

Deactivation of soybean agglutinin by enzyme hydrolysis and identification of active peptides from soy proteins

by

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LIST OF ABBREVIATIONS

ACE	Angiotension-converting enzyme
AEP	Aqueous extraction processing
BAPA	Benzoyl-L-arginine- <i>p</i> -nitroanilide hydrochloride
BBi	Bowman-Birk inhibitor
CCK	Cholecystokinin
CHO	Carbohydrate
CONT	High protein control
CP	Crude protein
DTT	Dithiothreitol
EAEP	Enzyme-assisted aqueous extraction processing
EGCG	Epigallocatechin gallate
FE	Feed efficiency
GalNAc	N-acetyl-D-galactosamine
GlcNAc	N-acetyl-D-glucosamine
Glu-C	Endoproteinase Glu-C
GI	Gastrointestinal
GuHCl	Guanidine hydrochloride
IMAC	Immobilized metal affinity chromatography
KTI	Kunitz trypsin inhibitor
LPC	Low protein control
LPSC	Low protein soy control
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
PER	Protein efficiency ratio
PVDF	Polyvinylidene difluoride
SBA	Soybean agglutinin
SBM	Soybean meal
SDS	Sodium dodecyl sulfate
SHR	Spontaneously hypertensive rats
SMBS	Sodium metabisulfite
SPC	Soy protein concentrates

SPH	Soy protein peptic hydrolysate
SPI	Soy protein isolates
SWF	Soy white flake
SWFE	Soy white flake saline extract
TBARS	Thiobarbituric-acid-reactive substances
TIs	Trypsin inhibitors
VOCs	Volatile organic compounds

CHAPTER 1. GENERAL INTRODUCTION

Literature Review

1. Soybean and processing

Soybeans are one of the most valuable and economical agricultural commodities in the world. Soybeans are not only a valuable source of edible oil, but also a very good source of proteins. Soybeans contain about 20% oil, the second highest among all food legumes, and about 40% protein, the highest among cereals and other legumes (Liu, 1997). Soy protein is a very good amino acid source for humans, especially for vegetarians, because its amino acid composition is similar to animal proteins, such as meat protein, and contains all the essential amino acids for humans. Soy protein is very high in lysine, which is deficient in most cereal proteins, but low in methionine (Liu, 1997). Soy protein is also known to have many beneficial health effects, for example, lowering plasma cholesterol and triacylglycerol (Baba et al., 2004), preventing cancer (Wu et al., 1998), diabetes, and obesity, protecting healthy digestive tract, and protecting bone, and kidney from irritants (Friedman and Brandon, 2001). In 1999, the FDA authorized the labeling on soy foods allowing a claim that soy protein reduces the risk of coronary heart disease. Soybeans are widely used in human and animal diets, including infant formulas, flours, protein isolates and concentrates, and textured fibers. There are also many soy foods available in market, for example soymilk, tofu, miso, natto, roasted soybeans, soybean sprouts, and vegetarian meat substitutes. New soy foods are continuously being developed (Friedman and Brandon, 2001).

Soy proteins

Soy proteins can be classified in different ways. Based on biological functions in plants, they can be divided into metabolic and storage proteins. Metabolic proteins are typically enzymes and structural proteins, whereas storage proteins are used in seed germination to provide sources of nitrogen and carbon skeletons for developing seedlings. The majority of soy proteins are storage proteins. Based on solubility, soy proteins can be classified as albumins, which are soluble in water, and globulins, which are soluble in salt solution. The most common classification is to separate the proteins based on their sedimentation coefficients using ultracentrifugation under appropriate buffer conditions. The protein fractions are known as 2S, 7S, 11S, and 15S (Liu, 1997). The 2S fraction consists of a number of enzymes, including trypsin inhibitors. The 7S fraction contains enzymes, lectin, and a major storage protein β -conglycinin or 7S globulin. The 11S fraction is the largest single fraction of total seed protein, which is composed of a major storage protein glycinin or 11S globulin. The 15S fraction contains urease and a dimer of glycinin (Liu, 1997).

There are three major soy protein products mainly used for food: defatted soy flours, soy protein concentrates, and soy protein isolates. Defatted soy flours are made by grinding the dehulled, defatted soybean flakes. They have about 45-50% protein and have other components such as carbohydrates. Soy protein concentrates (SPC) are further depleted of soluble carbohydrates by alcohol or acid precipitation of protein, and have at least 65% protein on moisture-free basis. Soy protein isolates (SPI) have more than 90% protein on dry-weight basis. SPI is extracted with water under alkaline conditions followed by acid precipitation; however, the alkali extraction process destroys lysine and cysteine (Lusas and Rhee, 1995). Another major application for soy is using the soybean meal (SBM) for animal feeding, with dehulled and solvent-extracted meal being most widely used. SBM contains 44-50% protein

and has excellent amino acid quality. In the United States, more than 50% of the SBM is used for poultry feeding and 26% is used for swine feeding. The rest is used in diets for ruminants, dogs, cats and others (Stein et al., 2008).

Anti-nutritional factors in soy protein products

Despite the beneficial health effects and wide use of soy proteins for food and feed, special attentions must be paid to the anti-nutritional factors in soybean. The major two anti-nutritional factors are trypsin inhibitors (TIs) and lectins. There are two types of trypsin inhibitors, the Kunitz trypsin inhibitor (KTI) and the Bowman-Birk inhibitor (BBI). KTI inhibits trypsin, whereas BBI inhibits both trypsin and chymotrypsin. The inhibition of digestive enzymes reduces the digestibility of the proteins. In addition, TIs can cause excessive secretion of cholecystokinin, which in turn leads to excessive secretion of pancreatic enzymes, thus causing pancreatic hypertrophy and hyperplasia (Yanatori and Fujita, 1976). Some hypothesized that the growth inhibition effect of TIs was due to the loss of amino acids from excessive secretion of pancreatic enzymes (Booth et al., 1960; Lyman and Lepkovsky, 1957). Native lectin is resistant to digestive enzymes and binds to the small intestinal brush boarder, causing increased weight of the small intestine and pancreatic hypertrophy.

Heat treatment is commonly used for deactivating anti-nutritional factors in soybeans. Lectin is readily eliminated by moist heat treatments, however, TIs are more resistant to heat. Heating at 120 °C for 30 min totally destroyed BBI activity, but left 20% of KTI activity (Friedman et al., 1991). In addition, excessive heat treatment can cause loss of essential amino acids in soy protein such as cysteine, lysine, and arginine. Other methods have been used to deactivate TIs. Sulfur-containing compounds, such as cysteine, N-acetyl-cysteine, glutathione,

and sodium sulfite, are effective in facilitating inactivation of TIs at lower temperature. The reason is that TIs contain substantial disulfide bonds; destroying them by sulfur-containing compounds would facilitate their deactivation (Herkelman et al., 1991; Wang et al., 2009).

Aqueous extraction processing and solvent extraction

Commercial soy oil is produced by solvent extraction, normally a mixture of hexanes. The solvent extraction process has high oil yield (99%) and high solvent recovery (over 95%). The relatively low cost makes the process much more commercially feasible than the aqueous extraction processing (AEP) (Rosenthal et al., 1996). Solvent extraction, however, is associated with environmental problems. Hexane contributes to the industrial emissions of volatile organic compounds (VOCs). Hexane reacts with pollutants, such as nitrogen oxides, to form ozone and other photochemical oxidants under sunlight. Excess ozone is undesirable at ground level, causing damage to crops (Rosenthal et al., 1996). In addition, hexane is highly flammable and has the danger for fire and explosion. Elaborate precautions need to be paid to avoid these dangers, which is very costly (Erickson, 1980). Furthermore, solvent extraction involves a large amount of heat treatment for removing the solvent, which reduces the quality of both oil and remaining proteins (Rosenthal et al., 1996).

AEP is a more environmentally favorable process. The process uses water as the extraction medium and is based on the insolubility of oil in water than on the dissolution of oil (Johnson and Lusas, 1983). Generally, the oil seed is ground or extruded and extracted in water. Enzymes, such as cellulase and protease, can be used to improve the oil yield (Lamsal et al., 2006) and facilitate the de-emulsification step (Jung et al., 2009; Wu et al., 2009). After centrifugal separation, three phases form: insoluble fiber fraction, protein-rich skim, and cream

and free oil. The two-stage countercurrent enzyme-assisted aqueous extraction processing was developed to achieve higher oil, protein, and solids extraction yields with one-half the amount of normal water usage. In this process, the insoluble fraction undergoes a second extraction stage, which is very similar to the first extraction. Similarly, after centrifugal separation, three fractions are obtained. The creams from two extractions are then demulsified to form crude oil, the skims are used for protein recovery, and the insolubles are used for ethanol production or dried for feeding (de Moura and Johnson, 2009; de Moura et al., 2009). The AEP process enables the simultaneous recovery of oil and protein (Cater et al., 1974) and the water-washed oil requires little further treatment before refining (Dominguez et al., 1995). Phospholipids are separated from the oil during AEP, therefore, degumming is not required for oil refining (Bocevska et al., 1993). In addition, AEP does not involve excessive heat treatment, avoiding damage to the proteins ensuring better functionality and nutritional value of the protein (Cater et al., 1974). Furthermore, AEP does not use organic solvents, which lowers the risk of fire and explosion and is less hazardous to humans and the environment (Rosenthal et al., 1996). Although has many benefits, the AEP is not yet used commercially. The oil yield is lower than solvent extraction and de-emulsification is required for oil recovery. The lack of heat treatment also preserves anti-nutritional factors in the soy protein, which need to be deactivated before human or animal consumption. AEP is more environmentally favorable, however, more research needs to be done to lower the processing cost, so that AEP can be used in industry.

2. Lectins

Lectins are a group of proteins widely distributed in almost all organisms, including plants, animals, bacteria, and viruses (Sharon and Lis, 2003a). Plant lectins are mainly located

in seeds but are also found in roots, stems, and leaves (Moreira et al., 1991). The seed lectins are located in the cotyledons (e.g., in legumes) or in the endosperm (e.g., castor bean), or in the primary axis (e.g., in wheat) (Damme et al., 1998). Lectins have unique properties of binding to specific carbohydrates with different affinities. In plants, lectins mainly participate in plant defense against the attacking of microorganisms or insects, as well as in physiological functions involving cell recognition (Sharon and Lis, 2003b).

General structure of soybean agglutinin

Soybean lectin, also known as soybean agglutinin (SBA), is a glycoprotein, and forms tetramer with 30-KDa subunits. Each subunit carries an N-linked carbohydrate unit $\text{Man}_9(\text{GlcNAc})_2$ attached to Asn-75, with molecular weight of 1866 Da. In addition, each subunit has a carbohydrate binding site, with highest affinity for N-acetyl-D-galactosamine (GalNAc) and second for galactose. GalNAc specifically inhibit the hemagglutination activity of SBA (Sharon and Lis, 2003c). Upon binding of oligosaccharides with terminal GalNAc or Gal residues, SBA forms unique cross-linked lattices with these oligosaccharides, which might be the bases for its ability to agglutinate cells (Olsen et al., 1997). Each subunit of SBA has tightly bound Ca^{2+} and Mn^{2+} ions or other transition metal ions, which are required for their carbohydrate binding activity of SBA (Jaffe et al., 1977). The structure of SBA monomer is shown in Fig. 1 (Dessen et al., 1995). For biosynthesis, a pre-SBA (285 amino acids) is first expressed from the gene. The pre-SBA then undergoes N-terminal truncation to form the mature SBA (253 amino acids), which is further truncated at the C-terminal to form isolectins with 240, 243, 246, and 252 amino acids, respectively (Sharon and Lis, 2003a). The isolectins have very similar properties and are immunochemically indistinguishable (Liener, 1994). This

might contribute to the multiple bands on the SDS-PAGE gel for SBA extract.

Anti-nutritional effects of lectins

Lectins are present in many edible plants, for example, legumes, such as soybeans, kidney beans, and lima beans, cereals, such as oats, barley, and rice, vegetables, such as celery, asparagus, potato, and tomato, fruits, such as banana, papaya, and apple, and others, such as coconut, mushroom, and garlic (Sharon and Lis, 2003d). Lectins are generally considered anti-nutritional factors, because they can survive the gastrointestinal (GI) tract and cause malfunctions. For example, Pusztai and Bardocz (1995) found that 50-90% concanavalin A (Jack bean agglutinin), PHA (kidney bean agglutinin), or WGA (wheat germ agglutinin) was recovered 1 h after intragastrically administered into rats. SBA and tomato lectin can also be recovered with lower yields. Intact lectins were found in the circulatory system with intact hemagglutinating and immunological activities in humans after consuming lectin-containing foods, for example, tomatoes (Kilpatrick et al., 1985), red kidney beans (Pusztai et al., 1989), and peanuts (Wang et al., 1998), indicating that these lectins have survived the acidity and digestive enzymes in the GI tract. Antibodies were also found in human serum for the lectins in peanuts, soybeans, and wheat germ (Tchernychev and Wilchek, 1996). There have been some cases of food poisoning that caused by consuming insufficiently cooked beans which had lectin in them. The person who ate uncooked kidney beans became acutely nauseated within 1 to 1.5 h and began to vomit, followed by diarrhea. After hospitalization, the recovery was rapid (Sharon and Lis, 2003d).

Native soybean agglutinin is resistant to digestive enzymes in the GI tract and has a unique property to bind to carbohydrate-containing molecules. As a result, SBA can survive

intestinal transit and bind to the intestinal epithelium, which in turn causes disruption of the brush boarder, atrophy of the microvilli and reduction in the viability of the epithelial cells. Because of the interaction with the epithelial surface of the small intestine, SBA can also cause hyperplasia of the crypt cells, thus increasing the weight of the small intestine in rats (Grant et al., 1987). The increasing in weight may be due to the accumulation of polyamines, mostly spermidine, which could stimulate cell proliferation (Deaizpurua and Russelljones, 1988). SBA can stimulate a peptide hormone cholecystokinin (CCK) release and pancreatic protein output in rats. CCK is a hormone secreted by the I-cells of the proximal small intestine, and causes secretion of digestive enzymes from pancreas and release of bile from gallbladder. As a result, prolonged excessive release of CCK causes pancreatic growth and enlargement of the pancreas (Grant et al., 1988; Jordinson et al., 1997). Some lectin can even cross the gut wall into circulation, generating antilectin antibodies (Deaizpurua and Russelljones, 1988). In addition, SBA can inhibit the disaccharidases and proteases in the intestines, interfere with the absorption of nonheme iron (Hisayasu et al., 1992) and lipid from the diet, lower the circulating insulin level and cause degenerative changes in the liver and kidneys (Liener, 1994).

SBA stability

Different lectins have different stabilities, but they are generally resistant to digestive enzymes. Lectins normally can be deactivated by moist heat treatment, but inadequate temperature leads to incompletely inactivated lectins. For example, slow cooking of kidney beans at 82 °C for 11 h or 91 °C for 5 h could not fully inactivate lectin (Sharon and Lis, 2003d). Lectins have been detected in roasted peanuts purchased from a local market (Wang et al.,

1998).

SBA is resistant to inactivation by dry heat treatment (Demuelenaere, 1964) but can be deactivated by moist heat treatment (Liener and Hill, 1953). The inability of dry heat treatment to fully deactivate SBA might account for detectable SBA activity in certain soy containing products (Delabarca et al., 1991). This low concentration of SBA might not be a risk to human health (Liener, 1994). For example, compared to 3600 ug/g lectin in raw soybeans, textured soy protein had 3.75 ug/g lectin (0.08% of original), milk substitute had 6.91 ug/g lectin (0.56% of original), cookies had 2.51 ug/g lectin (6.97% of original), whole wheat bread had 5.68 ug/g lectin (7.89% of original) (Delabarca et al., 1991). Demuelenaere (1964) found that dry heat at 100 °C for 30 min did not significantly reduce SBA activity, whereas autoclaving at 100 °C for 30 min fully deactivated SBA. Dai et al. (2003) found that after dry heat treatment at 120 °C for 60 min, SBA still had considerable activity, whereas moist heat treatment of 95 °C for 30 min, 100 °C for 20 min or 105 °C for 10 min was enough to fully deactivate SBA. Moist heat deactivates both SBA and trypsin inhibitors, and the hemagglutination activity can be used to measure the improvement in nutritional value of the soy protein (Liener and Hill, 1953). Readily deactivated by moist heat treatment, SBA is not a concern in the properly processed soy foods.

Liener and Wada (1956) made several chemical modifications of SBA. They found that modifying the α -amino groups only slightly reduced activity of SBA, whereas modifying the ϵ -amino groups (such as in lysine) resulted in significant loss of activity. In addition, the modification of phenol groups (such as in tyrosine) caused considerable loss in activity. Furthermore, the removing of the C-terminal amino acids by carboxypeptidase did not decrease SBA activity. They concluded that ϵ -amino and phenolic groups of SBA played

important roles in activity.

The literature showed contradictory results about the effect of deglycosylation on the activity of SBA. Nagai and Yamaguchi (1993) found that the intramolecular high-mannose oligosaccharide chains were essential for proper folding and activity of SBA. On the other hand, Adar et al. (1997) found that the SBA expressed by *Escherichia coli*, which lacks the ability to glycosylate proteins, had the same activity as native SBA and the SBA still formed a tetramer.

Bajpai et al. (2005) used zinc alginate beads to remove SBA and trypsin inhibitors from an aqueous extract of soy flour, and obtained 94 and 95% removal of SBA and trypsin inhibitors, respectively. The mechanism of the removal was immobilized metal affinity chromatography (IMAC) based on the special coordinate covalent bond of metal ions to amino acids such as histidine and cysteine.

Carbohydrate binding specificity for SBA

Researchers have studied the carbohydrate (CHO) binding specificity of SBA. SBA has highest affinity for N-acetyl-D-galactosamine (GalNAc) and glycosides and oligosaccharides containing terminal GalNAc. SBA also binds galactose (Gal) and derivatives of Gal with less affinity (Pereira et al., 1974). Hammarstrom et al. (1977) used CHO-protein conjugates for precipitation analysis and simple sugars for inhibition of precipitation to study the binding affinities of different carbohydrates to SBA. They had similar results as Pereira et al. (1974). In addition, SBA did not bind N-acetyl-D-glucosamine (GlcNAc) at all, and weakly bound other sugars like raffinose, stachyose, lactose, D-fucose and L-arabinose. Hammarstrom et al. (1977) also used monosaccharides derivatives to study the binding sites for sugar. They

found that sugar must be in the pyranose form and probably at 4C_1 chair conformation to have binding affinity. In addition, C-2 hydroxyls played an important role in the interaction between SBA and sugar. SBA has slow kinetics for CHO binding (slow binding and dissociation). The low dissociation rate of SBA–CHO complex might be favorable for the binding of SBA to multiple cells, causing topological reorganization and agglutination of the cells (De Boeck et al., 1984; Swamy et al., 1986). The interaction between lectin and monosaccharide is relatively weak and often not as specific as enzyme-substrate interactions. Higher binding affinity and specificity were observed for binding of lectin to oligosaccharides of cell surface glycoproteins and glycolipids, which might be due to multiple binding of lectin to different branch chains of the oligosaccharides. This higher binding affinity might account for proper recognition and binding of lectins to their receptors on cell surfaces in the biological processes (Sharon and Lis, 2003c).

Analytical methods for testing the activity of lectin

The activity of lectin can be tested by using different methods, all based on the CHO binding property. The most commonly used method is hemagglutination assay, which is based on the ability of lectin to agglutinate erythrocytes (Liener, 1994). In this method, lectin samples are serially diluted and mixed with erythrocytes from different species based on the specificity of a certain lectin. In the case of SBA, the most frequently used erythrocytes are from rabbit. The end point is given by the highest dilution of lectin that still causes clumping of the cells, which can be tested visually (Liener and Hill, 1953), by photometric measurement (Liener, 1955), or by examination under a microscope (Pennell et al., 1984). The visual test directly tells the end point, which is easy but subjective. The photometric measurement

measures the density of the layer of unsedimented red blood cells. The method is objective but can be influenced by the adhesion of cells to the glass tube wall and is more tedious than the visual test. Examination by microscopy is similar to the visual test, but is more tedious.

The erythrocytes can be treated with different enzymes or chemicals to improve the sensitivity of the test. Trypsin and papain are commonly used enzymes in the case of testing SBA (Liener, 1994), and glutaraldehyde is an example of a chemical used for treating erythrocytes (Turner and Liener, 1975). In addition to increased sensitivity, glutaraldehyde also permits the storage of blood cells for long periods of time.

The CHO- or glycoprotein-bound polystyrene latex beads is a substitute for the red blood cells. This method uses similar method as the hemagglutination assay and also uses visual inspection of the end point. This method avoids the use of fresh blood, which may not always be available (Kaul et al., 1991). For SBA, the covalent coupling of the latex beads to GalNAc or lactosamine is the most commonly used method. The replacement of blood obviates the influence of the age of the blood, the activity of the enzymes used to treat red blood cells, but introduces the inconsistency of the coupling procedure.

