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ORIGINAL PAPER

Identification and Validation of Soy Peptides with In-vitro Hemagglutination Activity

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Abstract We previously demonstrated that different enzyme hydrolysates of soybean agglutinin (SBA), β-conglycinin and glycinin-rich fractions had in-vitro hemagglutination activities. In the present study, the three proteins were subjected to trypsin hydrolysis, and *N*-acetyl-D-galactosamine (GalNAc)-agarose beads were used to isolate the active peptides. Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI−TOF) Mass Spectrometry (MS) was used to determine the mass of the peptides and the molecular weights were compared to the peptide profiles given by theoretical cleavage of the proteins, so that the peptide sequence could be identified. Two peptides from SBA, 24 peptides from β-conglycinin and 16 peptides from glycinin were identified from the active peptide extracts. The two SBA peptides, three peptides from β-conglycinin having strong MS signal intensity and three peptides from glycinin with strong MS intensity were synthesized and their activities were assessed by using the in-vitro hemagglutination assay. These peptides were proven to have hemagglutination activity, whereas a synthesized control peptide from SBA did not. These results validated our previous observation of various soy protein fractions having hemagglutination activities.

Keywords Bioactive peptides · β-Conglycinin · Glycinin · Hemagglutination activity · Lectin · Soybean agglutinin

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Introduction

Bioactive peptides in food have been extensively studied. Soybeans are considered to contain various active peptides. Soy protein hydrolysates from different enzymes have different activities, such as inhibiting leukemia cells in-vitro [1], inhibiting lipid accumulation in adipocytes in-vitro [2], suppressing appetite by stimulating cholecystokinin release in rats [3], inhibiting fatty acid synthase and in-vitro adipogenic response of human adipocytes [4], antioxidative activity [5], and suppressing of colon and liver tumorigenesis [6]. The enzyme hydrolysates of glycinin also have a bile acid-binding ability, which might partially account for the hypocholesterolemic effect of soy protein [6]. Many peptides from soybeans have been identified, synthesized and shown to have different activities. For example, Chen et al. [7, 8] identified four anti-hypertensive peptides: IA, GYLAGNQ, FFL, and IYLL. A peptide from glycinin (LPRPR) reduced serum cholesterol after oral administration to mice [9]. Nishi et al. [10] identified the β 51−63 fragment (VRIRLLQRFNKRS) of β-conglycinin as having appetite control activity. A peptide from the α' subunit of β-conglycinin with the sequence MITLAIPVNKPGR has phagocytosis-stimulating activity [11]. Although soy protein hydrolysates have been extensively studied, none has shown lectin-like (hemagglutination) activity.

Soybean agglutinin (SBA, a tetramer with a 30-kDa subunit) belongs to the lectin family and has the unique property of binding to carbohydrates. SBA can bind to the carbohydrate moiety of cell surfaces and cause cells to agglutinate, and this activity is referred to as hemagglutination activity [12]. SBA is normally considered to be an anti-nutritional factor due to its ability to bind to the brush border, causing increased intestinal weight and pancreatic

hypertrophy [12]. Lectins have also been shown to have potential in drug delivery due to the carbohydrate-binding specificity [13]. However, there are problems associated with the use of lectins for drug delivery. Lectins usually have molecular weights of more than 10 kDa, which are likely to cause toxicity and immunogenicity [13]. Small lectins might overcome this problem, and several of them have been found. For example, *Selenocosmia huwena* lectin-I purified from Chinese bird spider *Selenocosmia huwena* [14], θ-defensin purified from the leukocytes and bone marrow of the rhesus macaque (*Macaca mulatta*) [15], and odorranalectin purified from skin secretions of the frog *Odorrana graham* [16]. These small active peptides or lectin-like peptides made from soy proteins may be used for therapeutic purposes.

Our research has shown that after heat denaturation at 100 °C for 20 min, SBA had 60% of hemagglutination activity remaining; after single enzyme (such as trypsin, chymotrypsin, and thermolysin) hydrolysis, SBA still had 50−60% activity; and the activity of SBA was fully eliminated by multi-enzyme hydrolysis (whenever thermolysin was used) [17]. We explained that native SBA forms a tetramer, displays full activity; heated SBA is denatured, the activity is reduced due to a three-dimensional structure change; when SBA is hydrolyzed by single enzymes, bioactive peptides are released, displaying some activity; after hydrolyzing by a combination of enzymes, the active peptides are fully destroyed, losing active sites and their binding capacity [17]. In the present study, we intended to identify which peptides in soybean proteins may have in-vitro hemagglutination activity.

SBA binds to the oligosaccharide moieties of cell surface glycoproteins to cause aggregation. This binding is carried out by the carbohydrate-binding sites in each subunit of SBA. These sites have the highest affinity for *N*-acetyl-D-galactosamine (GalNAc) [18]. We expected that the active peptides should also carry the binding sites for GalNAc, so we used GalNAc-agarose beads to extract these peptides in trypsin hydrolysates, and employed MALDI−TOF MS (Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) to identify the bound peptides. Once the peptide sequence was identified, we synthesized some of the identified peptides and tested their activities.

