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Abstract

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Keywords

antinutritional factor, deglycosylation, enzymatic hydrolysis, hemagglutination activity, lectin, nonthermal treatment, soybean agglutinin

Disciplines

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Deactivation of Soybean Agglutinin by Enzymatic and Other Physical Treatments

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The main objective of this study was to eliminate the hemagglutination activity of an antinutritional factor in soybeans, soybean agglutinin (SBA). A series of experiments was designed to enzymatically modify SBA structure and to use other physical treatments to reduce activity. SBA extract was prepared from soy flour and used as the substrate for all treatments. Deglycosylation by enzyme decreased activity of SBA by 21%, but not to the level of denaturation by heat or by denaturing reagents (47–77% residual activity). Single enzymes, such as trypsin, chymotrypsin, thermolysin, and endoproteinase Glu-C, did not hydrolyze native SBA, but they hydrolyzed heat- or organic solute-denatured SBA. Even after hydrolysis, SBA still had 44–62% residual activity. Combinations of enzymes with thermolysin fully deactivated heat- or guanidine hydrochloride- and urea-treated SBA. Pepsin and pancreatin hydrolysis fully deactivated not only heated but also native SBA. Tea polyphenols, metal ions, and chelating agents were also tested, and they showed no significant effect on SBA activity. *N*-Acetylgalactosamine–agarose beads specifically but not fully removed SBA from the soy protein mixture. In general, SBA needs to be denatured first for an effective enzymatic hydrolysis, and multiple enzymes are needed to fully deactivate SBA. Pepsin and pancreatin treatment showed great promise in fully reducing SBA activity, and it would be further tested using soy flour as a model system.

KEYWORDS: Antinutritional factor; deglycosylation; enzymatic hydrolysis; hemagglutination activity; lectin; nonthermal treatment; soybean agglutinin

INTRODUCTION

Soybeans contain antinutritional factors. If soybeans are consumed by humans and animals without heating, they can cause growth depression (1). The most prominent antinutritional factors are the trypsin inhibitors and lectin (2), also known as soybean agglutinin (SBA). They are responsible for 40% and 50% of the growth inhibition of raw soybeans, with the remaining 10% being incomplete digestion of the protein (3). Native SBA is resistant to digestive enzymes in the gastrointestinal (GI) tract and has a unique property of binding to carbohydrate-containing molecules. It binds to the intestinal epithelium, which causes disruption of the brush border (4) and poorer growth of spleen and kidneys (5). Some lectins can cross the gut wall into circulation, generating antilectin antibodies (6). Lectin is therefore a group of proteins that participate in plant defense.

SBA is a glycoprotein and forms a tetramer with 30 kDa subunits. Each subunit carries an N-linked carbohydrate unit, $\text{Man}_9(\text{GlcNAc})_2$, with a molecular weight of 1.9 kDa. Each subunit has a carbohydrate-binding site, with the highest affinity for *N*-acetyl-D-galactosamine (GalNAc) (7). Different methods have been used to deactivate SBA, with heat treatment being the

most commonly used. Dry heat treatment is not as effective as moist heat in deactivating SBA (8,9). With regard to the role of the carbohydrate moiety of SBA in its activity, the literature shows contradictory results. Whereas Nagai and Yamaguchi found that the mannose oligosaccharide chains were essential for proper folding and activity (10), Adar et al. found that the SBA lacking such sugar groups had the same activity as native SBA (11). Therefore, additional study on the role of the carbohydrate moiety in its activity is needed.

Recently, there have been significant efforts to develop aqueous extraction process to extract oil from soybeans (12). This process uses much less heat than the traditional process; therefore, protein functionality is preserved. However, such a non-thermal process does not deactivate SBA. Therefore, our main research objective was to find practical methods to deactivate SBA with minimum heating. We wanted to examine treatment methods such as nonthermal denaturation, protein hydrolysis, polyphenols, metal ion addition, and sugar binding for reducing SBA activity. Polyphenols (13) and metal ions (14) have been found to show some effect.

The activity of lectin can be tested by different methods, all based on the carbohydrate-binding property. The most commonly used method is the hemagglutination assay, which is based on the ability of lectin to agglutinate erythrocytes (2). The end point is given by the highest dilution of lectin that still causes clumping of cells, which can be tested visually (9), by photometric

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Table 1. Experimental Conditions for Examining the Effect of Denaturing Buffer and Heat on Deglycosylation of Soybean Agglutinin by Enzyme

treatment	extract of SBA (mg)	10× glycoprotein denaturing buffer (μL)	H ₂ O (μL)	heat
1	1	15	10	100 °C, 10 min
2	1	0	25	100 °C, 10 min
3	1	15	10	unheated
4	1	0	25	unheated

measurement (15), or by examination by microscopy (16). We chose to use the most practiced rabbit red blood cells for the hemagglutination assay and visual examination of the end points.

