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ORIGINAL PAPER

In Vitro Hemagglutination Activity of β-Conglycinin and Glycinin Fractions and Feeding Study of Non-Thermal Treated Soy Protein

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Abstract The purpose of this study was to use non-thermal means to reduce hemagglutination activities of soy proteins and thereby improve the feeding quality of the resulting treated proteins. Two storage proteins in soybeans, β-conglycinin- and glycinin-rich fractions, were shown to have in vitro hemagglutination activity. The activity of the β-conglycinin fraction was not reduced by hydrolysis with single proteases, but the hemagglutination activity was fully eliminated by multiple enzyme treatments if it was first heated. However, the activity of glycinin fraction was not fully eliminated by either single or multiple enzyme hydrolysis. Pepsin and pancreatin hydrolysis, which was effective in eliminating hemagglutination activity of soybean agglutinin (SBA), was used to generate a feed material for in vivo evaluation of nutritional quality of soy white flake (SWF). SBA in reducing agent-treated then pepsin–pancreatin treated SWF was deactivated on analytical scale experiment, but not in the feed material. The treated SWF feed material did not improve chick growth performance compared with the raw SWF. However, chicks did not show enlargement of the pancreas or the intestines compared to the raw SWF feed, indicating deactivation of anti-nutritional factors. The trypsin inhibitors seemed to play a more important role than the hemagglutination activity of soy proteins in this nutritional test.

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Abbreviations

AEP	Aqueous extraction processing
BBI	Bowman–Birk inhibitor
CONT	High protein control
CP	Crude protein
EAEP	Enzyme-assisted aqueous extraction processing
FE	Feed efficiency
Glu-C	Endoproteinase Glu-C
GI	Gastrointestinal
KTI	Kunitz trypsin inhibitor
LPC	Low protein control
LPSC	Low protein soy control
PER	Protein efficiency ratio
SBA	Soybean agglutinin
SDS	Sodium dodecyl sulfate
SMBS	Sodium metabisulfite
SWF	Soy white flake
TIs	Trypsin inhibitors

Introduction

Hexanes are commonly used for soybean oil extraction because of the high oil recovery and low cost. However, the drawbacks of using organic solvents, including flammability, solvent residue in the protein meal, and emission to the atmosphere [1] pose safety, sustainability, and environmental issues. Aqueous extraction processing

(AEP) uses water as a medium to extract oil, which is more environmentally favorable [2]. In the AEP process, seeds are ground in water and oil is released and floats as free oil [3]. A two-stage countercurrent enzyme-assisted aqueous extraction process improved oil, protein, and solids extraction yields with less water usage compared to the standard AEP [4, 5]. The proteins remaining after oil extraction are expected to have unique properties because of minimal heat exposure. Such proteins may have better functionalities and nutritional values [2]. Although the protein fraction can be used to produce soy protein products for human consumption, the major use is still expected to be for animal feed. In the present study, we proposed to evaluate the feeding performance of non-thermal treated proteins to those with additional treatments for deactivating anti-nutritional factors. We used defatted soy white flake (SWF) as a model, because it is subjected to minimal heat treatment during solvent extraction.

The minimal heating of the AEP process does not deactivate the anti-nutritional factors in soybeans: soybean agglutinin (SBA, or lectin) and trypsin inhibitors (TIs). SBA can bind to the carbohydrate moiety of cell surfaces and cause the cells to agglutinate, and such activity is referred to as hemagglutination activity. SBA can bind to the brush border, causing an increase in the intestine weight and pancreatic hypertrophy. Intact SBA can be absorbed into blood stream, generating antilectin antibodies, as discussed in our earlier work [6]. We have previously used pepsin followed by pancreatin treatment to fully deactivate SBA with minimum heating [6]. In this study, we used the same enzyme treatment for deactivation of SBA to eliminate the hemagglutination activity in SWF, and then did a feeding study with the raw and treated SWF.

There are different proteins in SWF. Based on their sedimentation coefficients, they can be classified into four fractions known as 2, 7, 11, and 15S [7]. The 7S globulin or β-conglycinin and 11S globulin or glycinin are the two major storage proteins in soybean. The 7S fraction comprises 35% of the total soluble proteins with about 85% being β-conglycinin. Glycinin makes up about 85% of the 11S fraction, which comprises 31–52% of the soluble soy protein. Due to high contents of β-conglycinin and glycinin, the main functionalities and nutritional values of soy protein come from β-conglycinin and glycinin, therefore, they also need to be considered in testing hemagglutination activity of soy products. In the present study, we evaluated enzyme hydrolysis of the main protein fractions in SWF, and assessed the activities using an in vitro hemagglutination assay.