We had also shown previously that the soybean β-conglycinin and glycinin fractions had in-vitro hemagglutination activity [19]. After single enzyme hydrolysis, they did not show any reduction in the hemagglutination activity, but hydrolysis by a combination of enzymes fully deactivated β-conglycinin, but not glycinin [19]. We hypothesized that some peptides in β-conglycinin and glycinin have hemagglutination activity. Because hemagglutination activities of these storage proteins were also inhibited by GalNAc (data not shown), we used the same GalNAc-agarose beads to separate these active peptides and used the same methods as for SBA to identify and synthesize them and to verify their activities.

Experimental Procedures

Materials

β-Conglycinin and glycinin-rich fractions were obtained from the Center for Crops Utilization Research (CCUR) at Iowa State University. They were fractionated by Deak et al. using a two-step soy-protein fractionation procedure with 5 mM SO_2 and 5 mM $CaCl_2$ [20]. The glycinin-rich fraction contained 96% protein with 85% purity and the β-conglycinin-rich fraction contained 90% protein with 81% purity. Rabbit blood in Alsever's solution was obtained from Hemostat Laboratories (Dixon, CA) and was used within two weeks. *N*-acetyl-D-galactosamine-agarose beads were purchased from Sigma–Aldrich, with ≥6 mg/mL binding capacity for SBA. Trypsin (porcine) was purchased from Sigma–Aldrich with 10,000 BAEE units per mg protein. Other reagents were from Fisher Scientific (Pittsburgh, PA) or Sigma–Aldrich (St. Louis, MO).

Extraction and Purification of SBA

The procedure for extracting and purifying SBA as described by Lis and Sharon [21] was used. Generally, 1 kg of soy flake was dispersed in 12 L of distilled water and the pH was adjusted to 4.6 with concentrated HCl and the dispersion was incubated overnight at 4 °C to precipitate the major storage proteins. Most of the supernatant fluid was poured out and collected, and the remaining supernatant was obtained by centrifugation (3,020×*g*, 15 min). Ammonium sulfate, 300 g, was added to each L of supernatant to precipitate other proteins. Vacuum filtration was used to remove the precipitate. To each L of filtrate, an additional 270 g of ammonium sulfate was added while stirring and the mixture was incubated overnight at 4 °C to precipitate the crude SBA. The precipitate was then collected by centrifuging and resuspended in 200 mL of water and dialyzed against water for 24 h at 4 °C with two changes of water. Any insoluble material, which was present after dialysis, was removed by centrifuging and was discarded. A second precipitation of SBA was done by adjusting the pH of the solution to 4.6 with 1 N HCl and adding ammonium sulfate (56 g/100 mL of solution). The precipitate was collected by centrifugation (3,020×*g*, 15 min) and redissolved in 100 mL of 0.05 M sodium phosphate buffer, pH 6.1. The solution was dialyzed against water for 24 h at 4 °C and then lyophilized and

weighed. The different isolectins were not separated, and SBA was not purified further. This SBA was used to prepare a SBA solution with desirable mg extract/mL concentration for various treatments.

Enzyme Hydrolysis of Soy Proteins

SBA treated at 100 °C for 20 min, and β-conglycinin and glycinin-rich fractions treated at 100 °C for 5 min (all proteins were dispersed in water for heating) were subjected to trypsin (8.0 and 37 °C, the pH and temperature optima) hydrolysis in PBS (phosphate-buffered saline, 10 mM sodium phosphate, 0.9% saline of pH 8.0) with enzyme:protein ratio (w/w) of 1:16 for 24 h. The hydrolysates were used for further separation of bioactive peptides.

Separation of Peptides Bound to GalNAc-Agarose Beads

Trypsin hydrolyzed SBA, β-conglycinin or glycinin-rich fractions (6.4 mg) were mixed with about 1 mL GalNAc-agarose beads separately, and the mixtures were incubated at 4 °C overnight. The unbound peptides were collected by centrifuging the beads at $850\times g$ for 1 min. The beads were then washed twice with 0.9% saline. The bound peptides were eluted from the beads by using 600 μL of 25 mg/mL GalNAc in 0.9% saline. Both unbound and bound peptides of SBA were subjected to SDS-PAGE analysis, hemagglutination assay and MALDI−TOF MS analysis. The bound peptides from β-conglycinin and glycinin were subjected to MALDI−TOF MS analysis.

SDS-PAGE Protein Characterization

SDS-PAGE was performed according to Laemmli [22] using minigels. Briefly, 12% separating gel and 5% stacking gel were used and the electrophoresis was performed at 100 V for 1.5 h. The gels were stained with Coomassie brilliant blue G-250. Broad range protein standards from Bio-Rad Laboratories were used.