MATERIALS AND METHODS

Materials. Soy white flake was obtained from Cargill (Minneapolis, MN) and was produced by hexane extraction of the oil and then flash-desolventizing to achieve soy protein with ≥85% dispersibility in water. Rabbit blood in Alsever's solution was obtained from Hemostat Laboratories (Dixon, CA) and used within 2 weeks. Endoglycosidase H_F (Endo H_F), glycoprotein denaturing buffer, and G5 reaction buffer were purchased from New England Biolabs (Ipswich, MA). Tea polyphenols were obtained from MP Biomedicals (Solon, OH, catalog no. 02193756). *N*-Acetyl-D-galactosamine-agarose beads were purchased from Sigma-Aldrich, with ≥6 mg/mL binding capacity for SBA. Other reagents were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO).

Extraction and Purification of SBA. The procedure described by Lis and Sharon (17) was used. Generally, 1 kg of soy flake was dispersed in 12 L of distilled water, 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 clear liquid was poured out, the remaining supernatant obtained by centrifugation (3020g, 15 min) was collected, and the two liquid fractions were combined. Ammonium sulfate, 300 g, was added to each liter of supernatant to precipitate other unwanted proteins. Vacuum filtration was used to remove the precipitate. To each liter of filtrate was added with stirring 270 g of ammonium sulfate, and the mixture was incubated overnight at 4 °C to precipitate the crude SBA. The precipitate was then collected by centrifugation, resuspended in 200 mL of water, and dialyzed against water for 24 h at 4 °C with two changes of water. Any insoluble material present after dialysis was removed by centrifugation and 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 (3020g, 15 min) and redissolved in 100 mL of 0.05 M phosphate buffer, pH 6.1. The SBA solution was dialyzed against water for 24 h at 4 °C and then lyophilized, and the SBA was not further purified.

Deglycosylation of SBA Using Enzyme Endo H_F. Deglycosylation of SBA by Endo H_F enzyme (1,000,000 units/mL) was done according to the protocol in the product information sheet provided by New England Biolabs. It was recommended that the protein be denatured with glycoprotein denaturing buffer and heat treated before deglycosylation. To identify a proper condition for deglycosylation of SBA with Endo H_F and test the effects of denaturing buffer and heating on deglycosylation, SBA samples were treated as shown in Table 1. SBA extract (8 mg of protein/mL in 0.9% saline) was mixed with or without 10× glycoprotein denaturing buffer (5% sodium dodecyl sulfate (SDS), 0.4 M dithiothreitol (DTT)) and then either subjected to heat treatment or not. Then 20 μL of 10× G₅ buffer (0.5 M sodium citrate, pH 5.5), 20 μL of H₂O, and 10 μL of Endo H_F were added. The samples were incubated overnight (18 h) at 37 °C and then stored at -20 °C until hemagglutination and SDS-PAGE assays.

SDS-PAGE. Gel electrophoresis was performed 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.

Effect of pH on Activity of SBA. Hydrochloric acid or sodium hydroxide was used to adjust the pH of SBA solutions to 1–13 at 0.5 intervals. The samples were stored at 4 °C overnight before further testing. Because red blood cells would lyse when the pH was <5 (14),

samples that have pH values of <5 were tested in two different ways: one was to adjust the pH back to 5–6 using PBS buffer (phosphate-buffered saline, 0.1 M phosphate, 0.9% saline, pH 8.0) immediately before the test, and the other was to do the hemagglutination assay directly.

Effects of Heating and Denaturing Agents on Activity of SBA. SBA (1 mL, 8 mg/mL) in an Eppendorf tube was treated in boiling water for 20 min or in an autoclave at 121 °C for 30 min. Sodium dodecyl sulfate (SDS) was added to SBA to make a final concentration of 0.5%. After incubating at ambient temperature for 18 h, the sample was subjected to the hemagglutination assay. Organic solutes, urea and guanidine hydrochloride (GuHCl), were added to SBA (1 mL, 8 mg/mL) to make final concentrations of 8 and 6 M, respectively, and the pH was adjusted to 10. After incubating at ambient temperature for 18 h, half of the solution was dialyzed against water for 2 days to remove urea or GuHCl. The samples with SDS, GuHCl, or urea were subjected to the hemagglutination assay to examine the effects of the denaturing agents on SBA activity. The samples with or without GuHCl or urea removed were also subjected to enzymatic hydrolysis as described in the following section.

