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Angiotensin Converting Enzyme Inhibitory Activity of Soy Protein Subjected to Selective Hydrolysis and Thermal Processing

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ABSTRACT: Soy protein isolate (SPI) and β-conglycinin- and glycinin-rich fractions were hydrolyzed using papain and pepsin. Protein denaturation, profiling, and peptide identification were carried out following DSC, SDS-PAGE, and liquid chromatography−tandem mass spectrometry (LC-MS/MS) analysis. The in vitro antihypertensive activity of the hydrolysates was compared by determining the angiotensin converting enzyme (ACE) inhibitory activity. SDS-PAGE and LC-MS/MS analysis confirmed pepsin selectivity to glycinin and papain partial selectivity to β-conglycinin when the protein is least denatured. Both the papain-hydrolyzed SPI and the papain-hydrolyzed β-conglycinin-rich fraction had more than double the ACE inhibitory activity of that of pepsin-hydrolyzed SPI and pepsin-hydrolyzed glycinin-rich fraction. This observation indicated that β-conglycinin is a better precursor for antihypertensive peptides than glycinin. Additionally, the inhibitory activity of the papain-hydrolyzed SPI was thermally stable. This work demonstrated, for the first time, that selective hydrolysis to release peptides with ACE inhibitory activity can be accomplished without inducing extensive hydrolysis and performing unnecessary fractionation.

KEYWORDS: *antihypertensive peptides, soy protein hydrolysate, β-conglycinin, glycinin, angiotensin converting enzyme (ACE)*

INTRODUCTION

Enzymatic production and use of functional and bioactive protein hydrolysates have gained prominence in the food industry due to escalating consumer awareness and demand for healthy foods. Enzymatic hydrolysis of food proteins results in the release of bioactive peptides with several physiological benefits.[1] Bioactive peptides have been isolated from several food protein sources including soy protein.[2] There is growing support for the hypothesis that soy bioactive peptides derived from soy storage proteins, β-conglycinin and glycinin, are among the major contributors to the health benefits of soy protein. Hydrolysis of soy storage proteins, utilizing various enzymatic systems, resulted in the production of peptides with antioxidant activity,[3] anticancer properties,[4] and antihypertensive activity.[5]

In the United States, over 73 million adults are affected by hypertension, which may ultimately lead to coronary heart disease, heart failure, stroke, kidney failure, and other health problems, thus raising the mortality rate to over 57000/year.[6] Consequently, antihypertensive peptides have gained prominence in the past decade. The proposed mechanism of action of antihypertensive peptides is via the inhibition of the angiotensin converting enzyme (ACE). ACE raises blood pressure by converting the inactive decapeptide angiotensin I to the active octapeptide angiotensin II, which is a potent vasoconstrictor hormone.[7] Antihypertensive drugs target ACE, either by direct inhibition (ACE inhibitors, such as Captopril) or by blocking the angiotensin II receptors (angiotensin receptor blockers). Given the potential for adverse side effects with these medications, there has been substantial interest in exploring natural foods with ACE-inhibitory activity.

Several peptides with ACE-inhibitory activity have been identified in fermented soybean products, in soy protein peptic digest, and in soy protein alcalase digest.[5,8−11] The potency of these antihypertensive peptides is normally high in isolated peptides. These peptides, derived from excessively hydrolyzed protein, are often isolated following ultrafiltration and chromatographic separations. Fractionation and isolation of these peptides would potentially cause accrual of high processing costs and generation of waste streams. Additionally, excessive hydrolysis results in a product with highly perceived bitterness and poor functionality. Therefore, an unfractionated soy protein hydrolysate, produced by minimal and selective enzymatic hydrolysis to release potent ACE-inhibitory peptides, would be a suitable and attractive alternative for industrial applications.

Producing a minimally hydrolyzed soy protein ingredient that retains good functionality and significant ACE-inhibitory activity could be challenging. We hypothesize that selective hydrolysis of a targeted protein component can result in the release of peptides with significant ACE-inhibitory activity. There has been some interesting research showing that β-conglycinin has greater potential than glycinin for reducing the risk of cardiovascular diseases,[12,13] inhibiting fatty acid synthase and lipid accumulation,[14−16] reducing inflammation,[17] and inhibiting growth of leukemia cells.[18] Apparently, β-conglycinin constitutes various peptide sequences that can have a number of physiological benefits upon release from the parent protein.

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Therefore, selective hydrolysis of the β-conglycinin component of soy protein may result in a product with significant ACE-inhibitory activity.

The release of antihypertensive peptides is not enough to ensure beneficial effect upon consumption. Soy foods, like many other foods, are subjected to various thermal processing conditions. Evaluation of the thermal stability of the released peptides upon processing and the retention of bioactivity have to be taken into account. Depending on the amino acid sequence of the released bioactive peptides, they may be involved in irreversible interactions with other peptides or sugars in the system, via disulfide linkage or Maillard conjugation, respectively. Irreversible interactions due to processing may result in loss of the peptides' bioactivity. Degradation and loss of activity of some peptides have been reported after certain thermal processes.[2]

Therefore, the objectives of this study were to (1) monitor the ACE-inhibitory activity of soy protein isolate (SPI) subjected to limited and selective hydrolysis and (2) determine the effect of various thermal treatments on the retention of ACE-inhibitory activity of the produced soy protein hydrolysates (SPH).

