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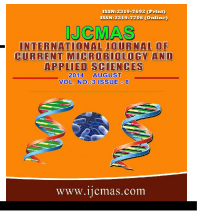

Original Research Article

Antimicrobial and antioxidant properties of β-conglycinin and glycinin from soy protein isolate

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Keywords

Soy protein isolate, glycinin, β-conglycinin, antimicrobial, antioxidant, biological activity

A B S T R A C T

Soybeans are legumes of global interest owing to their high protein content (40%) and high productivity. Bioactive peptides present in soybeans have been recognized to prevent some diseases; therefore, the goal of the present study was to produce a soy protein isolate and to isolate 2 proteins, glycinin and β-conglycinin, hydrolyze these proteins, and test the resulting peptides for their *in vitro* antimicrobial and antioxidant activity. Results showed that glycinin peptides had better activity against most microbial strains; however; β-conglycinin peptides and glycinin peptides had equivalent antimicrobial activity against *Escherichia coli*. Assays showed that antioxidant activity was dependent on the concentrations of glycinin and β-conglycinin peptides. In both groups of peptides, the highest concentrations produced the strongest antioxidant effects, but glycinin peptides were more effective than β-conglycinin peptides. Therefore, glycinin peptides exhibited stronger antioxidant effects than β-conglycinin peptides, as well as stronger antimicrobial activity against all tested microbes, with the exception of *E. coli*. These peptides represent an excellent natural source for antimicrobial and antioxidant compounds with potential for use as therapeutic agents.

Introduction

Soybeans (*Glycine max*) are an abundant source of proteins that have been recognized for their high nutritional value and bioactive properties. Soybeans are legumes, and possess high protein content (approximately 40%) as compared to most other legumes (Aguirre *et al*., 2008; Yimit *et al*., 2012). The health benefits of soybeans are attributed to the presence of bioactive compounds, and in particular proteins. Glycinin and β-conglycinin are globulins that are the major components of soy protein isolates (SPIs), accounting for approximately 85% of soybean protein content (Scilingo and Añon, 2004; Vernaza *et al*., 2012). Glycinin is a polypeptide that is composed of 2 acidic and basic subunits that are connected with disulfide bonds. β-

conglycinin is a glycoprotein with mannose and glucosamine residues (Barac *et al*., 2004; Castro and Fontes, 2005; Kuippers and Gruppen, 2008).

All plants produce peptides for antimicrobial control (Kokoska *et al*., 2002). These antimicrobials represent a fertile untapped source of natural compounds that can be used as therapeutic agents (Zasloff, 2002). The growing problem of resistance to conventional antibiotics and the necessity for new antibiotics has stimulated an interest in the development of antimicrobial peptides (AMPs) as human therapeutics (Maróti, 2011). AMPs are peptides that can kill microorganisms, and they often exhibit a broad spectrum of activity against gram-positive and gram-negative bacteria (Kamatou *et al.*, 2005). In recent years, natural molecules with antioxidant activity, especially antioxidant peptides, have drawn the attention of researchers due to their low molecular weights, good absorption profiles, and strong biological activities (Xie *et al.*, 2008).

Thus, research on the antimicrobial and antioxidant activities of plant constituent compounds, and especially the exploration of phytochemicals with low cytotoxicity, has become an important branch of biomedicine. The goal of the present study was to further elucidate the antimicrobial and antioxidant activities of glycinin and β-conglycinin extracted from SPI.

Materials and Methods

Materials

Defatted soybean flour containing 46% protein was kindly provided by IMCOPA-Importação, Exportação e Indústria de Óleos Ltda, Araucária-Paraná, Brazil. Hyaluronic acid, hyaluronidase enzyme (Type IV-S bovine), pepsin, TTC (2,3,5-triphenyltetrazolium chloride), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), β-carotene, pepsin, linoleic acid (99%), potassium persulfate, Tween 40, and isoflavone standards (genistin, daidzin, glicitin, genistein, daidzein, and glycitein) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Preparation of SPI

The SPI was prepared with defatted soybean flour. Briefly, SPI was extracted with water and the pH was adjusted to 9.5 (flour/water ratio of 1:20, w/v), after which the mixture was stirred at 10,000 rpm for 24 h at 25 °C. The suspension was then filtered through gauze and the filtrate was centrifuged at 9,000 rpm for 20 min at 4 °C. The supernatant was adjusted to pH 4.5 with citric acid and stirred at 8,500 rpm for 24 h at 25 °C. The precipitate that formed was separated by centrifugation at 10,000 rpm for 30 min at 4 °C. The precipitate was then suspended in distilled water and the pH adjusted to 7.0. The SPI suspension was dialyzed with a dialysis membrane (Spectra/Por® 6- 3.500 kDa cut-off) in distilled water for 48 h, after which it was freeze-dried and stored at 4 °C until the moisture and ash content were determined. The protein content of the SPI was measured by the Kjedahl method and multiplied by a conversion factor of 6.25 to calculate the total protein content (AOAC, 1995).