Enzymatic Hydrolysis of SBA To Reduce Hemagglutination Activity. All enzyme treatments were carried out according to the product information sheet provided by Sigma-Aldrich. Native SBA and SBA treated with heat, autoclaving, and organic solutes were subjected to the same enzyme hydrolysis procedure. For endoproteinase Glu-C (Glu-C, 500–1000 units/mg of enzyme solid) hydrolysis at the Glu site, 3.2 mg of SBA protein (400 μL of SBA extract) was treated in 0.1 M ammonium bicarbonate, pH 8.0 (buffer 1) with an enzyme/protein ratio of 3:80 for 18 h at 37 °C. For Glu-C hydrolysis at the Glu and Asp sites, the same treatment was done except the buffer was 0.1 M sodium phosphate buffer, pH 7.8 (buffer 2). The chymotrypsin (≥40 units/mg of enzyme) treatment was done by treating 3.2 mg of SBA protein in 100 mM Tris-HCl, 10 mM CaCl₂, pH 7.8 at an enzyme/protein ratio of 1:64 for 24 h at 30 °C. The thermolysin (50–100 units/mg of enzyme) treatment was done in the same buffer as chymotrypsin with an enzyme/protein ratio of 1:100 for 24 h at 70 °C. For trypsin (>250 units/mg of enzyme) hydrolysis, 3.2 mg of SBA protein was treated in PBS (phosphate-buffered saline, 10 mM phosphate, 0.9% saline), pH 8.0, with an enzyme/protein ratio of 1:16 at 37 °C for 24 h. Pancreatin (a mixture of enzymes all meeting USP specifications) hydrolysis was carried out by treating 3.2 mg of SBA protein in the same buffer as chymotrypsin at an enzyme/protein ratio of 1:25 for 24 h at 37 °C. For Pronase (4 units/mg of enzyme solid) hydrolysis, 3.2 mg of SBA protein was treated in 50 mM ammonium bicarbonate buffer, pH 8.0, with an enzyme/protein ratio of 1:50 at 37 °C for 24 h.

For multienzyme hydrolysis, 3.2 mg of SBA protein was mixed with 1 M Tris-HCl containing 100 mM CaCl₂, and then different combinations of enzymes were added. For the combinations of trypsin + thermolysin, trypsin + chymotrypsin + thermolysin, Pronase + pancreatin + thermolysin, and pancreatin + thermolysin, the mixture was incubated at 37 °C for 22 h and then at 70 °C for 2 h. For the combination of trypsin + chymotrypsin, the mixture was incubated at 37 °C for 24 h. For the combination of chymotrypsin + thermolysin, the mixture was incubated at 30 °C for 22 h and then at 70 °C for 2 h.

For pepsin (400–800 units/mg of enzyme) followed by pancreatin hydrolysis, 3.2 mg of SBA protein (400 μL extract) was mixed with 10 μL of 1 M HCl to adjust the pH to 2.0; then pepsin was added to make an enzyme/substrate ratio of 1:5. The mixture was incubated at 37 °C for 2 h. Tris-HCl (1 M) was then added to adjust the pH to 7.5, and pancreatin was added to make an enzyme/substrate ratio of 1:50. The mixture was then incubated at 37 °C for 22 h. All samples were heated 100 °C for 10 min to deactivate the enzyme and then stored at -20 °C until the hemagglutination assay.

Effect of Tea Polyphenols (TP) on Activity of SBA. The TP used in this experiment had 99.6% polyphenols, which contain 82.8% catechins and other components such as caffeine and ash. Different volumes of 100 mg/mL TP were added to 400 μL of SBA protein extract (3.2 mg), and the final concentration of TP was from 2.5 to 20 g/L, with concentration intervals being 2.5 g/L. The mixtures were incubated at room temperature for about 1.0 h and either subjected to hemagglutination assay directly or centrifuged (12395g for 2 min) to obtain the supernatant for hemagglutination assay.

Effect of Metal Ions on Activity of SBA. Different metal ions (Fe₂(SO₄)₃, FeSO₄, MgSO₄, CaCl₂, ZnSO₄, CuSO₄, and AlCl₃) were added to

the SBA samples to make a final concentration of 0.01 M. $\text{Fe}_2(\text{SO}_4)_3$ was also added to SBA samples to various final concentrations (0.25, 0.2, 0.1, 0.05, and 0.01 M). The samples were incubated at 4 °C overnight before assaying for hemagglutination activity.

Effect of Chelating Agents on Activity of SBA. Different chelating agents (1 M, pH 4.0, citric acid, acetic acid, phosphoric acid) were added to SBA to make final concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 M. Ethylenediaminetetraacetic acid (EDTA, 1 M, pH 8) was added to SBA to make the same final concentrations, and the pH was adjusted to 4.0 by HCl. The samples were incubated at 4 °C for 24 h before assaying for hemagglutination activity.

Effect of Removing of SBA from SBA Extract Using GalNAc–Agarose Beads. To remove SBA protein from the extract, SBA (500 μL , 8 mg/mL) was mixed with different amounts of GalNAc–agarose beads, gently mixed at 4 °C overnight, and then centrifuged (850g for 1 min) to obtain the supernatant for hemagglutination assay.

Effect of Removing SBA from Isoelectric Extract Using GalNAc–Agarose Beads. To test the specific binding of GalNAc–agarose beads to SBA in a mixed protein system, pH 4.6, isoelectric protein extract was used, assuming the isoelectric extract has concentrated SBA and many other proteins. Soy white flake (5 g) was dispersed in 50 mL of 0.9% NaCl solution, and the pH was adjusted to 4.6 using 2 M HCl to make a 10% (w/v) soy dispersion; the mixture was incubated overnight at 4 °C. The dispersion was then centrifuged for 10 min at 3000g to obtain an isoelectric soluble protein extract (100 mg of protein/mL). The extract was then incubated with 1 mL of GalNAc–agarose beads at 4 °C overnight and then centrifuged (850g for 1 min) to obtain the supernatants for hemagglutination assay.