Identifying Peptides Using MALDI−TOF MS Analysis

MALDI−TOF MS analysis was conducted in the protein facility of Iowa State University. Generally, the separated peptides were desalted and concentrated by ZipTip U-C18 from Millipore (catalog #ZTC18MO96). The peptides were then washed off the ZipTip by using 0.75 μL matrix solution [α-cyano-4-hydroxycinnamic acid (ACH), 5 mg/mL in 70% acetonitrile, 0.1% TFA in water], and loaded on the sample target of the mass spectrometer. Any remaining peptides were washed off the ZipTip by 0.5 μL 70% ACN (acetonitrile) with 0.1% TFA (trifluoroacetic acid) solution and dispensed onto the same sample target, which was dried at room temperature. The samples were then loaded into the mass spectrometer and analyzed. MALDI−TOF MS analysis was performed on a PerSeptive Biosystems Voyager DE-PRO Biospectrometry workstation equipped with a nitrogen laser ($\lambda = 337$ nm). Measurement was carried out by delayed extraction and laser-desorbed positive ions were analyzed after accelerating at 20 kV in the linear mode. External calibration was performed according to the instrument standard operating procedure with a mix of angiotensin I, adrenocorticotropic hormone clip 1−17, 18−39 and 7−38, and insulin. At least 50 laser shots were obtained for each spectrum and at least three positions on a spot were analyzed for each sample.

Amino acid sequences of SBA, glycinin and β-conglycinin were obtained from ExPASy database (http://ca.expasy.org/). The sequence was subjected to a theoretical trypsin digestion using MS-Digest of University of California, San Francisco (http://prospector.ucsf.edu/cgi-bin/msform.cgi?form=msdigest). The *m*/*z* identified by MALDI−TOF MS analysis were compared with the theoretically digested peptides to obtain the peptides that have the same molecular weight, and thus giving the sequences of these peptides.

Peptide Synthesis

Peptides were synthesized at the protein facility of Iowa State University using solid phase peptide synthesis developed by Merrifield [23]. Applied Biosystems Model 433A and 432A peptide synthesizer were used. Resin was used to anchor the peptide chain as each additional alpha-amino acid is attached. FMOC (9-fluorenylmethoxycarbonyl) was used to protect the alpha-amino group of the amino acid during the reaction. Stable blocking groups were used to protect the reactive functional group of an amino acid. The peptide was cleaved from the resin using trifluoroacetic acid (TFA) and extracted with ether to remove unwanted materials such as the scavengers used in the cleavage reaction.

Hemagglutination Assay

The hemagglutination assay was performed as previously described [17], according to the method of Lis and Sharon [21] with some modification. Briefly, rabbit red blood cells collected in Alsever's solution were washed three times using 0.9% saline with two fold dilution, and subjected to trypsin hydrolysis in 10 mM phosphate buffered 0.9% saline (pH 7.4) (PBS). The red blood cells were then washed four times using 0.9% saline, and finally resuspended at 3% (v/v) in 0.9% saline. Protein samples or the

synthesized peptide samples were serially diluted in a 96-well round bottom plate with saline to give a final volume of 0.1 mL. Then 0.1 mL of 3% trypsinized red blood cells was added to each well. The plates were kept at 37 °C for 2 h for agglutination to occur. The hemagglutination units (HU) per g of sample were determined by the equation [24]:

$$\text{HU/g} = \frac{D_A \times D_B \times S}{V}$$

Where D_A is the dilution factor of the first well, D_B is the dilution factor of the well containing 1 HU (the last dilution that causes cell agglutination), S is the amount of extract in mL per gram of sample (inverse of the initial concentration), and V is the volume of extract added. Each sample was done in duplicate. The HU/g values were then expressed as log (HU/g)/log 2 to normalize the data for the two-fold dilution. Because the results may be affected by the age of the blood, the activity of the trypsin, and other factors, SBA was used as a standard in each set of assays. The results were expressed as relative activity to SBA.

Slide Agglutination Test

The agglutination assay was done according to Pennell et al. [25]. Briefly, samples were serially diluted according to the hemagglutination assay. Then 5 μL of each diluted sample was placed on a slide, followed by 5 μL of trypsin-treated 3% rabbit red blood cells (the same red blood cells as in the hemagglutination assay). They were mixed with a pipette tip and rotated by hand for 10−20 s. The mixture was then observed under a microscope to examine hemagglutination. Saline was included as a negative control. The samples with clumps of cells were considered positive and the ones with uniformly distributed cells were considered negative. The same calculation of HU as that for the hemagglutination assay was used. All tests were duplicated.

Statistical Analysis

Data were analyzed by General Linear Model (GLM) of analysis of variance (ANOVA), and the least significant differences (LSD) were calculated at the 5% level to compare treatment means using the SAS system (version 9.1, SAS Institute Inc., Cary, NC).