Extraction of glycinin and β-conglycinin proteins

Glycinin and β-conglycinin were extracted from the SPI according to the methods of

Nagano *et al.* (1992) and Giora (2009). The SPI was dispersed in distilled water (1:10, w/v), dry $NaHSO_3$ was added (0.98 g/L), and the pH was adjusted to 6.4. The mixture was kept overnight at 4 °C, after which the suspension was centrifuged at 9,500 rpm for 30 min at 4 °C. The glycinin precipitate was suspended in distilled water and the pH was adjusted to 7.0. NaCl (0.25 mol/L) was added to the supernatant and the pH was adjusted to 5.5. The insoluble fraction was removed by centrifugation at 9,500 rpm for 30 min at 4 °C. The pH of the supernatant was adjusted to 4.8 and it was centrifuged at 9,500 *g* for 30 min at 4 °C. The β-conglycinin precipitate was suspended in distilled water and the pH was adjusted to 7.0. Glycinin and β-conglycinin were dialyzed with a dialysis membrane (Spectra/Por® 6- 3,500 kDa cut-off) in distilled water for 48 h.

The moisture and ash content were determined by previously described methods (AOAC, 1995). The protein concentrations were determined by the Lowry method (Lowry *et al*., 1951). The suspensions were freeze-dried and stored at 4 °C.

Hydrolysis of SPI, glycinin, and β-conglycinin

The hydrolysis protocol was performed as described by Penã-Ramos and Xiong (2002), as well as by Roblet *et al.* (2012), with optimization for our samples. SPI, glycinin, and β-conglycinin were hydrated with distilled water (2% w/v) and pre-heated for 30 min at 37 °C. The pH was then adjusted to 1.3 with 2N HCl and a pepsin solution that was diluted in 0.01N HCl and added to a final enzyme-substrate ratio of 1:100, and the mixture was hydrolyzed for 2 h. The reaction was stopped by increasing the pH to 7.0 with 2N NaOH. After SPI, glycinin and β-conglycinin protein hydrolysis, the proteins were centrifuged at 8,000 rpm for 10 min to remove unhydrolyzed residues. All hydrolysates were dialyzed with a dialysis membrane (Spectra/Por® 6- 3,500 kDa cut-off) in distilled water for 48 h, after which the suspensions were freeze-dried and stored at 4 °C.

Gel electrophoresis of SPI, glycinin, and β-conglycinin hydrolysates

Gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970). The samples were prepared in reduced conditions with β-mercaptoethanol using 12% separating gel and 5% stacking gel, and known commercial molecular weight markers (Amersham full-range rainbow) were used to determine molecular mass. Electrophoresis was performed at 200 V for 5 h. The gels were stained with Coomassie Brilliant Blue R-250 overnight and destained with a solution of methanol and glacial acetic acid. Images were taken using a Transilluminator Loccus L.PIX Molecular Imager® (Loccus Biotecnologia, Cotia, São Paulo, Brazil).

Pathogenic bacterial strains and growth conditions

Gram-positive and gram-negative bacteria used in this study were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). All bacterial strains were maintained in 20% glycerol stock at -20 °C. *Escherichia coli* ATCC 26922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were reactivated in Mueller Hilton broth and incubated at 37 °C for 48 h. *Salmonella enterica* ATCC 13312 and *Klebsiella pneumonie* ATCC 13883 were reactivated in nutrient broth and incubated at 37 °C for 48 h. *Streptococcus mutans* ATCC 25175 was reactivated in tryptic soy broth and

incubated at 37 °C for 48 h. *Propionibacterium acnes* ATCC 6919 was reactivated in reinforced clostridial broth and incubated at 37 °C for 7 days. All strains were regenerated twice before use in the tests (Yaltirak *et al*., 2009).