MATERIALS AND METHODS

Materials. Defatted soy flour (7B, 53% protein) and soy protein isolate (SPI, 90% protein) were kindly provided by Archer Daniels Midland (ADM) (Decatur, IL, USA). Glycinin and β-conglycinin were supplied by EPL Bio Analytical Services (Niantic, IL, USA). Papain (24 units/mg), pepsin (3200 units/mg), angiotensin converting enzyme (ACE, from rabbit lung, 5.5 units/mg), *N*-[3-2-(2-furyl)-acryloyl]-L-phenylalanyl-glycyl-glycine (FAP-GG), and Captopril were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). Prestained broad-range molecular weight standard, Laemmli sample buffer, 10× Tris/glycine/SDS running buffer, ammonium persulfate, 40% acrylamide/Bis solution, 37.5:1 (2.6% C), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad (Hercules, CA, USA). Analytical reagent grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Sigma Chemical Co.

Preparation of SPI. Minimally heat-treated and defatted soy flour was used to prepare SPI following the method outlined by Tsumura et al.[19] A soy flour and deionized distilled water (DDW) dispersion (1:10 w/w) was adjusted to pH 7.5 with 2 N NaOH, stirred at room temperature for 1 h, and centrifuged at 5000*g* for 30 min to remove the insoluble material. The supernatant was adjusted to pH 4.5 with 2 N HCl and centrifuged at 5000*g* for 10 min to precipitate the protein. The pellet was redispersed (1:4 w/w) in DDW, neutralized with 2 N NaOH, and lyophilized. The protein content of the lyophilized sample was 93% as determined following the Dumas method (AOAC 990.03) using a nitrogen analyzer (LECO, St. Joseph, MI, USA). The prepared SPI sample was kept at −20 °C until further analysis.

Fractionation of β-Conglycinin and Glycinin from Defatted Soy Flour. β-Conglycinin and glycinin were fractionated from minimally heat-treated and defatted soy flour according to the simplified fractionation procedure outlined by Deak et al.[20] Briefly, 120 g of defatted soy flour was mixed with DDW (15:1 water-to-flour ratio), and the pH was adjusted to 8.5 with 2 N NaOH. The slurry was stirred for 1 h at 25 °C followed by centrifugation at 14000*g* for 30 min at 15 °C. To the supernatant were added sodium metabisulfite and calcium chloride to obtain 5 mM SO_2 and 5 mM Ca^{2+}, respectively, and the pH was adjusted to 6.4 with 2 N HCl. The slurry was stirred for 12−16 h in a walk-in cooler at about 4−6 °C and then centrifuged at 14000*g* for 30 min at 4 °C. The precipitated curd (glycinin-rich fraction) was neutralized with 2 N NaOH, desalted, lyophilized, and stored at −20 °C for later use. The supernatant was adjusted to pH 4.8 with 2 N HCl, stirred for 1 h at 25 °C, and centrifuged at 14000*g* for 30 min at 4 °C. The precipitated curd (β-conglycinin-rich fraction) was neutralized with 2 N NaOH, desalted, lyophilized, and stored at −20 °C for later use. The protein contents of the β-conglycinin fraction and glycinin fraction were ∼88 and 97%, respectively, as determined following the Dumas method (AOAC 990.03) using a nitrogen analyzer (LECO).

Preparation of Hydrolyzed SPI, β-Conglycinin, and Glycinin. Dispersions (5 g of protein in 100 mL) of SPI (both the commercial and the prepared SPI), β-conglycinin-rich fraction, and glycinin-rich fraction, prepared individually in DDW, were subjected to enzymatic hydrolysis, in triplicate, by either papain or pepsin. Papain and pepsin were dissolved in DDW to concentrations of 9.975 and 1770 units/mL, respectively. For hydrolysis by papain, the protein dispersion was adjusted to pH 7.0 with 2 N NaOH, heated to 70 °C for 10 min prior to papain enzyme solution (2 mL) addition, and then incubated at 70 °C for 45 min. For hydrolysis by pepsin, the protein dispersion was adjusted to pH 2.0 with 2 N HCl and heated to 37 °C for 10 min prior to pepsin enzyme solution (2 mL) addition. The mixture was incubated at 37 °C for 60 min followed by pH adjustment to 7.0 with 2 N NaOH. The pH of each system was maintained throughout the incubation period, by the addition of either 2 N NaOH or 2 N HCl. After incubation, the hydrolysates were subjected to boiling for 5 min to inactivate the enzyme and then were lyophilized. Control samples were prepared and incubated under the same conditions without enzyme addition. The lyophilized samples were analyzed for protein content using a nitrogen analyzer and kept at −20 °C until further analysis. The protein content of the produced hydrolysates ranged from 92 to 96%. The hydrolysis conditions were established on the basis of preliminary work to maintain limited (<8%) and similar degrees of hydrolysis among the different hydrolysates.

Differential Scanning Calorimetry (DSC). Thermal denaturation of glycinin and β-conglycinin of the commercial SPI, the prepared SPI sample, and the β-conglycinin-rich and glycinin-rich fractions was monitored using a DSC 7 instrument (Perkin-Elmer, Waltham, MA, USA) according to the method of Tang et al.,[21] with slight modification. Duplicates of commercial and prepared SPI samples were solubilized to a 15% protein solution (w/v) in 0.05 M potassium phosphate buffer (pH 7.0) overnight to equilibrate and transferred to aluminum solid pans, which were hermetically sealed. The pans were held in the sample chamber at 20 °C for 5 min and then heated from 20 to 120 °C at 5 °C/min increments. Indium was used to test the calibration of the instrument. A sealed empty pan was used as reference.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). To monitor the pattern of protein hydrolysis, SDS-PAGE was performed on the basis of the method outlined by Laemmli.[22] Each sample was dissolved in DDW (∼10.60 mg protein/mL), and an aliquot (100 μL) was mixed 1:1 (v/v) with Laemmli buffer under reducing conditions (0.75 M β-mercaptoethanol) and boiled for 5 min. Prestained broad -range MW standard, glycinin and β-conglycinin standards, controls, and hydrolyzed samples (5 μL; containing ∼26.5 ug protein) were loaded onto 18-well hand-cast 15% acrylamide and 4% stacking gels. The gels were electrophoresed at a constant voltage of 200 V for approximately 1 h. The gels were stained using Coomassie Brilliant Blue for another hour followed by destaining. Molecular Imager Gel Dox XR system (Bio-Rad Laboratories) was used to scan the gels. To determine protein band intensities, gels were stained with Deep Purple total protein stain (GE Healthcare) per the manufacturer's protocol. An image of each gel was acquired with a Typhoon 8610 Variable Mode Imager (GE Healthcare) at a photomultiplier voltage of 550 V, excitation at 532 nm, and a bandpass (BP) emission filter of 610 BP 30. The densitometry analysis was performed with ImageQuant v5.2 software (GE Healthcare). The relative quantity of each band was determined as a percentage of the total protein bands in the lane. All measurements were carried out in triplicates (i.e., measurements were obtained from three gels and were averaged).