Several ligand blotting methods have also been developed for assessing lectin activity. Typically, lectin is blotted onto a membrane, such as polyvinylidene difluoride (PVDF), either by dot-blotting or by electroblotting after SDS-PAGE; a probe is then used to detect the lectin. Commonly used probes are horseradish peroxidase (HRP)-glycoproteins (Ueno et al., 1991) and CHO-conjugated biotinylated polyacrylamide-type probes (Kamemura and Kato, 1998) which has higher specificity. This method is more sensitive than the hemagglutination assay, but is much more tedious. After SDS-PAGE, the lectin is denatured, which might influence the CHO binding activity. Based on the ability of lectins to bind to CHO, other methods were also

developed. CHO-protein conjugates were used to precipitate lectins, and the protein content in the precipitate was tested (Hammarstrom et al., 1977). Surface plasmon resonance (SPR) was used to detect the binding affinity of CHO to lectins (Paidassi et al., 2008).

Carbohydrate-coupled affinity chromatography (Itin et al., 1996) and enzyme-linked solid-phase assays (Duk et al., 1994) were also used.

Although these *in vitro* detection methods are normally easy and quick, they might not truly reflect the *in vivo* binding of lectins to the intestinal epithelial cells. Hendriks et al. (1987) purified bovine small intestinal brush boarder membranes, and coated them to the bottom of a microtiter plate. The lectin was conjugated with peroxidase, and the binding of lectin to brush boarder was quantified by the peroxidase activity; however, this method cannot be used for the crude extract of lectin.

Animal feeding studies were also used to assess the improvement in nutritional quality of protein. Broiler chicks are normally fed treated soy protein for a certain length of time and different parameters are tested and compared to commercial soy flour and raw soy flour. For example, body weight gain and feed intake were improved for the heat-treated soybean meal compared with raw soybean meal (Liener and Hill, 1953; Liener, 1953). Marsman et al. (1997) found that extrusion significantly improved feed conversion ratio and apparent ileal digestibilities of crude protein and nonstarch polysaccharides when compared with toasting. Higher extrusion rate resulted in increase in water-holding capacity, chyme viscosity, and the concentration of soluble nonstarch polysaccharides in chyme. The intestine weight and pancreas weight were higher in rats fed with kidney bean lectin (PHA) transgenic rice than the normal rice (Poulsen et al., 2007). However, all these treatments have interferences. The heat treatments deactivated both SBA and trypsin inhibitors, and the detection methods did not tell

how much improvement was due to SBA deactivation. Fasina (2004) solved this problem by feeding turkey poults with a corn starch-casein based lectin-free diet with different amounts of SBA added. They found that adding 0.024% of SBA did not have any significant detrimental effects on turkey growth, pancreatic weight, activity of brush boarder enzymes, and they did not find any SBA antibody in circulation. Adding 0.048% SBA gave inconsistent results for feed efficiency and brush boarder enzyme levels.

Applications of lectins

Despite the anti-nutritional effects, lectins have many beneficial effects and can be used in certain areas. The applications are mainly based on the CHO-binding property of lectins.

Lectins can be used in cancer treatments. The mechanism of the anti-tumor effect of lectin is not clear, but there are several hypotheses. The surface glycosylation of tumor cells are different from normal cells, and certain CHOs on primary cancer cells are associated with metastasis. The specific binding of lectins to certain carbohydrates enables differentiation and specific binding of lectin to tumor cells other than normal cells. Thus, lectins can be used as prognostic markers for different types of cancers. In addition, lectins can also decrease protein synthesis and induce apoptosis, thus causing cytotoxicity to tumor cells, reduce cell proliferation, and stimulate the immune system (De Mejia and Prisecaru, 2005). Some lectins have also been shown to have mitogenic activity, that is, induce quiescent lymphocytes to grow and divide, which might be a reason for their stimulation of the immune system. The mitogenic activity is hypothesized to happen in two steps, binding of lectin to T-cell antigen receptor complex, and activating a signal transduction pathway (Rhodes and Milton, 1998).

Lectin can be used in cell identification and separation. Different lectins can recognize

cells with different surface glycosylation. In addition, the binding of lectins to cells is reversible, which enables the undamaged recovery of both lectin-binding and non-binding cells, with high vitality and yield (Sharon and Lis, 2003e). For example, SBA is used in clinical bone marrow transplantation for fractionation of bone marrow cells. SBA specifically binds to bone marrow mononuclear cells, whereas leaving human hematopoietic cells intact, thus enabling a simple and efficient way of enriching CD34⁺ hematopoietic progenitor cells (Nagler et al., 1999). SBA also specifically binds to leukemic T-cells and leaves normal lymphocytes intact. Bakalova and Ohba (2003) used SBA affinity chromatography to separate leukemic T-cells from normal lymphocytes.

Lectins can be used as detection tools. They can be used for detection, isolation, and structural studies of glycoproteins and glycolipids; as histochemistry and cytochemistry tools to study the carbohydrates on cells or subcellular organelles; mapping the neuronal pathways, and studying protein glycosylation. Lectins also have some clinical uses, such as blood typing, evaluation of immunocompetence, karyotyping, and enzyme replacement therapy (Sharon and Lis, 2003e).

3. Bioactive peptides from soybean

Bioactive peptides in foods have been studied extensively. These peptides normally contain 2 to 9 amino acids (Kitts and Weiler, 2003), but can be extended to 20 or more (Korhonen and Pihlanto, 2003). There are also some exceptions such as lunasin from soybean that contains 43 amino acids with a molecular weight of 5.4 KDa (Jeong et al., 2002). Dairy products are the most extensively studied source of bioactive peptides (Floris et al., 2003), however, other foods are also good sources, such as eggs, fish, oysters, cereal, soybeans, and

radish seeds (Wang and De Mejia, 2005). Bioactive peptides from soybean have also been extensively studied.

Antihypertensive peptides

Extensive studies have been published about the antihypertensive peptides in foods. The peptides carry out their functions by inhibiting the angiotension-converting enzyme (ACE). This enzyme converts angiotensin I to angiotensin II, a potent vasoconstrictor, thus causing antihypertensive effect. ACE also inactivates a vasodilator bradykinin, thus increasing blood pressure. The antihypertensive peptides inhibit the activity of ACE, so that reduce of blood pressure (Shahidi and Zhong, 2008).

Several antihypertensive peptides have been found in soybeans. Chen et al. (2002, 2004) separated four ACE inhibition peptides from pepsin digested soy proteins: Ile-Ala, Tyr-Leu-Ala-Gly-Asn-Gln, Phe-Phe-Leu, and Ile-Tyr-Leu-Leu. They were orally given to spontaneously hypertensive rats (SHR) and they lowered blood pressure. Wu and Ding (2001) separated antihypertensive peptides from alcalase digests of soy protein. They were effective in decreasing blood pressure of SHR, but not the normotensive rats. Koderá and Nio (2002) digested soy proteins with protease D3 and obtained five peptides with ACE inhibition activity and favorable taste of the hydrolysates. In addition to the natural antihypertensive peptides in soybean, an artificial peptide was introduced into three homologous sites in α' subunit of soybean β -conglycinin by site-directed mutagenesis. This introduction of peptide enhanced the antihypertension activity of the α' subunit (Yoshikawa et al., 2002). Fermented soy products also have ACE inhibitory peptides, for example, soybean paste (Shin et al., 2001), soy sauce (Okamoto et al., 1995), natto and tempeh (Gibbs et al., 2004).

Hypocholesterolemic peptides

Soybeans have long been known for their cholesterol-lowering effects. As a result, the U.S. Food and Drug Administration (FDA) recommended a daily intake of 25 g of soy protein for lowering cholesterol and reducing of the risk of cardiovascular disease (Shahidi and Zhong, 2008). This activity was first attributed to isoflavones because the removal of isoflavones from soy protein isolate by alcohol washing decreased the atherosclerosis inhibition effect (Adams et al., 2002). Fukui et al. (2002), however, found no cholesterol-lowering effect for isoflavones alone. As a result, it was hypothesized that the interaction between isoflavones and soy proteins is responsible for this activity (Hsu et al., 2001).

Soy protein peptic hydrolysate (SPH) has stronger cholesterol-lowering effects in rats than intact soy protein (Sugano et al., 1990). This effect might be due to the binding of the peptides to bile acids, thus suppressing micellar solubility of cholesterol, which leads to decreased absorption of cholesterol (Nagaoka et al., 1999). In addition, soy peptides might bind to phospholipids and lower cholesterol absorption in humans (Hori et al., 2001). The α and α' subunits of β -conglycinin are more effective than glycinin in regard to the LDL receptor up-regulation effects in human hepatoma cells (HepG2) (Lovati et al., 1992). A synthetic peptide from β -conglycinin (LRVPAGTTTFYVVNPDNDENLRMIA) was found to increase LDL uptake and degradation in HepG2 cells (Lovati et al., 2000). A peptide from glycinin (LPYPR) was also found to decrease serum cholesterol after oral administration to mice. This peptide is homologous to enterostatin, which also has hypocholesterolemic activity (Yoshikawa et al., 2000).

Antioxidative peptides

Some proteins, peptides and amino acids are considered to be antioxidants. Saito et al. (2003) studied the antioxidative properties of series of tripeptide libraries. They examined the antioxidative activity against linoleic acid peroxidation, free radical scavenging activity, reducing activity, and peroxynitrite scavenging activity. They found that peptides having Trp or Tyr at the N-terminus had strong radical scavenging activities, and synergistic effects were observed for peptides with other antioxidants such as phenolic compounds. It was also hypothesized that the His-containing peptides may act as metal ion chelators, active-oxygen quenchers, and hydroxyl radical scavengers (Saito et al., 2003).

Soy peptides have higher antioxidant activity than soy protein (Chen et al., 1998) and heating did not change the activity of the proteins carrying such peptides. This result indicates that peptide release is more critical than maintaining protein structure, and after hydrolysis, more active side chains of amino acids are exposed, which results in increased activity. Pena-Ramos and Xiong (2002) studied the inhibitory activity of soy peptides against formation of thiobarbituric-acid-reactive substances (TBARS) in a liposome-oxidation system. They used pepsin, papain, chymotrypsin, Alcalase®, Protamex®, and Flavourzyme® to hydrolyze native or heated soy protein isolate to different degree of hydrolysis, and the resulting peptide mixtures showed different antioxidant properties. This result indicates the important role of hydrolysis condition.

Antiobesity peptides

Soybeans are normally considered to be beneficial in the management of obesity. Soybean might carry out this function by appetite control, increasing metabolic rate, inhibiting

the absorption of dietary lipids, accelerating lipid metabolism, and decreasing body fat, which lead to weight loss, and decreased plasma and hepatic triacylglycerols (Shahidi and Zhong, 2008; Wang and De Mejia, 2005). Zhang et al. (1998) found the soy protein hydrolysates from an alkaline protease decreased blood lipids and body weight. A peptide from soy glycinin A5A4B3 subunit with the sequence of LPYPR was identified as having anorectic activity (the ability of reducing food intake) (Takenaka et al., 2000). Peptides with anorectic activity were shown to decrease food intake, fat, and body weight (Challis et al., 2004). Soy β -conglycinin pepsin hydrolysates were shown to directly act on rat small intestinal mucosal cells, thus suppressing food intake and gastric emptying (PupoVac and Anderson, 2002). The inhibition of food intake was also observed by Nishi et al. (2003b), and the inhibition can be abolished by devazepide, a selective peripheral CCK receptor antagonist, indicating the role of CCK in the inhibition effect. They also identified the β 51-63 fragment of β -conglycinin to have appetite control activity (Nishi et al., 2003a).

Anticancer peptides

The anticancer activities of soy protein and peptides are not well established, however, there is evidence for anticancer peptides from soybeans (Wang and De Mejia, 2005). Peptides purified from a thermolase hydrolysate of defatted soybean meal were shown to have *in vitro* cytotoxicity on mouse monocyte macrophage cell line, and the peptides arrested cells in G2/M phases of the cell cycle (Kim et al., 2000). Glycopeptides from bromelain-hydrolyzed soy protein have strong cytotoxic activity against P388D1 mouse lymphoma cells. Lunasin is a well-studied natural peptide from soybeans, which is in the 2S fraction of soy protein. Lunasin can bind to non-acetylated H3 and H4 histones and prevent their acetylation (de Lumen, 2005).

In addition, lunasin can suppress carcinogenic transformation induced by chemicals in mammalian cells (Lam et al., 2003).

Immunomodulatory peptides

Certain peptides from enzyme digests of soy protein prevent alopecia (hair loss) induced by cancer chemotherapy (Wang and De Mejia, 2005). Several peptides isolated from trypsin digests of soy proteins were found to have phagocytosis-stimulating activity, for example, a peptide from the α' subunit of β -conglycinin with the sequence of MITLAIPVUNKPGR (Maruyama et al., 2003).

In summary, soy protein is a good source for bioactive peptides that have diverse functions; however, the study is not as thorough as for the dairy protein. More research still need to be conducted to elucidate the sources, functions, and mechanisms of active peptides.

Thesis Organization

This thesis contains a general introduction chapter, followed by three research papers and a general conclusion chapter. The papers are in the journal format required by Journal of Agricultural and Food Chemistry.

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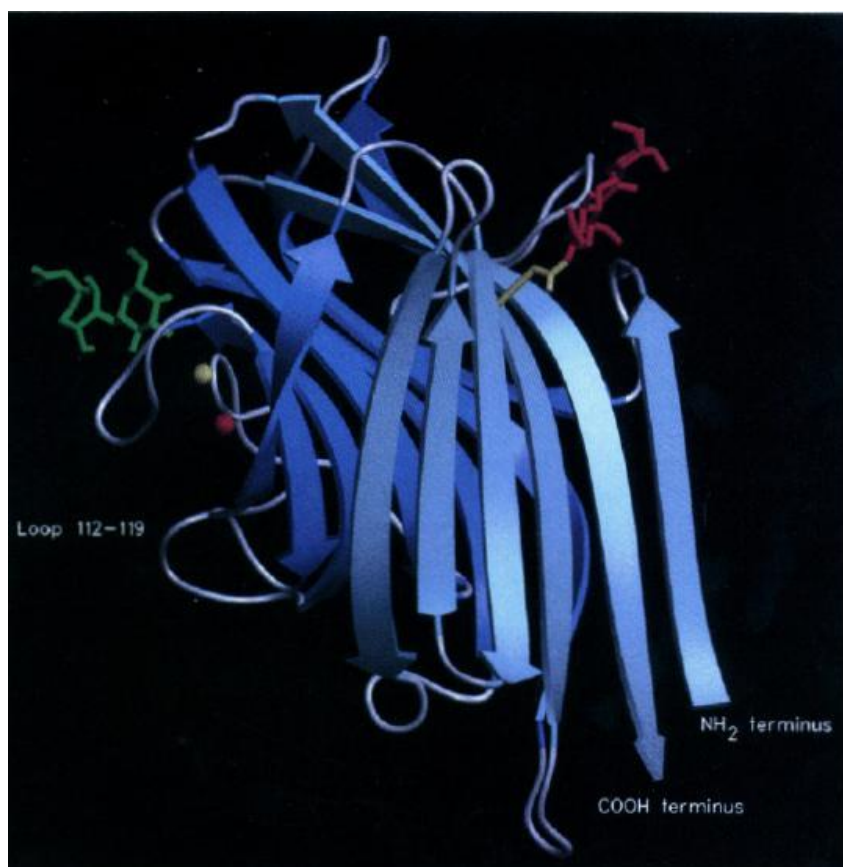


Figure 1. Schematic ribbon diagram of the SBA structure with bound Ca^{2+} (yellow), Mn^{2+} (red), one LacNAc arm of the noncovalent sugar (green), and the two GlcNAc moieties of the Man_9 chain (red) covalently bound to Asn 75 (yellow). The blue arrows are β -sheets. Source: Dessen et al., 1995

CHAPTER 2. DEACTIVATION OF SOYBEAN AGGLUTININ USING NON-THERMAL TREATMENTS

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Abstract

A series of experiments was conducted to eliminate the hemagglutination activity of the anti-nutritional factor, soybean agglutinin (SBA). Deglycosylation decreased activity of SBA by 21%, but not as much as denaturation by heat or denaturing reagents (47-77% residual activity). Single enzymes, such as trypsin, chymotrypsin, thermolysin and Endoproteinase Glu-C, did not hydrolyze native SBA, but hydrolyzed heat- or organic solute-denatured SBA. Even after hydrolysis, activity of SBA still was not fully eliminated (44-62% residual activity). Combinations of enzymes with thermolysin fully deactivated heat- or organic solute-, such as guanidine hydrochloride and urea, treated SBA. Pepsin and pancreatin hydrolysis fully deactivated native SBA. Tea polyphenols, metal ions, and chelating agents were also tested, and they showed no significant effect in SBA activity. N-acetyl-galactosamine-agarose beads specifically removed SBA from protein mixture, but not fully and the activity was not fully eliminated. In conclusion, SBA needs to be denatured first for enzyme hydrolysis and multiple enzymes are needed to fully deactivate SBA.

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KEYWORDS: Anti-nutritional factor; deglycosylation; enzyme hydrolysis; hemagglutination activity; soybean agglutinin

Introduction

Soybeans contain anti-nutritional factors. If soybeans are consumed by humans and animals without heating, they can cause growth depression, and pancreatic hypertrophy, and hyperplasia and adenoma can occur in some animal species (1). There are also reports that some soy protein causes allergies in human (2). The most prominent anti-nutritional factors are the trypsin inhibitors and lectin (3). It was estimated that the trypsin inhibitors accounted for about 40% of the growth inhibition of raw soybeans, while the lectin was responsible for 50%. The remaining 10% is likely due to the incomplete digestion of the undenatured protein (4).

Lectin is a group of proteins that participate in plant defense. In soybean, lectin is called soybean agglutinin (SBA). SBA is a glycoprotein and forms tetramer with 30-KDa subunits. Each subunit carries an N-linked carbohydrate unit $\text{Man}_9(\text{GlcNAc})_2$ attached to Asn-75, with a molecular weight of 1866 Da. In addition, each subunit has a carbohydrate binding site, with the highest affinity for N-acetyl-D-galactosamine (GalNAc), which specifically inhibits hemagglutination activity of SBA, and second highest affinity for galactose (5). Each subunit of SBA has tightly bound Ca^{2+} and Mn^{2+} ions or other transition metal ions that are required for their carbohydrate binding activity (6). SBA exists in different forms are called isolectins. In soybeans, four truncated forms, with 240, 243, 246, and 252 amino acids, were identified, in

addition to the native form with 253 amino acids (7). The isolectins have very similar properties and are immunochemically indistinguishable (3). These isolectins might contribute to the multiple bands of SBA extract on SDS-PAGE gels.

Native SBA is resistant to digestive enzymes in the gastrointestinal (GI) tract, and has a unique property of binding to carbohydrate-containing molecules. SBA is generally considered as an anti-nutritional factor because it can survive intestinal transit and bind to the intestinal epithelium, which, in turn, causes disruption of the brush border, atrophy of the microvilli, reduced viability of the epithelial cells, increased intestine weight (8), and pancreatic hypertrophy (9,10). Some of the lectin can even cross the gut wall into circulation, generating antilectin antibodies (11). SBA can also inhibit the disaccharidases and proteases in the intestine, interfere with the absorption of nonheme iron (12) and lipid from the diet, lower the circulating insulin concentration, and cause degenerative changes in the liver and kidney (3).

Different methods have been used to deactivate SBA, with heat treatment being the most commonly used one. Dry heat treatment is not effective in deactivating SBA (13), but moist heat treatment is (14). Demuelenaere (13) found that dry heating at 100 °C for 30 min did not significantly decrease activity of SBA, whereas autoclaving at the same conditions fully deactivated SBA. Dai et al. (15) found that, after dry heat treatment at 120 °C for 60 min, the SBA still had considerable activity, whereas moist heat treatment of 95 °C for 30 min, 100 °C for 20 min or 105 °C for 10 min was enough to fully deactivate SBA. Chemical modifications have been used to study the crucial amino acids for activity of SBA. Liener and Wada (16)

found modifying α -amino groups and removing the C-terminal amino acids by using carboxypeptidase did not decrease activity of SBA, whereas modifying the ε -amino groups (such as in lysine) and phenol groups (such as in tyrosine) caused significant loss in activity. Dai et al. (15) added different metal ions to SBA and observed reduced SBA activity for some of the metal ions, such as $\text{Th}(\text{NO}_3)_4$, AlCl_3 , $\text{Fe}(\text{NO}_3)_3$, $\text{Pb}(\text{NO}_3)_2$, FeSO_4 , and AgNO_3 . Bajpai et al. (17) used zinc alginate beads to remove SBA and trypsin inhibitors from an aqueous extract of soy flour, and obtained 94 and 95% removal of SBA and trypsin inhibitors, respectively. The literature reports contradictory results about the role of the carbohydrate moiety in SBA. Nagai and Yamaguchi (18) found that the intramolecular high-mannose oligosaccharide chains were essential for proper folding and activity. On the other hand, Adar et al. (19) found that the SBA expressed by *Escherichia coli*, which lacks the ability to glycosylate proteins, still had the same activity as native SBA, and SBA still formed a tetramer.

Recently, there have been on-going efforts to develop aqueous extraction process using water to replace hexane to extract oil from soybeans (20). This process uses much less heat than the traditional process; therefore, protein functionality is preserved. However, such nonthermal processes do not deactivate SBA. Our main research objective was to find practical methods to deactivate SBA with minimum heating. Our ultimate goal was to use non-thermal treatments to improve feeding quality of soybean for animals.