Agar well diffusion technique

The agar well diffusion technique was performed with slight modifications (Kalemba and Kunicka, 2003; Yaltirak *et al*., 2009; Oliveira et al., 2011). The experimental design involved 3 concentrations (1,000, 450 and 50 µg/mL) of glycinin and β-conglycinin peptides, the antibiotic positive control tetracycline (30 µg/mL), and sterile distilled water as a negative control. Briefly, the peptide suspensions in distilled water (pH 7.0) were sterilized by filtering through a 0.22-µm membrane (Millipore, Billerica, MA, USA). The bacterial suspensions in NaCl solution (0.85%) were adjusted by comparison against the 0.5 McFarland standards. Petri dishes containing 20 mL of culture medium and 1 mL of bacterial suspensions were prepared; after the agar had solidified, wells (9.0 mm) were made in the agar and 150 µL of each sample, or the positive or negative controls, were added. The plates were incubated at 37 °C for a time period appropriate for each strain. The experiment was carried out with 3 replicate determinations and the antibiosis effects were determined by measuring inhibited halos (mm) that formed around the wells.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined according to the method of Oliveira *et al.* (2011) with slight modifications. The glycinin and β-conglycinin peptides suspensions (50, 25, 12.5, 6.25, 3.12 µg/mL) and bacterial suspensions were prepared using the same methods that were used for the agar well diffusion technique. In a 24-well plate, 1.8 mL of culture medium, 100 µL of bacterial suspensions, and 100 µL of the appropriate concentration of each peptide were added to each well. For *P. acnes* and *S. mutans*, this technique was performed in glass tubes. The plates and glass tubes were incubated for 48 h, except for *P. acnes*, which was incubated for 7 days.

After incubation, 20 µL of 1% TTC was added to all wells and plates and glass tubes were incubated again for 30 min. Following incubation, the wells that showed a pink color were considered to contain culture-resistant bacteria (+) and those with no color change were considered to contain culture-sensitive bacteria (-). Non-inoculated broth and cells suspensions of bacteria were used as negative and positive controls, respectively. The experiment was carried out in 3 replicates for each strain. The MIC was defined as the lowest concentration of the glycinin or β-conglycinin peptides that inhibited visible bacterial growth.

Antioxidant capacity

DPPH radical scavenging assay

The DPPH radical scavenging assay was performed according to previously described methods (Rufino *et al.*, 2007a; Predes, *et al*., 2011). Suspensions of glycinin and β-conglycinin peptides were prepared (1000, 500, 250, 125, 62.5, 31.3, 15.6 µg/mL). A DPPH solution (200 µL) and methanol (50 µL) were added to each well of a 96-well plate, followed by the control or sample dilutions and plates were incubated in the dark for 20 min at room temperature, after which the absorbance at 517 nm was compared with the methanol control sample using a microplate reader (Power Wave XS;

Biotek, Winooski, VT, USA). Results were determined every 10 min for 80 min.

The percentage inhibition of the DPPH radical was calculated as follows (equation 1):

(1) DPPH radical scavenging activity (%) = (*Ac* - *A* / *Ac*) x 100

Where *Ac* is the absorbance of the control solution and *A* is the absorbance of the samples. Results were plotted and analyzed by exponential regression to obtain the concentration of antioxidant necessary to decrease the initial DPPH concentration by 50% (IC_{50}).

ABTS assay

The ABTS assay was performed according to previously described methods (Rufino *et al*., 2007b; Zhu *et al*. 2011). The concentrations of glycinin and β-conglycinin peptides were identical to those used in the DPPH assay. ABTS solution (200 µL) and Trolox (50 µL) were added to each well of a 96-well plate, followed by the control or sample dilutions, and the plate was incubated in the dark for 6 min at room temperature, after which the absorbance at 734 nm was measured with a microplate reader (Power Wave XS; Biotek, Winooski, VT, USA). The antioxidant activity was calculated throughout the range of the dose-response curve of Trolox (µM Trolox/g of sample) and expressed as Trolox equivalent antioxidant capacity (TEAC).