Degree of Hydrolysis (DH). Each control or hydrolyzed sample was solubilized (1:1000 w/v) in DDW and centrifuged at 10000*g* for 10 min. The protein content of the resulting supernatant was

determined using a micro BCA protein assay kit following the manufacturer's instruction. The DH was measured following the *o*-phthaldialdehyde (OPA) method as outlined by Nielsen et al.,[23] using DDW as the sample blank and L-serine as standard. The DH (%) was calculated following the formula reported by Adler-Nissen.[24]

Thermal Treatment of Hydrolysates. Hydrolysates were subjected to various heat treatments in completely crossed three factor−factorial experimental design, with heating temperature (two levels), time (two levels), and moisture content (two levels) as variables. Each of the lyophilized hydrolysates (1 g), in triplicate, was placed in screw-cap test tubes and either 0.0018 or 4 mL of DDW was added, to reach a moisture content of ∼0.18 or 80%, respectively. Samples were heated in an oil bath at either 120 or 175 °C for 10 or 15 min and then cooled on ice for 5 min. Heating conditions were chosen to simulate several processing conditions of dry and moist soy systems. After heat treatment, samples were lyophilized and stored at −20 °C until further analysis.

Measurement of ACE Inhibitory Activity. Measurement of ACE-inhibitory activity of the hydrolysates was conducted, in triplicate, following previous assays.[25,26] Sodium borate buffer (0.1 M borate, 0.3 M chlorine ion, pH 8.30) was used instead of 50 mM Tris-HCl buffer (pH 7.5). Sodium borate buffer delivers higher enzyme activity at pH 8.3 than Tris buffer.[27,28] Captopril was chosen as a reference inhibitor to determine the suitability of the chosen assay. The measured IC_{50} value (concentration that results in 50% inhibition of the ACE activity) of Captopril (1.738 ng/mL) fell within the reported range of 0.035−4.780 ng/mL obtained following similar in vitro ACE assays.[29] Hydrolysate solutions were prepared in DDW, centrifuged at 10000*g* for 10 min, and filtered through 0.45 μm syringe filters. The filtered solutions were diluted to various concentrations of 5.0, 7.5, 10.0, 12.5, and 15.0 mg/mL for the papain-hydrolyzed protein fractions and 10.0, 12.5, 15.0, 17.5, and 20.0 mg/mL for the pepsin-hydrolyzed protein fractions. The reaction mixtures were prepared in preheated microplates at 37 °C and placed in a Biotek Synergy HT microplate reader (Winooski, VT, USA). The reaction mixture of the control blank constituted 160 μL of sodium borate buffer and 10 μL of ACE (0.288 unit/mL in DDW), whereas that of the control constituted 10 μL of sodium borate buffer, 150 μL of FA-PGG (0.88 nM in sodium borate buffer), and 10 μL of ACE. The reaction mixture of the sample blank constituted 150 μL of sodium borate buffer, 10 μL of Captopril (10.0 nM in sodium borate buffer) or 10 μL of hydrolysate solution, and 10 μL of ACE;, whereas that of the sample constituted 150 μL of FA-PGG, 10 μL of Captopril or 10 μL of hydrolysate solution, and 10 μL of ACE. The final protein concentration in the well of papain-hydrolyzed protein fractions was 0, 0.101, 0.151, 0.201, 0.251, or 0.302 mg/mL and that of pepsin-hydrolyzed fractions was 0, 0.2, 0.26, 0.31, 0.36, or 0.41 mg/mL. During incubation at 37 °C, absorbance at 340 nm was recorded every 30 s for 30 min. The ACE activity was expressed as the slope of decrease in absorbance at 340 nm taken from 10 to 25 min of incubation. IC_{50} values were determined from plots of percent ACE inhibition versus inhibitor concentration (concentration of the protein content in the well). The percent ACE inhibition by the various inhibitory solutions was calculated as follows: % ACE inhibition = $((\text{slope}_{\text{control}} - \text{slope}_{\text{inhibitor}})/\text{slope}_{\text{control}}) \times 100\%$.