Despite all the contradictory data about the effects of deglycosylation on activity of SBA as mentioned earlier, the carbohydrate unit of SBA may play some role in the activity. We

wanted to deglycosylate SBA and test whether this deglycosylation is an effective way to deactivate SBA with minimum heating. In addition to deglycosylation, we also tested other factors that contribute to activity of SBA, such as protein structure, hydrolysis, polyphenols, and metal ions.

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 (3). The end point is given by the highest dilution of lectin that still causes clumping of cells, which can be tested visually (14), by photometric measurement (21), or by examination under the microscopy (22). In this study, we chose to use trypsin-treated 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-desolventing to achieve soy protein with $\geq 85\%$ dispersibility in water. Rabbit blood in Alsever's solution was obtained from Hemostat Laboratories (Dixon, CA) and used within two 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). Other reagents were purchased from Fisher Scientific (Pittsburg, PA) or Sigma-Aldrich (St.

Louis, MO).

Extraction and purification of SBA. The procedure described by Lis and Sharon (23) 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 obtained by centrifugation (3020 x g, 15 min) was saved. 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, 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 centrifugation 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 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 (3020 x g, 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. The different isolectins were not separated, and the SBA was not further purified.

Deglycosylation of SBA using enzyme Endo H_f Deglycosylation of SBA by EndoH_f

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 the best 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 (8 mg/mL in 0.9% saline) was mixed with or without 10 x glycoprotein denaturing buffer (5% sodium dodecyl sulfate (SDS), 0.4 M dithiothreitol (DTT)), then subjected to heat treatment or not. Then 20 µL of 10 x G₅ buffer (0.5 M sodium citrate, pH 5.5), 20 µL of ddH₂O and 10 µL of Endo H_f (1,000,000 U/mL) were added. The samples were then incubated overnight (18 h) at 37 °C and then stored at -20 °C until hemagglutination assay.

Effect of pH on activity of SBA. Hydrochloric acid or sodium hydroxide was used to adjust pH of SBA solutions to 1-13 on 0.5 intervals. The samples were stored at 4 °C overnight before further testing. Dai et al. (15) found that red blood cells would lyse when pH < 5. So, samples that have pHs lower than five were separated to two parts: one was adjusted to pH 5-6 using 10 x PBS buffer (phosphate-buffered saline, 0.1 M phosphate, 0.9% saline, pH 8.0), the other was subjected to hemagglutination assay directly.

Effects of denaturing agents on activity of SBA. Sodium dodecyl sulfate 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, 8 M urea or 6 M guanidine hydrochloride (GuHCl), was added to SBA (1 mL, 8 mg/mL) and the pH was adjusted to 10. After incubating at ambient temperature for 18 h, half of the solution was dialyzed against water for two 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 enzyme hydrolysis.

Enzyme hydrolysis of SBA to reduce hemagglutination activity. All enzyme treatments were carried out according to the product information sheet provided by Sigma-Aldrich (St. Louis, MO) and the information of enzymes is summarized in Table 2. Native SBA, SBA treated with heat, autoclaving, and organic solutes (with or without removal) were subjected to the same enzyme hydrolysis procedure. For Endoproteinase Glu-C (Glu-C) hydrolysis at the Glu site, 3.2 mg SBA protein was treated in 0.1 M ammonium bicarbonate pH 8.0 (buffer 1) with enzyme:protein ratio of 3:80 for 18 h at 37 °C. For Glu-C hydrolysis at the Glu and Asp site, the same treatment was done except the buffer was 0.1 M sodium phosphate buffer, pH 7.8 (buffer 2). The chymotrypsin treatment was done by treating 3.2 mg SBA protein in 100 mM Tris-HCl, 10 mM CaCl₂, pH 7.8 at enzyme:protein ratio of 1:64 for 24 h at 30 °C. The thermolysin treatment was done in the same buffer as chymotrypsin with enzyme:protein ratio of 1:100 for 24 h at 70 °C. For trypsin hydrolysis, 3.2 mg SBA protein

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 hydrolysis was carried out by treating 3.2 mg SBA protein in the same buffer as chymotrypsin, and enzyme:protein ratio of 1:25 for 24 h at 37 °C. For pronase hydrolysis, 3.2 mg SBA protein was treated in 50 mM ammonium bicarbonate buffer (pH 8.0) with enzyme:protein ratio of 1:50 at 37 °C for 24 h.

For multi-enzyme hydrolysis, 400 µL SBA protein (3.2 mg) was mixed with 1 M Tris-HCl containing 100 mM CaCl₂, and then different combination of enzymes were added. For the combination of trypsin + thermolysin, trypsin + chymotrypsin + thermolysin, pronase + pancreatin + thermolysin, and 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.

For pepsin followed by pancreatin hydrolysis, 400 µL SBA protein (3.2 mg) was mixed with 10 µL 1 M HCl to adjust pH to 2.0; then pepsin was added to make 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 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 100 °C for 10 min to deactivate the enzyme and stored at -20 °C until the hemagglutination assay.

Effect of tea polyphenols on activity of SBA. Tea polyphenols had 65.4%

epigallocatechin gallate (EGCG), 82.8% catechins, 1.2% caffeine, and other components such as moisture and ash. Different volumes of 100 mg/mL tea polyphenols were added to 400 μ L of SBA protein (3.2 mg), and the final concentration of polyphenols 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 (12,395 x g for 2 min) to obtain the supernatant for the hemagglutination assay.

Effect of metal ions on activity of SBA. Different metal ions ($\text{Fe}_2(\text{SO}_4)_3$, FeSO_4 , MgSO_4 , CaCl_2 , ZnSO_4 , CuSO_4 and AlCl_3) were added to the SBA samples to make a final concentration of 0.01 M. $\text{Fe}_2(\text{SO}_4)_3$ was 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 $^\circ\text{C}$ 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 0.5, 0.4, 0.3, 0.2, and 0.1 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 in 4 $^\circ\text{C}$ for 24 h before assaying for hemagglutination activity.

Effect of removing of SBA from SBA extract using GalNAc-agarose beads. To

remove SBA from the extract, SBA (500 μ L, 8 mg/mL) was mixed with different amount of GalNAc-agarose beads, gently mixed at 4 $^{\circ}$ C overnight, then centrifuged (850 x g for 1 min) to obtain the supernatant for the 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 system, pH 4.6 isoelectric extract was used, assuming the isoelectric extract has concentrated SBA and 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 and the mixture was incubated overnight at 4 $^{\circ}$ C. The dispersion was then centrifuged for 10 min at 3000 x g to make an isoelectric soluble protein extract (10%, 100 mg/mL). The extract was then incubated with 1 mL GalNAc-agarose beads 4 $^{\circ}$ C overnight and then centrifuged (850 x g for 1 min) to obtain the supernatants for the hemagglutination assay.

Hemagglutination assay. The hemagglutination assay was performed according to a method of Lis and Sharon (23) with some modification. Briefly, rabbit red blood cells collected in Alsever's solution were centrifuged for 5 min at 410 x 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 for 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 1.0 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 two-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 in 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 negative and the ones did not form “tear-drop” were considered positive. The hemagglutination units (HU) per g of sample were determined by using the equation (14):

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

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 mL 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 $\log(HU/g)/\log 2$ to normalize the data for the two-fold dilution. Because the results may be affected by 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 the influence of experimental conditions.

Statistical analysis. The data were analyzed by Analysis of Variance (ANOVA) and General Linear Model (GLM), 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

Lectin activity assay

Various methods for testing activity of lectin have been used, and hemagglutination assay is the most commonly used one. Variations to this method exist, for example, the erythrocytes can be treated with different enzymes or chemicals to improve the sensitivity of the test. Trypsin and papain are commonly used enzymes for SBA testing (3), and glutaraldehyde is an example of a chemical used for treating erythrocytes (24). The carbohydrate or glycoprotein-bound polystyrene latex beads is a substitute for the red blood cells. This method uses similar method as the hemagglutination assay and also uses visual inspection of the end point. This method avoids the use of fresh blood, which may not always be available (25). For SBA, the covalent coupling of the latex beads to GalNAc or lactosamine is commonly used. The replacement of blood obviates the influence of the age of the blood and the activity of the enzymes, but it introduces inconsistency of the coupling procedure. There are also other methods used to detect activity of lectin. Carbohydrate-protein conjugates were

used to precipitate lectins, and the protein content in the precipitate was tested (26). Surface plasmon resonance (SPR) was used to detect the binding affinity of carbohydrates to lectins (27). Ligand blotting method (28), carbohydrate-coupled affinity chromatography (29) and enzyme-linked solid-phase assays (30) were also used. All of the methods are much more tedious than hemagglutination assay. Although these *in vitro* detection methods are normally easy and quick, they might not truly reflect the *in vivo* binding of lectins to the intestinal epithelial cells. Hendriks et al. (31) purified bovine small intestinal brush boarder membranes and coated them to the bottom of a microtiter plate. The lectin was conjugated with peroxidase, and the binding of lectin to brush boarder was quantified by the peroxidase activity. However, this method cannot be used for the crude extract of lectin. Among all these methods, the hemagglutination assay is the most commonly used and easiest test for lectins. We need to do a large number of treatments for SBA, which requires an efficient analysis method to carry out the activity test, so we chose hemagglutination assay.

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. SBA has a Man₉(GlcNAc)₂ chain attached to Asn-75 of each subunit with N-linkage; so, SBA is substrate for Endo H_f. The SBA monomer is about 30 KDa, and the carbohydrate chain is about 1.9 KDa. After deglycosylation, SBA should have a molecular size of 28.1 KDa. Fig. 1 shows that

deglycosylated SBA was present in all the 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 (Lane 4 and 6) had both deglycosylated and native SBA, which indicated that SBA was not fully deglycosylated. These results indicate that heat treatment is not an effective pretreatment for deglycosylation of SBA by Endo H_f; however, the denaturing buffer containing SDS and DTT is an effective pretreatment for the hydrolysis reaction to complete. 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% activity, which was higher than for the heat treatment alone (56%) (Fig. 2). The activity is expressed as relative activity to native SBA, that is, the $\log(\text{HU/g})/\log 2$ value of the sample divided by the value for native SBA. This result indicates that the carbohydrate part may not play a crucial role in activity of SBA, because removal of this part does not fully decrease activity. This result is partially in agreement with Adar et al. (19) 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 lead to the proposal that proper three-dimensional structure of SBA may be crucial for its activity.

Effect of pH and denaturing agents on activity of SBA

Protein structure plays important roles in the function. An improperly folded protein has no function, or functions abnormally. For example, improper folding of a protein called prion causes various types of transmissible neurodegenerative spongiform encephalopathies in animals and human (32). Protein function could be lost when its secondary, tertiary or quaternary structure is changed (33). There are many methods to denature protein, for example, heat treatment, organic solutes, detergents, and extreme pHs. Proteins tend to unfold at very high or low pH, because of the strong repulsion between like charges (33). Heat can destabilize major noncovalent interactions, causing protein denaturation. Detergents, such as sodium dodecyl sulfate (SDS), bind to protein, causing a shift in equilibrium between the native and denatured states. SDS binds so tightly that denaturation is irreversible. Organic solutes, such as urea and GuHCl, solubilize hydrophobic amino acid residues by breaking the hydrogen bonded structure of water, making it a better solvent for nonpolar residues. At low concentration, this denaturation is reversible (33). In fact, urea and GuHCl are used for solubilizing and refolding of inclusion bodies, which is produced by over expression of protein in bacteria. GuHCl and urea can denature and solubilize the proteins, and upon removal, the proteins can fold back to original form (34). GuHCl is a more powerful denaturant than urea because it is ionic, which binds to protein easier (33). We wanted to examine whether these protein denaturation methods could fully deactivate SBA.

In order to determine the susceptibility of SBA to pH and determine whether extreme pHs can deactivate SBA, we tested the activities of SBA under different pHs. High or low pH

caused red blood cells (RBCs) to lyse; however, as samples were diluted, the cells were not lysed. Typically, after four to six dilutions, the cells were normal, and SBA agglutinated RBCs occurred after about 15 dilutions, so the effect of pH on the hemagglutination assay can be disregarded. In addition, the samples with low pH were tested twice, either adjusted back to pH 6 or tested directly, and the two samples showed similar activities, which was additional evidence that the effect of pH on this assay was little. After several dilutions of a sample or when the pH of a sample was adjusted back to 6, however, the pH of the sample was no longer the ones we intended to test. This might be a concern about whether the results showed true pH effects. Fig. 3 shows that activities of SBA did not decrease in the low pH range, whereas the activity decreased in the high pH range. This observation is not surprising because SBA can survive the very acidic environment in the stomach and the acidic condition only caused reversible structure change. On the contrary, high pH may cause the SBA tetramer to irreversibly dissociate and/or cause SBA monomer to partially unfold, thus decreasing activity; however, even at pH above 11, SBA still showed > 40% activity. Extremely high pH cannot be used in food system because high pH may destroy the nutritional value of food, for example, the formation of lysinoalanine at high pH lowers nutritional value of protein and causes kidney disease in rats (35). Changing pH is not suitable for practical use.

Heat treatment and three denaturing reagents, SDS, urea and GuHCl were expected to fully destroy the quaternary, tertiary and secondary structure of SBA, leaving unfolded protein. Fig. 4 shows that all these treatments decreased activity of SBA, but to different extents. This

result indicates that structure plays an important role in activity of SBA. Even after denaturation, however, SBA still had at least 47.1% activity (the 100 °C for 20 min). Therefore, denaturation alone is not effective to fully deactivate SBA.

Effect of enzyme hydrolysis on activity of SBA

From previous results, we found that the carbohydrate part and protein structure both play some roles in activity of SBA; however, neither of the modifications are fully responsible for activity of SBA. There may be other factors influencing activity of SBA. Protein functions as a whole unit, and destroying the protein may decrease the activity. Therefore, we tried destroying SBA by cutting it into small pieces. Four main hydrolytic enzymes were chosen from various sources. Trypsin is one of the most commonly used enzyme, and is readily available. This enzyme specifically hydrolyzes peptide bonds at the carboxyl side of arginine or lysine residues. The hydrolysis rate decreases if there is an acidic residue present on either side of the cleavage site. If a proline residue is on the carboxyl side of the cleavage site, hydrolysis will not occur (36). There are some consensus sequences in legume lectins (37), which might be important for their activities, so we chose three additional enzymes 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. When proline is on the carboxyl side, no cleavage will occur (38). Glu-C will not cut the protein too short, but can cut at the consensus sequence, so the enzyme is perfect to explore the influence of consensus sequences on activity of SBA. Liener and Wada (16) found that the modification of tyrosine decreased activity of SBA significantly, which indicated the possible involvement of tyrosine residue in activity of SBA, so we also chose chymotrypsin. Normally chymotrypsin hydrolyzes peptide bonds on the C-terminal side of tyrosine, phenylalanine, tryptophan, and leucine; but, secondary hydrolysis can also occur on the C-terminal side of methionine, isoleucine, serine, threonine, valine, histidine, glycine, and alanine (39). Thermolysin hydrolyzes protein bonds on the N-terminal side of hydrophobic amino acid residues, with preferential cleavage as follows: X-(cleavage site)-Y-Z, where X is any amino acid, Y is Leu, Phe, Ile, Val, Met or Ala, Z is any amino acid other than Pro. Because of the low substrate specificity, thermolysin can cut the protein into very small pieces. Also, thermolysin is active in a wide pH range (pH 5 - 9.5) and high temperature (optimal 70 °C), which is important in order to hydrolyze a tough protein like SBA. Another enzyme, pronase, from *Streptomyces griseus*, is a mixture of at least 10 proteases and is very nonspecific (39). We included pronase as a mixture of enzymes, trying to fully hydrolyze SBA and eliminate the activity. In addition to these enzymes, we also used pancreatin from porcine pancreas as a mixture of pancreatic enzymes.

Effect of single enzyme hydrolysis on activity of native SBA. Fig. 5 shows that after hydrolysis of native SBA, there were still SBA bands on the gel, which indicates that none of the hydrolytic enzymes we used can fully hydrolyze native SBA. The activities of enzyme-treated SBA did not decrease (Table 3). These results indicate that native SBA is resistant to hydrolytic enzymes. SBA has a very compact structure, which inhibits the access of enzymes to the cleavage site. We hypothesized we may need to destroy this structure before hydrolysis.

Effect of single enzyme hydrolysis on activity of heat treated SBA. In order to enable the access of enzymes to active sites in SBA, we heated at 100 °C for 20 min or autoclaved at 121 °C for 30 min to denature SBA first and then we treated the protein with different enzymes. Fig. 6 shows that after trypsin, chymotrypsin, or thermolysin hydrolysis of heat-treated SBA, there were no SBA bands on the gel, indicating that SBA was fully hydrolyzed. Glu-C cannot fully hydrolyze heated SBA in both buffer 1 (0.1M ammonium bicarbonate, pH8.0, hydrolyzes at the carboxyl side of glutamyl residue) and buffer 2 (0.1M sodium phosphate buffer, pH 7.8, hydrolyzes at the carboxyl side of either glutamyl or aspartyl residue). However, even after being hydrolyzed, activity of SBA did not further decrease and the residue activity was still similar to the heat treatment alone (Table 3). Single enzyme hydrolysis was not sufficient to fully deactivate SBA. In addition, there was no significant difference between the two heat treatments (moist heat and autoclave), so we used 100 °C for 20 min in future treatments.

Effect of single enzyme hydrolysis on activity of organic solutes treated SBA. We demonstrated that after denaturing with heat, SBA can be hydrolyzed by proteases; however, we want to avoid heat treatment of the proteins. Organic solutes such as GuHCl and urea can also denature SBA and decrease the activity to similar extent (Fig. 4), so we treated SBA with organic solutes including urea or GuHCl, and then with proteases. From previous results, we found SBA was more susceptible to basic conditions, so we conducted the urea or GuHCl treatment at pH 10, where SBA was more likely to unfold. 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 4). They all had similar activities as GuHCl treatment alone, which was in agreement with the heat treatment data (Table 3). Removing GuHCl or not did not affect the activities. Urea treatment displayed a similar pattern. After enzyme hydrolysis, they had similar activities with urea treatment alone (Table 4); however, there was significant difference between the urea removal or not, which was different from the GuHCl treatment. These results indicate that GuHCl and urea could replace heat treatment to denature SBA before enzyme hydrolysis.

Effect of multiple enzymes hydrolysis on activity of SBA. After single enzyme hydrolysis, SBA was not fully deactivated, so we tried to cut SBA into smaller pieces by using

a mixture of multiple enzymes. A combination of enzymes cannot hydrolyze native SBA, and the activity did not decrease (Table 5), which again proved that native SBA is resistant to hydrolytic enzymes. A combination trypsin, chymotrypsin and thermolysin, however, fully deactivated heat-treated SBA (Table 5). This indicates that after being cut into smaller pieces, SBA can be fully deactivated. In addition, any combination of enzyme treatments with thermolysin could fully deactivate SBA. This result indicates that thermolysin might cut the crucial sites for activity of SBA. However, thermolysin alone did not fully deactivate SBA, indicating that trypsin and chymotrypsin also cut at crucial sites, although not completely. This result was in agreement with the result that combination of trypsin and chymotrypsin did not fully deactivate SBA. Furthermore, pancreatin, which contains trypsin and chymotrypsin, did not fully deactivate SBA. This further supported the result that combination of trypsin and chymotrypsin could not fully deactivate SBA.

To fully deactivate SBA with minimum heating, GuHCl or urea were used to replace heat treatment. Before removing GuHCl, a combination of three enzymes (trypsin, chymotrypsin, and thermolysin) did not fully deactivate SBA. After GuHCl treatment and removal, the combination of the three enzymes fully deactivated SBA (Table 5). These results indicate that GuHCl may denature both SBA and the enzymes, so that GuHCl interfered with enzyme hydrolysis. In addition, GuHCl can irreversibly denature SBA, so after removal, SBA did not fold back to the original configuration, which enabled the enzyme hydrolysis that led to full deactivation of SBA. Urea behaved differently. After removal of urea, combination of

three enzymes still cannot fully deactivate SBA, which indicates that urea may not irreversibly denature SBA. After removal, some SBA folded back to original structure; thus, hydrolysis and deactivation were not as complete. This result also can explain the data in Tables 4 and 5. After removal of urea, activity of SBA was higher than before removal. In addition, single enzyme hydrolysis of SBA after removal of urea displayed higher activities than before removal. The decrease of activity for single enzyme hydrolysis in the presence of urea might be due to the denaturation of SBA, not hydrolysis. After removal of urea, some SBA folded back, inhibiting enzyme hydrolysis, so the activities were higher than SBA that denatured by urea (Table 4).

Table 6 shows the effects of other enzymes on SBA activity. Although pronase is capable of hydrolyzing casein into more than 70% amino acids (39), it did not fully deactivate the heated SBA (Table 6). 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, which was in agreement with our earlier results that thermolysin was able to cut the active sites of SBA.

