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Improving Digestibility of Soy Flour by Reducing Disulfide Bonds with Thioredoxin

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The Kunitz trypsin inhibitor (KTI) and the Bowman−Birk inhibitor (BBI) of trypsin and chymotrypsin contain disulfide bonds. Glycinin, the major storage protein in soybeans also contains disulfide bonds. Treatment of soy white flour with a NADP−thioredoxin system (NTS) effectively reduced disulfide bonds in soy flour and increased protein digestibility by trypsin and pancreatin as measured by the pH stat method. Treatment of soy flour with NTS increased the digestibility compared to soy white flour by 29.3 and 60.6% for trypsin and pancreatin, respectively. NTS-treated soy flour had similar digestibility by trypsin to autoclaved soy flour and casein, but digestibility by pancreatin was less than autoclaved soy flour and casein. The degree of reduction by NTS was highly correlated to the degree of hydrolysis (DH) by trypsin ($R^2 = 0.93$) and pancreatin ($R^2 = 0.99$). The DH of NTS-treated soy flour by trypsin is reflective of both inactivation of trypsin inhibitors and overall protein digestibility while pancreatin hydrolysis is reflective of only overall protein digestibility.

KEYWORDS: Disulfide bond; digestibility; soy; thioredoxin; trypsin; pancreatin; pH stat

INTRODUCTION

Current processing of soybeans utilizes high temperatures to inactivate trypsin inhibitors and increase protein digestibility. Soybean proteins are reported to have beneficial health effects, including lowering of plasma cholesterol and prevention of cancer, diabetes, and obesity, but without heat inactivation of trypsin inhibitors, growth depression and pancreatic hypertrophy, hyperplasia, and adenoma can occur in some species (*1*). However, heat treatment alone is not sufficient to inactivate trypsin inhibitors completely. For example, 20% of Kunitz trypsin inhibitor (KTI) activity remained in the soy flour after heating at 120 °C for 30 min, although all the Bowman−Birk trypsin inhibitor (BBI) was inactivated (*2*). In addition, heating soy to inactivate trypsin inhibitors may destroy important amino acids, such as methionine, cystine, arginine, and lysine (*3–6*). Recently, there has been an ongoing effort to develop aqueous extraction process using water to replace hexane to extract oil from soybeans (*7*). This process uses much less heat than the traditional process; therefore, protein functionality is preserved. One major difficulty to overcome is the treatment of the protein after the oil has been removed to inactivate the trypsin inhibitors and to increase the digestibility without excessive heating. One method to inactivate trypsin inhibitors to increase protein digestibility is disulfide bond reduction for altered protein structure and, therefore, reduced inhibitor activities.

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The BBI of chymotrypsin and trypsin and the KTI are major antinutritional factors in soybeans, and they contain disulfide bonds. BBI is an 8 kDa protein containing seven intramolecular disulfide bonds, and KTI is a 21 kDa protein containing two intramolecular disulfide bonds (*8–10*). Glycinin, a major storage protein of soybeans, is a hexamer with a molecular weight of 300−380 kDa. Each subunit of glycinin is composed of an acidic polypeptide with a molecular weight of ∼35 kDa and a basic polypeptide with a molecular weight of ∼20 kDa linked together by a disulfide bond (*11*). In addition to these six intermolecular disulfide bonds, glycinin is rich in intramolecular disulfide bonds. Several values for the free sulfhydryl and half-cystine content have been reported for glycinin, and they ranged from 0−1.9 mol free sulfhydryl/mol protein and 33.0−45.9 mol half-cystine/mol protein based on a molecular weight of 350 kDa (*11–19*). **Table 1** contains a summary of the sulfhydryl, disulfide, and half-cystine contents of BBI, KTI, and glycinin as well as their amounts in soy flour. Proteins high in disulfide bonds are generally less susceptible to proteolysis and more heat-stable as well as are potential food allergens. Reduction of disulfide bonds has shown an increase in susceptibility to proteolysis and decreases heat stability in certain proteins (*22–25*).