Liquid Chromatography−Tandem Mass Spectrometry (LC-MS/MS) Analysis. Identification of peptides in the hydrolysates was done following LC-MS/MS analysis. Hydrolysates were rehydrated in water/acetonitrile/formic acid 95:5:0.1 and loaded using a Paradigm AS1 autosampler system (Michrom Bioresources, Inc., Auburn, CA, USA). The column used was a 12 cm × 75 μm i.d., 5 μm, 200 Å Magic RP-18 capillary column, with a 50 × 0.15 mm i.d. Paradigm Platinum Peptide Nanotrap guard column (Michrom Bioresources, Inc.) of the same material. The flow rate was set at 250 nL/min. Peptides were fractionated on a 60 min (10−40% acetonitrile) gradient on an MS4 flow splitter (Michrom Bioresources, Inc.). Mass spectrometry (MS) was performed on a linear ion trap quadrupole (Thermo Electron Corp., San Jose, CA, USA). Ionized peptides eluting from the capillary column were subjected to an ionizing voltage (2.0 kV) and selected for MS/MS using a data-dependent procedure alternating between an MS scan followed by five MS/MS scans for the five most abundant precursor ions. All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 27, rev. 12). Sequest was set up to search the rs_Soybean_cRAP_v20120223 database (45358 entries) assuming the digestion enzyme was nonspecific. Sequest was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 1.00 Da. Oxidation of methionine was specified in Sequest as a variable modification. Scaffold version 3.3.1(Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95.0% probability as specified by the Peptide Prophet algorithm[30] and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[31] Proteins that contained similar peptides and could not be differentiated on the basis of MS/MS analysis alone were grouped to satisfy the principles of parsimony. Spectra exports were made from Scaffold to compare similar and distinct peptides in sample sets using an in-house Perl script. Spectra were further inspected manually to verify the assignment.

Statistical Analysis. Analysis of variance (ANOVA) was carried out utilizing SPSS 15 for Windows.[32] When a factor effect or an interaction was found to be significant, indicated by a significant *F* test ($P \leq 0.05$), differences between the respective means (if more than two means) were determined ($P \leq 0.05$) using the Tukey−Kramer multiple-means comparison test.

RESULTS AND DISCUSSION

Effect of Protein Denaturation State on Enzyme Selectivity. DSC analysis was performed to determine the protein denaturation state of the commercial SPI versus prepared SPI and that of β-conglycinin-rich and glycinin-rich fractions (Figure 1). The commercial SPI was constituted

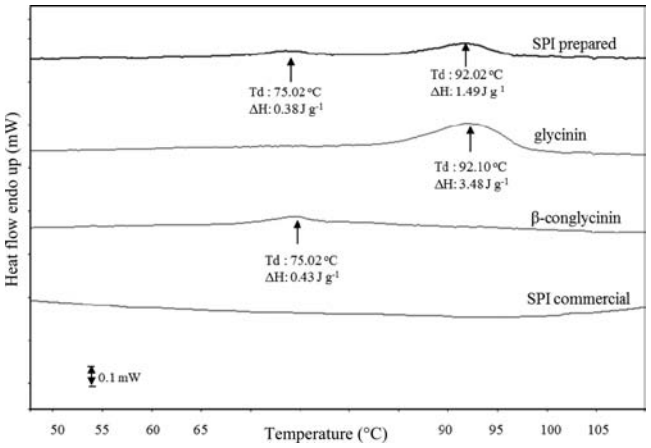

Figure 1. Differential scanning calorimetry of commercial SPI, prepared SPI, and fractionated glycinin and β-conglycinin. Td, denaturation temperature; ΔH, enthalpy.

completely of denatured proteins, as endothermic peaks (peaks that correspond to the onset of denaturation) of the β-conglycinin and glycinin components were not apparent. Both glycinin and β-conglycinin proteins of the commercial SPI have been denatured, possibly due to prior heat processing. However, β-conglycinin and glycinin components of our prepared SPI were not denatured. Two endothermic peaks, corresponding to the onset denaturation temperature of β-conglycinin and glycinin components of the prepared SPI, were observed at 75.02 and 92.02 °C, respectively. The identity of the endothermic peaks was confirmed by comparison to literature values.[21,33] Similarly, the β-conglycinin-rich and glycinin-rich fractions had distinctive endothermic peaks for

each protein, respectively. However, the enthalpy corresponding to each protein was higher in the isolated fractions than that of the prepared SPI, especially that of glycinin (Figure 1). Given that samples were analyzed at the same protein concentration, the observed greater enthalpy of glycinin in the glycinin-rich fraction can be attributed to the retention of the protein native form more than its counterpart in the prepared SPI. Apparently, the process followed to prepare SPI caused partial unfolding but not complete denaturation.

The effect of the protein denaturation state on enzyme selectivity was visualized using SDS-PAGE. The denatured β-conglycinin and glycinin components of the commercial SPI were hydrolyzed by both papain and pepsin enzymes (Figure 2, lanes 4 and 5). Following the hydrolysis of commercial SPI by either enzyme, protein bands corresponding to the subunits of both glycinin and β-conglycinin (as confirmed by running standard glycinin and β-conglycinin on the same gel) disappeared almost completely upon hydrolysis, with a subsequent release of peptides with various, but lower, molecular weights.

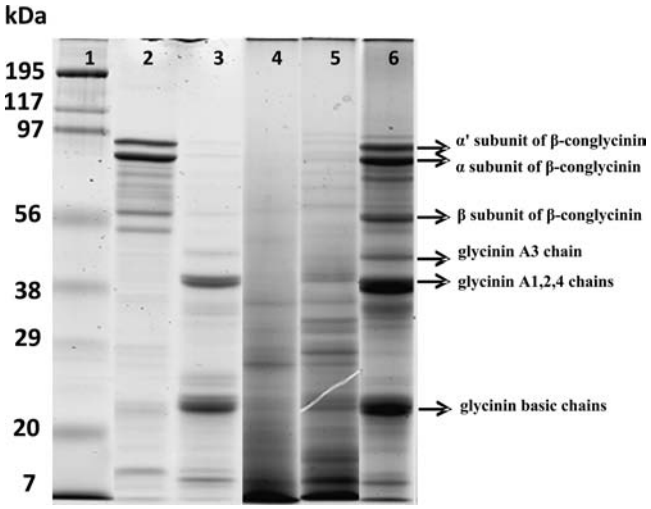

Figure 2. SDS-PAGE visualization of commercial SPI hydrolysis pattern. The amount of protein loaded in each lane is ~26.5 μg. Lanes: 1, molecular weight marker; 2, β-conglycinin standard; 3, glycinin standard; 4, papain-hydrolyzed SPI; 5, pepsin-hydrolyzed SPI; 6, commercial SPI.