In addition to SBA, soybean also has other anti-nutritional factor, i.e., TIs, including Kunitz trypsin inhibitor and Bowman–Birk trypsin inhibitor. TIs can cause excessive secretion of the cholecystokinin, which leads to excessive secretion of pancreatic enzymes, causing pancreatic hypertrophy and hyperplasia [8]. The anti-nutritional effects of TIs will interfere with our feeding trial using SWF, so this interference needs to be removed first. It has been shown that TIs can be deactivated by treating the proteins with reducing agents at mild temperatures [9, 10]. Wang et al. [11] used sodium metabisulfite (SMBS) to inactivate TIs and improved the degree of in vitro trypsin hydrolysis of soy protein. The weight gain:-feed ratio and protein efficiency ratio (PER) also increased. In the present study, we used the same SMBS conditions to inactivate TIs first, then applied enzyme treatment to deactivate SBA for reducing hemagglutination activity of soybean proteins.

Materials and Methods

Materials

The β-conglycinin- and glycinin-rich soy fractions were produced from the Center for Crops Utilization Research at Iowa State University. They were fractionated according to a two-step soy protein fractionation procedure with 5 mM SO_2 and 5 mM $CaCl_2$ [12]. The glycinin-rich fraction contained 85% glycinin and the β-conglycinin-rich fraction contained at least 81% β-conglycinin. The remaining proteins in each fraction are β-conglycinin and glycinin, respectively, due to incomplete separation. SWF was obtained from Cargill (Minneapolis, MN, USA), and was produced by hexane extraction of the oil and then flash-desolventizing to achieve soy protein with ≥85% dispersability in water. Rabbit blood in Alsever's solution was obtained from Hemostat Laboratories (Dixon, CA, USA) and it was used within 2 weeks. Other reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich (St. Louis, MO, USA).

Enzyme Hydrolysis of Different Soy Proteins to Reduce Hemagglutination Activity

All enzyme treatments were carried out according to the product information sheet provided by Sigma-Aldrich (St. Louis, MO, USA). The chymotrypsin (≥40 units/mg enzyme) treatment was conducted by treating 8 mg β-conglycinin- or glycinin-rich fraction (native or heat treated) in 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.8 at enzyme:protein ratio of 1:80 for 24 h at 30 °C. The thermolysin (50–100 units/mg 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 (10,000 BAEE units/mg enzyme) hydrolysis, 8 mg of β-conglycinin- or glycinin-rich fraction (native or heat

treated) was treated in PBS (phosphate-buffered saline, 10 mM phosphate, 0.9% saline), pH 8.0 with enzyme: protein ratio of 1:16 at 37 °C for 24 h. Pancreatin (4× USP specifications) hydrolysis was carried out by treating 8 mg β-conglycinin- or glycinin-rich fraction (native or heat treated) in the same buffer as chymotrypsin, and enzyme:protein ratio of 1:25 for 24 h at 37 °C.

For multi-enzyme hydrolysis, 8 mg β-conglycinin- or glycinin-rich fraction (native or heat treated) was mixed with 1 M Tris-HCl containing 100 mM $CaCl_2$, and then different combinations of enzyme were added. For the combination of trypsin + thermolysin, trypsin + chymotrypsin + thermolysin, pancreatin + thermolysin, the mixture was incubated at 37 °C for 22 h and then 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 70 °C for 2 h. The combination of enzymes has been used and reported in numerous studies without mentioning of proteases hydrolyzing other enzymes, so we did not test enzyme compatibility.

For pepsin (400–800 units/mg enzyme) followed by pancreatin hydrolysis, 8 mg β-conglycinin- or glycinin-rich fraction (native or heat treated), or SWF was adjusted to pH 2.0 with HCl, 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 added to adjust pH to 7.5, and pancreatin was added to make enzyme:substrate ratio of 1:50. The mixture was then incubated at 37 °C for 22 h.

All samples were heated at 100 °C for 10 min to deactivate the enzyme and stored at −20 °C until the hemagglutination assay.

SDS-PAGE

SDS-PAGE was performed according to Laemmli [13] 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 (14.5–200 kDa).

Effect of SMBS on Hemagglutination Activity of SWF

The SMBS treatment of SWF was done according to Wang et al. [11]. Briefly, 10% SWF was dispersed in 10 mM phosphate buffered 0.9% saline (pH 7.8), and 1.0 mmol/2 g SWF of SMBS was added. The mixture was then stirred and incubated at 55 °C for 1 h and dialyzed against water for 3 days. The dialyzed protein was then subjected to pepsin followed by pancreatin hydrolysis or hemagglutination assay.

Preparation of Soy Protein Samples for Feeding Trial

Three treatments were carried out for SWF: (1) SMBS treatment: SWF, 500 g, was dispersed in 2.5 L of 10 mM phosphate buffered 0.9% saline (pH 7.5), and 47.5 g SMBS (equivalent to 1 mmol/2 g SWF) was added. The mixture was then incubated at 55 °C for 1 h, and dialyzed against water for 4 days and lyophilized. (2) Enzyme-treated SWF: after treated by SMBS and dialysis, the pH of SWF was adjusted to 2.0, and pepsin was added to make substrate:enzyme ratio of 5:1 and 2 ppm Lactrol® was added to inhibit the growth of microorganisms. The mixture was then incubated at 37 °C for 18 h and the pH was then adjusted to 7.5. Pancreatin (100:1, substrate:enzyme ratio) was added and incubated at 37 °C for 20 h. The hydrolysate was then lyophilized. (3) Autoclaved SWF:SWF was mixed with water (1:2) and autoclaved at 121 °C for 40 min. The mixture was then oven-dried at 50 °C, and used as a negative control for feeding. A commercial toasted soy flour was also used as a control.