Thioredoxin is a 12 kDa protein capable of reducing protein disulfide bonds with the associated protein thioredoxin reductase and NADPH (*26*). The NADP−thioredoxin system (NTS) has been used to reduce the disulfide bonds in BBI and KTI. Treatment of BBI and KTI with NTS led to their inactivation and increased their heat and protease susceptibility (*24*). Treatment of β-lactoglobulin with NTS decreased its allergenicity and increased its digestibility (*27*). Treatment of wheat

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Table 1. Summary of Sulfhydryl, Disulfide, and Half Cystine Content (μmol/g) of KTI, BBI, and Glycinin and Their Amounts as a Percentage of Soy Flour (g protein/100 g soy flour)

protein	% soy flour	SH	SS	half cystine/mol	ref
inhibitors	3				*19*
BBI		0	7.0	14.0	*7*
KTI		0	2.0	4.0	*8*
glycinin[a]	25.5				*21*
		1.9	15.6	33.1	*10*
		1.5	21.9	45.3	*11*
		0	18.0	36.0	*13*
		1.8	21.8	45.4	*15*
				45.9	*16*
				44.0	*17*
				35.4	*18*
		1.1			*12*
		0.5			*14*

[a] Sulfhydryl (SH), disulfide (SS), and half-cystine content adjusted for a molecular weight of 350 kDa.

proteins with NTS decreased their allergenicity and increased their susceptibility to proteolysis and heat (*28*).

Although it has been demonstrated that thioredoxin is effective in reducing the disulfide bonds in several proteins, its ability to reduce protein disulfide bonds in a mixture of protein and other components has not been fully investigated. Thus, it is important to determine if treatment of soy flour with thioredoxin is effective in reducing disulfide bonds and increases the nutritive value of soy protein so that we can better evaluate its potential for use in an aqueous processing scheme for soybeans. The objective of our research was to determine the effect of reduction of disulfide bonds in soy flour by NTS on digestibility measured by the pH stat method and the relationship between digestibility and sulfhydryl content.

MATERIALS AND METHODS

Materials. Soy white flour was obtained from Cargill (Minneapolis, MN) by hexane extraction of the oil and then flash-desolventized with a protein dispersibility index ≥85%. Bovine trypsin, BSA, casein, cysteine, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), dithiothreitol, guanidine thiocyanate, NADPH, porcine pancreatin, thioredoxin, and thioredoxin reductase were purchased from Sigma-Aldrich (St. Louis, MO). Mercaptoethanol was purchased from MP Biomedicals (Solon, OH). All other reagents and supplies were purchased from Fisher Scientific (Pittsburgh, PA).

Disulfide Bond Reduction by NTS. Soy white flour (400 mg) was dissolved in 19.0 mL of 0.85% NaCl in 20 mM phosphate buffer (pH 7.5) with 10 mM EDTA (PBS). To this, 160 μL of thioredoxin (1 mg/mL), 73 μL of thioredoxin reductase (2.17 mg/mL), 320 μL of NADPH (50 mg/mL), and 447 μL of water were added to make the total volume 20.0 mL. A NTS control sample was also prepared the same as the NTS-treated sample, but without thioredoxin, thioredoxin reductase, and NADPH. Samples were incubated at 37 °C for 45 min at 125 rpm shaking. Two additional samples were prepared with either 80 μL of thioredoxin, 36 μL of thioredoxin reductase, 160 μL of NADPH, and 724 μL water or 40 μL of thioredoxin, 18 μL of thioredoxin reductase, 80 μL of NADPH, and 862 μL of water to soy white flour (400 mg) in PBS to examine the extent of disulfide bond reduction on digestibility. Samples were incubated under the same conditions. Soy white flour (400 mg) was also dissolved in 20.0 mL of PBS and either left unheated or was autoclaved for 40 min. Autoclaving of soy flour was based upon trypsin inhibitor content and chick growth for soybean meal autoclaved for 40 min as reported by Herkelman et al. (*29*). Casein was also prepared by dissolving in 1 M NaOH according to the manufacture's directions. All treatments were repeated three times.

Protein Analysis. The crude protein (percent nitrogen × 6.25) content of soy white flour was determined by the micro Kjeldahl method (*30*).