On the other hand, the pattern of enzymatic hydrolysis was more distinctive for the prepared SPI and the β-conglycinin-rich and glycinin-rich fractions (Figure 3). Upon subjecting the prepared SPI and glycinin-rich and β-conglycinin-rich fractions to papain hydrolysis (Figure 3, lanes D, E, and F), β-conglycinin subunits disappeared almost completely, whereas those corresponding to the glycinin subunits remained partially intact in the prepared SPI (Figure 3, lane D) and mostly intact in the glycinin-rich fraction (Figure 3, lane F). Upon subjecting the prepared SPI and glycinin-rich and β-conglycinin-rich fractions to pepsin hydrolysis (Figure 3, lanes G, H, and I), glycinin subunits disappeared almost completely, whereas those corresponding to the β-conglycinin subunits in the prepared SPI (Figure 3, lane G) and the β-conglycinin-rich fraction (Figure 3, lane H) remained almost intact compared to β-conglycinin bands in the respective controls (Figure 3, lanes C and A).

The selectivity of papain toward β-conglycinin and that of pepsin toward glycinin was further illustrated by determining protein percentage distribution of the various subunits in the prepared SPI, β-conglycinin-rich fraction, glycinin-rich fraction, and their respective hydrolysates (Table 1). The percentage of β-conglycinin subunits was significantly reduced compared to that of glycinin subunits in the prepared SPI and β-conglycinin-rich fraction subjected to papain hydrolysis. In contrast, the percentage of glycinin subunits was significantly reduced compared to that of β-conglycinin subunits in the prepared SPI and glycinin-rich as well as β-conglycinin-rich fractions subjected to pepsin hydrolysis.

The DSC results coupled with the SDS-PAGE results highlighted the significance of the protein denaturation state on the selectivity of both papain and pepsin enzyme toward β-conglycinin and glycinin subunits, respectively. Selectivity is more pronounced when the protein retains most of its native form, as illustrated, for instance, by the less hydrolyzed glycinin subunits in the glycinin-rich fraction versus prepared SPI

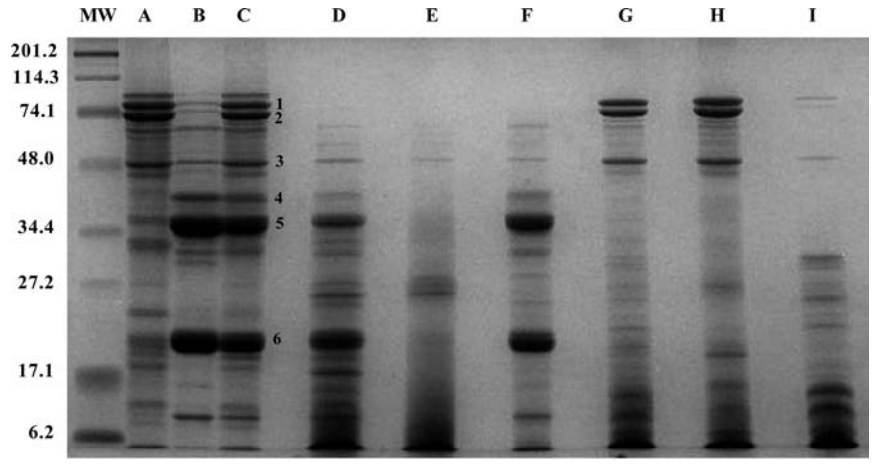

Figure 3. SDS-PAGE visualization of the hydrolysis pattern of prepared SPI, β-conglycinin-rich fraction, and glycinin-rich fraction. The amount of protein loaded in each lane is ~26.5 μg. Lanes: MW, molecular weight marker; A, β-conglycinin-rich fraction; B, glycinin-rich fraction; C, prepared SPI; D, papain-hydrolyzed SPI; E, papain-hydrolyzed β-conglycinin-rich fraction; F, papain-hydrolyzed glycinin-rich fraction; G, pepsin-hydrolyzed SPI; H, pepsin-hydrolyzed β-conglycinin-rich fraction; I, pepsin-hydrolyzed glycinin-rich fraction. Bands: 1, α' subunit of β-conglycinin; 2, α subunit of β-conglycinin; 3, β subunit of β-conglycinin; 4, glycinin A3 chain; 5, glycinin A1, 2,4 chains; 6, glycinin basic chains.