Results and Discussion

Activities of Separated Peptides from SBA

Trypsin is specific, only cleaving at the carboxyl side of arginine or lysine residues, and giving a small number of peptides, which made further analysis feasible. Therefore, trypsin was chosen to hydrolyze SBA to produce the starting peptide mix. Trypsin cannot hydrolyze SBA without heat treatment, so SBA was first treated at 100 °C for 20 min. After binding of the peptide mix to the GalNAc beads, we found that trypsin was not bound to the beads (Fig. 1), and in the bound fraction, there was no visible SBA, indicating an effective hydrolysis. The unbound peptides showed no hemagglutination activity, which suggests that the bound peptides might have full activity of the hydrolysate. However, there was concentrated GalNAc in the bound peptides mix when the peptides were eluted from the beads, which inhibited activities of the peptides, so we were unable to assay the activity of such peptides directly. Instead, slide agglutination test is suitable in such situation.

Activities of Identified and Synthesized Peptides from SBA

After separating peptides bound or unbound to beads, MALDI−TOF MS analysis was used to identify these peptides. Unbound and bound peptides gave different peptide profiles (Fig. 2), indicating that GalNAc specifically bound to certain peptides. The unbound fraction (Fig. 2 B) contained fewer but larger peptides, and the bound fraction (Fig. 2 A) had more and lower-molecular-weight peaks. From the results of bound peptides, the peak with m/z of 987.9 and 1456.6 were identified as SBA peptides by comparing with the protein database and by

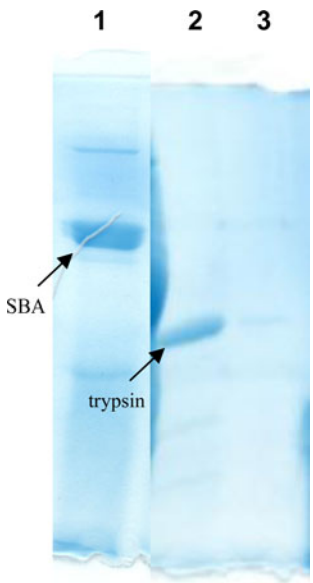

Fig. 1 SDS-PAGE of separation of peptides from soybean agglutinin trypsin hydrolysates that were bound or unbound to GalNAc beads. *Lane* 1, SBA extract; *lane* 2, peptides that were unbound to beads; *lane* 3, peptides that were bound to beads