Extraction of SBA from the Feed Materials

The classical procedure described by Lis and Sharon [14] was used. Briefly, 3 g of feed material was dispersed in 40 mL 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. The supernatant was collected by centrifugation (3,020*g*, 15 min). Ammonium sulfate, 0.3 g, was added to each mL of supernatant for precipitating other proteins. Vacuum filtration was used to remove the precipitate. To each mL of filtrate, an additional 0.27 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 centrifugation and resuspended in 1 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 centrifugation and discarded. A second precipitation of SBA was done by adjusting the pH of the solution to 4.6 with 1N HCl, and adding $(NH_4)_2SO_4$ (0.56 g/mL of solution). The precipitate was collected by centrifugation (3,020*g*, 15 min) and redissolved in 0.5 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. The dried material was dissolved in 0.9% saline to make a final concentration of 6–17 mg/mL and subjected to assay for hemagglutination activity.

Hemagglutination Assay

The hemagglutination assay was performed according to the classical and widely used method of Lis and Sharon [14] with some modification. Briefly, rabbit red blood cells collected in Alsever's solution were centrifuged for 5 min at 410*g*. After estimating the volume of the cells, 5 mL of 0.9% saline per mL of cells was added to wash the cells. Centrifugation was used to collect the cells. After washing three times, red blood cells were suspended at 4% (v/v) in 10 mM phosphate buffered 0.9% saline (pH 7.4) (PBS), then one volume of 1% trypsin (w/v) in PBS was added to 10 volumes of this suspension and the mixture was incubated for 1 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 twofold dilutions 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 were added to each well. The plates were placed in 37 °C for 2 h for agglutination to occur. The plates were then tilted to about 45°, the samples with erythrocytes streamed in a "tear-drop" fashion were considered negative and the ones that did not form a "tear-drop" were considered positive. The hemagglutination units (HU) per gram of protein were determined by the equation [15]:

$$\mathrm{HU/g} = \frac{D_\mathrm{A} \times D_\mathrm{B} \times S}{V}$$

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 mL of extract per gram of protein (inverse of the initial concentration), and V is the volume of extract added. Each sample was tested in duplicate. The HU/g values were then expressed as log (HU/g)/log 2 to normalize the data for the twofold dilution. Because the results may be affected by the age of the blood, the activity of the trypsin, and other factors, a SBA extract was used as a standard in every set of treatments. The results are expressed as relative activity to SBA.

Trypsin Inhibitor Activity Test

TIs were assayed by Eurofins Scientific Inc. (Des Moines, IA, USA). Briefly, the sample was extracted in a dilute NaOH solution, and centrifuged to obtain the supernatant. The sample was then reacted with acetic acid, trypsin solution, and benzoyl-L-arginine-*p*-nitroanilide hydrochloride. The sample was then read versus a blank and TIU/g was calculated. TIU was defined as the amount of inhibitor required to inhibit 1 unit of trypsin activity.

Animal Feeding Trial

To determine the digestibility of the treated soy proteins, 7-day-old male chicks were used to determine PER using formulated diets with low protein content. PER was calculated as previously described [11]. Ninety-weeks-old male chicks (Ross × Ross 708) were weighed and blocked on initial body weight into 25 pens consisting of five pens per treatment (3 chicks/pen). The treatments consisted of (1) high protein control (CONT): 23% crude protein (CP) diet containing commercial toasted defatted soy flour; (2) low protein control (LPC): 17.25% CP diet containing commercial toasted defatted soy flour; (3) low protein soy control (LPSC): 17.25% CP diet containing untreated SWF; (4) low protein SMBS soy (SMBS): 17.25% CP diet containing SMBS-treated SWF; and (5) low protein enzyme-treated SWF (ENZYME): 17.25% CP diet containing ENZYME-treated SWF. All diets were formulated to meet or exceed NRC [16] standards for vitamins and minerals, and the low protein diets were 75% of the NRC CP recommendations. The compositions of these diets are listed in Table 1. The diets were fed ad libitum for 9 days, all chicks were euthanized by carbon dioxide asphyxiation and their pancreases and small intestines (pyloric sphincter to illeocecal valve) were removed and weighed. All procedures outlined in this experiment were approved by the Iowa State University Animal Care and Use Committee.

Statistical Analysis

The treatment replicate number (N) from different experiments is different and the N values are given in tables or figures. The data were analyzed by 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, USA). Growth performance, PER, and pancreas and intestinal weights were also analyzed by ANOVA, with the experimental unit being a pen of three chicks.