Although treating with GuHCl and multiple enzymes can fully deactivate SBA with minimum heating, it is not a feasible way to treat large amounts of material for feeding trials. We want to identify other more practical ways to denature SBA. Low and high pHs can denature SBA. However, enzymes are also denatured at extreme pHs. There are several exceptions and pepsin is one. The optimal pH for pepsin is 2 to 4, in which SBA might be

denatured. Pepsin preferentially cleaves C-terminal to Phe, Leu and Glu, and does not cleave at Val, Ala or Gly. Pepsin cannot cleave the protein into very small peptides. We then included another non-specific enzyme pancreatin to hydrolyze SBA into small pieces. After hydrolysis with pepsin followed by pancreatin, the SBA band disappeared on SDS-PAGE (Fig. 7) and the activity of both native and heat-treated SBA was reduced to zero (Table 6). Pepsin and pancreatin are enzymes available in large quantity and low price, so it is feasible to use this combination to treat soy white flakes (SWFs) and generate feeding materials.

From the results above, we learned that altering SBA structure could decrease its activity. Native SBA was resistant to proteases, but when denatured first, either by heating or denaturing reagents, SBA can be hydrolyzed. Even after being hydrolyzed by individual enzymes, SBA still had some activity, whereas hydrolyzing by using multiple enzymes could fully deactivate SBA. Thus, we proposed our active peptide hypothesis: active SBA is a tetramer, having full activity; denatured SBA cannot form tetramer, so activity is reduced; single enzyme hydrolyzes SBA into small peptides, but some of the peptides still have activity; multiple enzymes hydrolyze SBA into even smaller peptides and destroy the active peptides, so SBA is fully deactivated. Fig. 8 illustrates the bioactive peptide hypothesis.

Other treatments used to decrease activity of SBA

In addition to the enzyme hydrolysis of SBA, we also considered some non-destructive methods, so that we may avoid the problem of trying to destroying the compact structure of

SBA.

Effect of tea polyphenols on activity of SBA. Tea polyphenols (TPs) are extracts from the green tea. They have been shown to complex with proteins (40) and influence their activities. Protein and polyphenols can form soluble complexes, which can grow to colloidal size and form haze, and if the complexes grow larger, can precipitate out of the solution. Hydrophobic interactions play important roles in this complexation, and proline is also shown to be crucial (40). TPs were shown to inhibit α -amylase and α -glucosidase (41), reduce activities of trypsin inhibitors (42), bind to and precipitate salivary proteins to cause the perception of astringent flavor (43). TPs carry out the function by forming networks (40), thus changing secondary structure of proteins (42). Zhu and Wang (44) found that the addition of tea polyphenols to concanavalin A (Jack bean agglutinin) decreased its activity. In this study, we tested the effect of TPs on SBA.

After adding TPs to SBA solution, they formed a complex with the protein and the complex precipitated out of solution. We found that the activities of precipitate and supernatant mixtures did not decrease, they even slightly increased at some TP concentrations (Table 7). We then tested the supernatant alone and found the activities decreased but not in responding to TP doses (Table 7). These results indicate that the effect of tea polyphenols on SBA is simply removing SBA from the mixture and such interaction may not be specific and strong. Such treatment is not practical because TP may not distinguish SBA from other soy proteins,

therefore, the addition of TP would precipitate all types of soy proteins, decreasing nutritional value of soy protein.

Effect of metal ions on activity of SBA. Jaffe et al. (6) found that the tetramer of SBA had four transition metal sites, with Mn^{2+} bound to them and Mn^{2+} is required for activity of SBA. In addition, the exchange of Mn^{2+} with Co^{2+} and Cd^{2+} did not influence activity of SBA, whereas the exchange for Ni^{2+} significantly increased activity. Dai et al. (15) found that adding certain metal ions other than Co^{2+} , Cd^{2+} and Ni^{2+} to SBA extraction solution could reduce activity, presumably by interfering with the binding of Mn^{2+} to SBA and the formation of the correct three-dimensional structure of SBA. Hisayasu et al. (12) found that SBA could complex with ferrous iron, thus inhibiting nonheme iron absorption in rats. We hypothesized that some divalent or trivalent metal ions could interfere with the binding of Mn^{2+} to SBA, thus decreasing the activity. These metal ions generally did not decrease activity of SBA considerably (data not shown). We then chose $\text{Fe}_2(\text{SO}_4)_3$ and used higher concentration; however, the activity did not decrease at all (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. In addition to the replacement of other metal ions, chelating agents can bind to metal ions, thus may be a possible way to remove Mn^{2+} in SBA. Different chelating agents with various concentrations were tested. Jaffe et al. (6) did

extensive dialysis of SBA using acetic acid buffer and achieved 70-80% loss of activity, so we also included acetic acid. Table 8 shows that citric acid, acetic acid and phosphoric acid in any concentrations did not decrease activity of SBA. EDTA decreased activity of SBA, but not in a dose-dependent manner, and the remaining activity was still high. The results were different from Jaffe et al. (6), who achieved 70-80% and even full loss of precipitating activity by extensive dialysis of SBA against chelating agents. They used both precipitating activity test (the ability of SBA to precipitate hog gastric mucin) and hemagglutination test. In certain cases, they achieved 75% reduction in precipitating activity whereas only 30% loss in hemagglutination activity. They suggested that the binding of SBA to the Mn^{2+} leached from the erythrocytes restored activity during the hemagglutination assay. We did not use extensive dialysis, instead, directly added chelating agents into SBA. We wanted to find practical ways to inactivate SBA. After dialysis and if soy protein is mixed with other food components, SBA will bind to Mn^{2+} in other food again, which can be overcome by directly adding chelating agents.

Effect of removing SBA from the protein mixture on activity. Different lectins bind to different carbohydrates with specificity. SBA binds to GalNAc with highest affinity. GalNAc-agarose beads have been used to purify SBA (23), because the beads can specifically bind SBA without binding to other soy proteins. We wanted to use GalNAc-agarose beads to specifically remove SBA from the soy protein mixture. We first tested this method with pure

SBA. As more beads were added, more SBA was removed from the mixture (Fig. 9), and the activity decreased more (Table 9). Even when as high as 1.5 mL beads were added, however, the activity was still 60%. The binding capacity of the beads was 6 mg/mL, 1.5 mL beads should be able to bind 9 mg SBA, there were only 4 mg SBA in the solution, so 1.5 mL beads was in large excess to bind all the SBA. The reason for the inability to remove all the SBA from the solution may be that the interaction or affinity between beads and SBA was not strong, so after centrifugation, some SBA dissociated from the beads. Then we tested removing SBA from a mixed system, the isoelectric extract. The activity of isoelectric extract decreased 28% after incubating with 1 mL of beads, when testing SBA alone, the activity also decreased 28%. So, other proteins in solution did not interfere with binding of SBA to beads. Although this method can specifically remove SBA, it is not effective to remove all the SBA.

There are also other treatments we conducted but without positive results. SBA can bind to GalNAc with highest affinity that specifically inhibits activity of SBA (5). We hypothesized that by adding excessive amount of this monosaccharide, the carbohydrate binding sites of SBA can be saturated, so that will not bind to the cell surface. We added GalNAc into SBA solution with different molar ratios of SBA to GalNAc: 1:10, 1:50, 1:100, 1:200, 1:300 and 1:400. We found the 1:10 ratio did not have any inhibition effect. When the ratio was equal to or above 1:50, the activity of SBA was inhibited, that is, the cells were not agglutinated. After serial dilution of the GalNAc-SBA sample, this inhibition effect disappeared, and the cells agglutinated again (data not shown). This indicates that the

interaction between GalNAc and SBA was not strong and they undergo association-dissociation equilibrium, and dilution shifted the binding equilibrium. This relatively weak binding of GalNAc to SBA cannot survive in the GI tract, so GalNAc is not a practical method for treating soy proteins.

From previous results, we found destroying structure of SBA decreased activity of SBA. High pressure (HP) seems to be a good method for destroying protein structure. We treated SBA with 400 MPa pressure (4,000 times of atmosphere pressure) for 5 min at room temperature and found no change in activity. In addition, trypsin did not hydrolyze HP-treated SBA (data not shown), which indicates that SBA was not sufficiently denatured to allow trypsin hydrolysis and activity reduction. Although higher pressure and temperature might be able to deactivate SBA, it is not practical.

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Table 1. Experimental condition for examining the effect of denaturing buffer and heat on deglycosylation of soybean agglutinin

Sample	Extract of SBA (μL)	10X Glycoprotein Denaturing Buffer (μL)	H ₂ O (μL)	Heat
1	125	15	10	100 °C, 10 min
2	125	0	25	100 °C, 10 min
3	125	15	10	Unheated
4	125	0	25	Unheated

Table 2. Enzymes used for hydrolysis of soybean agglutinin

Enzyme	Activity	Optimum reaction conditions	Source
Endoglycosidase H _f	Cleaves high mannose and some hybrid types of N-linked carbohydrates on a protein	pH 5.5, 37 °C, 1-5 µL of Endo H _f hydrolyzes 1-20 µg of protein	New England Biolabs
Endoproteinase Glu-C	Cleave peptide bonds at the carboxyl side of Asp and Glu residues or only Glu residues	pH 4.0 to 8.0, 37 °C, a ratio of 3/100 (w/w) of enzyme to substrate	Sigma-Aldrich
Chymotrypsin	Hydrolyzes peptide bonds on the C-terminal side of tyrosine, phenylalanine, tryptophan, and leucine. A secondary hydrolysis occurs on the C-terminal side of methionine, isoleucine, serine, threonine, valine, histidine, glycine, and alanine	100 mM Tris-HCl-10 mM CaCl ₂ , pH 7.8, 30 °C, a ratio of 1:60 (w/w) of enzyme to substrate	Sigma-Aldrich
Thermolysin	X-(cleavage site)-Y-Z, where X is any amino acid, Y is Leu, Phe, Ile, Val, Met or Ala, Z is any amino acid other than Pro	pH 8.0, 70 °C	Sigma-Aldrich
Trypsin	Hydrolyzes peptide bonds at the carboxyl side of arginine or lysine residues	pH 8.0, 37 °C	Sigma-Aldrich
Pancreatin	Mixture of enzymes from porcine pancreas, such as amylase, trypsin, lipase, ribonuclease and protease	pH 7.5, 40 °C for protein	Sigma-Aldrich
Pepsin	Preferentially cleaves C-terminal to Phe, Leu and Glu; does not cleave at Val, Ala or Gly.	pH 2-4, 37 °C	Sigma-Aldrich
Pronase	A mixture of at least 10 proteases, very non-specific	pH 7-8, 37 °C	Sigma-Aldrich

Table 3. Effect of single enzyme hydrolysis of native or heated soybean agglutinin on hemagglutination activity

Enzyme treatments	Native SBA	100 °C-treated SBA	Autoclaved SBA
No enzyme	100 a*	47.1 \pm 2.8 bc	48.0 \pm 2.5 c
Glu-C 1	104.4	53.2 \pm 5.6 bc	53.1 bc
Glu-C 2	104.4	44.8 \pm 2.5 bc	53.1 bc
Trypsin	97.6	53.7 \pm 4.1 bc	54.6 \pm 3.8 bc
Chymotrypsin	100.7	35.1 \pm 32.4 c	51.7 \pm 3.7 bc
Thermolysin	95.5 \pm 8.1	62.2 \pm 1.4 b	59.0 \pm 6.6 b
LSD _{0.05}		17.2	

* The values are the relative activity to native SBA (%) of the samples. N=3. Data are presented as Mean \pm SD. Different letters within the column of 100 °C treated and autoclaved SBA represent significant differences ($P \leq 0.05$), and native SBA was used as a control. 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 4. Effect of single enzyme hydrolysis of organic solutes-treated soybean agglutinin

Enzyme treatments	Native SBA	GuHCl	Remove GuHCl	Urea	Remove urea
No enzyme	100.0 Aa*	52.8 \pm 2.6 B	49.5 \pm 13.9 B	48.7 \pm 3.2 de	75.0 \pm 26.1 bc
Trypsin	–	53.0 \pm 2.8 B	54.5 \pm 19.1 B	48.5 \pm 2.1 de	88.3 \pm 2.3 ab
Chymotrypsin	–	48.0 \pm 8.5 B	59.5 \pm 10.6 B	40.5 \pm 2.1 e	89.7 \pm 3.1 ab
Thermolysin	–	60.0 \pm 2.8 B	62.0 \pm 2.8 B	59.5 \pm 2.1 cd	82.0 \pm 1.7 ab
LSD _{0.05}		18.1		18.3	

* The values are the relative activity to native SBA (%) of the samples. N=3. Data are presented as Mean \pm SD. Different letters within the column of GuHCl and remove GuHCl, urea and remove urea represent significant differences ($P \leq 0.05$), and native SBA was used as a control. GuHCl: GuHCl-treated SBA; Remove GuHCl: SBA treated with GuHCl then removal of GuHCl; Urea: urea-treated SBA; Remove urea: SBA treated with urea then removal of urea.

Table 5. Effect of combination of enzymes hydrolysis of differently treated soybean agglutinin

Enzyme treatments	Native SBA	100 °C 20 min	GuHCl	Remove GuHCl	Urea	Remove urea
No enzyme	100.0 ± 0.0 Aa*	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			

* The 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 native SBA and 100 °C 20 min, GuHCl and remove GuHCl and urea and remove urea represent significant differences ($P \leq 0.05$), native SBA was used as a control for both. T: trypsin; C: chymotrypsin; Th: thermolysin. P: pancreatin. See table 4 for the abbreviations.

Table 6. Effect of multiple-enzyme hydrolysis of native or heated soybean agglutinin

Enzyme treatments	Native SBA	100 °C 20 min
No enzyme	100.0 ± 0.0 a*	65.5 ± 16.0 ab
Pro	96.0 a	34.9 ± 30.3 bc
Pep then P	0.0 ± 0.0 c	0.0 ± 0.0 c
Pro+P+Th	80.7 a	0.0 c

* The values are the relative activity to native SBA (%) of the samples. N=3. Data are presented as Mean ± SD. Different letters represent significant differences ($P \leq 0.05$). $LSD_{0.05}$ is 36.9. pro: pronase; pep: pepsin; P: pancreatin; Th: thermolysin.

Table 7. Effect of tea polyphenol on activity of soybean agglutinin

Final tea polyphenol concentration (g/L)	Supernatant + precipitate	Supernatant
0.0	100.0 \pm 0.0 c*	100.0
2.5	103.8 \pm 0.2 bc	66.9
5.0	122.7 \pm 10.8 a	74.8
7.5	115.0 \pm 7.2 ab	76.8
10.0	99.2 \pm 10.7 c	68.9
12.5	98.2 \pm 1.5 c	68.9
15.0	100.2 \pm 6.4 c	77.2
17.5	95.5 \pm 0.2 c	73.2
20.0	90.8 \pm 3.5 c	65.7

* The values are the relative activity to native SBA (%) of the samples. N=2 for mixture and N=1 for supernatant. Data are presented as Mean \pm SD. Different letters represent significant differences ($P \leq 0.05$). $LSD_{0.05}$ is 13.9.

Table 8. Effect of chelating agents on activity of soybean agglutinin

Final buffer concentration (M)	citric acid	acetic acid	phosphoric acid	EDTA
0.1	108.1 \pm 13.3 abc*	111.1 \pm 3.0 abc	118.8 \pm 7.9 ab	64.1 \pm 3.0 f
0.2	107.9 \pm 6.3 abc	112.0 \pm 3.0 abc	113.2 \pm 4.8 abc	68.4 \pm 3.0 ef
0.3	107.7 \pm 7.9 abc	104.3 \pm 15.1 abc	115.0 \pm 6.0 abc	67.9 \pm 4.8 ef
0.4	106.4 \pm 7.3 abc	103.0 \pm 12.1 bc	114.1 \pm 6.0 abc	69.2 \pm 9.1 ef
0.5	105.6 \pm 4.2 abc	105.6 \pm 10.3 abc	120.5 \pm 10.9 a	84.6 \pm 3.0 de

*The values are the relative activity to native SBA (%) of the samples. N=2. Data are presented as Mean \pm SD. Different letters represent significant differences ($P \leq 0.05$). $LSD_{0.05}$ is 16.9.

Table 9. Effect of GalNAc beads on activity of soybean agglutinin

Beads added (mL)	0	0.1	0.3	1	1.5
SBA	100*	92	74	72	60
Isoelectric extract	81	–	–	58	–

*N=1. The values are the relative activity to native SBA (%) of the samples.

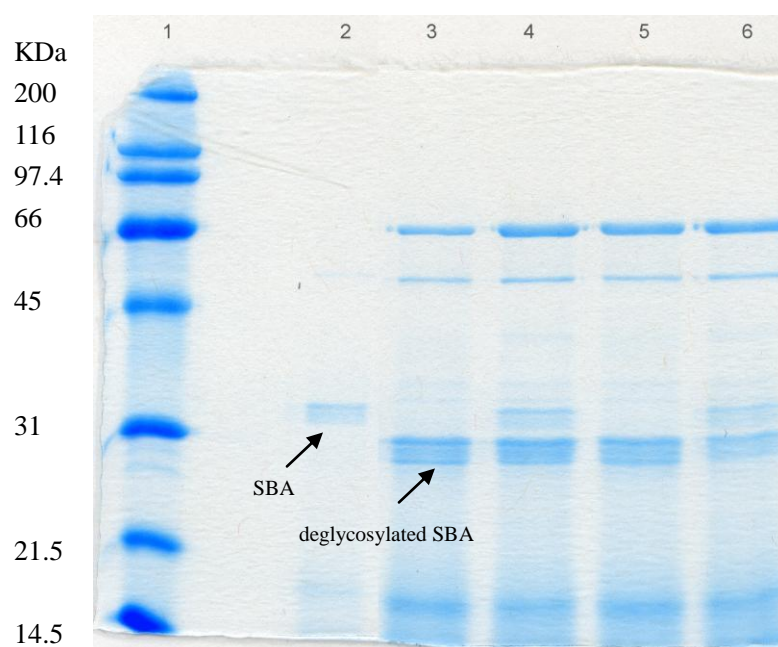


Figure 1. Deglycosylation of soybean agglutinin using enzyme Endo H_f. Lane 1, molecular weight marker; lane 2, SBA extract; lane 3, SBA treated with denaturing buffer and heating, then with Endo H_f; lane 4, SBA treated with heating then with Endo H_f; lane 5, SBA treated with denaturing buffer then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

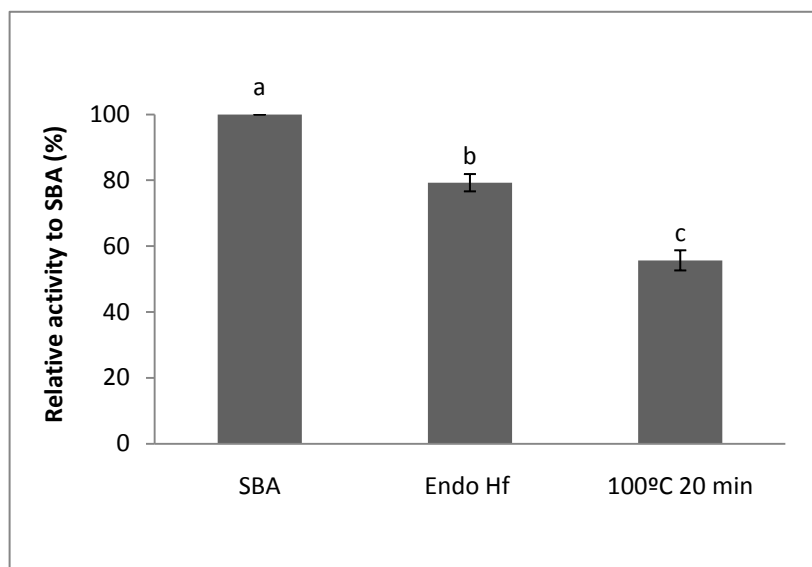


Figure 2. Effects of deglycosylation and heating on hemagglutination activity of soybean agglutinin. N=4. Data are presented as Mean \pm SD. Different letters represent significant differences ($P \leq 0.05$). Least significant difference at 95% confidence level ($LSD_{0.05}$), is 3.8.

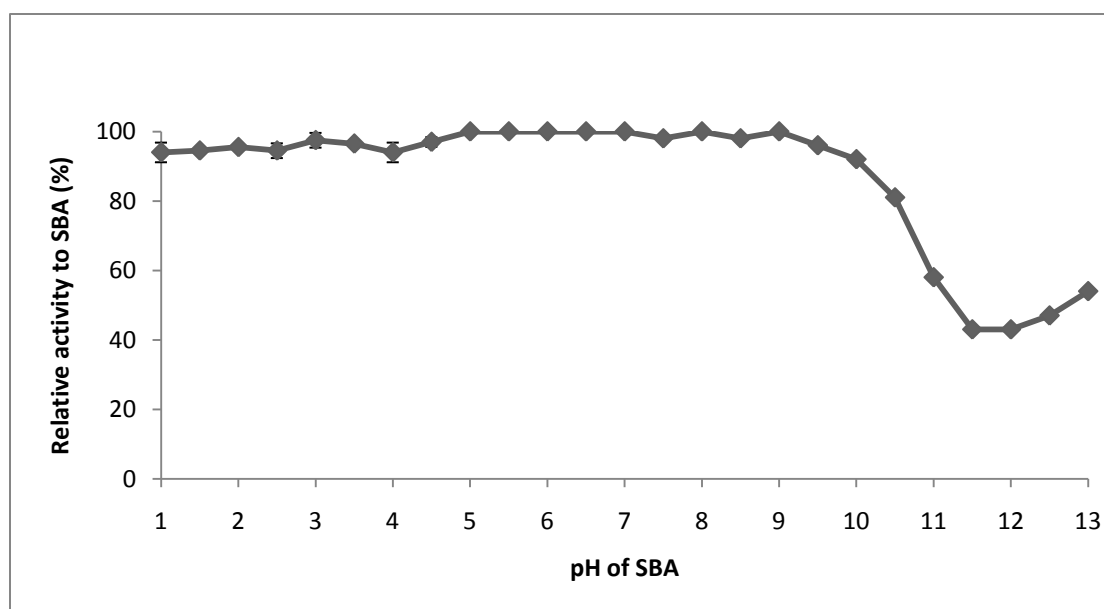


Figure 3. Effect of pH on hemagglutination activity of soybean agglutinin. N=2. Data are presented as Mean \pm SD.

