.11	9.36±0.18	9.35±0.21	9.34±0.11
Methionine	3.20±0.18^{ns}	3.20±0.16	3.09±0.21	3.10±0.16	3.09±0.19	3.09±0.08
Lysine	53.95±0.34^{ns}	53.97±0.19	52.07±0.32	52.20±0.22	52.12±0.29	52.08±1.01
Arginine	65.09±0.41^{a}	65.12±0.15^{a}	62.82±0.23^{b}	62.98±0.20^{b}	62.88±0.18^{b}	62.83±0.29^{b}
Histidine	22.59±0.11^{ns}	22.60±0.12	21.80±0.28	21.85±0.22	21.82±0.22	21.80±0.27
Phenylalanine	44.04±0.21^{a}	44.06±0.22^{a}	42.50±0.81^{b}	42.61±0.18^{b}	42.54±0.32^{b}	42.51±0.13^{b}
Tyrosine	30.02±0.31^{ns}	30.03±0.29	28.98±0.12	29.05±0.19	29.00±0.27	28.98±0.28
Proline	32.08±0.19^{a}	32.09±0.11^{a}	30.96±0.36^{b}	31.04±0.21^{b}	30.99±0.23^{b}	30.97±0.31^{b}
TAA[3]	820.08±1.21^{a}	820.38±1.41^{a}	791.46±1.11^{b}	793.45±1.32^{b}	792.23±1.81^{b}	791.59±1.62^{b}
EAA[4]	306.02±1.13^{a}	306.09±1.12^{a}	295.30±1.06^{b}	296.04±1.02^{b}	295.58±1.22^{b}	295.34±1.25^{b}
EAA/TAA	0.3732	0.3733	0.3685	0.3689	0.3688	0.3686

Data are presented as the mean±SD of three separate experiments.
Values with different letters (a, b) within the same row differ significantly (P<0.05).
[ns]Not significant.
[1)]No treatment.
[2)]Only high temperature pre-treatment without enzymatic hydrolysis.
[3)]Total amino acids.
[4)]Essential amino acids (valine, leucine, isoleucine, threonine, methionine, lysine, phenylalanine, and histidine).

Table 5. Emulsion capacity and stability for beany flavor-removed soy protein isolate hydrolysates produced by combined high temperature pre-treatment and enzymatic hydrolysis using different hydrolysis times (unit: %)

Hydrolysis time (min)	Emulsion capacity	Emulsion stability
Control[1]	8.26±1.33^{e}	14.63±2.26^{b}
0[2]	11.25±1.36^{e}	15.26±2.33^{b}
10	24.33±2.15^{d}	22.15±1.32^{a}
20	28.25±1.85^{c}	24.32±2.15^{a}
30	31.38±1.28^{b}	24.16±1.85^{a}
40	34.25±1.66^{a}	27.33±2.50^{a}

Data are presented as the mean±SD of three separate experiments.
Values with different letters (a-e) within the same column differ significantly (P<0.05).
[1)]No treatment.
[2)]Only high temperature pre-treatment without enzymatic hydrolysis.

the present study that the significantly improved emulsion capacity and stability of beany flavor-removed SPI hydrolyzates could be related to the increase exposure inside hydrophobic groups and protein solubility and decrease in molecular weight during the heat and enzymatic treatments. Therefore, the SPI hydrolyzates obtained from the present study could be a candidate for a new emulsifier in the food industry.