Hemagglutination Assay. The assay was performed according to a method of Lis and Sharon (17) with some modification. Briefly, rabbit red blood cells collected in Alsever's solution were centrifuged for 5 min at 410g. After estimation of the volume of the cells, 5 mL of 0.9% saline/mL of cells was added to wash the cells. Centrifugation was used to collect the cells. After three washings, red blood cells were suspended at 4% (v/v) in 10 mM phosphate-buffered 0.9% saline, pH 7.4 (PBS), and then 1 volume of 1% trypsin (w/v) in PBS was added to 10 volumes of this suspension and the mixture was incubated for 1.0 h at 37 °C to increase the sensitivity of the assay. The red blood cell suspension was then washed four times with 0.9% saline, and finally cells were resuspended at 3% (v/v) in 0.9% saline.

Protein samples were serially diluted with 2-fold dilution 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 placed at 37 °C for 2.0 h for agglutination to occur. The plates were then tilted about 45°; the samples with erythrocytes streamed in a “tear-drop” fashion were considered to be negative and the ones that did not form a “tear-drop” were considered to be positive. The hemagglutination units (HU) per gram of sample were determined by using the equation (9)

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

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 milliliters of extract per gram of sample (inverse of the initial concentration), and V is the volume of extract added. Each sample was done in duplication. The HU/g values were then expressed as $\log(\text{HU/g})/\log 2$ to normalize the data for the 2-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 every set of treatments. The results were expressed as relative activity to SBA to eliminate any influence of experimental conditions.

Statistical Analysis. The treatment replicate numbers (N) for different experiments are different, and the N values are given in the tables and figures. The 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

Purity of SBA Extract. As shown in Figure 1 (lane 2), in the SBA extract the main protein is SBA with about a 30 kDa molecular size. There are also a few lower molecular weight

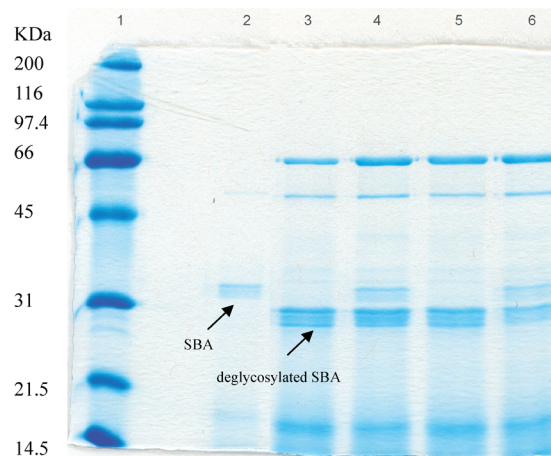


Figure 1. Deglycosylation of soybean agglutinin using enzyme Endo H_f . Lanes: 1, molecular weight marker; 2, SBA extract; 3, SBA treated with denaturing buffer and heating, then with Endo H_f ; 4, SBA treated with heating, then with Endo H_f ; 5, SBA treated with denaturing buffer, then with Endo H_f ; 6, SBA treated with Endo H_f .

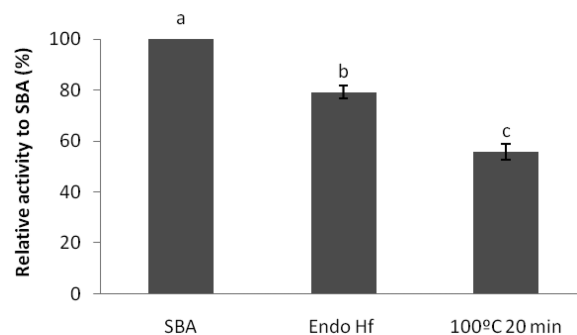


Figure 2. Effects of deglycosylation and heating on hemagglutination activity of soybean agglutinin. $N = 4$. Data are presented as mean \pm SD. Different letters represent significant differences ($P \leq 0.05$). Least significant difference at 95% confidence level ($\text{LSD}_{0.05}$) is 3.8.

proteins. Further purification of SBA could not be feasibly done, so we used such extract as the SBA protein for all treatments. Because all results are expressed relative to the untreated SBA activity, these evaluations are not affected by SBA purity in the extract. There are also multiple bands of SBA before and after Endo H_f treatment (Figure 1), and these are isolectins, as reported by Sharon and Lis (7).

Effect of Deglycosylation on Activity of SBA. Endo H_f is an enzyme that specifically cleaves high-mannose and certain hybrid types of N-linked carbohydrates on proteins and leaves an GlcNAc residue on the protein. After deglycosylation, SBA theoretically should have a molecular size of 28.1 kDa. Figure 1 shows that deglycosylated SBA was present in all samples. Samples in lanes 3 and 5, which were treated with denaturing buffer and with or without heating, were fully deglycosylated. The ones without denaturing buffer and with or without heating (lanes 4 and 6) had both deglycosylated and native SBA, which indicates that SBA was not fully deglycosylated. These results indicate that heat treatment is not as an effective pretreatment as the denaturing buffer for deglycosylation of SBA by Endo H_f . Therefore, we treated SBA with denaturing buffer before Endo H_f hydrolysis in all subsequent experiments.