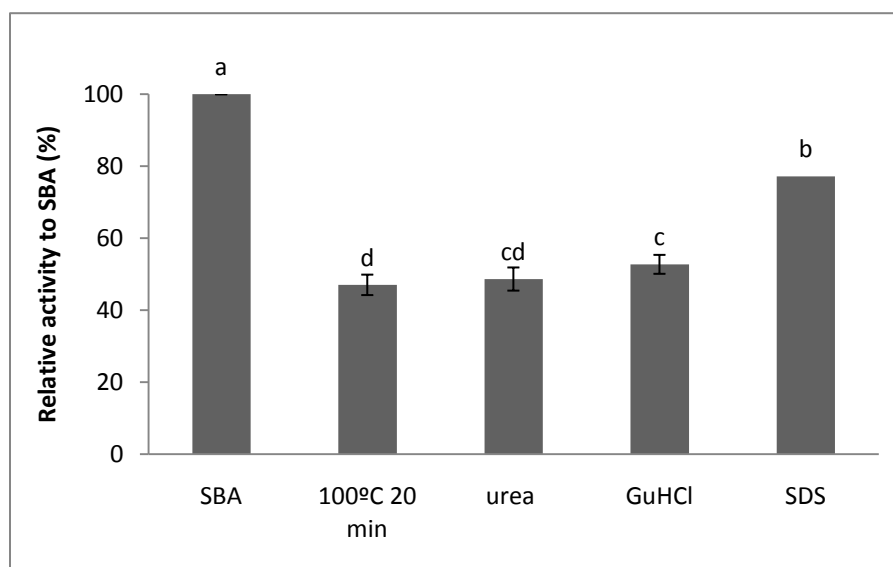


Figure 4. Effect of denaturation treatment on hemagglutination activity of soybean agglutinin. N=4. Data are presented as Mean \pm SD. Different letters represent significant differences ($P \leq 0.05$). $LSD_{0.05}$ is 4.9.

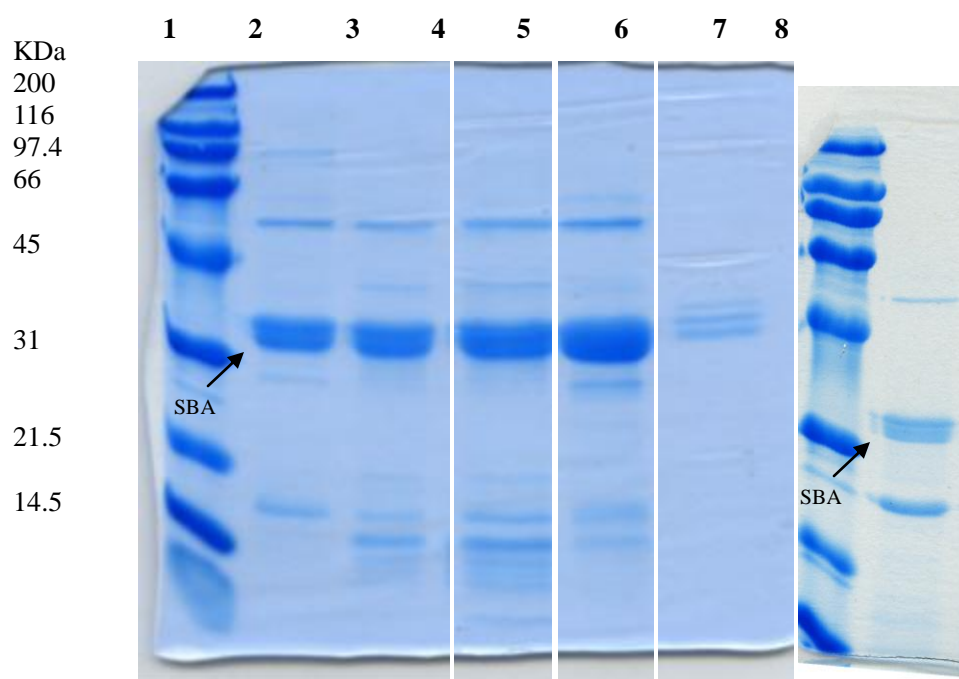


Figure 5. Different enzyme hydrolysis of native soybean agglutinin. Lane 1, molecular weight marker; lane 2, SBA extract; lane 3, SBA treated by endoproteinase Glu-C in buffer 1 (0.1 M ammonium bicarbonate, pH8.0); lane 4, SBA treated by endoproteinase Glu-C in buffer 2 (0.1M sodium phosphate buffer, pH 7.8); lane 5, SBA treated by chymotrypsin; lane 6, SBA treated by thermolysin; lane 7, molecular weight marker; lane 8, SBA treated by trypsin.

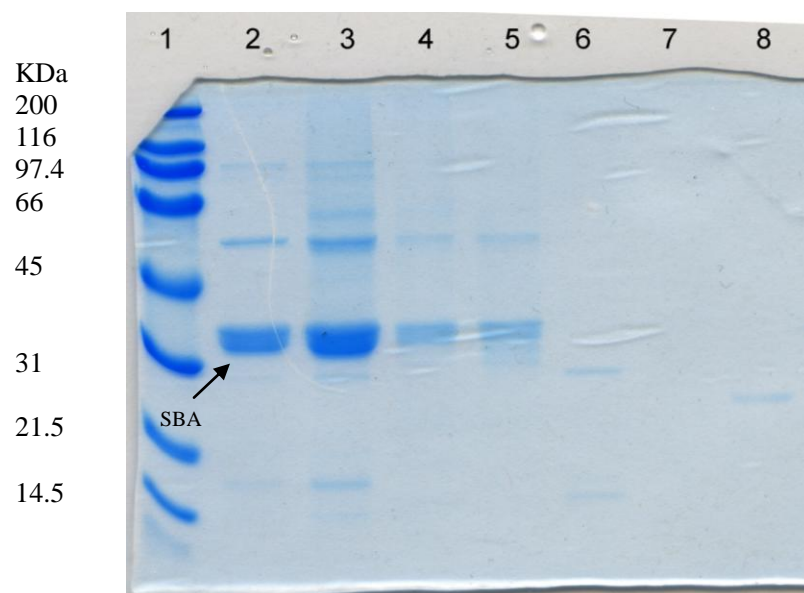


Figure 6. Single enzyme hydrolysis of 100 °C for 20 min-treated soybean agglutinin. Lane 1, molecular weight marker; lane 2, SBA extract; lane 3, SBA treated at 100 °C for 20 min; lane 4, SBA treated at 100 °C for 20 min then with Glu-C in buffer 1; lane 5, SBA treated at 100 °C for 20 min then with Glu-C in buffer 2; lane 6, SBA treated at 100 °C for 20 min then with chymotrypsin; lane 7, SBA treated at 100 °C for 20 min then with thermolysin; lane 8, SBA treated at 100 °C 20 min then with trypsin.

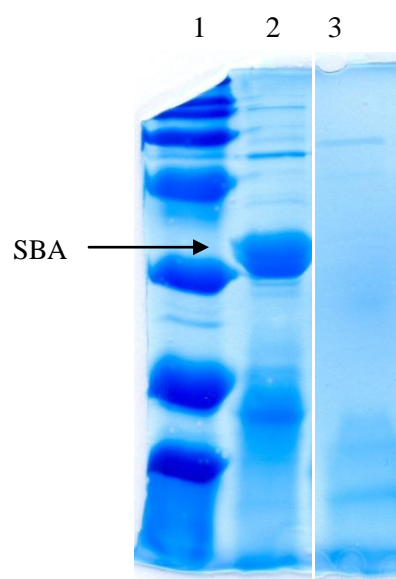


Figure 7. Effect of pepsin then pancreatin hydrolysis on activity of soybean agglutinin. Lane 1, molecular weight marker; lane 2, SBA extract; lane 3, native SBA treated by pepsin then pancreatin.

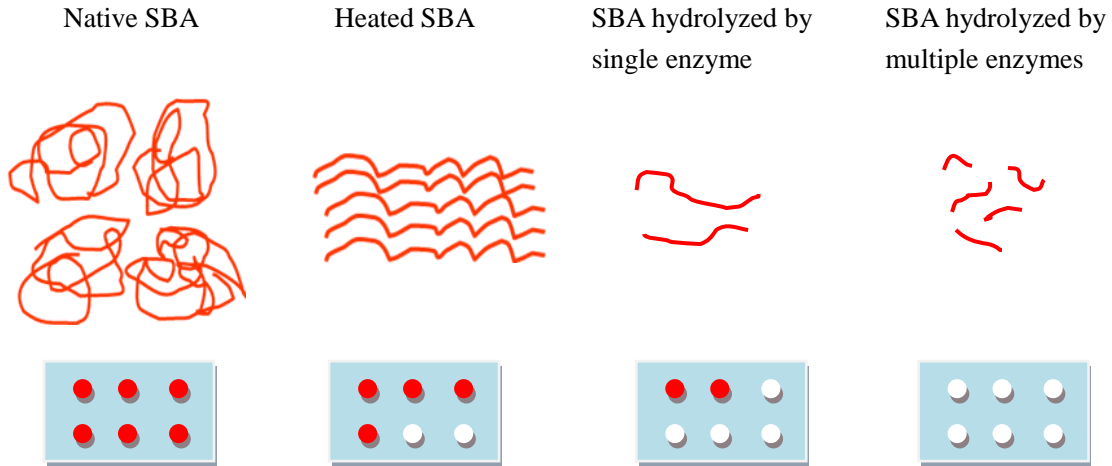


Figure 8. Bioactive peptide hypothesis. Native SBA forms tetramer, displaying full activity; heated SBA is denatured, the activity is reduced; When SBA is hydrolyzed by single enzyme, some active peptides have activity; after hydrolyzing by combination of enzymes, the active peptides are destroyed, showing no activity. The plate with red dots indicate blood agglutination.

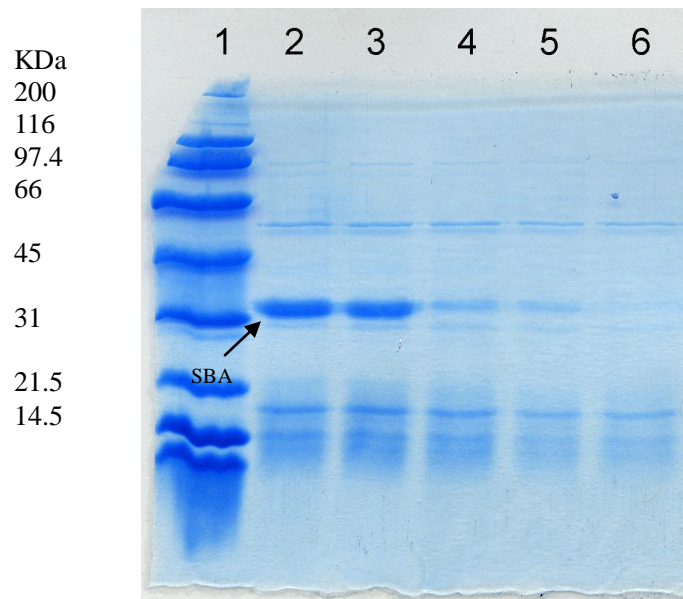


Figure 9. Effect of GalNAc beads on soybean agglutinin removal. Lane 1, molecular weight marker; lane 2, SBA extract; lane 3-6: the remaining supernatant after being treated with different amounts of GalNAc beads: lane 3, 100 μ L; lane 4, 300 μ L; lane 5, 1 mL; lane 6, 1.5 mL. Equal amount of samples were loaded on the gel.

CHAPTER 3. HEMAGGLUTINATION ACTIVITY OF β -CONGLYCININ AND GLYCININ AND FEEDING STUDY OF LECTIN-DEACTIVATED SOY WHITE FLAKES

A paper to be submitted to the *Journal of Agricultural and Food Chemistry*

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Abstract

The purpose of this study was to use non-thermal means to reduce hemagglutination activity of soy proteins and to improve the feeding quality of soy proteins. Two storage proteins in soybeans, β -conglycinin and glycinin, were shown to have hemagglutination activity. The activity of β -conglycinin was not reduced by hydrolysis with a single protease, but was fully eliminated by multiple enzymes; however, the activity of glycinin was not fully eliminated by either single or multi enzyme hydrolysis. Similarly, the hemagglutination activities of soy white flakes (SWFs) and soy protein isolate (SPI) were not reduced by single or combination of enzyme hydrolysis. Pepsin and pancreatin treatment, which was effective in eliminating hemagglutination activity of soybean agglutinin (SBA) with minimum heating, was used to generate a feed material for *in vivo* evaluation of nutritional quality. SBA in pepsin and pancreatin-treated SWF was deactivated at analytical scale, but not in the feed material.

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Pepsin and pancreatin-treated SWF did not improve chick growth performance compared with the raw SWF. Chicks did not show any pancreas enlargement or intestine weight increase compared with the raw soy feed or a commercial diet, indicating deactivation of SBA.

KEYWORDS: β -Conglycinin; enzyme hydrolysis; glycinin; hemagglutination activity; soybean agglutinin

Introduction

Organic solvents, such as hexanes, are commonly used for soybean oil extraction because of the high oil recovery and low cost; however, hexane has many drawbacks, such as flammability, solvent residual in the meal, and emission to the atmosphere (1). Aqueous extraction processing (AEP) uses water as medium to extract oil, which is more environmentally favorable (2). In this process, seed is ground in water, the oil is released and floats as emulsified or free oil (3). Although two-stage countercurrent enzyme-assisted aqueous extraction processing (EAEP) improved oil, protein and solids extraction yields than standard EAEP with less water usage (4,5), the process is still not as economically feasible as organic solvent extraction. The utilization of the remaining proteins after oil extraction can be a positive aspect of EAEP because they are subjected to minimal heat treatment, so they are not as damaged as that from commercial solvent extraction, therefore, the proteins may have better functionalities and nutritional values (2). Our research goal is to utilize such proteins as food or

feed with minimum heating. Although the protein can be used to produce soy protein products for human consumption, the major use is still expected to be for animal feeding. In the present study, we evaluated the feeding performance of such proteins alone and with additional treatments. We used defatted soy white flakes (SWFs) as a model, because SWF is subjected to minimal heat treatment.

The minimal heating of the AEP process does not deactivate the anti-nutritional factors in soy protein: soybean agglutinin (SBA) 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 also bind to the brush boarder, causing an increase in the intestine weight and pancreatic hypertrophy. We have previously used pepsin followed by pancreatin treatment to fully deactivate SBA with minimum heating (6). In this study, we wanted to use the same enzyme treatment for SBA to treat SWF, trying to eliminate hemagglutination activity of SWF.

There are different proteins in SWF. Based on their sedimentation coefficients, they can be classified into four fractions known as 2S, 7S, 11S, 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 soluble proteins with about 85% being β -conglycinin, and other proteins such as agglutinin and some enzymes. Glycinin makes up about 85% of the 11S fraction, which comprises 31 to 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, so they are two important factors we also need to consider in testing hemagglutination activity. In the present study, we evaluated enzyme hydrolysis of the main protein fractions in SWF, and assessed the activities by using an *in vitro* hemagglutination assay and an *in vivo* feeding trial.

In addition to SBA, soybean also has other anti-nutritional factor, trypsin inhibitors (TIs). There are two types of TIs: the Kunitz trypsin inhibitor (KTI) inhibits trypsin activity, while the Bowman-Birk trypsin inhibitor (BBI) inhibits both trypsin and chymotrypsin activities. The inhibition of digestive enzymes reduces the digestibility of the proteins. In addition, TIs can cause excessive secretion of the cholecystokinin, which in turn, leads to excessive secretion of pancreatic enzymes, causing pancreatic hypertrophy, and hyperplasia (8). Anti-nutritional effects of TIs will interfere with our feeding trial using SWF. TIs are rich in disulfide bonds and various thiol-containing compounds, such as cysteine, N-acetyl-cysteine, glutathione and sodium sulfite, were used to facilitate the inactivation TIs at lower temperature (9,10). Wang et al. (11) used sodium metabisulfite (SMBS) to inactivate TIs and improved the *in vitro* degree of hydrolysis of soy protein from 1.2 to 8.5%, which was even higher than for an autoclaved sample (7.9%). The gain:feed ratio and protein efficiency ratio (PER) increased 27 and 57%, respectively, compared with raw soy flour. In the present study, we used the same SMBS conditions to inactivate TIs first, then applied enzyme treatment to denature SBA and reduce hemagglutination activity of soybean storage proteins.

Materials and Methods

Materials. β -conglycinin- and glycinin-rich fractions were produced from the Center for Crops Utilization Research (CCUR) 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 fractions contained 96% protein with 85% purity and β -conglycinin-rich fraction contained 90% protein and 81% purity. Soy protein isolate (SPI) was obtained from Archer Daniels Midland (Decatur, IL) with 92.8% protein (dry weight basis). Soy white flake was obtained from Cargill (Minneapolis, MN), and it was produced by hexane extraction of the oil and then flash-desolventing to achieve soy protein with $\geq 85\%$ dispersibility in water. Rabbit blood in Alsever's solution was obtained from Hemostat Laboratories (Dixon, CA) and it was used within two weeks. Other reagents were purchased from Fisher Scientific (Pittsburg, PA) or Sigma-Aldrich (St. Louis, MO).

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). The chymotrypsin treatment was done 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 treatment was done in the same buffer as chymotrypsin with enzyme:protein ratio of 1:100 for 24 h at 70 °C. For trypsin 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 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 hours at 37 °C. For pronase hydrolysis, 8 mg β -conglycinin-, glycinin-rich fraction, soy white flake saline extract (SWFE) or soy protein isolate (SPI) (native or heat treated) was treated in 50 mM ammonium bicarbonate buffer (pH 8.0) with enzyme:protein ratio of 1:50 at 37 °C for 24 h.

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 added different combination of enzymes. For the combination of trypsin + thermolysin, trypsin + chymotrypsin + thermolysin, pronase + pancreatin + 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.

For pepsin followed by pancreatin hydrolysis, 8 mg β -conglycinin-, glycinin-rich fraction, SWFE, SPI or SWF (native or heat treated) 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. For pepsin

then pronase + pancreatin + thermolysin hydrolysis, the procedure were essentially the same as pepsin then pancreatin treatment, except that pronase, pancreatin and thermolysin were added after adjusting pH to 7.5. All samples were heated 100 °C for 10 min to deactivate the enzyme and stored at -20 °C until hemagglutination assay.

Effect of SMBS on hemagglutination activity of SWF. The SMBS treatment of SWF was done according to Wang et al. (11). Generally, 10% SWF was dispersed in 10 mM phosphate buffered 0.9% saline (pH 7.8), and 1.0 mmol/2 g SWF of sodium metabisulfite was added. The mixture was then stirred and incubated at 55 °C for 1 h and dialyzed against water for 3 days, and subjected to enzyme hydrolysis or hemagglutination assay.

Preparation of soy protein samples for feeding trial. Three treatments were carried out for SWF: (1) SMBS treatment of SWF: 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) was added and incubated at 37 °C for 20 h. The hydrolysate was then

lyophilized. (3) To produce 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. Commercial toasted soy flour was also used as a control.

Extraction of SBA from the feed materials. The procedure described by Lis and Sharon (13) was used. Generally, 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 saved by centrifugation (3020 x 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, 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 (NH₄)₂SO₄ (0.56 g/ mL of solution). The precipitate was collected by centrifugation (3020 x 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 a method of Lis and Sharon (23) with some modification. Briefly, rabbit red blood cells collected in Alsever's solution were centrifuged for 5 min at 410 x 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 for 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 two-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 in 37 °C for 2 h for agglutination to occur. The plates were then tilted about 45 °, the samples with erythrocytes streamed in a “tear-drop” fashion were considered negative and the ones did not form “tear-drop” were considered positive. The hemagglutination units (HU) per g of sample were determined by the equation (14):

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

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 mL of extract 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 $\log(\text{HU/g})/\log 2$ 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 every set of treatments. The results were expressed as relative activity to SBA.

Trypsin inhibitor activity test. TIs were assayed by Eurofins Scientific Inc. (Des Moines, IA). 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 (BAPA). The sample was then read versus a blank and TIU/g was calculated. TIU was defined as the amount of inhibitor required to inhibit one 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 the protein efficiency ratio (PER) using formulated diets with low protein content. PER was calculated as previously described (11). Ninety week-old male chicks (Ross x Ross 708) were weighed and blocked on initial body weight into 25 pens consisting of five pens per treatment (three 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 (15) 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 for nine days, all birds were euthanized by carbon dioxide asphyxiation and their pancreases and small intestines (pyloric sphincter to illeocecal valve) were removed and weighed.