Sulfhydryl Quantification. The sulfhydryl content of soy white flour was determined using a modified procedure initially described by Ellman (*31*) and again by Robyt et al. (*32*). In a 15 mL centrifuge tube, 1.0 mL of sample in PBS was mixed with 1.0 mL of 4 M guanidine thiocyanate 6 mM EDTA, 0.1 mL of 1 M Tris 1 M phosphate buffer (pH 8.1), and 0.5 mL 2 mM DTNB (in 10 mM phosphate buffer, pH 8.1). The mixture was incubated at room temperature for 30 min. Concurrently, a sample was incubated without DTNB as a blank. Samples were centrifuged for 5 min at 3000*g*, 200 μL of supernatant was added in triplicate to a 96-well plate, and the absorbance was read at 412 nm. The absorbance of the sample with DTNB minus the absorbance of the sample without DTNB was used for sulfhydryl determination (eq 1) to account for the contribution of the soy flour to absorbance due to turbidity. From each replicate of each treatment, three measurements were taken.

$$A_{412}\text{corrected} = A_{412}w/\text{DTNB} - A_{412}w/o\ \text{DTNB} \quad (1)$$

Determination of Molar Extinction Coefficient. The molar extinction coefficient of CNT (3-carboxylate-4-nitrophenolate), the product of the reaction of DTNB with sulfhydryls, was experimentally determined using cysteine, dithiothreitol, and mercaptoethanol as standards following the procedure above. Six dilutions of standards were used with a sulfhydryl concentration of 10−200 μM, and three measurements per dilution were made.

Sample Turbidity Interference of Sulfhydryl Quantification. BSA was used as an internal control to determine the effect of centrifugation on sulfhydryl determination. Four dilutions of three samples were measured: soy white flour, BSA, and soy white flour with BSA added. For each dilution, the measurement of [soy] plus [BSA] should equal that of [soy + BSA]. Sulfhydryl content was determined as described above with and without centrifugation. Several treatments and centrifugation speeds were evaluated to improve the accuracy of sulfhydryl quantification. The difference (D_i) of the sulfhydryl content of soy flour, BSA, and soy flour with BSA added is defined as

$$D_{i(i=0\text{ or }1)}(\%) = \frac{|(a_i + b_i - c_i)|}{\frac{1}{2}(a_i + b_i + c_i)} \quad (2)$$

where a, b, and c are defined as the mole sulfhydryl determined for soy white flour, BSA, and soy white flour with BSA added, respectively. D_0 is defined as the difference of samples without centrifugation, and D_1 is defined as the difference of samples with centrifugation. The improvement (I) in sulfhydryl determination by centrifugation was calculation as follows:

$$I = \frac{|D_1 - D_0|}{D_0} \times 100 \quad (3)$$

Protein Hydrolysis by Trypsin and Pancreatin Enzymes. The susceptibility of the protein samples to trypsin and pancreatin hydrolysis was measured using a 718 Stat Titrino (Metrohm, Herisau, Switzerland). Samples were diluted to approximately 40 mL with water, and the temperature and pH were stabilized at 37 °C and 8.5, respectively. Trypsin hydrolysis was performed by adding 0.25 mL of 1 mg/mL trypsin (in 1 mM HCl, 20 mM $CaCl_2$) and monitoring the consumption of 20 mM NaOH for 1 h. For all hydrolyses with trypsin, the enzyme-to-substrate ratio (g enzyme:g protein) was at 1:100. Pancreatin hydrolysis was performed by adding 0.5 mL of 1 mg/mL pancreatin (in 10 mM Tris pH 8.5) and monitoring the consumption of 20 mM NaOH for 30 min. For all hydrolyses with pancreatin, the enzyme-to-substrate ratio was at 1:50. The degree of hydrolysis (DH) for each sample was determined as follows:

$$\text{DH}(\%) = B \times N_B \times \frac{1}{\alpha} \times \frac{1}{\text{MP}} \times \frac{1}{h_{tot}} \times 100 \quad (4)$$

where B is the base consumption in mL, N_B is the normality of the base, α is the average dissociation of the α-NH group, MP is the mass of the protein in g, and h_{tot} is the total number of peptide bonds in the protein substrate. The values for α and h_{tot} are 0.92 and 7.8, respectively (*33*).