β-carotene bleaching assay

The β-carotene bleaching assay was performed according to previously described methods (Duarte-Almeida *et al*., 2006; Rufino *et al*., 2010; Tyug, Prasad, and Ismail, 2010) with slight modifications. The concentrations of glycinin and β-conglycinin peptides were identical to those used in the DPPH assay. The reaction mixture (250 µL) and methanol (20 µL) were added to each well of a 96-well plate, followed by the control or sample dilutions, and the plate was kept at 37 °C for 5 min, after which the absorbance at 470 nm was measured with a microplate reader (Power Wave XS; Biotek, Winooski, VT, USA). An ascorbic acid standard was used and results were determined every 15 min for 100 min. Antioxidant activity was calculated using oxidation inhibition percentages as follows (equations 2–4):

(2) $Ac = Abs_{initial} - Abs_{final}$

(3) $Aam = Abs_{initial} - Abs_{final}$

(4) *AA* (%) = (*Ac* - *Aam* / *Ac*) x 100

Where *Ac* and *Aam* are the measured absorbance of the control and experimental samples, respectively.

Isoflavones of glycinin and β-conglycinin peptides

The extraction of isoflavone compounds was carried out according to previously described methods (Mantovani *et al*., 2013). The glycinin and β-conglycinin peptides were diluted in 15 mL of 80% methanol, and the resulting mixture was centrifuged for 15 min at 5,000 rpm. The supernatant was filtered with Whatman filter paper (grade 1) and through a filter with a 0.45-µm membrane pore size (Millipore, Billerica, MA, USA).

Isoflavone identification and quantification were carried out via HPLC, which was conducted in a Shimadzu chromatograph through a Hypersil ODS C 18 (250 mm x 4.6 mm i.d. x 5 µm) column (Shimadzu, Kyoto, Japan) with a quaternary pump (Shimadzu LC-10ADVP) and the Shimadzu CLASS-VP® Release (version 6.14 SP1) software system, with a UV wavelength of 254 nm and a mobile phase flow rate of 1 mL min^{-1}. Isoflavones content was expressed as µg/mg of sample.

Amino acid analysis

The samples were hydrolyzed with 6N hydrochloric acid containing 0.2% phenol and 0.2% sodium azide (w/v). The hydrolysis was conducted at approximately 105 °C for 24 h (Fountoulakis and Lahm, 1998). The solution was then filtered with a membrane with a 0.22-µm pore size (Millipore, Billerica, MA, USA) to remove all particles in suspension and evaporated to eliminate the acid. The supernatant was then dissolved with solution buffer and analyzed with a Sykam amino acid analyzer (model S433; Sykam GmbH, Eresing, Germany).

Statistical analysis

The results obtained in the study were expressed as mean ± standard deviation from 3 replicate determinations. Differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. P-values < 0.05 were considered to be statistically significant.

Results and Discussion

Protein content of SPI and the β-conglycinin and glycinin

Table 1 shows the characteristics of SPI and the β-conglycinin and glycinin protein. The SPI protein content was 94.6% as determined by the Kjedahl method, and was thus considered to be a protein isolate, because it was greater than 90% protein on a dry basis (Deak and Johnson, 2007). The isolated β-conglycinin and glycinin samples were 83.8% and 88.4% protein, respectively, which were similar to the levels described by Puppo *et al*. (2011), who reported protein content of 85.9% for β-conglycinin and 88.2% for glycinin, and Tarone *et al*. (2013), who reported protein content of 84.3% for β-conglycinin and 83.0% for glycinin; these groups extracted β-conglycinin and glycinin from SPI by the same methods that were used in this study. In contrast, Samoto *et al*. (2007) reported protein levels of 87.0% for the β-conglycinin fraction and 93.1% for the glycinin fraction, which were obtained using a different method of extraction, in which sulfuric acid was used for protein isolation from defatted soybean flour.

Gel electrophoresis of SPI, β-conglycinin and glycinin hydrolysates

The electrophoretic profiles of SPI and the proteins after hydrolysis are shown in Figure 1. The molecular weight of β-conglycinin constituents ranged from 48 to 75 kDa. The glycinin constituents with molecular weights measured at approximately 30 kDa and 20 kDa were the acidic and basic subunits, respectively. According to Sitohy *et al*. (2012), the isolation of these 2 subunits indicated good separation. This electrophoretic procedure shows only the constituent subunits, because it was conducted in the presence of β-mercaptoethanol. The SDS-PAGE profiles were in accordance with the available literature, including Medrano and Del Castillo (2011) for SPI and glycinin, Souza (2000) for all analyses, Fassini (2010) and Bittencourt *et al*., (2007) for glycinin and β-conglycinin.