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Table 1. Protein Percentage Distribution, As Determined by SDS-PAGE, of Various β-Conglycinin and Glycinin Subunits in Nonhydrolyzed and Hydrolyzed SPI, β-Conglycinin-Rich Fraction, and Glycinin-Rich Fraction

	% of total protein[a]								
storage protein detected	A	B	C	D	E	F	G	H	I
α′ subunit of β-conglycinin	9.31 b	1.64 d	6.84 c	0.26 d	0.32 d	0 d	14.5 a	14.4 a	5.69 c
α subunit of β-conglycinin	10.7 b	1.33 de	8.62 c	0.31 e	0.41 e	0 e	15.1 a	15.9 a	3.15 d
β subunit of β-conglycinin	7.31 cd	3.07f	6.01 cde	3.35 ef	5.59 def	3.62 ef	10.7 ab	12.1 a	8.59 bc
glycinin A3 chain	0 d	6.04 a	4.91 a	3.51 b	0 d	6.10 a	1.77 c	0 d	0 d
glycinin A1,2,4 chains	2.35 d	15.0 a	10.8 b	10.6 b	6.35 c	14.2 a	0.41 d	1.63 d	0 d
glycinin basic chains	10.3 e	47.4 a	28.2 c	40.3 b	19.9 d	53.0 a	8.39 e	5.36 e	8.08 e
total β-conglycinin	27.3 b	6.04 d	21.5 c	3.91 d	6.32 d	3.62 d	40.3 a	42.4 a	17.4 c
total glycinin	12.7 e	68.5 a	43.8 c	54.4 b	26.2 d	73.3 a	10.6 e	5.74 e	8.08 e

[a]Means in each row with different lower case letters are significantly different according to the Tukey−Kramer multiple-means comparison test ($P \leq 0.05$); $n = 3$. A, β-conglycinin-rich fraction; B, glycinin-rich fraction; C, SPI; D, papain-hydrolyzed SPI; E, papain-hydrolyzed β-conglycinin; F, papain-hydrolyzed glycinin; G, pepsin-hydrolyzed SPI; H, pepsin-hydrolyzed β-conglycinin; I, pepsin-hydrolyzed glycinin.

(Figure 3; compare lane F to lane D) when papain was used. Glycinin in the glycinin-rich fraction had higher enthalpy than that of its counterpart in the prepared SPI (Figure 1), thus explaining its greater resistance to hydrolysis by papain (Figure 3 and Table 1; compare F to D).

Unfolding of the protein causes increased susceptibility to enzymatic hydrolysis and ultimately reduces enzyme selectivity.[19,34] When selective hydrolysis is desired, as in this case, it is crucial to start with a minimally denatured protein fraction. Therefore, papain hydrolysates of prepared SPI and β-conglycinin-rich fraction and pepsin hydrolysates of prepared SPI and glycinin-rich fraction were further analyzed to determine their DH and ACE inhibitory activities.

Degree of Hydrolysis and ACE-Inhibitory Activity of Soy Protein Hydrolysates. Protein hydrolysis that results in a DH of >8% normally causes the release of a significant amount of bitter peptides.[35] Therefore, to prevent the liberation of an excess amount of bitter peptides, enzymatic hydrolysis was controlled to limit the DH to levels below 8% (Table 2).

Table 2. Degree of Hydrolysis (DH) and IC_{50} Values of Protein Hydrolysates

protein hydrolysate	%DH[a]	IC_{50}[a,b] (mg protein/mL)
papain-hydrolyzed SPI	4.50 ± 0.29 b	0.177 ± 0.01 c
papain-hydrolyzed β-conglycinin-rich fraction	7.74 ± 0.53 a	0.170 ± 0.03 c
pepsin-hydrolyzed SPI	4.57 ± 0.07 b	0.361 ± 0.01 b
pepsin-hydrolyzed glycinin-rich fraction	7.56 ± 1.12 a	0.588 ± 0.05 a

[a]Means in each column with different lower case letters are significantly different according to Tukey− Kramer multiple-means comparison test ($P \leq 0.05$); $n = 3$. [b]The lower the IC_{50} value, the higher the potency.

On the basis of the findings thus far, it is assumed that the peptides in the papain-hydrolyzed SPI and β-conglycinin-rich fraction mostly originate from the β-conglycinin subunits and those in the pepsin-hydrolyzed SPI and glycinin-rich fraction from the glycinin subunits. Therefore, the ACE-inhibitory activityies of these four hydrolysates were compared.

The IC_{50} of the mentioned hydrolysates ranged between 0.177 and 0.588 mg protein/mL, falling within the reported range of IC_{50} values for peptides with ACE-inhibitory activity (0.046−0.930 mg protein/mL).[36−40] Although there was no significant difference in their %DH, papain-hydrolyzed SPI had a significantly lower IC_{50} than pepsin-hydrolyzed SPI (Table 2). A lower IC_{50} value indicates higher ACE inhibitory activity because less protein is required to cause 50% inhibition. Similarly, at equivalent %DH, the papain-hydrolyzed β-conglycinin-rich fraction had a significantly lower IC_{50} than the pepsin-hydrolyzed glycinin-rich fraction. Both papain-hydrolyzed SPI and papain-hydrolyzed β-conglycinin-rich fraction had more than double the ACE-inhibitory activity of the pepsin-hydrolyzed SPI and pepsin-hydrolyzed glycinin-rich fraction.

When β-conglycinin subunits were hydrolyzed, the ACE activity of the released peptides was more pronounced. Because papain and pepsin had limited activity on glycinin and β-conglycinin, respectively, the peptides contributing to the higher ACE inhibitory activity most likely originated from β-conglycinin. This was partially confirmed by the similar IC_{50} values of both papain-hydrolyzed SPI and papain-hydrolyzed β-conglycinin-rich fraction (Table 2).

Identification of Peptides in Soy Protein Hydrolysates Using LC-MS/MS. To confirm the origin of peptides in the different hydrolysates, LC-MS/MS was employed to compare and contrast peptides in papain-hydrolyzed SPI versus papain-hydrolyzed β-conglycinin-rich fraction and those in pepsin-hydrolyzed SPI versus pepsin-hydrolyzed glycinin-rich fraction.