Results and Discussion

Effect of Enzyme Hydrolysis on Hemagglutination Activity of the β-Conglycinin-Rich Fraction

β-Conglycinin and glycinin fractions were initially used as negative controls, because they were not expected to have hemagglutination activity. Most of the SBA should have been removed from the storage proteins during the pH 4.5 precipitation step [14] of the protein isolation process, therefore, these two storage protein fractions should be free

Table 1 Composition of experimental diets for chicks

Ingredient (%)	High protein control (CONT)	Low protein control (LPC)	Low protein soy control (LPSC)	Low protein SMBS soy (SMBS)	Low protein enzyme soy (ENZYME)
Corn	51.84	68.96	70.80	74.95	67.64
Commercial toasted soy flour	37.10	22.50	–	–	–
Raw soy flour (untreated)	–	–	20.95	–	–
SMBS soy flour	–	–	–	17.47	–
ENZYME soy flour	–	–	–	–	23.70
Soybean oil	6.67	4.07	3.79	3.14	4.25
Di-calcium phosphate	1.98	2.07	2.08	2.10	2.06
Limestone	1.15	1.22	1.22	1.24	1.21
Vitamin mix[a]	0.50	0.50	0.50	0.50	0.50
Mineral mix[b]	0.10	0.10	0.10	0.10	0.40
Sodium chloride	0.40	0.40	0.40	0.40	0.10
DL-Methionine	0.21	0.13	0.11	0.05	0.09
Selenium	0.05	0.05	0.05	0.05	0.05
Total	100.00	100.00	100.00	100.00	100.00
Calculated nutrient content					
Metabolizable energy (kcal/kg)	3200.00	3200.00	3200.00	3200.00	3200.00
Crude protein (%)	23.00	17.25	17.25	17.25	17.25
Methionine (%)	0.55	0.40	0.40	0.38	0.38
Methionine + cysteine (%)	0.90	0.68	0.68	0.85	0.68
Lysine (%)	1.23	0.83	0.84	0.98	0.83

[a] Composition of vitamin mix (per pound): vitamin A, 10,00,000 IU; D3, 2,50,000 IU; E, 5,000 IU; B12, 5.0 mg; riboflavin, 1,500 mg; niacin, 7,500 mg; and *d*-pantothenic acid, 4,000 mg

[b] Composition of mineral mix: calcium, 2.6–3.6%; copper, 1.75%; iron, 17.5%; manganese, 6%; zinc, 15%; and iodine, 2,000 ppm

of SBA. However, we found they also had activity as shown in Table 2. Since our ultimate goal was to eliminate the hemagglutination activity of SWF in order to improve its feeding quality, we have to examine and reduce such activity of all protein components in the SWF, including β-conglycinin and glycinin. This is the reason that β-conglycinin and glycinin fractions were treated with the similar treatment strategy as for SBA as described by Ma and Wang [6], in which various individual enzymes and enzyme combinations were tested.

Table 2 shows a tendency that heating reduced the hemagglutination activity of β-conglycinin-rich fraction, however, single enzyme hydrolysis, whether native or heated, did not fully eliminate the β-conglycinin's hemagglutination activity. This result was similar to enzyme hydrolysis of SBA (47.1% residual activity after heating and 35–62% residual activity after heating and single enzyme treatment). However, the activities were similar for enzyme hydrolyzed native, heated or autoclaved β-conglycinin, which was different from SBA (enzymes could not hydrolyze native SBA), indicating that native β-conglycinin was not resistant to enzyme hydrolysis.

SDS-PAGE also showed that enzymes can hydrolyze native β-conglycinin (Fig. 1 lane 4). This result indicates that enzyme hydrolysis might have released active peptides in β-conglycinin, that gave the hemagglutination activity. It also suggests that unlike SBA, native β-conglycinin is susceptible to enzyme hydrolysis and it may not survive in the GI tract digestion. It may be fully hydrolyzed by multiple enzymes and may not cause serious anti-nutritional effects as native SBA would.

Table 2 Effects of single enzyme hydrolysis on hemagglutination activity of β-conglycinin- and glycinin-rich fractions

Enzyme used	None	Trypsin	Chymotrypsin	Thermolysin	$LSD_{0.05}$
β-Conglycinin					
Native	61.7 a	60.6 a	60.2 a	51.2 ab	38.1
100 °C 20 min	26.9 ab	51.5 a	34.6 ab	38.6 ab	
Autoclave	13.4 b	46.5 ab	24.1 ab	26.4 ab	
Glycinin					
Native	40.1 ab	57.2 a	56.3 a	45.2 a	29.4
100 °C 20 min	14.8 bc	63.7 a	54.4 a	43.6 ab	
Autoclave	0.0 c	68.4 a	55.8 a	50.3 a	

The values are the relative activity to native SBA (%). Data are means of three replicates. Different letters within all treatments for each individual protein represent significant differences ($P \leq 0.05$)