Even fully deglycosylated by Endo H_f , SBA still had 79% of its original activity, which was higher than the sample with heat treatment alone (56%) (Figure 2). This result indicates that the

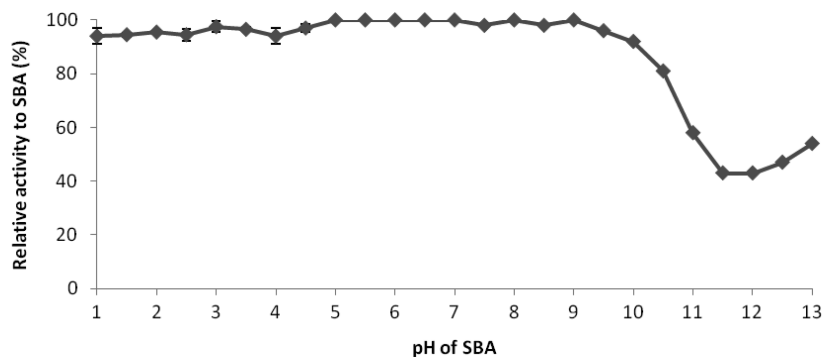


Figure 3. Effect of pH on hemagglutination activity of soybean agglutinin. $N = 2$. Data are presented as mean \pm SD.

carbohydrate moiety may not play a crucial role in the activity of SBA. This result is partially in agreement with Adar et al. (11), who found that the SBA expressed by *Escherichia coli*, which lacked the ability to glycosylate proteins, still had the same activity as native SBA. The reduction in activity of the Endo H_F deglycosylated SBA may also be due to the denaturing step before deglycosylation. These results led to the hypothesis that a proper three-dimensional (3-D) structure of SBA may be crucial for its activity.

Effect of pH and Denaturing Agents on Activity of SBA. To examine the effect of 3-D structure on the activity of SBA, different pH values, SDS, and organic solutes such as urea and GuHCl were used to treat SBA.

Activities of SBA were tested under different pH values. Extremely high or low pH caused red blood cells to lyse; however, as samples were diluted as the assay requires, the cells were no longer lysed. Typically, after four to six dilutions, the cells became intact, and SBA agglutinated blood cells occurred after about 15 dilutions, so the effect of pH on cell lysis is not a factor in the assay. In addition, the samples with low pH were tested in two different ways, either pH adjusted back to 5–6 or tested directly. Because the two samples showed similar activity results, they serve as additional evidence that the effect of pH on cell lysis can be neglected. It may be a concern that after several dilutions of a sample or when the pH of a sample was adjusted back to 6, the pH of the sample was no longer the pH at which we intended to test. However, the fact that the effect of pH on activity was clearly observed indicates the testing method is valid.

Figure 3 shows that activity of SBA did not decrease in the low pH range, whereas it decreased in the high pH range. This observation indicates the acidic condition may only cause reversible structure change. On the contrary, high pH may have caused the SBA tetramer to irreversibly dissociate and/or cause SBA monomer to partially unfold, thus decreasing activity. However, even at pH > 11, SBA still showed > 40% activity. Extremely high pH values cannot be used in food systems because it may lower the nutritional value of food. For example, the formation of lysinoalanine at high pH lowers the nutritional value of protein and causes kidney disease in rats (18). Elevating the pH is not suitable for practical use.

Figure 4 shows that heat treatment and three denaturing reagents, SDS, urea, and GuHCl, decreased the activity of SBA, but to different extents. Even after denaturation, SBA still had at least 47% activity. This result indicates that 3-D structure plays an important role in the activity of SBA. However, denaturation alone is not effective to fully deactivate SBA.

Effect of Enzymatic Hydrolysis on Activity of SBA. On the basis of the evidence above, we hypothesized that destroying the primary structure of protein may decrease the activity. Four main proteases were chosen. Trypsin is readily available and specifically

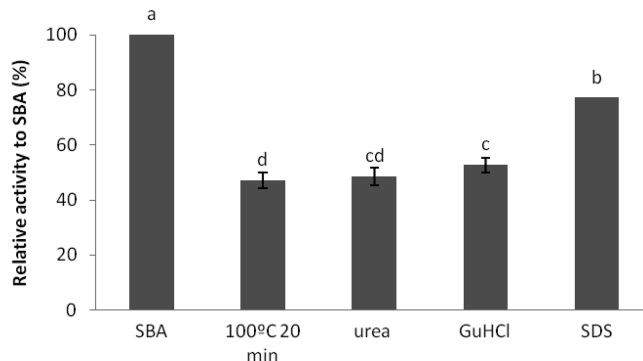


Figure 4. Effect of denaturing agent on hemagglutination activity of soybean agglutinin. $N = 4$. Data are presented as mean \pm SD. Different letters represent significant differences ($P \leq 0.05$). $LSD_{0.05}$ is 4.9.