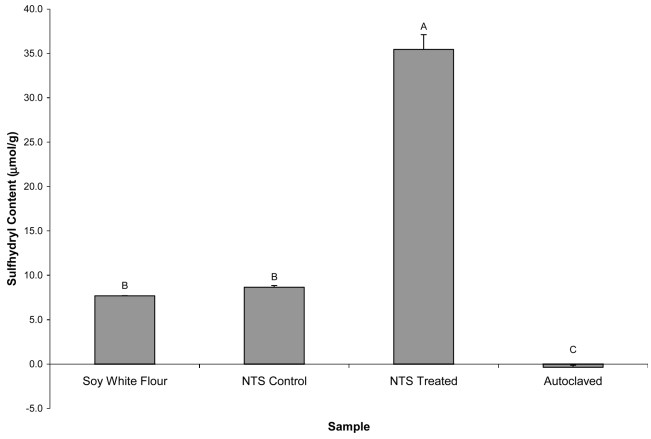

Figure 1. Effect of treatment on the sulfhydryl content of soy white flour. Data is presented as mean ± standard error. Different letters represent significant differences ($P \leq 0.05$).

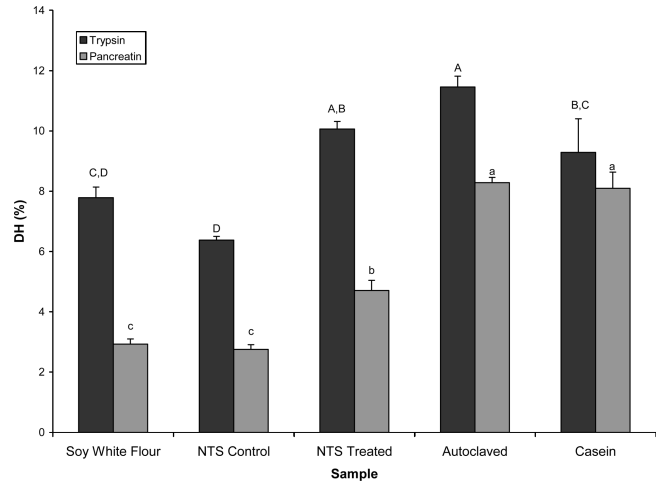

Figure 2. Effect of treatment on degree of hydrolysis (DH) by trypsin and pancreatin enzymes. Data is presented as mean ± standard error. Different letters represent significant differences ($P \leq 0.05$).

Statistics. Data are expressed as means ± standard errors. Statistical analyses were performed using SAS JMP (SAS Institute, Cary, NC). Means were compared and they were considered different when $P \leq 0.05$.

RESULTS AND DISCUSSION

Sulfhydryl Quantification. The experimentally determined molar extinction coefficient for CNT was 14 300 ± 150 L/mol/cm, which is in good agreement with previously reported values (*12*). The turbidity of dispersed soy flour interferes with sulfhydryl quantification significantly, even when the absorbance of a sample without DTNB is used as a blank. Several centrifugation speeds were tested to reduce the turbidity of soy flour and, thus, the interference in sulfhydryl determination. It was determined that centrifugation of samples at 3000*g* after incubation with DTNB sufficiently reduced the turbidity due to soy flour and that higher centrifugation speeds do not reduce the turbidity of soy flour significantly more. To determine the effect of centrifugation on sulfhydryl quantification, BSA was used as an internal control. In theory, the sum of the sulfhydryl content of the BSA sample and the soy white flour sample would equal the sulfhydryl content of the soy white flour with BSA added. Without centrifugation, the samples were 24.9% different (D_0), and with centrifugation, the samples were 12.6% different (D_1), as calculated using eq 2. Using BSA as an internal control, it was determined that centrifugation at 3000*g* for 5 min improved sulfhydryl quantification by 49.3%.