DPPH radical scavenging activity

DPPH radical scavenging activity was significantly increased by approximately 8- or 10-fold after combined high temperature pre-treatment and enzymatic hydrolysis (Table 6). Moreover, DPPH radical scavenging activity was significantly elevated when the enzymatic hydrolysis time was increased from 10 to 40 min. Generally speaking, antioxidant activities of protein hydrolyzates are affected by the protease and hydrolysis conditions, such as time, temperature, and pH (29). A variety of the smaller peptides and free amino acids can be produced during hydrolysis, depending upon enzyme specificity. Variations in composition, size, and level of free amino acids and small peptides affect the antioxidant activity. de Castro and Sato (30) found that whey protein hydrolysates which were produced by Flavorzyme had higher DPPH radical scavenging activity than the native whey protein. Liu and Zhao (1) reported that the thermal and chemical treatments significantly increased smaller peptides in SPI hydrolysates, which significantly enhanced the DPPH radical scavenging activity of the hydrolyzates. Therefore, it is suggested in the present study that the combined high temperature pre-treatment and enzymatic

Table 6. DPPH radical scavenging activity of beany flavor-removed soy protein isolate hydrolysates produced by combined high temperature pre-treatment and enzymatic hydrolysis using different hydrolysis times (unit: %)

Concentration (mg/mL)	Control[1)]	Hydrolysis time (min)				
		0[2)]	10	20	30	40
2.0	2.16±0.12^{Ef}	3.72±0.25^{Ee}	14.96±0.12^{Ed}	15.06±0.15^{Ec}	15.59±0.22^{Eb}	17.79±0.23^{Ea}
4.0	3.15±0.31^{Df}	4.51±0.61^{De}	24.81±0.17^{Dd}	27.1±0.21^{Dc}	31.92±0.19^{Db}	36.77±0.21^{Da}
6.0	4.81±0.11^{Cf}	5.66±0.22^{Ce}	40.25±0.14^{Cd}	42.25±0.17^{Cc}	45.97±0.21^{Cb}	49.44±0.32^{Ca}
8.0	6.05±0.21^{Bf}	7.15±0.03^{Be}	56.05±0.12^{Bd}	56.46±0.11^{Bc}	58.17±0.24^{Bb}	64.12±0.21^{Ba}
10.0	7.12±0.18^{Af}	9.12±0.17^{Ae}	62.52±0.21^{Ad}	65.54±0.16^{Ac}	70.51±0.31^{Ab}	74.20±0.33^{Aa}
IC_{50} (mg/mL)	76.73	71.43	7.63	7.34	6.82	6.22

Data are presented as the mean±SD of three separate experiments.
Values with different letters within the same column (A-E) and the same row (a-f) differ significantly (P<0.05).
[1)]No treatment.
[2)]Only high temperature pre-treatment without enzymatic hydrolysis.

Table 7. Superoxide radical scavenging activity of beany flavor-removed soy protein isolate hydrolysates produced by combined high temperature pre-treatment and enzymatic hydrolysis using different hydrolysis times (unit: %)

Concentration (mg/mL)	Control[1)]	Hydrolysis time (min)				
		0[2)]	10	20	30	40
2.0	3.85±0.22^{Ef}	5.84±0.32^{Ee}	16.63±0.25^{Ed}	17.18±0.61^{Ec}	20.06±0.25^{Eb}	22.66±0.25^{Ea}
4.0	6.28±0.35^{Df}	7.51±0.71^{De}	22.67±0.27^{Dd}	24.11±0.28^{Dc}	27.36±0.36^{Db}	29.64±0.33^{Da}
6.0	9.44±0.27^{Cf}	10.06±0.36^{Ce}	30.68±0.33^{Cd}	31.69±0.37^{Cc}	33.69±0.11^{Cb}	37.08±0.72^{Ca}
8.0	11.21±0.36^{Bf}	12.15±0.48^{Be}	38.22±0.43^{Bd}	40.26±0.63^{Bc}	42.22±0.51^{Bb}	44.96±0.23^{Ba}
10.0	12.72±0.18^{Af}	15.62±0.35^{Ae}	44.86±0.31^{Ad}	46.03±1.02^{Ac}	51.08±0.80^{Ab}	53.82±0.53^{Aa}
IC_{50}	43.20	38.86	11.38	10.91	9.93	9.19

Data are presented as the mean±SD of three separate experiments.
Values with different letters within the same column (A-E) and the same row (a-f) differ significantly (P<0.05).
[1)]No treatment.
[2)]Only high temperature pre-treatment without enzymatic hydrolysis.

hydrolysis could increase the amounts of small peptides in the hydrolyzates, consequently leading to the improved DPPH radical scavenging activity.