hydrolyzes peptide bonds at the carboxyl side of arginine or lysine residues (19). There are some consensus sequences in legume lectins (20), which might be important for their activities; therefore, three additional enzymes were chosen to target these sequences: Glu-C from *Staphylococcus aureus* V8, α -chymotrypsin from bovine pancreas, and thermolysin from *Bacillus thermoproteolyticus* rokko. Glu-C hydrolyzes peptide bonds at the carboxyl side of glutamyl and aspartyl residues. In ammonium acetate, pH 4.0, or ammonium bicarbonate, pH 8.0, the enzyme preferentially cleaves glutamyl bonds, whereas in phosphate buffer, pH 7.8, the enzyme cleaves at either glutamyl or aspartyl sites (19). Glu-C will not cut the protein too short, but can cut at the consensus sequence. With low substrate specificity, thermolysin can cut protein into very small pieces. Thermolysin is also active in a wide pH range (pH 5.0–9.5) and high temperature (optimal 70 °C), which is important in hydrolyzing a tough protein such as SBA. Liener and Wada (21) found that the modification of tyrosine decreased the activity of SBA significantly, so we also chose chymotrypsin. Another nonspecific enzyme, Pronase, from *Streptomyces griseus*, and a mixture of at least 10 proteases (22) was also tested. In addition, pancreatin from porcine pancreas was used due to its high availability.

Effect of Single-Enzyme Hydrolysis on Activity of Native and Heated SBA. After hydrolysis of native SBA, there were still SBA bands on the SDS-PAGE gel (data not shown), which indicates that none of the hydrolytic enzymes tested can fully hydrolyze native SBA. The activities of enzyme-treated SBA did not decrease (Table 2). These results indicate that native SBA is resistant to enzyme hydrolysis. SBA has a very compact structure, which may inhibit the access of enzymes to the cleavage site.

To enable the access of enzymes to active sites in SBA, the samples were heated at 100 °C for 20 min or autoclaved at 121 °C for 30 min to denature SBA first and then treated with different

enzymes. After trypsin, chymotrypsin, or thermolysin hydrolysis of heat-treated SBA, there were no SBA bands on the gel (data not shown), indicating that SBA was fully hydrolyzed. Glu-C did not fully hydrolyze heated SBA in either buffer 1 or buffer 2. However, even after hydrolysis, the activity of SBA did not further decrease and the residual activity was still similar to the heat treatment alone (Table 2). In addition, there was no significant difference between the two heat treatments (boiling and autoclaving), so 100 °C and 20 min was used in future treatments.

Effect of Single-Enzyme Hydrolysis on Activity of Organic Solutes-Treated SBA. To develop the nonthermal treatment method, we used organic solutes GuHCl and urea to denature SBA before protease treatment. Previous results showed that SBA was more susceptible to basic conditions, so urea and GuHCl treatment was done at pH 10. Organic solutes cannot be left in food, so they were removed by dialysis against water before enzyme hydrolysis. We found single-enzyme hydrolysis after GuHCl treatment (either with GuHCl removed or not) did not fully deactivate SBA (Table 3). They all had similar activities as GuHCl treatment alone, which was in agreement with the heat

treatment data in Table 2. Urea treatment displayed a similar pattern; however, there was significant difference between the urea removal and without removal treatment, which was different from the GuHCl treatments. These results indicate that GuHCl and urea could replace heat treatment to denature SBA before enzymatic hydrolysis.

Effect of Multiple-Enzyme Hydrolysis on Activity of SBA. To cut SBA into smaller pieces, mixtures of enzymes were used. The combination of enzymes did not hydrolyze native SBA, and the activity did not decrease as shown in Table 4, which again proved that native SBA is resistant to hydrolytic enzymes. A combination of trypsin, chymotrypsin, and thermolysin, however, fully deactivated heat-treated SBA. This indicates that after being cut into smaller pieces, SBA can be fully deactivated. In addition, any combination of enzyme treatments with thermolysin fully deactivated SBA. This result indicates that thermolysin might cut the crucial sites for activity of SBA. However, thermolysin alone did not fully deactivate SBA, suggesting that trypsin and chymotrypsin also cut at crucial sites, although not completely. Furthermore, pancreatin, which contains trypsin and chymotrypsin, did not fully deactivate SBA. This further supports the result that the combination of trypsin and chymotrypsin could not fully deactivate SBA.