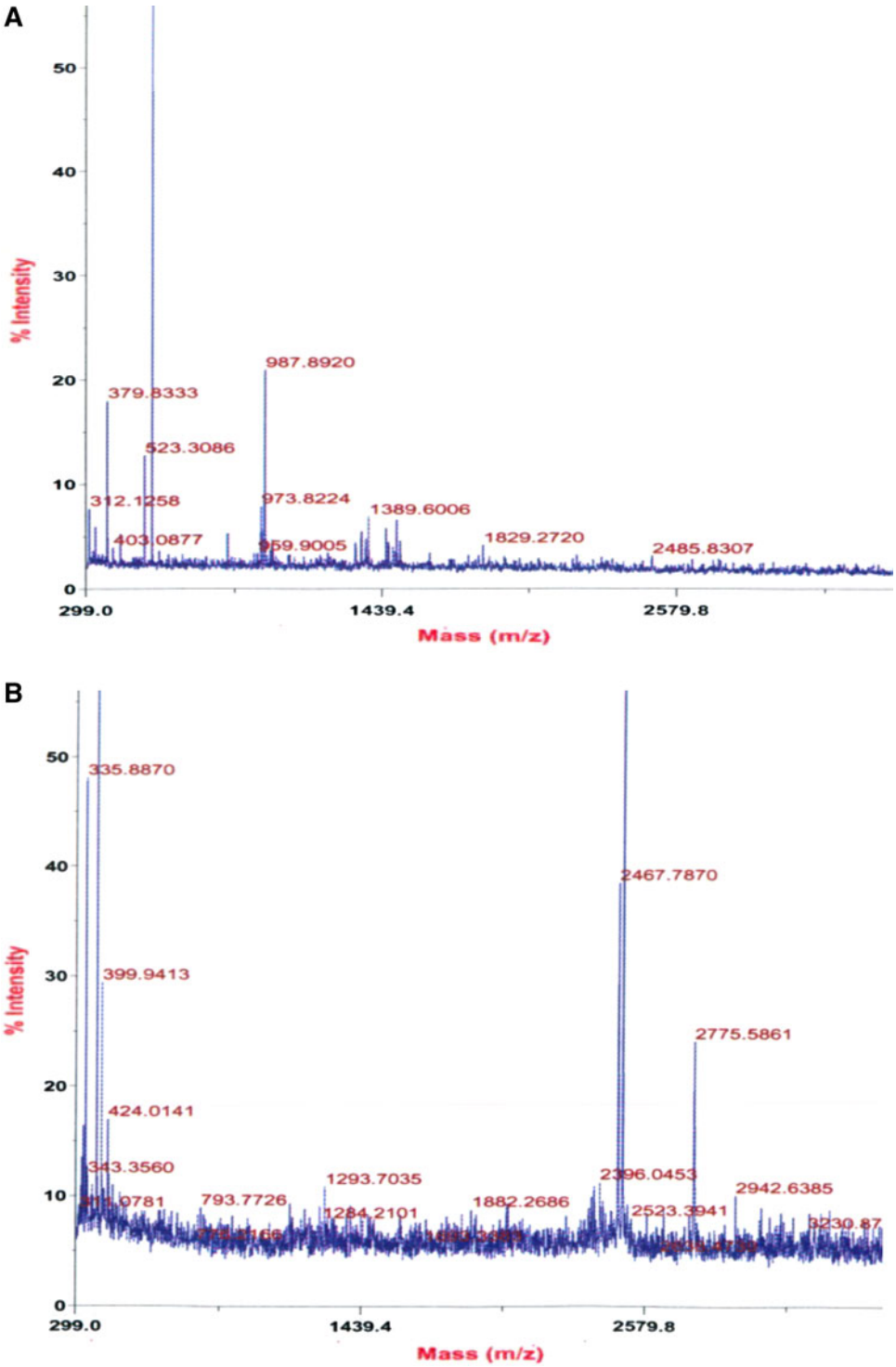

Fig. 2 MALDI−TOF MS analysis results for peptides from soybean agglutinin trypsin hydrolysates that were bound (*A*) or unbound (*B*) to GalNAc beads

theoretical cleavage by trypsin. One peptide is SBA 197−206, with the sequence of KTSLPEWVRI, and the other peptide is SBA 36−51 with the sequence of KVDENGTPKPSSLGRA. The two peptides were then synthesized and their activities were assessed. One peptide from unbound peptides: SBA 15−24 with the sequence of QPNMILQGDA was also synthesized as a negative control.

Figure 3 shows that the positive control SBA (A) had red blood cell clumps on the slide, whereas the negative control saline (B) had uniformly distributed red blood cells. For sample SBA 197−206, (C) shows the last dilution had activity, while the next dilution (D) did not. Similarly, (E) shows the last dilution for SBA 36−51, which had activity, and the next dilution (F) did not. (G) shows that a lesser dilution of SBA 36−51 had more obvious clumps than (E).