Superoxide radical scavenging activity

The superoxide radical scavenging activity, one of the most common tests used for *in vitro* antioxidant capacity of food products due to its biological relevance to *in vivo* antioxidant efficacy (31,32), was selected as a measure to evaluate and compare the potential of beany flavor-removed SPI hydrolyzates as a source of antioxidants. In the present study, significant increase of the superoxide radical scavenging activity was achieved after combined high temperature pre-treatment and enzymatic hydrolysis (Table 7). In addition, superoxide radical scavenging activity was significantly affected by elevating enzymatic hydrolysis time from 10 to 40 min. Based on the results obtained from DPPH radical scavenging activity and superoxide radical scavenging activity, it is indicated that the enzymatic hydrolysis in combination with high temperature pre-treatment can improve the antioxidant capacity of the native SPI and can allow for the production of beany flavor-removed SPI hydrolysates with superior antioxidant functionalities.

Table 8. Steady shear rheological properties of beany flavor-removed soy protein isolate hydrolysates produced by combined high temperature pre-treatment and enzymatic hydrolysis using different hydrolysis times

Hydrolysis time (min)	$\eta_{a,100}$ (Pa·s)	K (Pa·s^n)	n	σ_{oc} (Pa)
Control[1)]	95.86±1.25^{a}	24.26±1.19^{a}	0.30±0.01^{d}	1.92±0.10^{a}
0[2)]	88.44±2.08^{b}	22.43±1.62^{b}	0.30±0.03^{d}	1.82±0.19^{b}
10	22.05±1.26^{c}	3.01±0.28^{c}	0.44±0.01^{c}	0.23±0.02^{c}
20	20.33±1.32^{c}	2.75±0.32^{c}	0.44±0.01^{c}	0.19±0.01^{c}
30	20.91±1.16^{c}	2.67±0.21^{c}	0.45±0.02^{b}	0.18±0.01^{c}
40	21.44±2.04^{c}	2.64±0.38^{c}	0.46±0.02^{a}	0.18±0.03^{c}

Data are presented as the mean±SD of three separate experiments.
$\eta_{a,100}$, apparent viscosity; K, consistency index; n, flow behaviour index; σ_{oc}, Casson yield stress.
Values with different letters (a-d) within the same column differ significantly (P<0.05).
[1)]No treatment.
[2)]Only high temperature pre-treatment without enzymatic hydrolysis.

Steady shear rheological analysis

The n indicates the extent of shear-thinning (pseudoplastic) behavior as it deviates from 1 (n=1 for Newtonian flow) and it was in the range of 0.30~0.46, subsequently indicating the pseudoplastic behavior of all the sam-