Agar well diffusion technique

Results from the agar well diffusion technique are shown in Table 2. Glycinin peptides showed stronger antibiotic activity than β-conglycinin peptides. Papagianni (2003) and Teixeira *et al*., (2012) have shown that a diverse group of peptides kills bacteria by permeabilization through the formation of stable pores through which the cellular contents leak, as well as by membrane thinning or micellization in a detergent-like manner. For peptides that have high disulfide bond content, such as

glycinin, positive charges presumably facilitate interaction with negatively charged bacterial phospholipid-containing. Halos of 33.0 mm were observed in the tetracycline-treated (30 μg/mL) positive control group, and cell shapes were unchanged in the sterile water-treated negative control group. Furthermore, the 1,000 μg/mL glycinin peptide treatment produced an inhibitory effect equivalent to that of 30 μg/mL tetracycline; therefore, high concentrations of glycinin peptides are required to produce an effect similar to that of commercial antibiotics.

Zasloff (2002) reported that gram-negative bacteria are susceptible to AMPs, because they possess lipopolysaccharides that have high levels of negative charge and facilitate linkage to cationic peptides, thus causing damage to the external membrane. Furthermore, Matsuzaki (2009) showed that gram-positive bacteria have negatively charged teichoic and lipoteichoic acids in their peptidoglycans; therefore, both strains were particularly susceptible to AMPs.

Determination of minimum inhibitory concentration (MIC)

MIC values of the glycinin and β-conglycinin peptides against all strains are shown in Table 3; lower MIC values are indicative of stronger antimicrobial activity. MIC values indicated that all bacterial strains were susceptible to the glycinin and β-conglycinin peptides. Only *E. coli* showed equivalent susceptibility to both fractions, and it was the most susceptible of the bacteria to the peptides, followed by *P. aeruginosa* and *S. aureus*. A concentration-dependent effect was observed for both fractions, but glycinin had lower MIC values than β-conglycinin in all bacteria, with the exception of *E. coli*, indicating stronger antimicrobial activity. Sitohy *et al*. (2012) determined the MICs of glycinin and β-conglycinin for other strains of bacteria and observed that glycinin had better antimicrobial activity than β-conglycinin. Similarly, in the current study, glycinin peptide was also found to be more effective than β-conglycinin peptides against both gram-negative and gram-positive strains. The basic subunit of glycinin may facilitate targeting to cell membranes, allowing electrostatic interactions between the positively charged cationic and the negatively charged phospholipid regions of cell membranes.

Isoflavones in glycinin and β-conglycinin peptides

The isoflavone levels for the glycinin peptide were 5.2 μg/mg daidzin, 11.7 μg/mg genistin, 1.3 μg/mg daidzein, and 2.4 μg/mg genistein, and the levels for the β-conglycinin peptide were 4.8 μg/mg genistin, 1.2 μg/mg daidzein, and 2.5 μg/mg genistein. An 80% methanol solution is the most commonly used reagent for isoflavone extraction in the literature, as reported by Kulling, Honig, and Metzler (2001), Zuo *et al*. (2008), and Mantovani *et al*., (2009). Wang *et al.* (1998) extracted isoflavones with 80% methanol and observed that isoflavones remain in the supernatant and are not found in the precipitated protein. Therefore, our use of this method likely contributed to the very low observed levels of isoflavones in this study. In particular, we observed a loss of daidzein in the β-conglycinin, because this isoflavone is more susceptible to changes in pH and was eliminated during the centrifugation steps.

The present study did not detect glycitin and glycitein, which is typical of studies of Brazilian soybeans, according to Lui *et al*. (2003), who showed that concentrations of isoflavones in grains are genetically

controlled and influenced by soil and climate conditions. Benassi and Prudencio (2013) reported that comparing results across different studies is difficult, because of the variability in cultivars, climate conditions (which vary by temperature, year, and location), and post-harvest processing (storage time, temperature, and humidity), which can influence isoflavones concentrations.

Amino acid analysis

The amino acid profiles of β-conglycinin and glycinin proteins are shown in Table 4. The amino acid valine was absent in β-conglycinin. Pickering and Newton (1990), as well as Fountoulakis and Lahm (1998), have reported that aliphatic amino acid residues, such as valine, can be resistant to acid hydrolysis, and therefore extra time may be necessary for hydrolysis; this was probably the case in the present study. The ammonia present in both proteins is residue from hydrolysis, which the equipment does not recognize as protein.