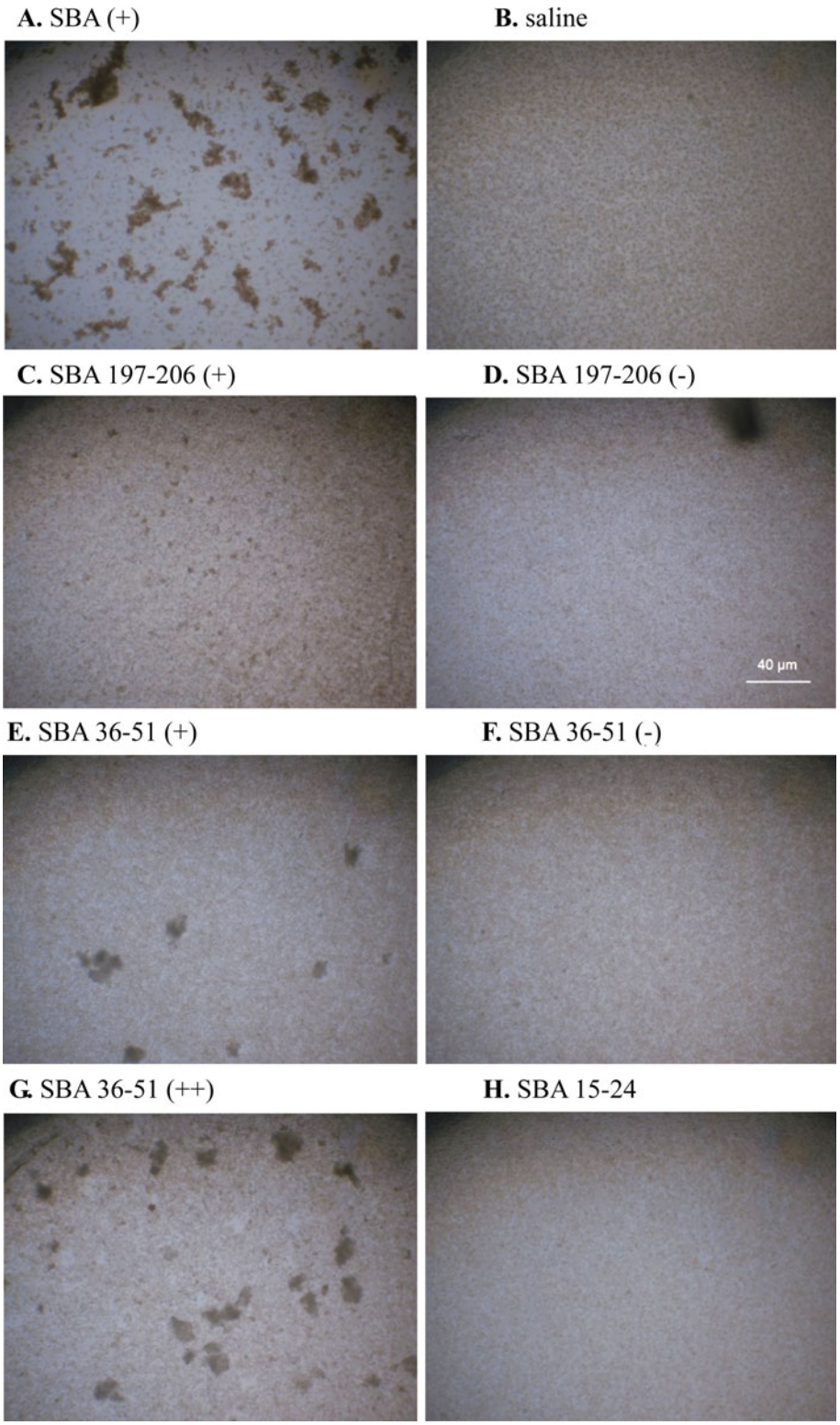

Fig. 3 Hemagglutination activities of active peptides from soybean agglutinin. The pictures are 3% rabbit red blood cells mixed with: A 2.5×10^{-3} mg/mL SBA; B 0.9% saline; C 26.8 mg/mL SBA 197−206; D 13.4 mg/mL SBA 197−206; E 3.18 mg/mL SBA 36−51; F 1.59 mg/mL SBA 36−51; G 12.7 mg/mL SBA 36−51; H 24 mg/mL SBA 15−24

On the other hand, the control peptide (SBA 15−24) did not have activity (H). The activities of the peptides were calculated according to the equation used in the hemagglutination assay and summarized in Table 1. Both of the synthesized SBA 36−51 and SBA 197−206 had activity, 38 and 49% relative to SBA, whereas the control peptide SBA 15−24 did not. This result confirmed our hypothesis that certain active peptides in SBA have in-vitro

Table 1 Hemagglutination activities of synthesized peptides from soybean agglutinin, β-conglycinin, and glycinin

Origin	*m/z* from MALDI-TOF MS	Calculated *m/z*	Peptides synthesized	Amino acid sequence[a]	Relative activity to SBA (%)	Minimum concentration for activity (mg/mL)
Soybean agglutinin	987.9	987.5	SBA 197−206	(K)TSLPEWVR(I)	38.1 ± 3.7	26.8
	1,456.6	1,456.7	SBA 36−51	(K)VDENGTPKPSSLGR(A)	48.8 ± 2.0	3.2
β-Conglycinin	842.6	842.5	Ba′ 566−575	(K)GPLSSILR(A)	40.5 ± 1.7	12.5
	726.5	726.4	Ba′ 24−31	(R)QQHGEK(E)	40.1 ± 1.7	13.6
	1,101.4	1,101.5	Ba 58−66	(R)EEQEWPR(K)	47.2 ± 2.0	4.1
Glycinin	1,407.7	1,407.7	GB2 1−13	(G)IDETICTMRLR(H)	46.1 ± 0.0	6.6
	1,424.6	1,423.7	GA1a 101−114	(R)GQSSRPQDRHQK(I)	47.0 ± 3.1	7.9
	629.3	629.4	GA4 236−242	(K)KTQPR(R)	45.2 ± 3.1	5.5
Unbound peptides			SBA 15−24	QPNMILQGDA	0	

[a] The amino acids in parenthesis are not part of the identified peptides, but they are adjacent to the identified peptides in the protein sequence, therefore were included in the synthesis of these peptides

Data are presented as means ± SD. N = 2. Ba′ 566−575: β-conglycinin α′ chain amino acid 566−575; Ba′ 24−31: β-conglycinin α′ chain amino acid 24−31; Ba 58−66: β-conglycinin α chain amino acid 58−66; GB2 1−13: glycinin subunit B2 amino acid 1−13; GA1a 101−114: glycinin subunit A1a amino acid 101−114; GA4 236−242: glycinin subunit A4 amino acid 236−242

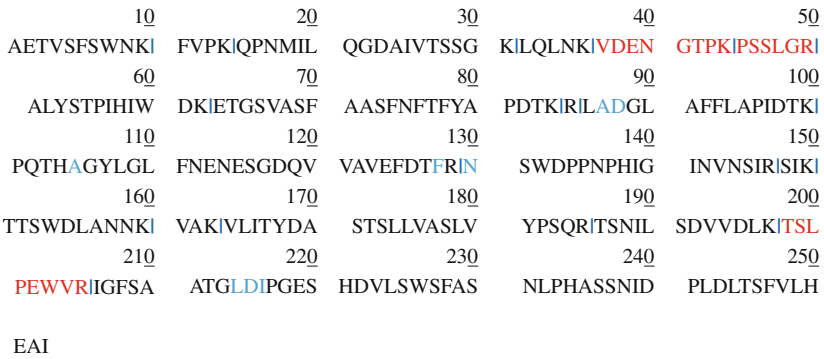

Fig. 4 Relative positions of the two active peptides from SBA in SBA sequence. The red lettered sequences are the two active peptides (SBA 36−51 and 197−206) that were identified and synthesized (included two more amino acids at the ends). The blue letters are amino acids that participate in carbohydrate binding according to Dessen et al. [26]. The *vertical blue lines* are the theoretical cutting sites of the enzyme

hemagglutination activity, and the activity may be due to the binding of peptides to the carbohydrates on red blood cell surfaces.