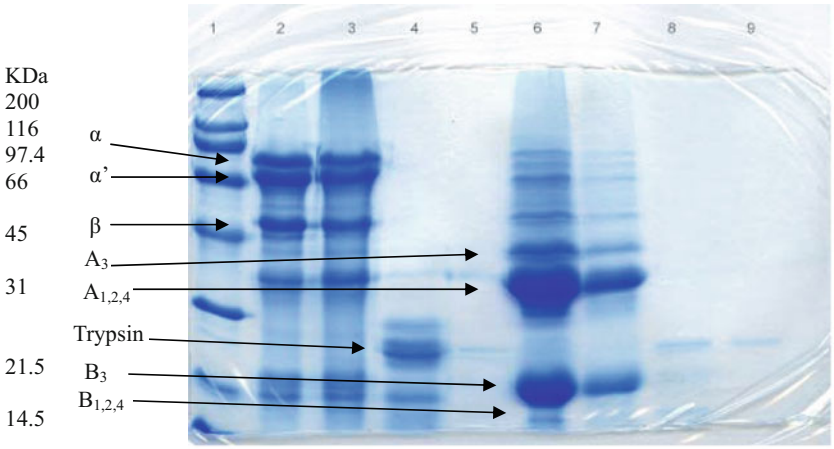

Fig. 1 Effects of enzyme treatment on β-conglycinin and glycinin. *Lane 1*, molecular weight marker; *lane 2*, β-conglycinin; *lane 3*, β-conglycinin treated at 100 °C for 20 min; *lane 4*, β-conglycinin treated with trypsin; *lane 5*, β-conglycinin treated at 100 °C for 20 min, then by trypsin; *lane 6*, glycinin; *lane 7*, glycinin treated at 100 °C for 20 min; *lane 8*, glycinin treated with trypsin; *lane 9*, glycinin treated at 100 °C for 20 min, then by trypsin

Table 3 Effects of multi-enzyme hydrolysis on activity of β-conglycinin and glycinin-rich fractions

Enzyme used	β-Conglycinin		Glycinin	
	Native	100 °C 20 min	Native	100 °C 20 min
No enzyme	65.4 a	44.2 b	38.7 ab	16.8 b
T + C	–[a]	50.5 ab	–	55.9 a
T + Th	–	0.0 c	–	54.0 a
C + Th	–	0.0 c	–	33.4 ab
T + C + Th	54.1 ab	0.0 c	57.8 a	58.5 a
P	54.7 ab	11.6 c	51.4 a	53.1 a
P + Th	45.7 b	0.0 c	47.8 ab	56.9 a
$LSD_{0.05}$	15.2		32.5	

The values are the relative activity to native SBA (%). Data are means of three replicates. Different letters within all treatments for each individual protein represent significant differences ($P \leq 0.05$)

T trypsin, *C* chymotrypsin, *Th* thermolysin, *P* pancreatin

[a] Not tested

Table 4 Effects of enzyme hydrolysis on activity of native or 100 °C for 20 min treated soy proteins

Enzyme used	None	Pep then P	$LSD_{0.05}$
β-Conglycinin			
Native	43.4 a	35.6 a	21.6
100 °C	0.0 b	38.0 a	
Glycinin			
Native	63.2 a	19.1 b	31.0
100 °C	37.7 ab	19.1 b	

The values are the relative activity to native SBA (%). Data are means of four replicates. Different letters within all treatments for each individual protein represent significant differences ($P \leq 0.05$)

Pep pepsin, *P* pancreatin

After multi-enzyme hydrolysis, activity of the native β-conglycinin fraction was not reduced to zero (Table 3). Like SBA, multi-enzyme hydrolysis with thermolysin fully deactivated heated β-conglycinin. This difference between native and heated β-conglycinin indicates that heating facilitated the hydrolysis of β-conglycinin, so it may be cleaved into smaller pieces. This also indicates that like SBA, thermolysin might cut at the crucial sites for activity of β-conglycinin.

Simplified conditions of the GI tract were also applied to the β-conglycinin fraction, i.e., hydrolysis was conducted with pepsin followed by pancreatin and the results are shown in Table 4. The activity of β-conglycinin did not decrease by this treatment, which indicates the inability of the two enzymes to cut active sites of β-conglycinin, therefore, β-conglycinin may have the possibility of showing anti-nutritional effect.

Effect of Enzyme Hydrolysis on Hemagglutination Activity of the Glycinin Fraction

The glycinin-rich fraction showed similar results as the β-conglycinin fraction in reponse to single enzyme hydrolysis. Heating slightly reduced hemagglutination activity, however, single enzyme hydrolysis did not decrease hemagglutination activity (Table 2), and, in fact, activity increased slightly following some single enzyme treatments. This result suggests that heating may cause protein to denature and aggregate so that the active sites may be buried. Partial enzyme hydrolysis might have opened up and released the active peptides, thus increasing the hemagglutination activity. The hydrolyzed native, heated or autoclaved glycinin had similar activity. SDS-PAGE showed that like β-conglycinin, native glycinin can be hydrolyzed by enzymes (Fig. 1 lane 8). These results indicate that glycinin may not survive in the GI tract if multiple enzymes can fully hydrolyze the protein.

However, unlike for β-conglycinin, none of the multi-enzyme hydrolyses fully deactivated glycinin, whether in the native or heated forms (Table 3). This result indicates

that these enzymes were unable to cleave all of the active sites in glycinin; whereas for β-conglycinin, the extensive hydrolysis with the enzymes may have destroyed the active sites on the peptides.