Statistical analysis. The data were analyzed by Analysis of Variance (ANOVA) and General Linear Model (GLM), 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). Growth performance, PER, and pancreas and intestinal weights were analyzed by ANOVA using the PROC GLM procedure of SAS, with the experimental unit being a pen of three male chicks.

Results and Discussion

Effect of enzyme hydrolysis on activity of β -conglycinin. The β -conglycinin and glycinin we used were not pure, they were β -conglycinin- and glycinin-rich fractions. In the

results, we used the term β -conglycinin and glycinin for simplification. At the beginning, we planned to use β -conglycinin and glycinin as negative controls, which were not expected to have hemagglutination activity; however, we found they also had activity (Table 2). Our ultimate goal was to treat SWF to improve feeding quality, so we wanted to eliminate the hemagglutination activities from all the components in the SWF including β -conglycinin and glycinin. Therefore, both β -conglycinin and glycinin were treated with the similar treatment strategy as for SBA.

Table 2 shows the tendency that heating reduced activity of β -conglycinin, single enzyme hydrolysis of β -conglycinin, either native or heated, did not fully deactivate it. 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 (enzyme could not hydrolyze native SBA), indicating that β -conglycinin was not resistant to enzyme hydrolysis, so native β -conglycinin was hydrolyzed similarly as the heated one. There were large variations in the data, which might be due to inconsistency of enzyme hydrolysis and the detection limit of the hemagglutination assay. In the hemagglutination assay, if the first dilution did not cause cell agglutination, the activity was considered to be 0, whereas if the first dilution caused cell agglutination and the second did not, the activity was about 40% (relative activity to SBA) depending on the starting concentration of the sample. For example, if two replications of a certain hydrolysis results are 0 and 40%

activity, the standard deviation was 28.3%, although the two results differed in one dilution. The enzyme hydrolysis was not consistent for some of the enzymes, so some of the data had high variation. Nonetheless, some general trends can be observed.

SDS-PAGE also showed that enzymes can hydrolyze native β -conglycinin (Fig. 1A lane 4, B lane 3, 4). This indicates that enzyme hydrolysis might have released active peptides in β -conglycinin. This result also indicates that unlike SBA, β -conglycinin is not resistant to enzyme hydrolysis and cannot survive in the GI tract digestion, that is, as β -conglycinin passes through the GI tract, it becomes hydrolyzed and may not cause serious anti-nutritional effects as SBA, if there are no bioactive peptides released.

After multi-enzyme hydrolysis, activity of native β -conglycinin did not reduce 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, and β -conglycinin may be cut into smaller pieces. This also indicates that like SBA, thermolysin might cut at the crucial sites for activity of β -conglycinin, and trypsin and chymotrypsin also played a role in reducing activity of β -conglycinin, although the enzymes were not as effective as thermolysin.

In order to deactivate all SBA-like components in SWF, we used the same treatment as for SBA (6), which was pepsin followed by pancreatin hydrolysis of β -conglycinin (Table 4). Unlike SBA, the activity of β -conglycinin did not decrease, which indicated the inability of the two enzymes to cut active sites of β -conglycinin. Also like SBA, the pronase could not fully

deactivate β -conglycinin, nor could the combination of enzymes with pronase. Although in previous results we showed that β -conglycinin could be fully deactivated by pancreatin and thermolysin (Table 3), combination of pronase, pancreatin and thermolysin did not fully deactivate it, which indicates the possible interference of pronase to thermolysin hydrolysis.

A common food protein, casein from bovine milk, was used as a negative control for the enzymatic treatments. Casein did not have any hemagglutination activity, nor did the enzyme hydrolysates of casein (Table 5) when the same conditions were used for hydrolysis. This indicates that the hemagglutination activity is indeed specific for soy protein.

Effect of enzyme hydrolysis on hemagglutination activity of glycinin. Glycinin showed similar results as β -conglycinin in repose to single enzyme hydrolysis. Heating slightly reduced hemagglutination activity, however, after single enzyme hydrolysis, activity did not decrease (Table 2), some even increased. This result suggests that heating may cause protein to aggregate so that the active sites are buried and partial enzyme hydrolysis might have released the active peptides, thus increasing the hemagglutination activity. Again, the single enzyme hydrolyzed native, heated or autoclaved glycinin had the same activity. In addition, SDS-PAGE showed that like β -conglycinin, native glycinin can be hydrolyzed by enzymes (Fig. 1A lane 8). These results indicate that glycinin may not survive in the GI tract, but the peptides released from glycinin could potentially cause anti-nutritional effects.

Unlike β -conglycinin, none of the multi-enzyme hydrolysis fully deactivated glycinin,

either native or heated (Table 3). This result indicates that these enzymes were unable to cut all of the active sites in glycinin; whereas for β -conglycinin, the extensive hydrolysis with the enzymes destroyed the active peptides.

We also used the same enzyme treatments that were effective for SBA to treat glycinin. The results were all similar to the ones for β -conglycinin. Pepsin followed by pancreatin hydrolysis did not fully deactivate glycinin, pronase did not decrease activity of glycinin, and combination of other enzymes (pepsin then pronase, pancreatin and thermolysin) did not fully deactivate glycinin (Table 4). These results indicated that these enzymes were unable to cut all the active peptides in glycinin.

Effect of enzyme hydrolysis on the hemagglutination activity of SWFE. The feed material was SWF, so the pepsin followed by pancreatin hydrolysis which was effective for inactivation of SBA was used for treating SWF saline extract. The extract contained the majority of the proteins in the SWF and was devoid of the insoluble components (fiber, undissolved proteins, and others), which was easier for treating with enzymes and assaying for hemagglutination activity. Similar to β -conglycinin and glycinin, pepsin and pancreatin treatment did not fully deactivate SWFE (Table 4), which was predictable because β -conglycinin and glycinin are two major components in SWFE and they were not fully deactivated by such treatment. Again like β -conglycinin and glycinin, pronase did not decrease activity of SWFE, and combination of pronase with other enzymes decreased activity of native

SWFE, but not the heated one. Unlike SBA, the activities of enzyme-treated native and heated SWFE were not significantly different, which was similar to the results for β -conglycinin and glycinin. All these similarities of the behavior of SWFE to β -conglycinin and glycinin may be due to the larger proportion of β -conglycinin and glycinin in the SWFE.

Effect of enzyme hydrolysis on hemagglutination activity of SPI. SWF contains not only proteins, but also carbohydrates. In order to examine the effect of enzyme hydrolysis in a protein system without other interferences, we carried out enzyme hydrolysis on SPI. The effects of enzymes on SPI were similar to that of SWFE. None of the enzymes fully eliminated hemagglutination activity of SPI, and the enzyme-hydrolyzed native and heated SPI had similar activities (Table 4). These results indicate that the other components in SWF did not influence enzyme hydrolysis or the activity assay.

Effect of enzyme hydrolysis on hemagglutination activity of SWF. We tried different enzymes, but still could not fully deactivate hemagglutination activity of SWF. Our previous results indicate that β -conglycinin and glycinin were not resistant to hydrolytic enzymes, so they could be hydrolyzed in the GI tract, which might not be a significant problem for human and animals to consume, if the hydrolyzed proteins do not have significant hemagglutination activities, or their activities are not high enough to cause apparent nutritional problems. SBA can survive the GI tract, so our goal was to fully deactivate SBA. Although pepsin and

pancreatin did not fully deactivate β -conglycinin and glycinin, the two enzymes fully hydrolyzed SBA, so this treatment was chosen as a non-thermal treatment for generating feed material from SWF. SWF, however, has trypsin inhibitors (TIs), which inhibit the activity of chymotrypsin and trypsin in animals. In addition, TIs also inhibit animal growth and cause pancreas enlargement, which interfere with our feeding evaluation. As a result, sodium metabisulfite (SMBS) was used for non-thermal deactivation of TIs, and then SWF was treated with pepsin and pancreatin.

Fig. 2 shows that after SMBS treatment, hemagglutination activity of SWF reduced slightly, and the activity was not as low as pepsin and pancreatin treated one, which indicates that SMBS only had 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 which composed of majority of the protein could not be deactivated by this treatment. Activity of heat-treated SWF significantly decreased. Heat followed by pepsin and pancreatin treatment of SWF had an increased activity, which may be due to 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 (16), as a result, excessive dose of enzyme may overcome the inhibition effect of TIs, causing the similar activities of enzyme-treated native or SMBS treated SWF.

Activities of SBA extracted from SMBS and enzyme-treated SWF. Our goal 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. The activity of SBA extracted from pepsin- and pancreatin-treated native SWF was not the same as in the model pure SBA system, whereas the one from SMBS-treated SWF was the same (Table 6) as in the model system. This result indicates that after SMBS deactivation of TIs, SBA was more easy to be hydrolyzed by pepsin and pancreatin. For these two treatments, whole SWF had similar activities (Fig. 2), but the extracted SBA had different activities (Table 6). This might be due to the contribution of β -conglycinin and glycinin to the SWF than SBA. Although the activities of the SBA portion were different for these two samples, the activities generated by β -conglycinin and glycinin hydrolysates masked this difference, and made the activities of whole SWF the same in the two samples.

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

Pepsin and pancreatin are naturally occurring enzymes in the GI tract of human and

animals, however, they cannot fully deactivate SBA *in vivo*. The amount of enzyme we used was much larger than that in GI tract. Different enzyme dosages were used to and pepsin:SBA ratio of 1:5 was the lowest that fully hydrolyzed SBA (data not shown), so that the pepsin in GI tract was not enough for deactivating SBA. In addition, SBA was consumed with other foods, which might bury SBA inside, so that SBA cannot be hydrolyzed by pepsin after entering the GI tract.

Hemagglutination activities of feed materials. After preparing large quantities of feed materials, some key factors were tested. Fig. 3A shows that the hemagglutination activity of SMBS-treated SWF did not have significant difference 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 in analytical scale. Similar as in analytical scale, the SBA extract from SMBS treated SWF still had similar activity as the one from SWF (Fig. 3B); however, the activity of SBA extracted from enzyme sample was not reduced to zero (Fig. 3B). This may be due to the incomplete hydrolysis of SBA in bulk materials, which may be a concern for treating large quantity of materials.

Trypsin inhibitors activities in feed materials. The TIs activity in soy white flake was 60,300 TIU/g; in SMBS was 2,800 TIU/g (4.6% residual activity); in the enzyme-treated sample was 3,900 TIU/g (6.5% residual activity); in autoclave was <2,000 TIU/g (<3.3%

residual activity). This result indicates that TIs were largely reduced in SMBS and enzyme-treated samples, and almost totally destroyed in autoclaved samples. Therefore the SMBS treatments were effective and TIs should not cause significant problems in the feeding evaluation. Autoclaving was a more effective way of destroying the TIs than the other treatments.

Hemagglutination activity evaluated by animal feeding study. Raw soy protein in the LPSC diet reduced the growth performance of chicks as expected (Table 7). Both the average daily gain and average daily feed intake were reduced compared to the LPC diet. Gain:feed and PER, however, were not 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 7). 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 any pancreas enlargement or increase in intestine weight. These results demonstrate that the SMBS treatment increased the nutritional value of the protein, as demonstrated previously (11).

The growth performance of chicks on the ENZYME diet was not improved compared

to the LPSC (Table 7). Average daily gain, gain:feed, 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 be lead to the lower growth performance than the LPC and SMBS diets. In addition, similar as SMBS, ENZYME did not cause any pancreas enlargement or intestine weight increase, which indicated that the anti-nutritional factors in raw SWF were deactivated by this treatment, at least to a level that does not influence the normal condition of the pancreas and intestine. Comparing the differences of ENZYME sample on chick growth performance and 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. So the residual SBA in ENZYME sample was able to inhibit chick growth, but not cause pancreas enlargement and increased intestine weight. This result was in agreement with Fasina et al. (17) who incorporated 0.024 and 0.048% of SBA into diets of turkey poults and did not find any pancreatic hypertrophy, whereas the 0.048% diet showed inconsistent results for feed efficiency (FE). The 0.048% diet showed higher FE for the feeding of female day-old turkey poults (Hybrid Converter strain) from day 0 to 12, but lower for the Nicholas 88 strain. In addition, their results also showed that incorporating 0.024 or 0.048% SBA decreased feed intake.

SMBS diet, which still had intact SBA but no TIs, performed as well as the control LPC diet. This suggests that SBA might not be a serious 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 of the chicks. This might be due to decreased palatability, such as unpleasant smell, texture or bitterness of the sample. The protein hydrolysates often have bitter taste, and especially these peptides containing neutral amino acids with large alkyl or aromatic side chains (18), which might account for the decreased feed intake. In addition, the excess pepsin and pancreatin in the ENZYME sample might lead to dysfunction of the digestive system of the chicks, which might be another reason for limited feed intake. After hydrolysis, β -conglycinin and glycinin still had hemagglutination activity and this activity was similar to hydrolyzed native and heat-denatured β -conglycinin and glycinin. Humans and animals have consumed heated soybeans and soy protein products for thousands of years and do not have any problem, indicating that the active peptides released from β -conglycinin and glycinin by enzyme hydrolysis might not be a serious problem. As a result, this remaining activity of β -conglycinin and glycinin hydrolysates might not be the reason for growth inhibition. Indeed, the small amount of SBA in feed (0.024%) was tolerated by turkey poult as illustrated by Fasina et al. (17) who incorporated 0.024% SBA in diets for turkey poult and did not find any antinutritional effect.

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 discarded. This observation is another indication that the texture of the feed material is important.

Collectively, these data suggest that while the SMBS-treated diet and the

ENZYME-treated diet protect against pancreatic hypertrophy and increased intestinal growth, and only the SMBS diet maintains the growth performance for the chicks when compared to the LPC diet.

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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*	0.50	0.50	0.50	0.50	0.50
Mineral Mix**	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

*Composition of vitamin mix: vitamin A, D3, E, B12, riboflavin, niacin, and d-pantothenic acid.

**Composition of mineral mix: calcium, copper, iron, manganese, zinc, and iodine.

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

Enzyme used		None	Trypsin	Chymotrypsin	Thermolysin	LSD _{0.05}
β -conglycinin	Native	61.7 \pm 7.8 a*	60.6 \pm 0.9 a	60.2 \pm 5.1 a	51.2 \pm 7.8 ab	38.1
	100 °C 20 min	26.9 \pm 25.4 ab	51.5 \pm 8.9 a	34.6 \pm 30.2 ab	38.6 \pm 33.9 ab	
	Autoclave	13.4 \pm 23.2 b	46.5 \pm 12.6 ab	24.1 \pm 34.1 ab	26.4 \pm 37.3 ab	
Glycinin	Native	40.1 \pm 29.3 ab	57.2 \pm 5.7 a	56.3 \pm 0.5 a	45.2 \pm 4.5 a	29.4
	100 °C 20 min	14.8 \pm 20.3 bc	63.7 \pm 17.1 a	54.4 \pm 2.3 a	43.6 \pm 5.5 ab	
	Autoclave	0.0 \pm 0.0 c	68.4 \pm 10.5 a	55.8 \pm 5.7 a	50.3 \pm 2.7 a	

*The values are the relative activity to native SBA (%) of the samples. N=3. Data are presented as Mean \pm SD. Different letters within all treatments for each individual protein (native, 100 °C treated, and autoclaved) represent significant differences ($P \leq 0.05$).

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

Enzyme used	β -Conglycinin		Glycinin	
	Native	100 °C 20 min	Native	100 °C 20 min
No enzyme	65.4 \pm 3.0 a*	44.2 \pm 9.1 b	38.7 \pm 22.8 ab	16.8 \pm 23.3 b
T+C	—	50.5 \pm 2.8 ab	—	55.9 \pm 5.2 a
T+Th	—	0.0 \pm 0.0 c	—	54.0 \pm 2.5 a
C+Th	—	0.0 \pm 0.0 c	—	33.4 \pm 47.2 ab
T+C+Th	54.1 \pm 5.5 ab	0.0 \pm 0.0 c	57.8 \pm 1.9 a	58.5 \pm 5.6 a
P	54.7 \pm 6.4 ab	11.6 \pm 20.0 c	51.4 \pm 3.1 a	53.1 \pm 0.3 a
P+Th	45.7 \pm 5.2b	0.0 \pm 0.0 c	47.8 \pm 8.1 ab	56.9 \pm 0.0 a
LSD _{0.05}	15.2		32.5	

*The values are the relative activity to native SBA (%) of the samples. N=3. Data are presented as Mean \pm SD. Different letters within all treatments for each individual protein (both native and 100 °C treated) represent significant differences ($P \leq 0.05$). T: trypsin; C: chymotrypsin; Th: thermolysin. P: pancreatin.

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

Enzyme used		None	Pep then P	Pronase	Pro+P+Th	Pep then pro+P+Th	LSD _{0.05}
β-Conglycinin	Native	43.4 ± 2.3 abc*	35.6 ± 23.8 c	63.9 ± 3.2 a	55.8 ± 1.8 abc	56.2 ± 0.0 abc	21.6
	100 °C	0.0 ± 0.0 d	38.0 ± 25.4 bc	64.7 ± 4.5 a	58.8 ± 6.0 ab	58.1 ± 2.7 ab	
Glycinin	Native	63.2 ± 2.0 a	19.1 ± 22.2 b	43.2 ± 29.1 ab	22.6 ± 27.4 b	28.5 ± 24.7 b	31.0
	100 °C	37.7 ± 3.2 ab	19.1 ± 22.2 b	41.8 ± 27.9 ab	22.6 ± 27.4 b	28.5 ± 24.7 b	
SWFE	Native	85.6 ± 5.6 a	54.7 ± 10.0 b	73.1 ± 5.5 a	59.1 ± 11.1 b	48.3 ± 10.4 b	13.8
	100 °C	50.4 ± 13.9 b	50.8 ± 9.1 b	58.8 ± 5.8 b	53.3 ± 6.9 b	49.6 ± 8.4 b	
SPI	Native	43.5 ± 24.4 b	41.0 ± 7.3 b	73.6 ± 4.7 a	53.7 ± 7.1 ab	36.0 ± 31.1 b	23.3
	100 °C	0.0 ± 0.0 c	43.4 ± 6.8 b	70.5 ± 3.6 a	53.8 ± 5.4 ab	35.2 ± 30.5 b	

*The values are the relative activity to native SBA (%) of the samples. N=4. Data are presented as Mean ±SD. Different letters within all treatments for each individual protein (both native and 100 °C treated) represent significant differences ($P \leq 0.05$). pro: pronase; pep: pepsin; P: pancreatin; Th: thermolysin; SWFE: soy white flake saline extract; SPI: soy protein isolate.

Table 5. Effect of enzyme hydrolysis on the hemagglutination activity of casein

Enzyme used	none	Trypsin	Chymotrypsin	Thermolysin	Pep then P	T+C+Th
Relative activity to SBA (%)	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0

N=2. T: trypsin; C: chymotrypsin; Th: thermolysin; Pep: pepsin; P: pancreatin.

Table 6. Activities of SBA extracted from differently treated SWFs at analytical scale

SBA extraction from	SWF	Pepsin and pancreatin treated SWF	SMBS then pepsin and pancreatin treated SWF
Relative activity to SBA (%)	100 \pm 0.0 a	36.4 \pm 1.1 b	0.0 \pm 0.0 c

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

Table 7. 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/d)	48.0a	42.2b	29.5c	38.6b	21.6d
Average daily gain (g/d)	36.9a	23.3b	12.9c	24.3b	10.7c
Gain:Feed	0.77a	0.55bc	0.43c	0.63b	0.49c
Protein Efficiency Ratio (PER)	3.4ab	3.2ab	2.5b	3.7a	2.9b
Pancreas weight (g/100 g BW)	0.37b	0.38b	0.86a	0.39b	0.41b
Intestine weight (g/100 g BW)	7.01b	6.77b	8.03a	6.82b	7.10b

N = 5 pens of 3 chicks per pen

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. Different letters in the same row represent significant differences ($P \leq 0.05$).