Figure 4 shows the relative position of the two active peptides (in red letters) in SBA sequence. Dessen et al. [26] cross-linked SBA with a synthetic biantennary analog of the blood group I carbohydrate antigen with structure of $(\beta\text{-LacNAc})_2\text{Gal-}\beta\text{-R}$, where R is $-O(CH_2)_5COOCH_3$. They found the following amino acids of SBA interacted with the carbohydrate side chains: Phe 128, Ile 216, Leu 214, Ala 105, Ala 87, Asn 130, Asp 88, and Asp 215 (blue letters in Fig. 4). None of these amino acids are in the two peptides we identified, probably due to the difference in carbohydrates (of the synthetic vs. that on the red blood cell surface), or method used for identifying the binding sites.

In Fig. 4, the vertical blue lines represent the theoretical cleavage sites in SBA by trypsin. There are two cutting sites within one of the two identified active peptides. The reason that we have recovered and identified the two active peptides may be that our enzyme hydrolysis was not complete due to enzyme accessibility. If more time or higher enzyme dose was used, we may not be able to identify these peptides. These observations also tell us that the fact that theoretical cutting by trypsin and the real hydrolysis yielded different peptides may be due to the enzyme purity or other unknown factors. Therefore, the study of making and identifying bioactive peptides may in general depend on the type of enzymes and experimental conditions used.

Activities of Identified and Synthesized Peptides from the β-Conglycinin Fraction

Twenty-four peptides from the β-conglycinin hydrolysate that were bound to GalNAc beads were identified as products by trypsin digestion when compared with the

theoretical cleavage. Table 2 shows 11 of the 24 peptides, because the others had low signal intensity thus low concentration. We selected three peptides which had the highest MS intensity to synthesize: α′ chain amino acid 566−575 (Ba′ 566−575) with sequence of KGPLSSILRA, α′ chain amino acid 24−31 (Ba′ 24−31) with sequence of RQQHGEKE, and α chain amino acid 58−66 (Ba 58−66) with sequence of REEQEWPRK. The activities of the synthesized peptides were tested using the slide agglutination test, as for SBA peptides. The hemagglutination activities of these peptides are summarized in Table 1. All three peptides from β-conglycinin have hemagglutination activity, ranging from 40 to 47% relative to SBA, confirming our bioactive peptide hypothesis.

Activities of Identified and Synthesized Peptides from the Glycinin Fraction

Sixteen peptides from the glycinin hydrolysate that were bound to GalNAc beads were identified as products by trypsin digestion when compared with the theoretical cut, and Table 3 shows eight of them. We selected three peptides with the highest MS signal intensity for synthesis: subunit B2 1−13 (GB2 1−13) with the sequence GIDETICTMRLRH; subunit A1a 101−114 (GA1a 101−114) with the sequence RGQSSRPQDRHQKI; subunit A4 236−242 (GA4 236−242) with the sequence KKTQPRR. The activities of the synthesized peptides were tested and the results are summarized in Table 1. This result of about 46% activity relative to native SBA confirmed that glycinin also carries peptides that have hemagglutination activity.

Nishi et al. [10] found that β 51−63 of β-conglycinin bound to the small intestinal brush border and stimulated cholecystokinin (CCK) release, suppressing food intake. Peptides with arginine (R) and glycine (G) had a strong affinity, but not with single R. All the peptides we identified have R and G, but mostly in a single R format. In addition, we did not find any similarities among the peptides we identified. Although there might be some specific amino acids involved in the carbohydrate binding, we do not yet know exactly what they are.

This report is the first to show that β-conglycinin and glycinin had hemagglutination activity and we synthesized peptides to verify our findings. These bioactive peptides can bind to cell surface, not only cause blood cells to agglutinate, but may also induce other cell biological or

Table 2 Eleven selected active peptides from 24 identified in soybean β-conglycinin hydrolysate

m/z from MALDI-TOF MS	Calculated *m/z*	Peptide synthesized	Amino acid sequence[a]
627.3	627.4	α Chain 367−373	(K)NPQLR(D)
955.6	955.5	α Chain 161−170	(R)SPQLQNLR(D)
1,101.4	1,101.5	α Chain 58−66	(R)EEQEWPR(K)
1,378.9	1,378.7	α Chain 89−101	(R)QFPFPRPPHQK(E)
762.5	762.4	β Chain 395−401	(K)EEGSKGR(K)
1,029.6	1,029.6	β Chain 190−197	(K)EQIRQLSR(R)
1,361.9	1,361.7	β Chain 345−358	(R)NFLAGEKDNVVR(Q)
627.3	627.4	α′ Chain 401−407	(R)NPQLR(D)
726.5	726.4	α′ Chain 24−31	(R)QQHGEK(E)
842.6	842.5	α′ Chain 566−575	(K)GPLSSILR(A)
1,022.5	1,022.4	α′ Chain 58−66	(K)EEHEWHR(K)