We also used pepsin and then pancreatin enzyme treatments that were effective in deactivating SBA [6] to treat the glycinin fraction. The results were similar to those for β-conglycinin, that is pepsin followed by pancreatin hydrolysis did not fully deactivate glycinin. Therefore, glycinin may also have the potential of causing anti-nutritional effect.

The β-conglycinin and glycinin fractions used for this study were not pure, and they are contaminated with each other, as shown in Fig. 1. However, we do not expect SBA contamination of these two fractions as mentioned previously. We believe that the hemagglutination activity we observed in the two storage proteins are indeed from within the proteins or peptides. As we have shown in another study, the peptides we identified from the hydrolysis products of β-conglycinin and glycinin and then synthesized from amino acids truly had hemagglutination activity [17].

There were large variations in the hemagglutination activity data, which might be due to the inconsistency of enzyme hydrolysis during the repeated treatments, or the detection and nature of the hemagglutination assay. This assay is a traditional method and its use was validated [6, 17] and compared to other methods [6]. Nonetheless, some general trends can still be observed. In addition, different batches of β-conglycinin and glycinin were used for various experiments, so the values of native and 100 °C 20 min-treated β-conglycinin and glycinin varied.

A common food protein, casein from bovine milk, was also used as a negative control for the enzymatic treatments and hemagglutination assay. Casein did not show any hemagglutination activity, nor did the enzyme hydrolysates of casein when the same conditions were used for protein hydrolysis (data not shown). This indicates that β-conglycinin and glycinin fractions truly had in vitro hemagglutination activity.

Effect of Enzyme Hydrolysis on Hemagglutination Activity of SWF

Although pepsin and pancreatin did not fully deactivate β-conglycinin and glycinin, the two enzymes did fully hydrolyze SBA [6], so this treatment was chosen as a non-thermal treatment for generating feed material from SWF. SWF, however, has trypsin inhibitors, which inhibit the activity of chymotrypsin and trypsin in animals. TIs also inhibit animal growth and cause pancreas enlargement, which interfere with our feeding evaluation. Therefore, SMBS was used for non-thermal deactivation of TIs, and then such SWF was treated with pepsin and pancreatin.

Figure 2 shows that after SMBS treatment, hemagglutination activity of SWF reduced slightly, and the activity was not as low as pepsin and pancreatin treated samples, which indicates that SMBS only had a slight effect on SBA. The pepsin and pancreatin treatment significantly reduced activity of SWF, but not to zero. This result was expected because β-conglycinin and glycinin, the major soy proteins, were not deactivated by this treatment. The activity of heat-treated SWF was significantly decreased. Heat followed by pepsin and pancreatin treatment of SWF had an increased activity, which may be due to the more effective protein hydrolysis and the release of active peptides. In addition, after pepsin and pancreatin treatment, native or SMBS-treated SWF had similar activities. TIs inhibit trypsin in a competitive manner [18], as a result, an excessive dose of pancreatin may overcome the inhibition effect of TIs, giving the similar hemagglutination activities of enzyme-treated native or SMBS-enzyme treated SWF.

Activities of SBA Extracted from SMBS and Enzyme-Treated SWF

Our initial goal of enzymatic treatment of SWF was to fully deactivate SBA; however, β-conglycinin and glycinin interfered with the hemagglutination test of SBA when testing the activity of the whole SWF. In order to evaluate whether SBA was fully hydrolyzed by enzyme treatment and eliminate the interference of β-conglycinin and glycinin, SBA was isolated from SWF samples that were treated differently. The activity of SBA extracted from pepsin and then pancreatin treated native SWF (36.4%) was not the same as in the model pure SBA system which was zero. Whereas, the SBA extracted from SMBS-treated SWF showed zero activity (Table 5). This result suggests that after SMBS deactivation of TIs, SBA was easier to

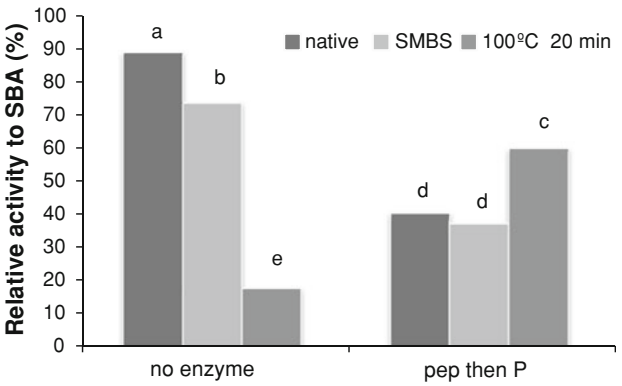

Fig. 2 Effects of SMBS and enzyme treatments of soy white flake (SWF). *SMBS* sodium metabisulfite treated SWF; *pep then P* pepsin then pancreatin treatments; data are mean of three replicates. Different letters represent significant differences ($P \leq 0.05$). $LSD_{0.05}$ is 13.4

hydrolyze by pepsin and pancreatin. For these two treatments, whole SWF had similar activities (Fig. 2), but the extracted SBA had different activities (Table 5). This must be due to the contribution of β-conglycinin and glycinin to the SWF.