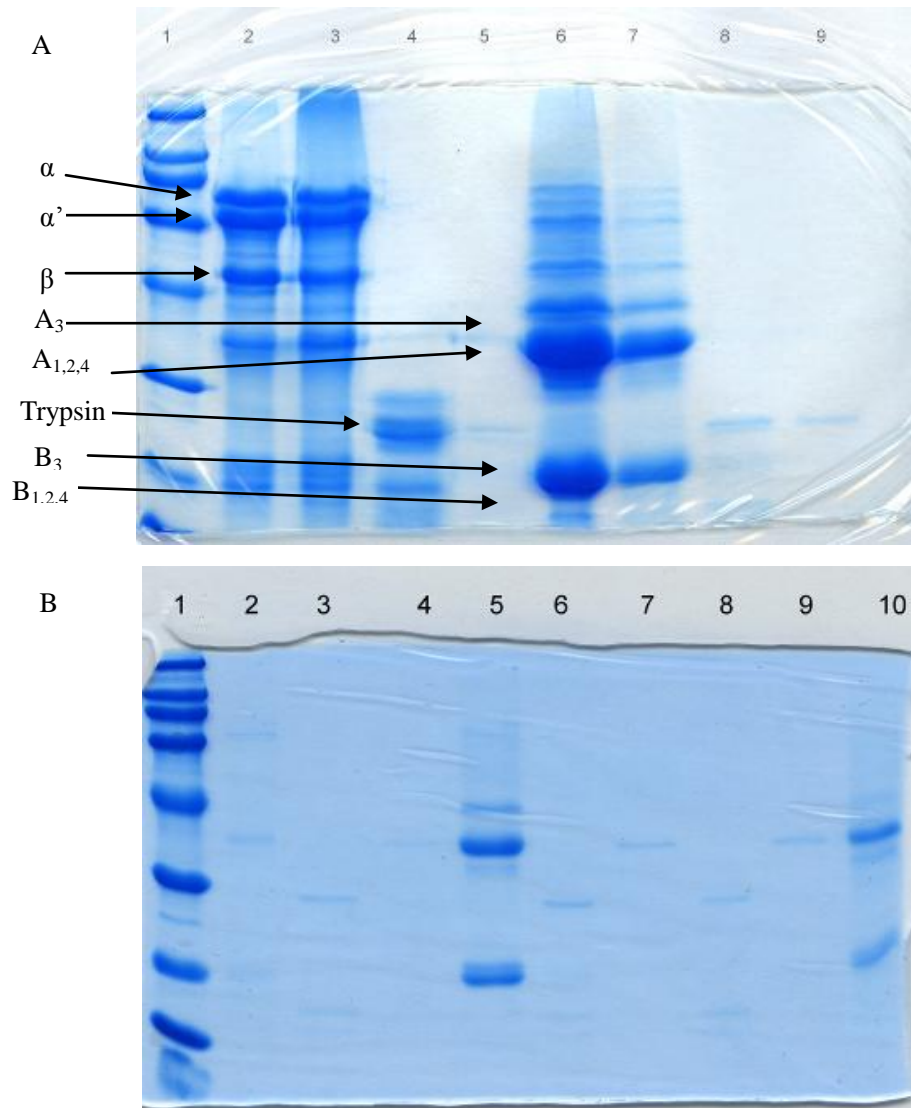


Figure 1. Effects of different enzyme treatments of β -conglycinin and glycinin. A. Lane 1, molecular weight marker; lane 2, β -conglycinin; lane 3, β -conglycinin treated at 100 °C for 20 min; lane 4, β -conglycinin treated with trypsin; lane 5, β -conglycinin treated at 100 °C for 20 min, then by trypsin; 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. B. Lane 1, molecular weight marker; lane 2, β -conglycinin; lane 3, β -conglycinin treated with chymotrypsin; lane 4, β -conglycinin treated with thermolysin; lane 5, β -conglycinin treated at 100 °C for 20 min; lane 6, β -conglycinin treated at 100 °C for 20 min then by chymotrypsin; lane 7, β -conglycinin treated at 100 °C for 20 min then by thermolysin; lane 8, autoclaved β -conglycinin treated with chymotrypsin; lane 9, autoclaved β -conglycinin treated with thermolysin; lane 10, autoclaved β -conglycinin

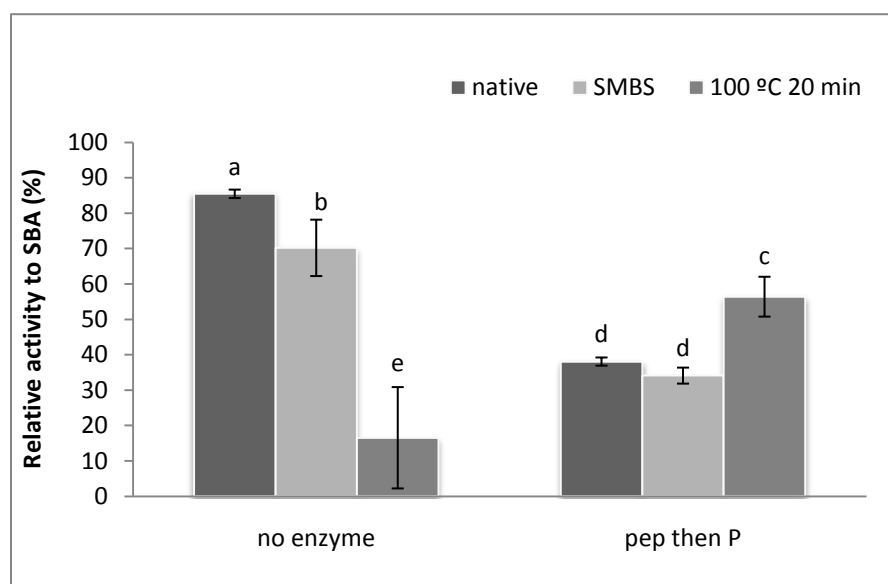
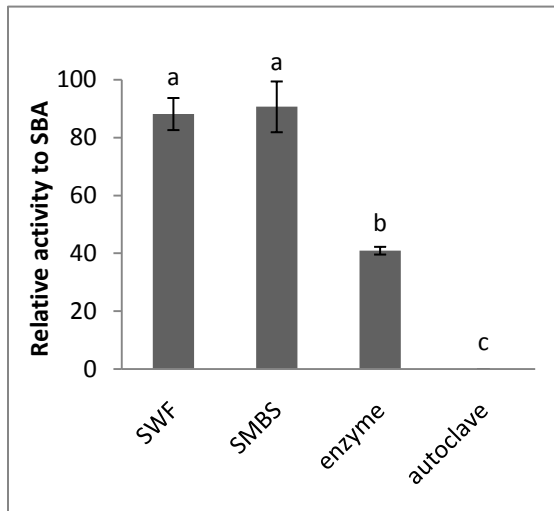


Figure 2. Effects of SMBS and enzyme treatments of soy white flake. N=3. SMBS: sodium metabisulfite treated SWF; pep then P: pepsin then pancreatin treatments; data are presented as Mean \pm SD. Different letters represent significant differences ($P \leq 0.05$). $LSD_{0.05}$ is 12.7.

A. Whole feeding material



B. SBA extract from feeding material

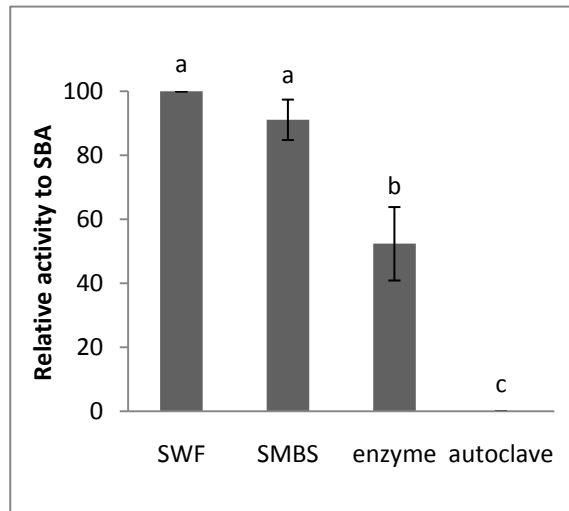


Figure 3. Hemagglutination activity of feeding materials. A. Hemagglutination activity of the whole feeding materials. SWF: soy white flake; SMBS: sodium-metabisulfite-treated SWF; enzyme: pepsin and pancreatin-treated SWF; autoclave: autoclave (121 °C 40 min) treated SWF. Data are presented as Mean \pm SD. Different letters represent significant differences ($P \leq 0.05$). $LSD_{0.05}$ is 8.9. B. Hemagglutination activity of SBA extracts from feeding materials. $LSD_{0.05}$ is 18.5.

CHAPTER 4. PEPTIDES FROM SOYBEAN AGGLUTININ, β -CONGLYCININ, AND GLYCININ HAVE HEMAGGLUTINATION ACTIVITY

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Abstract

We previously demonstrated different enzyme hydrolysates of soybean agglutinin (SBA), β -conglycinin and glycinin had 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) was used to identify the mass of the peptides and the masses were compared to the peptide profiles given by theoretical cleavage of the proteins, so that the peptides could be identified. Two peptides from SBA, 24 peptides from β -conglycinin and 16 peptides from glycinin were identified from the active peptide extracts. In addition, 2, 3 and 3 peptides from SBA, β -conglycinin and glycinin, respectively, were synthesized and their activities were assessed by using hemagglutination assay. These peptides had hemagglutination activity whereas a synthesized control peptide from SBA did not. These results confirmed our hypothesis that there are active peptides in soy proteins that have

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hemagglutination activity.

KEYWORDS: Active peptides; β -conglycinin; glycinin; hemagglutination activity; lectin; soybean agglutinin;

Introduction

Bioactive peptides in food have been extensively studied. Soybeans are considered embedding various active peptides. Soy protein hydrolysates by different enzymes have different activities, for example, inhibiting leukemia cells *in vitro* (1), inhibiting lipid accumulation in 3T3-L1 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), anti-oxidative activity (5), and suppressing of colon and liver tumorigenesis (6). The hydrolysates of glycinin also have 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 antihypertensive 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 to have appetite control activity. A peptide from the α' subunit of β -conglycinin with the sequence of MITLAIPVKNKPGR has phagocytosis-stimulating activity (11). Although

soy protein hydrolysates have been extensively studied, none have lectin-like (hemagglutination) activity.

Soybean agglutinin (SBA) 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. SBA is normally considered to be an anti-nutritional factor due to the ability to bind to the brush boarder, 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. There are problems associated with the use of lectins for drug delivery. Lectins usually have molecular weight of more than 10 KDa, which are likely to cause toxicity and immunogenicity (13). Small lectins might overcome this problem. Several small lectins have been found. Purified from Chinese bird spider *Selenocosmia huwena*, *Selenocosmia huwena* lectin-I is composed of 32 amino acids and has three disulfide bonds (14). θ -defensin is circular, tetracyclic peptides with three disulfide bonds and is purified from leukocytes and bone marrow of the rhesus macaque (*Macaca mulatta*) (15). θ -defensin has antimicrobial activity and protects cells from *in vitro* infection by HIV-1. Odorranalectin, which was purified from skin secretions of the frog *Odorrana graham*, is composed of 17 amino acids with the sequence of YASPKCFRYPNGVLACT (16).

After heat denaturation, SBA had remaining 60% hemagglutination activity; after single enzyme hydrolysis, SBA still had 50-60% activity; and activity of SBA was fully

eliminated by multi-enzyme hydrolysis (17). We hypothesized that native SBA forms tetramer, displays full activity; heated SBA is denatured, the activity is reduced; when SBA is hydrolyzed by multiple enzymes, some active peptides still have some activity; after hydrolyzing by combination of enzymes, the active peptides could be fully destroyed (17). In the present study, we identified which peptide(s) may have this 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 carbohydrate-binding sites have highest affinity for N-acetyl-D-galactosamine (GalNAc) (18). We hypothesized that the active peptides may have binding sites for GalNAc and these sites are responsible for the hemagglutination activity. In the present study, we used GalNAc-agarose beads to separate these peptides in trypsin hydrolysates from the unbound peptides, and used MALDI-TOF to identify the bound peptides. Once the peptide sequence was identified, we synthesized some of the identified peptides and tested activities of them.

After single enzyme hydrolysis, β -conglycinin and glycinin did not show any reduction on the hemagglutination activity, and combination of enzyme hydrolysis fully deactivated β -conglycinin, but not glycinin (18). We also hypothesized that some peptides in β -conglycinin and glycinin have hemagglutination activity. Because β -conglycinin and hemagglutination activities of glycinin were also inhibited by GalNAc (data not shown), we used the same GalNAc-agarose beads to separate these peptides and used the same methods as for SBA to identify them, and synthesized the identified peptides for activity assay.

Materials and Methods

Materials. β -conglycinin- and glycinin-rich fractions were produced from the Center for Crops Utilization Research (CCUR) in 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 (20). The glycinin-rich fractions contained 96% protein with 85% purity and β -conglycinin-rich fraction contained 90% protein and 81% purity. Rabbit blood in Alsever's solution was obtained from Hemostat Laboratories (Dixon, CA) and was used within two weeks. Other reagents were purchased from Fisher Scientific (Pittsburg, PA) or Sigma-Aldrich (St. Louis, MO).

Extraction and purification of SBA. The procedure for extraction and purifying SBA 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 saved by centrifugation (3020 x 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, 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 resuspending 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 1N HCl and adding ammonium sulfate (56 g/100 mL of solution). The precipitate was collected by centrifugation (3020 x g, 15 min) and redissolved in 100 mL of 0.05 M phosphate buffer, pH 6.1. The solution was dialyzed against water for 24 h at 4 °C and then lyophilized. The different isolectins were not separated, and SBA was not purified further.

Enzyme hydrolysis of soy proteins. SBA treated at 100 °C for 20 min, or β -conglycinin- or glycinin-rich fraction treated at 100 °C for 5 min was subjected to trypsin hydrolysis 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. The hydrolysates were used for further separation of peptides.

Separation of peptides that bound to GalNAc-agrose beads. Trypsin hydrolyzed SBA, β -conglycinin- or glycinin-rich fractions (6.4 mg) were mixed with about 1 mL GalNAc-agrose beads, respectively, and incubated at 4 °C overnight. The unbound peptides were collected by centrifuging the beads at 850 x 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 μ L 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. The bound peptides for β -conglycinin and glycinin were subjected to MALDI-TOF.

Identifying the peptides using MALDI-TOF. MALDI-TOF was done 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 μ L Matrix solution matrix (α -Cyano-4-hydroxycinnamic acid (ACH), 5 mg/mL), and loaded on the sample target of mass spectrometer. The remaining peptides were washed off the ZipTip by 0.5 μ L 70% ACN (acetonitrile) with 0.1% TFA (trifluoroacetic acid) solution and dispensed onto the same sample target, which were dried at room temperature. The samples were then loaded into the mass spectrometer and analyzed. MALDI-TOF 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 with a mix of angiotensin I, adrenocorticotrophic 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.

Sequences of SBA, glycinin and β -conglycinin were obtained from ExPASy databases

(<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 were compared with the theoretical digestion peptides to obtain the peptides that have the same molecular weight, and gave the sequences of these peptides.

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 for 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 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 placed in 37 °C for 2 h for agglutination to occur. The hemagglutination units (HU) per g of sample were determined by the equation (22):

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

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 mL 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 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 assay. The results were expressed as relative activity to SBA.

Slide agglutination test. The agglutination assay was done according to Pennell et al. (23). Generally, samples were serially diluted according to 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 in hemagglutination assay). They were mixed with a pipette tip and rotated by hand for 10 to 20 sec. The mixture was then observed under microscope to examine hemagglutination. Saline was included as 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 hemagglutination assay was used.

Statistical analysis. The data were analyzed by Analysis of Variance (ANOVA) and General Linear Model (GLM), 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 cleaves at the carboxyl side of arginine or lysine residues, and gives small number of peptides, which is easier to analyze. Therefore, trypsin was chosen to hydrolyze SBA as the starting peptide mix. Trypsin cannot hydrolyze SBA without heat treatment, so SBA was treated at 100 °C for 20 min. After trypsin hydrolysis of heated SBA, there were some precipitates. The supernatant and precipitate were separated and hemagglutination activities of them were tested. We found that the supernatant had activity whereas the precipitate did not, so the supernatant was used to bind to the GalNAc beads. We found that trypsin was not bound to the beads (Fig. 1), which indicated that GalNAc beads specifically bound to peptides. The unbound peptides showed no hemagglutination activity, which indicates that the other part (bound peptides) might have full activity. 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 in them.

Activities of identified and synthesized peptides from SBA. After separating peptides bound or unbound to beads, MALDI-TOF was used to identify these peptides. Unbound and bound peptides gave different peptide profiles (Fig. 2), which indicated that GalNAc specifically bound to certain peptides. From the results of bound peptides, two

peptides belonging to SBA were identified. One is SBA 197-206, with the sequence KTSLPEWVRI, another is SBA 36-51 with the sequence KVDENGTPKPSSLGRA. The two peptides were then synthesized and activities of them were assessed. One peptide from unbound peptides: SBA 15-26 with the sequence QPNMILQGDA was also synthesized as a negative control.

Fig. 3 shows that the positive control SBA (A) had clumps on the slide, whereas negative control saline (B) had uniformly distributed 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 higher dilution of SBA 36-51 had more obvious clumps than E. On the other hand, the control peptide (SBA 15-26) did not have activity (H), which was just like saline (B). The activities of the peptides were calculated according the equation used in hemagglutination assay and summarized in Table 1. Both of SBA 36-51 and SBA 197-206 had activity, whereas the control peptide SBA 15-26 did not. This result confirmed our hypothesis that some active peptides in SBA have hemagglutination activity, and the activity is due to the binding of peptides to the carbohydrates on cell surfaces. Fig. 4 shows the relative position of the two active peptides in SBA sequence. Dessen et al. (24) 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 $-\text{O}(\text{CH}_2)_5\text{COOCH}_3$. They found the following amino acids 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 the amino acids are in the two peptides we identified, probably due to the different carbohydrates and methods used for identifying the binding sites.

Activities of identified and synthesized peptides from β -conglycinin. Twenty-four peptides from β -conglycinin were identified that were bound to GalNAc beads and Table 2 shows 11 of them. We selected three peptides which had the highest intensity and hitting times to synthesize: α' chain amino acid 566 to 575 (Ba' 566-575), α' chain amino acid 24 to 31 (Ba' 24-31), and α chain amino acid 58 to 66 (Ba 58-66). The activities were tested using the slide agglutination test. Fig. 5 shows slide agglutination results for these peptides. For peptide Ba' 566-575, A is the last dilution that had the activity, whereas B is the next dilution that did not have activity. By similarity, C and E are the dilutions that Ba'24-31 and Ba 58-66 had activity, whereas D and F are the next dilutions the two peptides did not have activity. The hemagglutination activities of these peptides are summarized in Table 1. All three peptides from β -conglycinin have hemagglutination activity, confirming our hypothesis.

Activities of identified and synthesized peptides from glycinin. Sixteen peptides from glycinin bound to GalNAc beads and Table 3 shows eight of them. We selected three peptides for synthesis: subunit B2 1-13 (GB2 1-13) with sequence GIDETICTMRLRH; subunit A1a 101-114 (GA1a 101-114) with sequence RGQSSRPQDRHQKI; subunit A4 236-242 (GA4 236-242) with sequence KKTQPRR. Fig. 6 shows the slide agglutination test

results. Fig. 6 A, C, and E are the last dilutions that GB2 1-13, GA1a 101-114, and GA4 236-242 had activity, whereas B, D, and F are the next dilutions the three peptides did not have activity. The activities are calculated and summarized in Table 1. This result 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 boarder and stimulated cholecystokinin (CCK) release suppressing food intake. Peptides with arginine (R) and glycine (G) had strong affinity, but not single R. All the peptides we identified have R and G, but mostly in a single R format. We did not find any similarities between the peptides we identified. Although there might be some specific amino acids involved in the carbohydrate binding, we do not know exactly what the rule is.

Hemagglutination assay and slide agglutination test. When we used the typical hemagglutination assay to test activities of synthesized peptides, the peptides caused cell lysis, for reasons not known. We looked for alternative methods and the slide agglutination test seems to be a good one. For the positive samples, the cells formed clump almost immediately after getting 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. We tested the same sample (SBA) using both the hemagglutination assay and slide agglutination assay. We found the results only differ in one dilution, so that the hemagglutination unit differed in one unit. This result shows the consistent results and 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. The slide agglutination assay is very tedious, because we had to put every diluted sample onto a slide and mix with red blood cells. This method is 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.

Although the peptides we identified had hemagglutination activity *in vitro*, we do not know how they would behave *in vivo*. Being able to bind to blood cells, there is a possibility that they can also bind to other cells, for example, the small intestinal brush boarder. This possible binding might cause anti-nutritional effects as SBA, but might 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 (CCK) 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 unknown. The ability for peptides to bind to the cells gives some indication of how the peptides carry out their functions, for example, bind to and enter the cells through endocytosis. More research needs to be done to study the *in vivo* effect of these lectin-like peptides and the possible links between the activity of binding to cells and other activities.

The potential use of these small lectin-like peptides in drug delivery needs to 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. The smallest peptide we identified only had seven amino acids, which was smaller than the smallest lectin peptides identified before (17 amino acids) (16) and easy to manipulate. The specificity of binding to GalNAc could be a potential for targeting special sites or cells.