Lysine, arginine, and histidine are cationic amino acids common to antimicrobial peptides (AMPs) that are important for electrostatic interactions with negatively charged pathogens. Furthermore, aromatic amino acids such as phenylalanine and tyrosine are considered to play an important role in antimicrobial effects by anchoring peptides to the bacterial membranes (Teixeira *et al*., 2012). Hydrophobic amino acids such as alanine, valine, leucine, isoleucine, and methionine aid in AMP binding, destabilization of phospholipid membranes, and antimicrobial action (Strömstedt *et al*., 2010). The amino acid profiles obtained herein are similar to those reported by Samoto *et al*. (2007), who extracted β-conglycinin and glycinin using a different method, with the exception of tryptophan, which was not identified in this study.

Antioxidant capacity

Antioxidant capacity was determined by DPPH, ABTS, and β-carotene methods (Table 5). For both peptides, the 1000 μg/mL concentration had the highest antioxidant capacity in the ABTS assay and the β-carotene bleaching assay, but the glycinin peptide produced stronger antioxidant effects than the β-conglycinin peptide. In the DPPH assay, low IC_{50} values indicate strong radical scavenging activity (Zhu *et al*., 2011). For both peptides, the 250 μg/mL concentration showed the strongest DPPH radical scavenging activity. We were unable to obtain results for the 1000 μg/mL and 500 μg/mL glycinin and β-conglycinin peptides concentrations in the DPPH assay, probably due to the intense staining of these samples.

Several studies have demonstrated the ability of proteins to inhibit lipid oxidation in foods. Proteins originating from milk and soybeans have been shown to exhibit antioxidant activity due to their abilities to inactivate reactive oxygen species, scavenge free radicals, chelate pro-oxidative transition metals, reduce hydroperoxides, enzymatically eliminate specific oxidants, and alter the physical properties of foods to separate reactive species (Elias *et al.,* 2008).

Some amino acids, such as histidine, tyrosine, methionine, and cysteine, have been reported to show antioxidant activity. In particular, histidine exhibited strong radical scavenging activity due to the decomposition of its imidazole (Xie *et al*., 2008). Chen *et al*. (2012) isolated alanine, aspartic acid, and phenylalanine from walnut (*Juglans regia*), and these hydrophobic amino acids showed antioxidant activity in the β-carotene bleaching assay.

Table.1 Characteristics of soy protein isolate, β-conglycinin, and glycinin proteins.

Sample	Protein* (%)	Moisture (%)	Ash (%)
SPI	94.6 ± 0.2**,a	2.6 ± 0.1[a]	2.1 ± 0.05[a]
β-conglycinin	83.8 ± 0.4[b]	2.2 ± 0.3[b]	1.6 ± 0.02[b]
glycinin	88.4 ± 0.2[c]	2.0 ± 0.2[c]	2.3 ± 0.03[c]

* Values in the same column followed by different letters are significantly different as determined by Tukey's test ($p < 0.05$).
** Mean ± standard deviation of 3 replicates.

Table.2 Antibiosis activity of glycinin (1) and β-conglycinin (2) peptides against gram-positive and gram-negative bacteria as indicated by halo inhibition (mm)

Strains	1000 μg/mL		450 μg/mL		50 μg/mL	
	1*	2	1	2	1	2
E. coli	32.0 ± 0.57**Aa	24.0 ± 0.57Db	18.0 ± 1.00Bc	14.0 ± 1.52Ed	11.0 ± 0.50Ce	08.5 ± 0.30Ff
K. pneumonie	30.5 ± 1.00Aa	23.0 ± 0.50Db	16.5 ± 0.00Bc	13.5 ± 0.00Ed	10.0 ± 0.30Ce	08.0 ± 0.30Ff
P. acnes	28.5 ± 0.76Aa	21.0 ± 0.57Db	13.5 ± 0.50Bc	09.0 ± 0.86Ed	07.0 ± 0.76Ce	04.0 ± 0.30Ff
P. aeruginosa	30.5 ± 0.86Aa	22.0 ± 0.57Db	17.0 ± 0.30Bc	12.0 ± 0.50Ed	09.5 ± 0.50Ce	08.0 ± 0.57Ff
S. aureus	29.0 ± 0.00Aa	23.0 ± 0.76Db	15.5 ± 0.00Bc	10.0 ± 0.57Ed	08.0 ± 0.30Ce	06.5 ± 0.50Ff
S. enterica	31.0 ± 0.30Aa	23.0 ± 1.05Db	17.5 ± 0.50Bc	14.0 ± 1.73Ed	09.0 ± 0.30Ce	07.0 ± 0.00Ff
S. mutans	27.0 ± 0.30Aa	22.5 ± 0.00Db	15.0 ± 0.00Bc	11.0 ± 0.76Ed	06.5 ± 0.30Ce	05.0 ± 0.00Ff

* Upper-case letters show significant differences between concentrations of the same peptide in the same strain, and lower-case letters across the rows show significant differences between peptide treatments in the same strain, as determined by Tukey's test ($p < 0.05$).
** Mean ± standard deviation of 3 replicates.