Disulfide bonds in soy white flour were effectively reduced by NTS (**Figure 1**). NTS treatment of soy white flour increased the sulfhydryl content compared to untreated soy white flour and the NTS control samples (35.44 vs 7.69 and 8.66 μmol/g, respectively). The free sulfhydryls in the autoclaved sample were either destroyed by heating or oxidized to disulfides or formed other compounds. Most likely, the free sulfhydryls were destroyed by severe heating. Data from Wang and Damodaran (*3*) shows that at temperatures above 90 °C, thermal destruction of cysteine and cystine become significant, as well as losses of other amino acids at high heating temperatures. If the sole contributor of sulfhydryls in soybean protein was considered to be glycinin, soy flour would contain approximately 29.7 μmol sulfhydryl/g soy flour when completely reduced based on the information in **Table 1**. This is an underestimate because of the high cysteine content of BBI and KTI as well as other sulfhydryl-containing proteins in soybeans. Nonetheless, on the basis of this crude estimate, treatment of soy white flour with NTS is an effective method for reducing the disulfide bonds to free sulfhydryls.

Protein Hydrolysis by Trypsin and Pancreatin Enzymes. NTS treatment of soy white flour increased digestibility by trypsin as compared to untreated soy white flour and NTS control samples (10.06% vs 7.78 and 6.38%, respectively) and was similar to autoclaved soy flour and casein (11.46 and 9.29%, respectively) (**Figure 2**). NTS treatment of soy white flour increased the digestibility by pancreatin as compared to untreated soy white flour and control samples (4.71% vs 2.93 and 2.75%, respectively). However, the NTS-treated samples were less digestible by pancreatin than autoclaved soy flour and casein (8.29 and 8.10%, respectively) (**Figure 2**). NTS treatment of soy flour increased the digestibility compared to the untreated soy flour by 29.3 and 60.6% for trypsin and pancreatin, respectively. Autoclaving soy white flour increased the digestibility of soy white flour by 47.2 and 182.7% for trypsin and pancreatin, respectively.

The NTS control sample had a slightly lower DH, although not statistically different ($P = 0.1085$), by trypsin than the original soy white flour. The initial rate of hydrolysis of the original soy white flour was higher than the NTS control and was similar to casein, autoclaved soy, and NTS-treated soy (data not shown). Hill et al. (*34*) investigated the rapid release of hydrogen ions of unheated soy flour upon the addition of trypsin to the reaction mixture. They suggested that adjusting the enzyme pH to assay pH would eliminate the rapid release of hydrogen ions upon the addition of trypsin to unheated soy flour. We propose that there is a time delay for the inhibition of trypsin by inhibitors during initial hydrolysis and that mild heating causes a decrease in the time delay, resulting in the slightly lower DH of the NTS control compared to the original soy white flour. In our experiment, trypsin was added to each assay in the same volume, and therefore, it would have the same effect on initial rates of hydrolysis. Additionally, the DH by pancreatin of the original and NTS control samples were similar. Pancreatin contains not only trypsin, but also other proteases; therefore, any time delay in the interaction between trypsin inhibitors and trypsin would only minimally affect DH by pancreatin.

Upon the basis of the casein hydrolysis by both trypsin and pancreatin (**Figure 2**), the pancreatin may contain less protease activity at our assay conditions than trypsin. Hydrolysis by

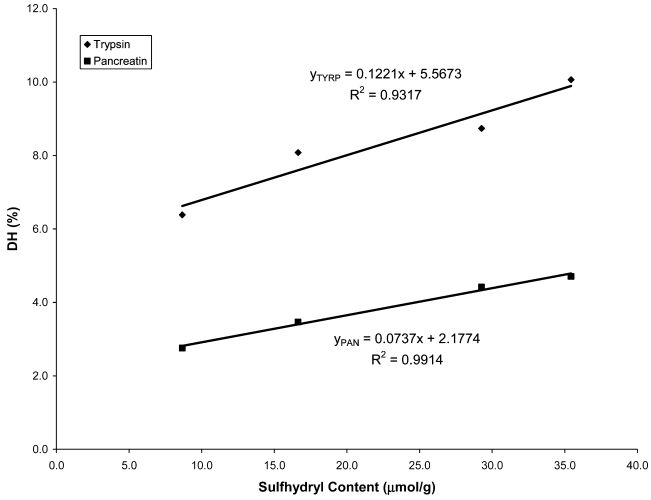

Figure 3. Correlation between sulfhydryl content of NTS control and NTS-treated samples and degree of hydrolysis by trypsin and pancreatin.

pancreatin was measured for only 30 min instead of 1 h, since the enzyme was buffered to assay pH of 8.5. However, twice as much pancreatin was used for hydrolysis than trypsin; therefore, the protease activity can be estimated between the two enzymes.