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Table 1. Activities of synthesized peptides from soybean agglutinin, β -conglycinin, and glycinin

Origin	Peptides	Sequence	Relative activity to SBA (%)
Soybean agglutinin	SBA 197-206	KTSLPEWVRI	38.1 \pm 3.7
	SBA 36-51	KVDENGTPKPSSLGRA	48.8 \pm 2.0
β -Conglycinin	Ba' 566-575	KGPLSSILRA	40.5 \pm 1.7
	Ba' 24-31	RQQHGEKE	40.1 \pm 1.7
	Ba 58-66	REEQEWPRK	47.2 \pm 2.0
Glycinin	GB2 1-13	GIDETICTMRLRH	46.1 \pm 0.0
	GA1a 101-114	RGQSSRPQDRHQKI	47.0 \pm 3.1
	GA4 236-242	KKTQPRR	45.2 \pm 3.1
Unbound peptides	SBA 15-26	QPNMILQGDA	0

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

Table 2. Selected identified active peptides from soybean β -conglycinin

Peptide	Sequence
α chain 367-373	KNPQLRD
α chain 161-170	RSPQLQNLRD
α chain 58-66	REEQEWPRK
α chain 89-101	RQFPFPRPPHQKE
β chain 395-401	KEEGSKGRK
β chain 190-197	KEQIRQLSRR
β chain 345-358	RNFLAGEKDNVVRQ
α' chain 401-407	RNPQLRD
α' chain 24-31	RQQHGEKE
α' chain 566-575	KGPLSSILRA
α' chain 58-66	KEEHEWHRK

K: Lysine (Lys); N: Asparagine (Asn); P: Proline (Pro); Q: Glutamine (Gln); L: Leucine (Leu); R: Arginine (Arg); D: Aspartic Acid (Asp); S: Serine (Ser); E: Glutamic Acid (Glu); W: Tryptophan (Trp); F: Phenylalanine (Phe); G: Glycine (Gly); A: Alanine (Ala); V: Valine (Val); I: Isoleucine (Ile); Y: Tyrosine (Tyr); H: Histidine (His); T: Threonine (Thr).

Table 3. Selected identified active peptides from soybean glycinin

Peptide	Sequence
Subunit B2 162-172	RQIKNNNPFKF
Subunit B2 90-101	RVFDGELQEGRV
Subunit B2 1-13	GIDETICTMRLRH
Subunit A1a 282-287	RGSQSK
Subunit A1a 101-114	RGQSSRPQDRHQKI
Subunit A4 236-242	KKTQPRR
Subunit A4 155-182	KWQEQQDEDEDEDEDEDEDEQIPSHPPRR
Subunit A2 242-250	RVTAPAMRK

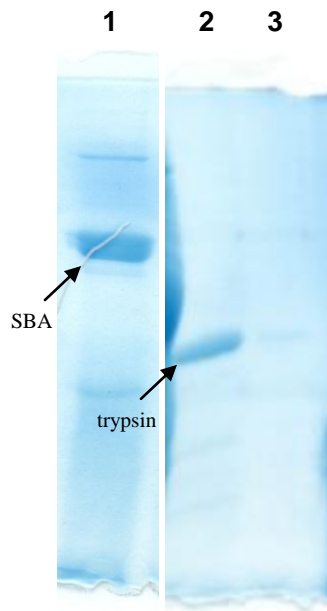


Figure 1. Separation of peptides from soybean agglutinin trypsin hydrolysates that were bound or unbound to GalNAc beads. Lane 1, SBA extract; lane 2, peptides that were unbound to beads; lane 3, peptides that were bound to beads.

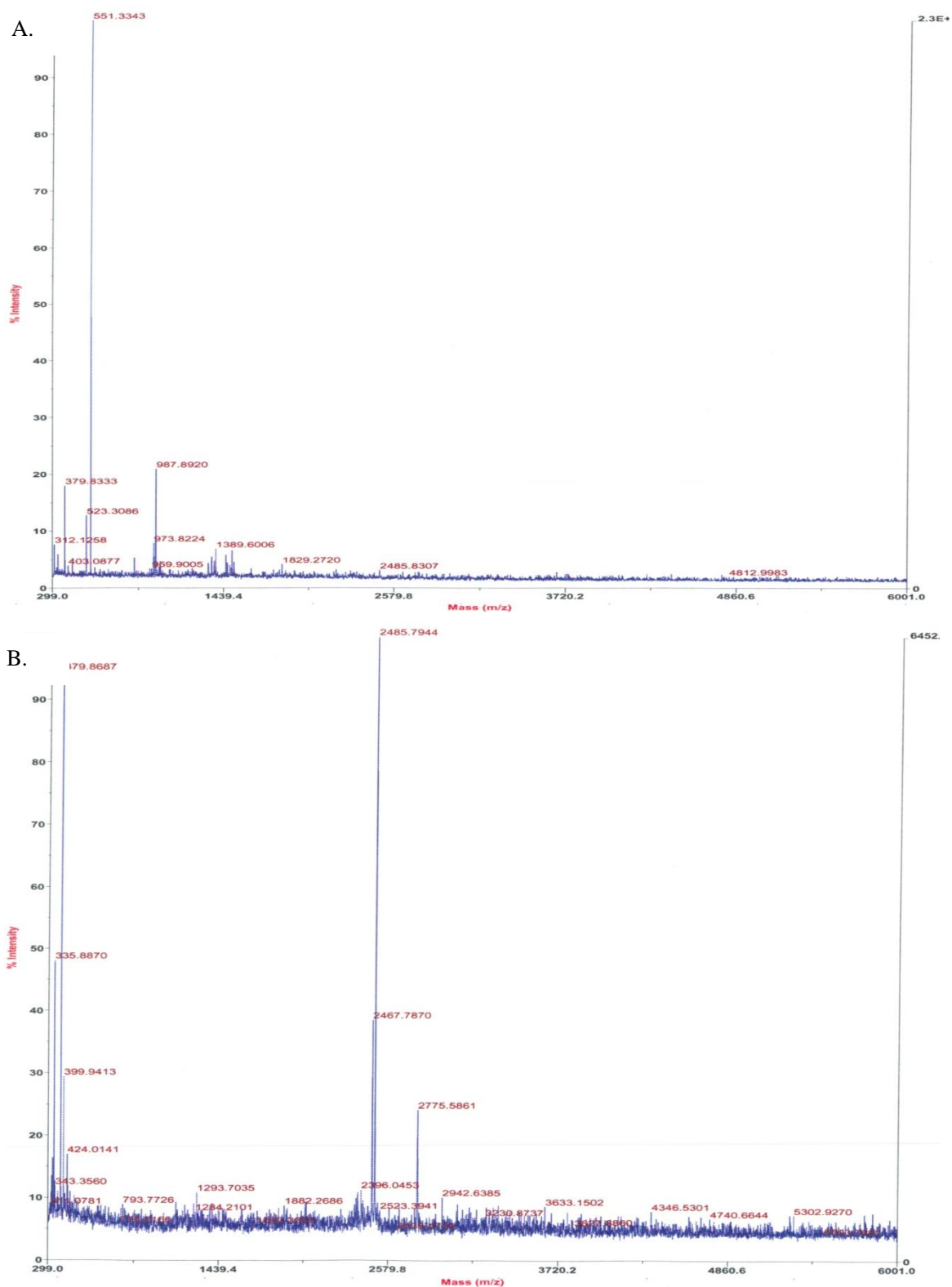


Figure 2. MALDI-TOF results for peptides from soybean agglutinin trypsin hydrolysates that were bound or unbound to GalNAc beads. A. Peptides that were bound to beads. B. Peptides that were unbound to beads.

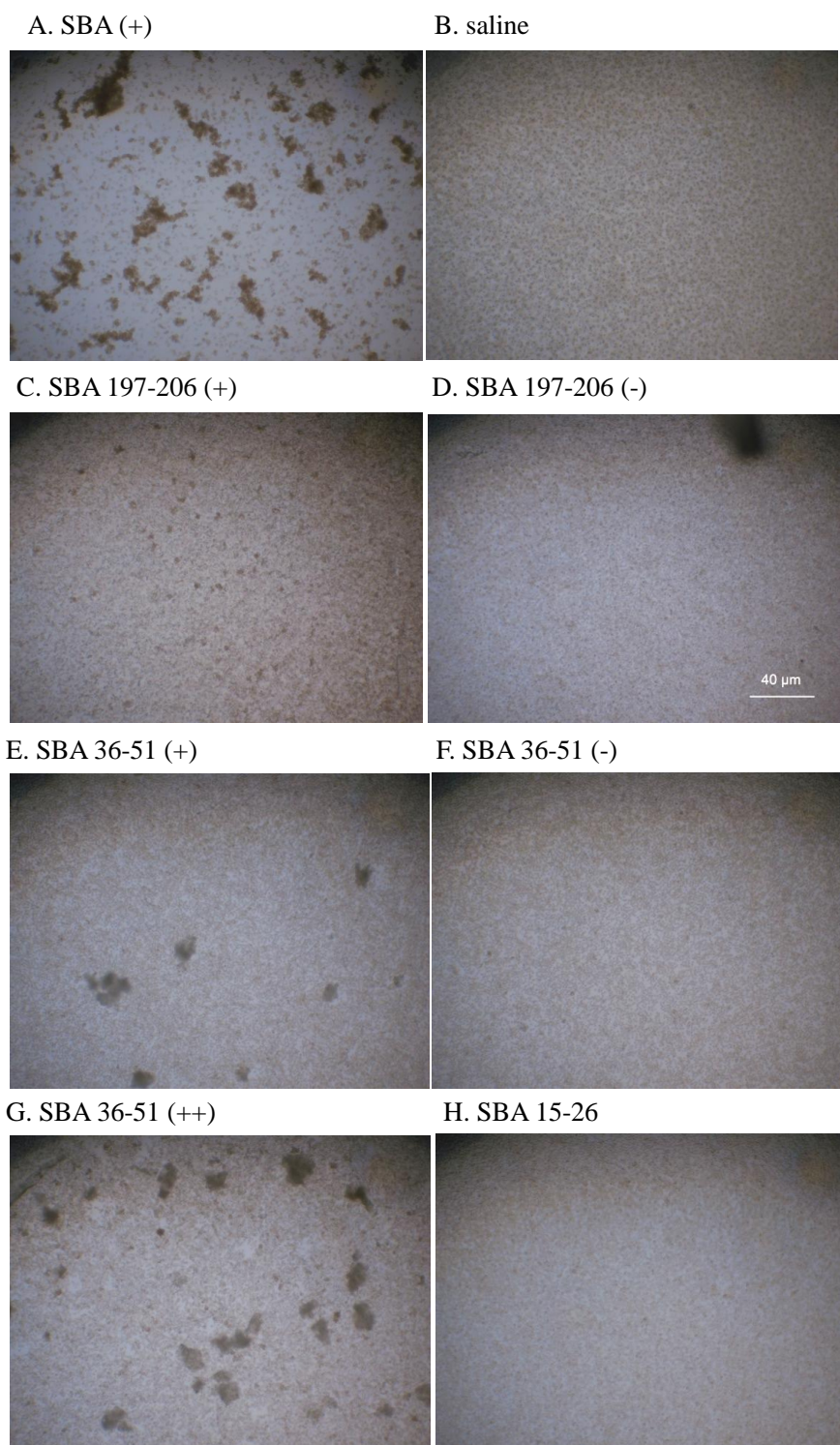


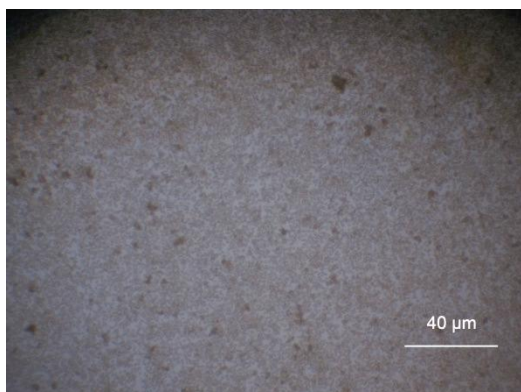
Figure 3. Hemagglutination activities of active peptides from soybean agglutinin. The pictures are 3% rabbit red blood cells mixed with: A. 2.5×10^{-3} mg/mL SBA; B. 0.9% saline; C. 26.8 mg/mL SBA 197-206; D. 13.4 mg/mL SBA 197-206; E. 3.18 mg/mL SBA 36-51; F. 1.59 mg/mL SBA 36-51; G. 12.7 mg/mL SBA 36-51; H. 24 mg/mL SBA 15-26.

<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>
AETVSFSWNK	FVPKQPNMIL	QGDAIVTSSG	KLQLN KVDEN	GTPKPSSLGR
<u>60</u>	<u>70</u>	<u>80</u>	<u>90</u>	<u>100</u>
AL YSTPIHIW	DKETGSVASF	AASFNFTFYA	PDTKRL AD GL	AFFLAPIDTK
<u>110</u>	<u>120</u>	<u>130</u>	<u>140</u>	<u>150</u>
PQTH AG YLGL	FNENESGDQV	VAVEFD TFRN	SWDPPNPHIG	INVNSIRSIK
<u>160</u>	<u>170</u>	<u>180</u>	<u>190</u>	<u>200</u>
TTSWDLANNK	VAKVLITYDA	STSLLVASLV	YPSQRTSNIL	SDVV DLK TSL
<u>210</u>	<u>220</u>	<u>230</u>	<u>240</u>	<u>250</u>
PEWVR IGFSA	ATG LDI PGES	HDVLSWSFAS	NLPHASSNID	PLDLTSFVLH

EAI

Figure 4. Relative positions of the two active peptides from SBA in SBA sequence. The red letters are the two active peptides: SBA 36-51 and 197-206. The blue letters are amino acids that participate in carbohydrate binding according to Dessen et al. (24).

A. Ba' 566-575 (+)



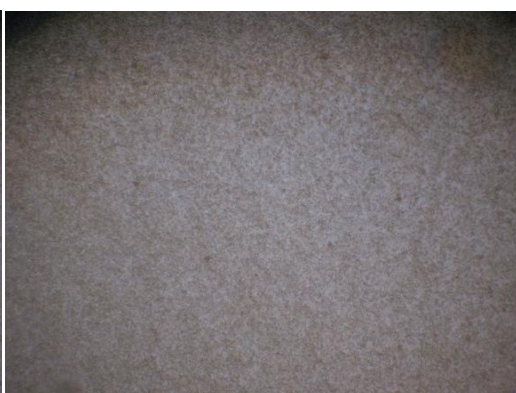
B. Ba' 566-575 (-)



C. Ba' 24-31 (+)



D. Ba' 24-31 (-)



E. Ba 58-66 (+)

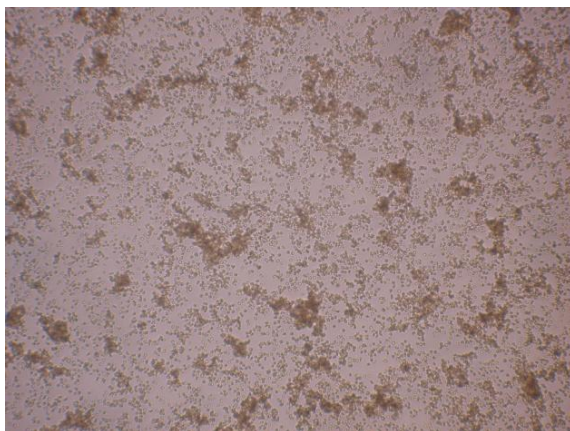


F. Ba 58-66 (-)



Figure 5. Hemagglutination activities of active peptides from β -conglycinin. The pictures are 3% rabbit red blood cells mixed with: A. 12.5 mg/mL Ba' 566-575; B. 6.25 mg/mL Ba' 566-575; C. 13.62 mg/mL Ba' 24-31; D. 6.81 mg/mL Ba' 24-31; E. 4.14 mg/mL Ba 58-66; F. 2.07 mg/mL Ba 58-66.

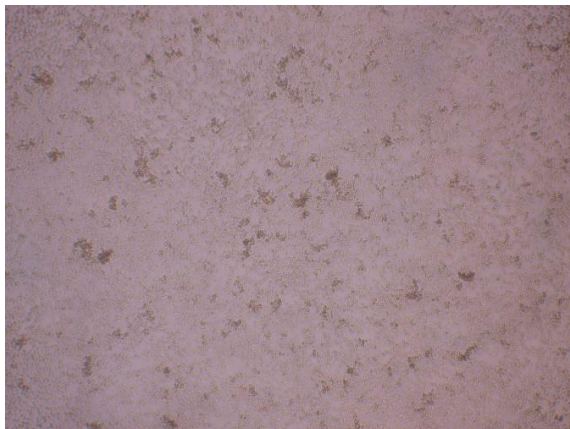
A. GB2 1-13 (+)



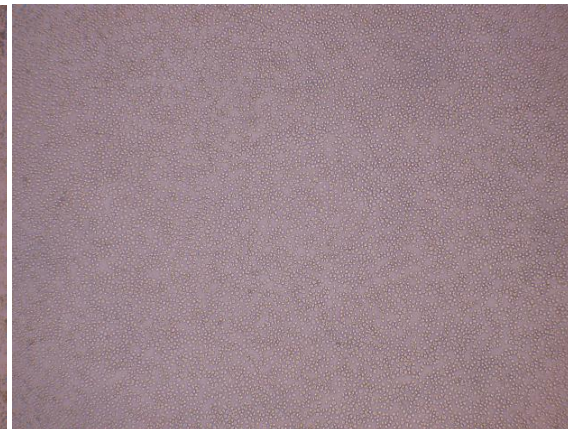
B. GB2 1-13 (-)



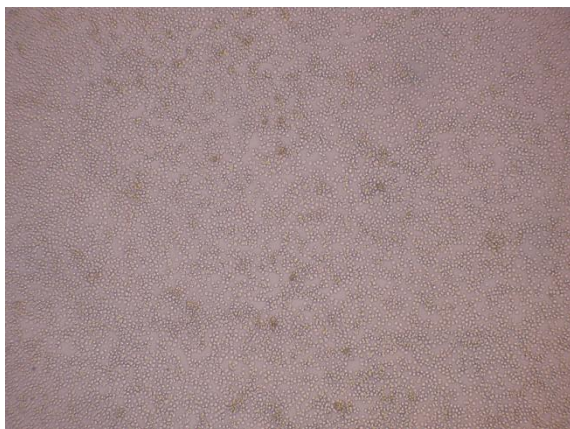
C. GA1a 101-114 (+)



D. GA1a 101-114 (-)



E. GA4 236-242 (+)



F. GA4 236-242 (-)



Figure 6. Hemagglutination activities of active peptides from glycinin. The pictures are 3% rabbit red blood cells mixed with: A. 6.65 mg/mL GB2 1-13; B. 3.33 mg/mL GB2 1-13; C. 7.93 mg/mL GA1a 101-114; D. 3.96 mg/mL GA1a 101-114; E. 5.49 mg/mL GA4 236-242; F. 2.74 mg/mL GA4 236-242.

CHAPTER 5. GENERAL CONCLUSIONS

Lectin is a group of protein that binds to carbohydrates. Soybean lectin, known as soybean agglutinin (SBA), causes growth inhibition, intestinal weight gain, and pancreatic hypertrophy, when consumed intact. SBA is readily deactivated by moist heat treatment, however, we want to utilize the protein fraction from the aqueous extraction process with minimum heating to preserve better functionality and nutritional quality of the soy proteins. In this study, a series of experiments was conducted to eliminate activity of SBA. Deglycosylation decreased activity of SBA by 21%, but not as much as denaturation (23-53%). Single enzymes did not hydrolyze native SBA, but hydrolyzed heat- or organic solutes-denatured SBA. Even after hydrolysis, activity of SBA still was not fully eliminated (44-62% residual). A combination of multiple enzymes with thermolysin fully deactivated heat- or organic solutes-treated SBA. Tea polyphenols, metal ions, chelating agents were also tested and had no significant effect on deactivating SBA. N-acetyl-galactosamine (GalNAc)-agrose beads specifically removed SBA from a protein mixture, but not fully and activity of SBA was not eliminated. Pepsin and pancreatin hydrolysis fully deactivated native SBA. This treatment uses minimum heating, as a result, it was chosen to treat soy white flake (SWF) for feeding trial to evaluate feeding quality.

During the preparation of feeding material, we surprisingly found that the two storage proteins in soybean, β -conglycinin and glycinin, also had hemagglutination activity. The activity of β -conglycinin was not reduced by single enzyme hydrolysis, but was fully

eliminated by a combination of multiple enzyme treatments. Activity of glycinin was not fully reduced by either single or a combination of multiple enzyme hydrolysis. Similarly, the activities of SWF and soy protein isolate (SPI) were not fully reduced by single or combination of multiple enzyme hydrolysis. Although pepsin and pancreatin treatment did not fully deactivate β -conglycinin, glycinin, SWF and SPI, this method was used to generate feed material for *in vivo* evaluation of nutritional quality due to the ability to fully deactivate SBA. The *in vitro* study showed that the SBA in SWF was deactivated by this treatment, but did not improve chick growth performance compared to the raw SWF, however, the chicks did not show any pancreas enlargement or intestine weight increase compared to the raw soy feeding or a commercial diet, indicating the deactivation of SBA in the material.

After enzyme hydrolysis, SBA, β -conglycinin and glycinin still had hemagglutination activity, this result caused us to hypothesize that certain peptides in these proteins had hemagglutination activity. In order to identify the active peptides, the three proteins were subjected to trypsin hydrolysis, and GalNAc-agarose beads were used to isolate the active peptides. MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time-of-Flight) was used to identify the masses of the peptides, and the masses were compared to the peptide profiles given by theoretical cleavage of the proteins, so that the peptides could be identified. Two peptides from SBA, 24 peptides from β -conglycinin and 16 peptides from glycinin were identified from the active peptide extracts. In addition, 2, 3 and 3 peptides from SBA, β -conglycinin and glycinin respectively were synthesized and their activities were assessed

using hemagglutination assay. These peptides have hemagglutination activity whereas a synthesized control peptide from SBA did not show any activity. This result confirmed our hypothesis that there are active peptides in soy protein that have hemagglutination activity.

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