To fully deactivate SBA with minimum heating, GuHCl or urea was used to replace heat treatment. Before removal of GuHCl, a combination of three enzymes (trypsin, chymotrypsin, and thermolysin) did not fully deactivate SBA. After GuHCl removal, the combination of the three enzymes fully deactivated SBA (Table 4). These results indicate that GuHCl may have denatured both SBA and the enzymes, so GuHCl interfered with enzymatic hydrolysis. In addition, GuHCl can irreversibly denature SBA, because after its removal, SBA did not fold back to the original configuration, which enabled the enzymatic hydrolysis that led to full deactivation of SBA. However, urea behaved differently. After removal of urea, the combination of three enzymes did not fully deactivate SBA (Table 4), which indicates that urea may not irreversibly denature SBA. After removal,

Table 2. Effect of Single-Enzyme Hydrolysis of Native or Heated Soybean Agglutinin on Hemagglutination Activity (%)

enzyme treatment	native SBA	100 °C treated SBA	autoclaved SBA
no enzyme	100 a ^a	47.1 ± 2.8 bc	48.0 ± 2.5 c
Glu-C 1	104.4	53.2 ± 5.6 bc	53.1 ± 0 bc
Glu-C 2	104.4	44.8 ± 2.5 bc	53.1 ± 0 bc
trypsin	97.6	53.7 ± 4.1 bc	54.6 ± 3.8 bc
chymotrypsin	100.7	35.1 ± 32.4 c	51.7 ± 3.7 bc
thermolysin	95.5 ± 8.1	62.2 ± 1.4 b	59.0 ± 6.6 b
LSD _{0.05}		17.2	

^aThe values are the relative activity to native SBA of the samples. *N* = 3. Data are presented as mean ± SD. Different letters represent significant differences (*P* ≤ 0.05). Glu-C 1, endoproteinase Glu-C in 0.1 M ammonium bicarbonate, pH 8.0; Glu-C 2, endoproteinase Glu-C in 0.1 M sodium phosphate buffer, pH 7.8.

Table 3. Effect of Single-Enzyme Hydrolysis of Organic Solutes-Treated Soybean Agglutinin on Hemagglutination Activity (%)

enzyme treatment	native SBA	GuHCl	remove GuHCl	urea	remove urea
no enzyme	100.0 Aa ^a	52.8 ± 2.6 B	49.5 ± 13.9 B	48.7 ± 3.2 de	75.0 ± 26.1 bc
trypsin		53.0 ± 2.8 B	54.5 ± 19.1 B	48.5 ± 2.1 de	88.3 ± 2.3 ab
chymotrypsin		48.0 ± 8.5 B	59.5 ± 10.6 B	40.5 ± 2.1 e	89.7 ± 3.1 ab
thermolysin		60.0 ± 2.8 B	62.0 ± 2.8 B	59.5 ± 2.1 cd	82.0 ± 1.7 ab
LSD _{0.05}		18.1		18.3	

^aThe values are the relative activity to native SBA of the samples. *N* = 3. Data are presented as mean ± SD. Different letters within the column of GuHCl and remove GuHCl and urea and remove urea represent significant differences (*P* ≤ 0.05), and native SBA was used as a control. GuHCl, GuHCl-treated SBA; remove GuHCl, SBA treated with GuHCl and then removal of GuHCl; urea, urea-treated SBA; remove urea, SBA treated with urea and then removal of urea.

Table 4. Effect of Combination of Enzymes Hydrolysis of Differently Treated Soybean Agglutinin on Hemagglutination Activity (%)

enzyme treatment	native SBA	100 °C, 20 min	GuHCl	remove GuHCl	urea	remove urea
no enzyme	100.0 ± 0.0 Aa ^a	64.7 ± 7.8 c	52.8 ± 2.6 BC	49.5 ± 13.9 BC	48.7 ± 3.2 BC	75.0 ± 26.1 AB
T + C		51.0 ± 2.8 cd				
T + Th		0.0 ± 0.0 e				
C + Th		0.0 ± 0.0 e				
T + C + Th	97.0 a	0.0 ± 0.0 e	49.7 ± 5.9 BC	0.0 ± 0.0 D	41.5 ± 2.1 C	25.3 ± 43.9 CD
P	92.0 ± 12.7 ab	45.3 ± 7.8 d				
P + Th	80.0 b	0.0 e				
LSD _{0.05}	14.6			30.1		

^aThe values are the relative activity to native SBA of the samples. *N* = 3. Data are presented as mean ± SD. Different letters within the columns of native SBA and 100 °C 20 min, GuHCl and remove GuHCl, and urea and remove urea represent significant differences (*P* ≤ 0.05). Native SBA was used as a control for both. T, trypsin; C, chymotrypsin; Th, thermolysin; P, pancreatin. See Table 3 for other abbreviations.

some SBA folded back to original structure, leading to incomplete hydrolysis and deactivation. This result also can explain the data in **Table 3** that after removal of urea, the activity of SBA was higher than before its removal.

For the effects of other enzymes on SBA activity, Pronase did not fully deactivate the heated SBA (35% original activity), although it is capable of hydrolyzing casein into > 70% amino acids (22). This result indicates the inability of Pronase to cut the active sites of SBA. The combination of Pronase, pancreatin, and thermolysin fully deactivated heated SBA (0%), which was in agreement with our earlier results that thermolysin was able to cut the active sites of SBA.