Effect of Disulfide Bond Reduction on Soy Protein Hydrolysis. Figure 3 shows the effect of the degree of disulfide reduction of soy flour on the DH by trypsin and pancreatin. Both The DH by trypsin and pancreatin are strongly correlated to the degree of disulfide reduction of soy flour by NTS. The higher slope of the linear regression for trypsin hydrolysis reflects the effect of treatment with thioredoxin on both total protein digestibility and trypsin inhibitor inactivation. Trypsin hydrolysis of soy flour is both a measurement of trypsin inhibitory activity and protein digestibility. Although pancreatin contains trypsin, the effect of trypsin inhibitors on pancreatin hydrolysis is small, and thus, the DH by pancreatin can be attributed to the increase in digestibility of total protein. The difference in the slopes of **Figure 3** for DH by trypsin and pancreatin can be used as an estimate of the effect disulfide reduction by NTS on digestibility by inactivation of trypsin inhibitors.

Rothenbuhler and Kinsella (*35*) suggested that the pH stat method can provide a sensitive method for trypsin inhibition detection in food proteins. The pH stat method is an effective method of estimating the digestibility of proteins. It has been used to determine the effects of heat treatment on initial rates of trypsin proteolysis of soy protein, to determine the effects of chemical treatment on trypsin proteolysis of glycinin and its subunits, to assess residual trypsin inhibitory activity after heating, and to evaluate the trypsin digestion of soy treated with *N*-acetyl-L-cysteine (*35, 34, 36–38*). The effect of protein concentration, enzyme−substrate ratio, pH, ionic strength, interfering food components, and protein modification has also been evaluated (*35*). Many researchers evaluated the digestibility of proteins using the pH stat method using the initial rate of hydrolysis. We evaluated soy protein by its degree of hydrolysis over a defined time period with the viewpoint that it was more reflective of in vivo digestion.

There are many potentially allergenic proteins in soybeans, including glycinin and KTI (*39*). NTS treatment of β-lactoglobulin and wheat proteins has been shown to decrease their allergenicity in highly allergenic dogs (*27, 28*). Although the allergenicity of proteins in NTS-treated soy flour was not evaluated, it is probable that allergenic disulfide bond-containing proteins in NTS-treated soy flour have a reduced potential to cause an allergic response in susceptible individuals. Additionally, the effect of NTS treatment on the anticarcinogenic effects of BBI is unknown.

Evaluation of NTS Treatment in an Aqueous Processing Scheme. NTS-reduced soy flour was nearly as digestible by trypsin as the autoclaved soy flour, but less digestible by pancreatin than either the autoclaved soy flour or casein. On the basis of these results, NTS-reduced soy is as effective or nearly as effective as heat treatment in inactivating trypsin inhibitors, but may not be as effective as heat treatment in increasing the overall digestibility of soy proteins. However, treatment with NTS does not cause negative heat-induced effects, such as amino acid destruction and Maillard browning reactions.

The cost of treating soy with NTS is prohibitive, despite its potential in improving its nutritive value with minimal heat. Using the reagents in our experiment, the cost of treating one gram of soy flour with NTS was ~$660. The cost of reagents purchased in larger quantities will be less expensive than our experiment, but nonetheless, it will still be a considerable amount. For larger scale treatment with NTS, thioredoxin and thioredoxin reductase may be produced using a yeast or bacterial overexpression system and purified. In addition, an enzymatic NADPH regeneration system could be utilized to reduce cost. Several researchers have developed a genetically modified thioredoxin reductase with specificity for the cofactor NADH instead of NADPH (*40*). The cost of NADH is significantly less than NADPH, and a NADH regeneration system could be utilized, as well. Our initial intent with the enzymatic reduction experiment was to provide evidence for effectiveness of such protein modification for aqueous processing of soybeans. Currently, we are evaluating the effect of chemical reduction of disulfide bonds and its effect on digestibility as measured by the pH stat method as well as the ability of the pH stat method to predict in vivo digestibility.

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