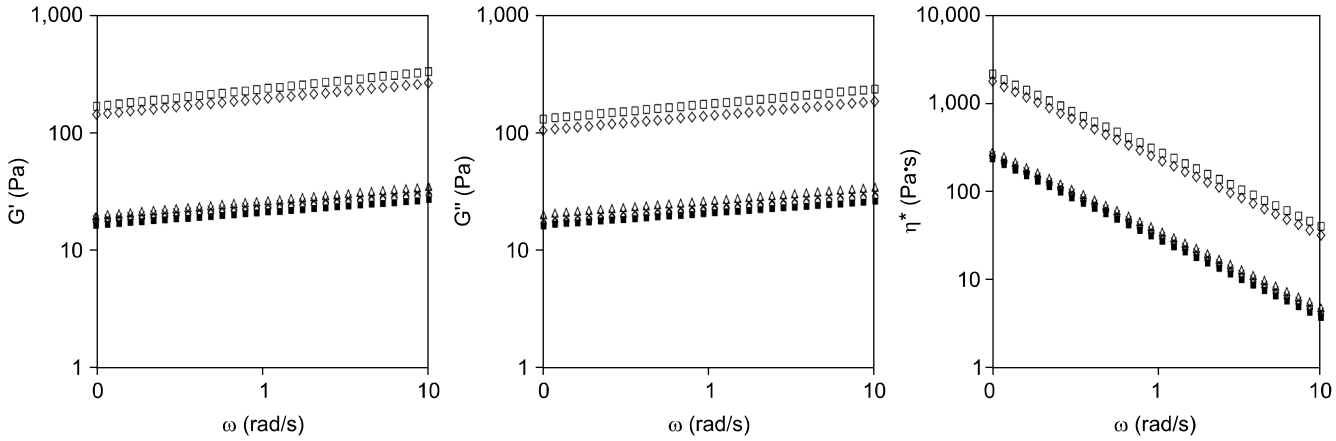

Fig. 2. Plots of log storage modulus (G'), log loss modulus (G''), and complex viscosity (η^*) versus log frequency (ω) of beany flavor-removed soy protein isolate hydrolysates produced by combined high temperature pre-treatment and enzymatic hydrolysis using different hydrolysis times. □, control; ◇, 0 min; △, 10 min; X, 20 min; ○, 30 min; ■, 40 min. Control, no treatment; 0 min, only high temperature pre-treatment without enzymatic hydrolysis.

ples (Table 8). The similar trend was also found in the study of soy protein hydrolyzates obtained from the enzyme bromelain (12). Compared to the control, all of the beany flavor-removed hydrolyzates showed significantly lower values of $\eta_{a,100}$, K, and σ_{oc}. This finding was consistent with the results of Tsumura et al. (33), who reported that the $\eta_{a,100}$ of β-conglycinin and glycinin papain hydrolysates from selective proteolysis was lower than that of unhydrolysed SPI. Lamsal et al. (12) noted that the decrease in $\eta_{a,100}$ and K of SPI hydrolysates can be associated with the reduction in molecular weight and the increase in the protein solubility as compared to the unhydrolysed SPI. Furthermore, in the present study, the reduction in the values of ηa,100 and K can be attributed in part to the elevation in degree of hydrolysis, which was shown in Table 1.

Dynamic shear rheological analysis

The changes in G', G'', and η^* as a function of the ω for the control, beany flavor-removed SPI, and beany flavor-removed hydrolyzates are illustrated in Fig. 2. The values of G' and G'' for the control were much higher than those for all the hydrolyzates, indicating that the elastic and viscous properties of SPI were decreased after enzymatic hydrolysis. Furthermore, the value of η^* was remarkably decreased when enzymatic hydrolysis was applied to SPI, which was the same as the results on the previous $\eta_{a,100}$ (Table 8). Based on the results on the steady and dynamic rheological properties, it was found that combined heat and enzymatic treatments can dramatically influence both of the steady and dynamic shear rheological properties of SPI.

CONCLUSIONS

Combined high temperature pre-treatment and enzymatic hydrolysis led not only to a decrease in residual lipoxygenase activity in SPI, but also to a reduction of some volatile compounds such as hexanol, hexanal, and pentanol in SPI, indicating that the removal of beany flavor in SPI can take place during combined heating and enzymatic treatments. The emulsion capacity and activity were significantly elevated during combined heating and enzymatic treatments. Both of DPPH radical scavenging activity and superoxide radical scavenging activity were significantly affected by enzymatic hydrolysis time (10～40 min). Furthermore, it was revealed that combined heat and enzymatic treatments can dramatically influence both of the steady and dynamic rheological properties of SPI. Based on the results, it was suggested that the combined high temperature pre-treatment and enzymatic hydrolysis could be the efficient technology to remove the beany flavor, modify rheological properties, and improve the emulsion and antioxidant activities of SPI.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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