[a] See Table 1 footnote

Table 3 Eight selected active peptides from 16 identified in soybean glycinin hydrolysate

m/z from MALDI-TOF MS	Calculated *m/z*	Peptide synthesized	Amino acid sequence[a]
1,085.6	1,085.6	Subunit B2 162−172	(R)QIKNNNPFK(F)
1,149.5	1,149.6	Subunit B2 90−101	(R)VFDGELQEGR(V)
1,407.7	1,407.7	Subunit B2 1−13	(G)IDETICTMRLR(H)
506.2	506.2	Subunit A1a 282−287	(R)GSQS(K)
1,424.6	1,423.7	Subunit A1a 101−114	(R)GQSSRPQDRHQK(I)
629.3	629.4	Subunit A4 236−242	(K)KTQPR(R)
3,210.6	3,210.3	Subunit A4 155−182	(K)WQEQQDEDEDEDEDDEDEQIPSHPPR(R)
745.4	745.4	Subunit A2 242−250	(R)VTAPAMR(K)

[a] See Table 1 footnote

biochemical reactions. Much more work is needed to further study bioactivity and study mechanism of action.

Although the peptides we identified and synthesized had hemagglutination activity in-vitro, we do not know how they would behave in-vivo. Being able to bind to blood cells that is the well-accepted model for testing cell agglutination, it is possible that the peptides can also bind to other cells, for example, the small intestinal brush border. This possible binding might cause anti-nutritional effects as SBA does if the peptide concentration is above a certain threshold level, but such binding may also have other beneficial effects. Binding can be the first step for several activities. For example, the β 51−63 fragment of β-conglycinin can bind to intestinal mucosal cells and stimulate cholecystokinin release, thus suppressing appetite [10]. Although extensive research about different functions of active peptides has been conducted, how the peptides interact with cells to trigger downstream signals is not well known. The ability of peptides to bind to the cells gives some indication of how the peptides may carry out their functions, for example, they may bind to and enter the cells through endocytosis. In addition, it is not known if the 50% hemagglutination activity of the peptides relative to SBA will produce a similar anti-nutritional effect in live animals as the native SBA would. More research needs to be conducted to study the in-vivo effect of these lectin-like peptides and the possible links between the activity of binding to cells and other biological activities. It is natural to question if native SBA, heat denatured SBA, and soy storage proteins will only be partially hydrolyzed in the gastrointestinal system to have these bioactive peptides or whether they may be more fully hydrolyzed to show no residual activities and thus no anti-nutritional effect at all. Our other studies may partially answer these questions [17, 19], but more in-depth study is needed.

The potential use of these small lectin-like peptides in drug delivery should be further examined. Lectin-mediated drug delivery was discouraged due to the toxicity and immunogenicity of the lectins, which can be overcome by small lectins or active peptides. The smallest peptide we identified only had seven amino acids, which was smaller than the smallest lectin peptides identified previously (17 amino acids) [16] and are easy to manipulate.

Hemagglutination Assay and Slide Agglutination Test

We have shown that the slide agglutination test gave results similar to the hemagglutination assay. When we used the typical in-vitro hemagglutination assay to test activities of synthesized peptides, the peptides caused cell lysis, possibly due to certain residual chemicals used for peptide synthesis. Purification methods were sought but they were not successful or feasible. The slide agglutination test was shown to be a good alternative test. For samples with a positive reaction, the cells formed clumps almost immediately after coming in contact with the protein or peptide (Fig. 3A), while the negative control saline formed uniformly distributed cells on the slide (Fig. 3B), which was easy to distinguish. The same sample (SBA) was tested using both the hemagglutination assay and slide agglutination assay, and the results only differed in one dilution, so that the hemagglutination unit differed in one unit. This test shows consistent results and there is high correlation between the two assays. The slide agglutination assay did not cause cell lysis, which may be due the short exposure time of samples to cells. The results were read very quickly after the sample was mixed with blood cells, so the cells may have agglutinated before lysed. However, the slide agglutination assay is more tedious and not as convenient as the hemagglutination assay. When the samples do not cause any issue to the cells, the hemagglutination assay is a better choice.

Conclusion

In summary, this study demonstrates that there are bioactive peptides from soy lectin and storage proteins that have in-vitro hemagglutination activity. However, we do not know if this level of activity (about half of the native SBA) will produce any in-vivo physiological effects, both as a positive aspect and as a negative anti-nutritional aspect. More thorough nutritional studies with these peptides at the cellular and systematic levels are desirable, and this study provides the necessary preliminary data and justification for doing so.

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