Table.3 Determination of minimum inhibitory concentrations (MIC) of glycinin (1) and β-conglycinin (2) peptides against bacterial strains

Strains	MIC (μg/mL)	
	1	2
E. coli	3.12	3.12
K. pneumonie	6.25	12.5
P. acnes	6.25	25.0
P. aeruginosa	3.12	12.5
S. aureus	3.12	6.25
S. enterica	6.25	12.5
S. mutans	12.5	25.0

Table.4 Amino acid profiles (% w/v) of glycinin (1) and β-conglycinin (2) peptides

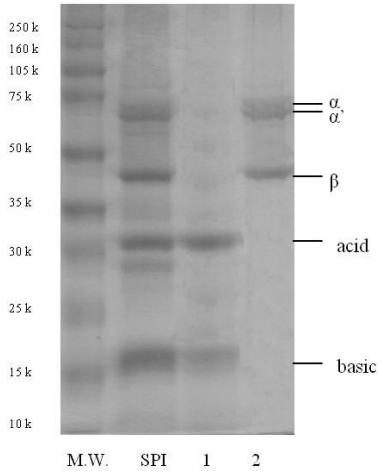
Amino acids	1	2
Aspartic acid	9.1	9.6
Threonine	3.9	4.2
Serine	6.3	5.7
Glutamic acid	8.5	8.2
Proline	0.4	0.2
Glycine	6.7	6.0
Alanine	4.8	4.5
Cysteine	5.7	5.4
Valine	1.1	-
Methionine	2.9	2.6
Isoleucine	6.1	6.3
Leucine	2.4	2.1
Tyrosine	2.6	2.5
Phenylalanine	4.6	4.4
Histidine	7.4	7.2
Lysine	11.3	10.1
Arginine	6.2	6.0
Ammonia	10.0	15.0

Table.5 Antioxidant capacity of glycinin and β-conglycinin peptides*

Peptides	DPPH IC_{50}	$ABTS^{\cdot+}$ μM Trolox/g	βCB % O.I.
glycinin	$46.8 \pm 1.2^{**a}$	28.5 ± 3.1^{a}	71.7 ± 2.0^{a}
β-conglycinin	71.6 ± 2.6^{b}	15.2 ± 2.5^{b}	56.3 ± 1.5^{b}

* Mean ± standard deviation of 3 replicates.
βCB: β-carotene bleaching.
O.I.: Oxidation inhibition.
**Values in the same columns followed by different letters are significantly different as determined by Tukey's test ($p < 0.05$).

Figure.1 SDS-PAGE of soybean protein isolate, glycinin (1), and β-conglycinin (2). The number on the left indicate the molecular weight markers (k)



Sarmadi and Ismail (2010) described the mechanisms of action of amino acids: aromatic amino acids convert radicals to stable molecules by donating electrons while keeping their own stability; hydrophobic amino acids enhance the solubility of peptides in lipids, which facilitates accessibility to hydrophobic radical species; and acidic and basic amino acids contain carboxyl and amino groups in their side chains, which act as chelators of metal ions and as hydrogen donors. Dietary use of antioxidants has been shown to promote health by increasing antioxidant capacity (Samaranayaka and Li-Chan, 2011).

Soy proteins are an excellent source of bioactive compounds. The biological activity of the glycinin and β-conglycinin hydrolysates was confirmed, and glycinin peptides were found to produce stronger antimicrobial and antioxidant effects than β-conglycinin peptides. We detected very small amounts of isoflavones, which displayed concentration-dependent antioxidant activities in 3 different tests *in vitro*. Further studies of the amino acid sequences of glycinin peptide constituents are necessary to understand the structure-activity relationships of these peptides, and thus to elucidate their antioxidative and antimicrobial mechanisms of action.

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