For the feeding trial, we treated SWF with SMBS and then pepsin and pancreatin to deactivate TIs and SBA; SMBS-treated SWF was used as a control for deactivating TIs but not SBA; and SWF was used as a control as untreated material. Herkelman et al. [9] found that heating soybeans to 121 °C for 40 min achieved maximum growth performance. We included this treatment as a control for the deactivation of all the anti-nutritional factors in SWF and for maximum performance.

Pepsin and pancreatin are naturally occurring enzymes in the GI tract of human and animals, however, these enzymes may not fully deactivate SBA in vivo. SBA is always consumed with other food components, which may serve as a barrier, so SBA may not be hydrolyzed in the GI tract. The amount of enzyme we used in this study may be much higher than that in GI tract. Different enzyme dosages were used in a screening test and pepsin:SBA ratio of 1:5 was the lowest that fully hydrolyzed SBA in SBA extract (data not shown).

Hemagglutination Activities of Feed Materials

After preparing large quantities of feed materials, some key samples were tested. Fig. 3a shows that the hemagglutination activity of SMBS-treated SWF did not differ significantly from the native SWF, which indicates the inability of SMBS to deactivate SBA. The activity of enzyme-treated SWF was significantly reduced, which was consistent with the results from the analytical scale test, in that the non-zero value could be the contribution of activities from the storage proteins. Similarly, the SBA extract from SMBS treated SWF still had similar activity as that from SWF (Fig. 3b); however, the activity of SBA extracted from the enzyme-treated sample was not reduced to zero (Fig. 3b). This may be due to the incomplete hydrolysis of SBA in the bulk SWF material.

Trypsin Inhibitor Activities in Feed Materials

The TI activity in SWF was 60,300 TIU/g whereas that in SMBS was 2,800 TIU/g (4.6% residual activity). TI in the enzyme-treated sample was 3,900 TIU/g (6.5% residual activity). TI in the autoclaved sample was <2,000 TIU/g (<3.3% residual activity). These results indicate that TIs were greatly reduced in SMBS and enzyme-treated samples, and almost totally destroyed in autoclaved samples. Therefore, the SMBS treatments were effective and TIs should not contribute significant problems in the feeding evaluation. Autoclaving was more effective in destroying the TIs than the other treatments.

Hemagglutination Activity Evaluated by an Animal Feeding Study

The initial weight of the chicks was 137.2 ± 1.7 g. Raw soy protein in the LPSC diet reduced the growth performance of chicks as expected (Table 6). Both the average daily gain and average daily feed intake of LPSC treatment were reduced compared to the LPC diet. Gain:feed and PER, however, were not significantly reduced compared to the LPC diet. The raw soy protein in the LPSC diet caused pancreatic hypertrophy due to TIs and SBA. Additionally, the LPSC diet caused increased intestinal mass as a percentage of body weight. This intestinal growth may be due to the need for increased absorption in a diet containing a nutritionally poor protein.

The SMBS diet did not reduce the growth performance of chicks (Table 6). The average daily gain, average daily feed intake, and gain:feed were not different from chicks on the LPC diet. Additionally, the PER for chicks on the SMBS diet was not different from chicks fed the CONT diet. Furthermore, the SMBS diet did not promote pancreas enlargement or an increase in intestine weight. These results demonstrate that the SMBS treatment improved the nutritional value of native soy protein, as demonstrated previously [11].

The growth performance of chicks on the ENZYME diet was not improved compared to the LPSC (Table 6). Average daily gain, gain:feed ratio, and PER were not different from the LPSC. The average feed intake of the ENZYME sample, however, was also significantly lower than the other samples, which might have led to the lower growth performance than the LPC and SMBS diets. However, similar to SMBS, ENZYME did not cause pancreas enlargement or intestine weight increase, which indicates that the anti-nutritional factors in the raw SWF were effectively deactivated by ENZYME treatment, at least to a level that does

Table 5 Activity (%) of SBA extracted from differently treated SWFs on analytical scale

SBA extraction from	SWF	Pepsin and pancreatin treated SWF	SMBS then pepsin and pancreatin treated SWF
Relative activity to SBA (%)	100 a	36.4 b	0.0 c

Different letters designate significant differences ($P \leq 0.05$). $LSD_{0.05}$ is 1.9. $N = 2$