Of 168 matching peptides identified, with >95% confidence, in both papain-hydrolyzed SPI and papain-hydrolyzed β-conglycinin-rich fraction, 131 originated from β-conglycinin subunits, whereas only 37 originated from glycinin subunits. The most predominant peptides with at least two hits and up to 20 hits are presented in Table 3. As shown earlier, the β-conglycinin-rich fraction did contain about 12% total glycinin (Table 1, column A). Whereas most of the identified peptides originated from β-conglycinin subunits, it is apparent that papain did partially hydrolyze some of the glycinin subunits. On the other hand, all 139 matching peptides identified, with >95% confidence, in both pepsin-hydrolyzed SPI and pepsin-hydrolyzed glycinin originated from glycinin subunits. The most predominant peptides with at least 2 hits and up to 20 hits are presented in Table 4.

These findings confirmed our previous discussion regarding pepsin selectivity to glycinin, and papain partial selectivity to β-conglycinin, when the protein is least denatured. In addition to

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Table 3. Predominant Protein Sequences Identified by LC-MS/MS[a] in both Papain-Hydrolyzed SPI and Papain-Hydrolyzed β-Conglycinin-Rich Fraction

putative sequence	exptl mass (Da)	theor mass (Da)	protein source	accession no.
ASYDTKFEEINKVLFG	1861.33	1859.93	*f* (360−375) α′ subunit (β-conglycinin)	gi 351724511
INAENNQRNFLAGSKDNVISQ	2331.95	2331.16	*f* (545−565) α′ subunit (β-conglycinin)	gi 351724511
REEGQQQGEERLQESVIVE	2242.91	2242.08	*f* (376−394) α′ subunit (β-conglycinin)	gi 351724511
SQSESYFVDAQPQQKEEGN	2170.89	2169.94	*f* (588−606) α′ subunit (β-conglycinin)	gi 351724511
YYVVNPDNDENLRMITL	2069.27	2067.99	*f* (309−325) α′ subunit (β-conglycinin)	gi 351724511
IPSQVQELAFPGSAQAVEKLLK	2353.49	2352.31	*f* (550−571) α subunit (β-conglycinin)	gi 356575853
NILEASYDTKFEEINKVL	2125.89	2125.09	*f* (340−357) α subunit (β-conglycinin)	gi 356575853
TEAQQSYLQGFSR	1514.79	1513.72	*f* (327−339) α subunit (β-conglycinin)	gi 356575853
TYYVVNPDNNENLRLITL	2,151.85	2,150.10	*f* (292−309) α subunit (β-conglycinin)	gi 356575853
VVNPDNNENLRLITL	1723.73	1722.93	*f* (295−309) α subunit (β-conglycinin)	gi 356575853
YVVNPDNNENLRLITL	1887.47	1885.99	*f* (294−309) α subunit (β-conglycinin)	gi 356575853
YYVVNPDNNENLRLITL	2049.97	2049.05	*f* (293−309) α subunit (β-conglycinin)	gi 356575853
INAENNQRNFLAGEKDNVVR	2301.52	2300.16	*f* (363−382) β subunit (β-conglycinin)	gi 356575855
NGPQEIYIQQGKGIFG	1748.07	1747.89	*f* (86−101) glycinin G1	gi 356505023
ALPEEVIQHTFNLK	1638.93	1637.88	*f* (444−457) glycinin G2	gi 351725363
ANSLLNALPEEVIQHTFN	2010.37	2009.02	*f* (438−455) glycinin G2	gi 351725363
ATSLDFPALWLLK	1474.31	1473.82	*f* (334−346) glycinin G2	gi 351725363
FAPEFLKEAFG	1255.11	1254.63	*f* (221−231) glycinin G2	gi 351725363
GANSLLNALPEEVIQHTFN	2067.89	2066.04	*f* (437−455) glycinin G2	gi 351725363
IVRNLQGENEEEDSGAIVTVK	2300.47	2299.17	*f* (236−256) glycinin G2	gi 351725363
LDFPALWLLK	1215.55	1214.71	*f* (337−346) glycinin G2	gi 351725363
TSLDFPALWLLK	1404.65	1402.79	*f* (335−346) glycinin G2	gi 351725363
VSIIDTNSLENQLDQMPR	2073.19	2072.02	*f* (160−177) glycinin G2	gi 351725363

[a]Peptide sequences listed were found predominant with more than 2 hits (some were up to 20 hits), with identity confirmed at a confidence of at least 95% ($P < 0.05$).

Table 4. Predominant Protein Sequences Identified by LC-MS/MS[a] in both Pepsin-Hydrolyzed SPI and Pepsin-Hydrolyzed Glycinin-Rich Fraction