Pepsin and then pancreatin treatment not only fully deactivated heated SBA (0% activity) but also fully deactivated the native SBA (0% activity). After such hydrolysis, the SBA band disappeared on SDS-PAGE gel. This enzyme combination is the only one from all enzymes tested that is effective toward the native SBA. Pepsin and pancreatin are enzymes available in large quantity and at low price, so it is feasible to use this combination to treat soy white flakes (SWFs) and generate feeding materials for further biological or animal testing.

From these results, we proposed our bioactive peptide hypothesis: Native SBA is a tetramer, binds to red blood cells, and forms network to cause cell agglutination. Denatured SBA cannot form tetramers and may not form an extensive network, so activity is reduced. A single enzyme hydrolyzes SBA into small peptides, but some of the peptides can still bind to red blood cells. The active peptides either bind multiple cells or bind each other to cause agglutination. Multiple enzymes hydrolyze SBA into even smaller peptides and destroy the red blood cell binding sites, so they can no longer cause agglutination. This hypothesis may need further experiments to specifically test the mechanism of cell binding.

Other Physical Treatments To Decrease Activity of SBA. *Effect of TP on Activity of SBA.* When TP were added to SBA samples, precipitates appeared. The activities of precipitate and supernatant mixtures did not significantly decrease, ranging from 100 to 91% activity at 0 to 20 g/L TP concentration. The supernatant alone was then tested, and the activity decreased but not in response to TP concentration (about 65% relative to control at 2.5–20 g/L concentration). These results indicate that the effect of TP on SBA may be simply removing SBA from the mixture, and such interaction may not be specific. Such treatment is not practical because TP would precipitate all soy proteins, decreasing their nutritional value. TP have been shown to complex with proteins and influence their activities (23). Zhu and Wang (13) found that the addition of TP to concanavalin A (jack bean agglutinin) decreased its activity, and this may be also simply due to protein precipitation.

Effect of Metal Ions on Activity of SBA. On the basis of Jaffe et al. (24), who found that SBA bound Mn^{2+} , which is required for SBA activity, and the hypothesis that the exchange of Mn^{2+} with other ions will reduce SBA activity, we tested some common divalent or trivalent metal ions. Our results showed that metal ions Fe^{2+} , Fe^{3+} , Mg^{2+} , Ca^{2+} , Zn^{2+} , Cu^{2+} , and Al^{3+} generally did not decrease activity of SBA considerably (data not shown). We then chose $Fe_2(SO_4)_3$ and used higher concentrations; however, the activity did not decrease (data not shown). The reason may be that Mn^{2+} binds to SBA too tightly to allow the replacement of other ions.

Effect of Chelating Agents on Activity of SBA. Chelating agents can bind to metal ions and, thus, may remove Mn^{2+} from SBA, reducing its activity. Citric acid, acetic acid, and phosphoric acid used in concentrations ranging from 0.1 to 0.5 M did not decrease the activity of SBA (data not shown). However, EDTA

decreased the activity of SBA (65–70% of the original activity), but not in a dose-dependent manner.

Effect of Removing SBA from the Protein Mixture on Activity. It is known that SBA binds to GalNAc with highest affinity (7), and GalNAc–agarose beads have been used to purify SBA (17). In the SBA extract system, as more beads were added, more SBA was removed from the mixture, and the activity of the supernatant gradually decreased, with 92, 74, 72, and 60% activity remaining after the addition of 0.1, 0.3, 1.0, and 1.5 mL of beads, respectively. The binding capacity of the beads was 6 mg/mL, so 1.5 mL of beads should be able to bind 9 mg of SBA. There was only 4 mg of SBA in the solution, so 1.5 mL of beads was in large excess for binding all of the SBA. The reason for the inability to remove all of the SBA from the solution may be that the interaction or affinity between beads and SBA was not very strong. In a mixed protein system, the activity of the isoelectric protein extract decreased 28% after incubation with 1.0 mL of beads, the same results as with SBA extract. Seemingly, other proteins in the isoelectric extract did not greatly interfere with binding of SBA to beads. Although this method may specifically remove SBA, it is not effective in removing all of the SBA.

In conclusion, this study demonstrates that enzymatic hydrolysis can be an effective means to deactivate soybean agglutinin, and pepsin and pancreatin combination treatment is especially effective. These results provided data for our future nonthermal treatment for deactivating soybean antinutritional factor. Certain enzymatic, chemical, and physical methods examined in this study may not seem to be practical at this stage of research; however, this study provides very useful information on SBA chemistry, structure, and biological activity. Soybean trypsin inhibitors can also be deactivated with nonthermal means, as shown in our previously published results (25, 26).

ABBREVIATIONS USED

C, chymotrypsin; DTT, dithiothreitol; Endo H_f, endoglycosidase H_f; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; Glu-C, endoproteinase Glu-C; GI, gastrointestinal; GuHCl, guanidine hydrochloride; P, pancreatin; Pep, pepsin; Pro, Pronase; SBA, soybean agglutinin; SDS, sodium dodecyl sulfate; SWF, soy white flake; T, trypsin; Th, thermolysin.

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