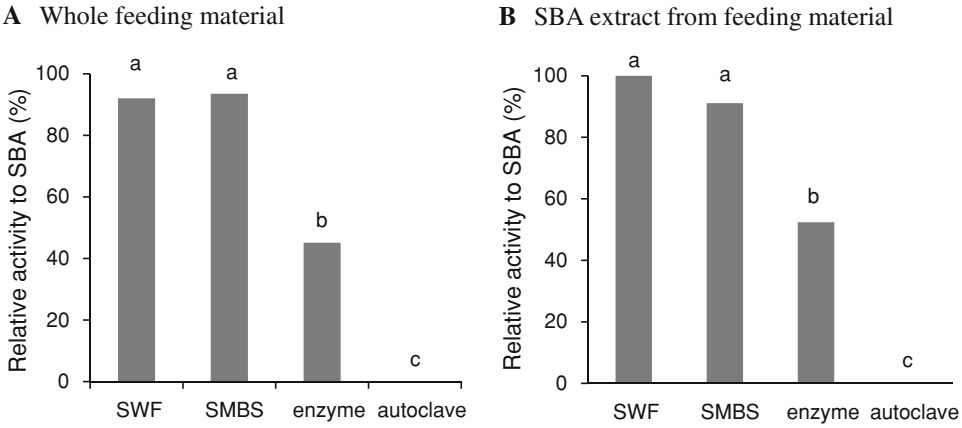

Fig. 3 Hemagglutination activity of feeding materials. **a** Hemagglutination activity of the whole feeding materials. *SWF,* soy white flake; *SMBS,* sodium-metabisulfite-treated SWF; *enzyme* pepsin and pancreatin-treated SWF; *autoclave* autoclave (121 °C 40 min) treated SWF. Data are means of three replicates. Different letters represent significant differences ($P \leq 0.05$). $LSD_{0.05}$ is 9.1. **b** Hemagglutination activity of SBA extracts from feeding materials. $LSD_{0.05}$ is 18.5

Table 6 Effects of diet containing SMBS and enzyme-treated soy protein on growth performance, protein efficiency ratio, and pancreas and intestinal weights

	CONT	LPC	LPSC	SMBS	ENZYME
Average daily feed intake (g/day)	48.0 a	42.2 b	29.5 c	38.6 b	21.6 d
Average daily gain (g/day)	36.9 a	23.3 b	12.9 c	24.3 b	10.7 c
Gain:feed	0.77 a	0.55 bc	0.43 c	0.63 b	0.49 c
Protein efficiency ratio (PER)	3.4 ab	3.2 ab	2.5 b	3.7 a	2.9 b
Pancreas weight (g/100 g BW)	0.37 b	0.38 b	0.86 a	0.39 b	0.41 b
Intestine weight (g/100 g BW)	7.01 b	6.77 b	8.03 a	6.82 b	7.10 b

$N = 5$ pens of 3 chicks per pen. Different letters in the same row represent significant differences ($P \leq 0.05$)

CONT, high protein control diet, 23% crude protein (CP); *LPC*, low protein control diet, 17.25% CP; *LPSC*, raw soy control diet, 17.25% CP; *SMBS*, low protein-SMBS-treated raw soy diet, 17.25% CP; *ENZYME*, low protein diet SMBS-and-enzyme-treated raw soy diet, 17.25% CP

not influence the function of the pancreas and intestine. Comparing the effect of ENZYME sample on chick growth performance versus pancreas and intestine weight, it seems that the growth inhibition can happen at a low level of SBA, whereas pancreas and intestine weight increase need higher levels of SBA to be affected. This result was in agreement with Fasina et al. [19].

However, chicks on SMBS diet, which still had intact SBA but no TIs, performed as well as those on control LPC diet. This suggests that SBA might not be a significant problem for chick growth compared to TIs. This makes it highly likely that the poor performance of the ENZYME sample was due to inadequate feed intake by the chicks, not the effect of BSA on nutrient absorption. The poor feed intake might be because of the decreased palatability, such as unpleasant smell, texture or bitterness of the sample. Protein hydrolysates often have a bitter taste, particularly peptides containing neutral amino acids with large alkyl or aromatic side chains [20]. Humans and animals have consumed heated soybeans and soy protein products for a long time without nutritional consequence, indicating that the active peptides released from β-conglycinin, glycinin, or SBA by enzyme hydrolysis might not be a significant problem. As a result, the remaining hemagglutination activity of β-conglycinin and glycinin hydrolysates might not be the reason for growth inhibition in this study, just as the small amount of SBA in feed (0.024%) was tolerated by turkey poults as illustrated by Fasina et al. [19].

The autoclaved SWF was in large chunks and could not be finely ground, so the chicks picked through the diet and did not eat as much. This makes the results of autoclaved SWF unreliable, so the data for this sample was not used. This observation is another indication that the texture of the feed material is important.

Collectively, these data suggest that TI inactivation protected chicks from pancreatic hypertrophy and from increased intestinal weight. TI inactivation also led to good growth performance. The data may also suggest that the hemagglutination activity has to reach some threshold value to exert anti-nutritional effect in biological systems.

Conclusion

This study is the first to show that soybean storage proteins may have hemagglutination activity and their activities can be reduced by enzyme hydrolysis just as for SBA. However, the non-thermal enzymatic treatment developed in this study with a certain degree of in vitro effectiveness may not be commercially feasible or necessary. We predict that heating is still the best means to deactivate most of the anti-nutritional factors. Nonetheless, this study does provide data and evidence of bioactive peptides and the need for further testing of the properties of these peptides from soybean storage proteins. The in vitro hemagglutination activity demonstrated in this study may not cause significant nutrient malabsorption, however, its effect on cell surface properties may induce other biological functions.

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