putative sequence	exptl mass (Da)	theor mass (Da)	protein source	accession no.
LTLPALRQFQLSAQ	1585.925	1584.898	*f* (415−428) glycinin G4 (A5, A4, B3)	gi 351734402
NALEPDHRVESEGGL	1623.605	1621.769	*f* (38−52) glycinin G4 (A5, A4, B3)	gi 351734402
NSLTLPALRQ	1112.565	1111.635	*f* (413−422) glycinin G4 (A5, A4, B3)	gi 351734402
NSLTLPALRQFQLSAQ	1786.945	1785.973	*f* (413−428) glycinin G4 (A5, A4, B3)	gi 351734402
NSLTLPALRQFQLSAQY	1949.965	1949.037	*f* (413−429) glycinin G4 (A5, A4, B3)	gi 351734402
RAIPSEVLAHSYNLRQSQVSEL	2497.718	2496.308	*f* (518−539) glycinin G4 (A5, A4, B3)	gi 351734402
VVAEQAGEQGFE	1262.825	1262.577	*f* (487−498) glycinin G4 (A5, A4, B3)	gi 351734402
IAVPTGVAWW	1098.965	1098.586	*f* (139−148) glycinin G2	gi 351725363
IAVPTGVAWWM	1230.725	1229.626	*f* (139−149) glycinin G2	gi 351725363
IIYALNGRAL	1103.385	1102.649	*f* (371−380) glycinin G2	gi 351725363
IYIQQGNGIF	1152.345	1151.597	*f* (88−97) glycinin G2	gi 351725363
KTNDRPSIGNLAGANSL	1727.905	1726.896	*f* (425−441) glycinin G2	gi 351725363
KTNDRPSIGNLAGANSLL	1840.345	1839.98	*f* (425−442) glycinin G2	gi 351725363
LKEAFGVNM	1008.285	1007.511	*f* (226−234) glycinin G2	gi 351725363
NALPEEVIQHTFNL	1624.845	1623.825	*f* (443−456) glycinin G2	gi 351725363
NSIIYALNGRAL	1304.565	1303.724	*f* (369−380) glycinin G2	gi 351725363
VSFKTNDRPSIGNLAGANSLL	2174.385	2173.149	*f* (422−442) glycinin G2	gi 351725363
VSIIDTNSLENQLDQMPRRF	2377.058	2375.19	*f* (160−179) glycinin G2	gi 351725363
VSIIDTNSLENQLDQMPRRFYLAG	2781.008	2779.396	*f* (160−183) glycinin G2	gi 351725363
YLAGNQEQEF	1198.325	1197.53	*f* (180−189 glycinin G2	gi 351725363
YVSFKTNDRPSIGNLAGANSL	2224.025	2223.128	*f* (421−441) glycinin G2	gi 351725363
YVSFKTNDRPSIGNLAGANSLL	2337.145	2336.212	*f* (421−442) glycinin G2	gi 351725363
KTNDTPMIGTLAGANSLL	1817.025	1815.94	*f* (435−452) glycinin G1	gi 356505023
PALSWLRL	955.4054	954.5646	*f* (350−357) glycinin G1	gi 356505023
TATSLDFPALSW	1308.545	1307.639	*f* (343−354) glycinin G1	gi 356505023
TSLDFPALSW	1136.465	1135.555	*f* (345−354) glycinin G1	gi 356505023
YVSFKTNDTPMIGTLAGANSLL	2313.665	2312.172	*f* (431−452) glycinin G1	gi 356505023

[a]Peptide sequences listed were found predominant with more than 2 hits (some were up to 20 hits), with identity confirmed at a confidence of at least 95% ($P < 0.05$).

common peptides identified in both papain-hydrolyzed SPI and papain-hydrolyzed β-conglycinin-rich fraction, few peptides were uniquely identified in each (data not shown). Most of the unique peptides identified in the papain-hydrolyzed SPI, and absent in the papain-hydrolyzed β-conglycinin-rich fraction, originated from glycinin. This observation indicated that papain, in addition to targeting the β-conglycinin subunits, did in fact hydrolyze some of the glycinin subunits within the SPI matrix. Thus, whereas both enzymes showed a preference to one protein component over another, pepsin was more selective than papain under the conditions tested in this study.

Effect of Thermal Treatment on the ACE-Inhibitory Activity of Papain-Hydrolyzed SPI. The overall goal is to avoid fractionation and produce a hydrolysate with significant ACE -inhibitory activity. Therefore, among the four hydrolysates, papain-hydrolyzed SPI was chosen for further investigation of its bioactive stability after thermal treatment. The IC_{50} of the papain-hydrolyzed SPI was not significantly affected by any of the thermal treatments or moisture levels (Table 5). Whereas some researchers have reported degradation and loss of activity of bioactive peptides after thermal processing,[2] others have reported thermal stability of potent ACE-inhibitory peptides isolated from soy protein hydrolysates.[40] In this study, the peptides responsible for the ACE-inhibitory activity did not seem to take part in any irreversible chemical interaction upon heating at both moisture levels. Therefore, the ACE-inhibitory activity of the produced hydrolysates using papain can be considered to be thermally stable under the processing conditions tested. Determining the sequence of the peptides released will provide further explanation for the observed stability.

Table 5. Effects of Heat Treatments on the ACE-Inhibitory Activity of Papain-Hydrolyzed SPI

treatment			
moisture (%)	temperature (°C)	time (min)	IC_{50}[a] (mg protein/mL)
lyophilized	22 (control)	0	0.181 ± 0.01 a
0.18	120	10	0.179 ± 0.01 a
		15	0.169 ± 0.02 a
	175	10	0.190 ± 0.02 a
		15	0.171 ± 0.01 a
80	120	10	0.174 ± 0.01 a
		15	0.176 ± 0.02 a
	175	10	0.212 ± 0.02 a
		15	0.193 ± 0.02 a

[a]IC_{50} means for each hydrolysate with different lower case letters are significantly different according to Tukey–Kramer multiple-means comparison test ($P \leq 0.05$); $n = 3$. The lower the IC_{50} value, the higher the potency.

The results of this work confirmed that β-conglycinin is a better precursor of ACE-inhibitory activity peptides than glycinin. Further investigation to identify the peptides with the potent antihypertensive activity, released upon papain hydrolysis of SPI, would be a natural follow-up to the present study. This work demonstrated, for the first time, that selective and targeted hydrolysis can be accomplished when the protein is not fully denatured, releasing ACE-inhibitory peptides without having to induce extensive hydrolysis and undergo unnecessary fractionation. This approach will not only result in a bioactive and functional ingredient, it will also reduce production costs and eliminate additional waste streams. With limited human trials to confirm the potency of the bioactivity observed in vitro, it is hard to make a generalized conclusion. However, this work provides the basis for further investigation to determine the dosage of such a protein ingredient that may elicit bioactivity in vivo, which is a crucial step prior to launching and advertising a bioactive ingredient or product.

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Notes

The authors declare no competing financial interest.

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