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ORIGINAL PAPER

Increased In Vitro and In Vivo Digestibility of Soy Proteins by Chemical Modification of Disulfide Bonds

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Abstract To improve protein digestibility of aqueously extracted soy proteins, an effective chemical treatment under mild conditions is needed. Soy proteins, including storage protein glycinin and antinutritional factors such as trypsin inhibitors, are rich in disulfide bonds. Reduction of these disulfide bonds by incubating soy proteins with sodium sulfite and sodium metabisulfite at 55 °C showed no net increase of free sulfhydryl groups after dialysis to remove the residual reducing agent. However, the in vitro digestibility measured by trypsin hydrolysis using the pH-Stat method was significantly increased. Sodium metabisulfite (SMBS) was more effective in increasing in vitro digestibility than sodium sulfite at the same molar concentration. The digestibility of soy protein treated by 0.5 mmol SMBS/g soy flour at 55 °C was more than doubled compared to that of the control without reduction treatment. Large-scale testing of soy proteins treated with SMBS for an in vivo animal feeding study showed similar in vitro digestibility by trypsin, e.g., the degree of hydrolysis of the treated sample was 8.5% compared to 1.6% of the control. These soy proteins were further evaluated using a chick growth model. The protein efficiency ratio (PER) increased by 57% when the chicks fed SMBS-treated soy were compared to the chicks fed raw soy flour. SMBS-fed chicks did not display any pancreatic hypertrophy compared to those fed with raw soy control. These results indicate that there is great potential to use safe chemicals and mild temperature to inactivate the anti-nutritional factors in soybeans and thus improve digestibility of soy proteins that are extracted with low-temperature aqueous process.

Keywords Anti-nutritional factors · Digestibility · Disulfide bond · Pancreatin · pH-Stat · Sodium metabisulfite · Sodium sulfite · Soy protein · Trypsin

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Introduction

In aqueous-extraction processes (AEP), water is used instead of conventional organic solvent to extract oil from oilseeds [1]. The tightening of emission standards in recent years by the U.S. Environmental Protection Agency has rekindled efforts to develop AEP technology for soybean processing [2]. The advantages of such a process include no hazardous solvent use, new oil and protein products with unique properties, and smaller but flexible production scale. Several technical hurdles have to be overcome before this technology can become economically viable. One is the utilization of the protein fraction after oil is removed by aqueous process. The proteins in this fraction are subjected to no or low heat treatment, thus the majority of the antinutritional factors remain native and active. Although the aqueous protein fraction can be an excellent starting material for the production of new value-added soy protein products for human consumption, its main use is

expected to be livestock feed. Further processing to increase its digestibility at a reasonable cost is vital to the success of the aqueous oil extraction process.

High temperature heating is the most common method to treat soy proteins in animal feed to denature the antinutritional factors, mainly the trypsin inhibitors, to improve digestibility. However, heat treatment alone is not sufficient to fully inactivate the antinutritional factors. For example, 20% of Kunitz trypsin inhibitor activity remained in the soy flour after heating at 120 °C for 30 min although all the Bowman–Birk trypsin inhibitor was inactivated [3]. Severe heating may also compromise the bioavailability of the sensitive amino acids such as cysteine, lysine, and arginine. Since soy proteins remain in the water slurry after oil is extracted in AEP, the challenge remains as to how the native proteins can be treated without conventional drying, such as spray-drying or concentration then drum-drying, which is expected to be prohibitive for small-scale commercial feed production. One possibility for feed use is based on the rationale that if the digestibility of the soy protein fraction can be improved without dewatering or further heat treatment, the liquid soy protein slurry can be used directly to feed animals. This is probably the most practical means to utilize the protein fraction from AEP process. In this study, an aqueous slurry of native soybean proteins made from white flakes was used as a model to study the effects of chemical treatment on soy protein digestibility since the slurry contains soybean protein components similar to that of AEP.

The major soy storage protein glycinin and trypsin inhibitors such as Kunitz trypsin inhibitor (KTI) and Bowman–Birk trypsin inhibitor (BBTI) are rich in disulfide bonds. Glycinin has both intra- and intermolecular disulfide bonds. Completely reduced glycinin has 41 sulfhydryl groups/mol protein while the value for native glycinin is about 1.7 [4]. The total number of disulfide bonds in glycinin is estimated to be 20/mol protein [5]. KTI contains two intramolecular disulfide bonds [6], while BBTI has seven [7]. Such covalent bonds are crucial for maintaining the native structural conformation and thus activity of the proteins. Disruption of the disulfide bonds, especially through reduction, may change the three-dimensional structure of the proteins, leading to the loss of bioactivity (such as trypsin inhibition activity or potential allergenicity) and improved protein digestibility [8].

Friedman et al. showed that the inactivation of trypsin inhibitors was enhanced dramatically by thiol reduction [9]. For example, after heating soy flour for 1 h at 65 °C, only 20% of the trypsin inhibitor activity was destroyed, whereas after incubation at the same condition with *N*-acetyl-L-cysteine (NAC), the same soy flour lost 91% of the trypsin inhibitor activity. Other thiols such as L-cysteine, reduced glutathione, and mercaptopropionylglycine had a similar inactivation effect, and higher temperature and pH facilitated the inactivation of soy trypsin inhibitors. The authors [9] believed that thiol-disulfide interchange triggered the rearrangement of the original disulfide bonds in soy trypsin inhibitors, altering the native structural conformation that is critical for the binding and inhibition of trypsin or chymotrypsin. An animal feeding study using rats demonstrated that treating soy flours at 45 °C in the presence of cysteine and NAC increased the protein efficiency ratio (PER) from 0.95 to 2.01 and 2.20, respectively [10]. The authors also examined the effect of non-thiol-disulfide cleaving reagents and showed that incubation of soy flour with sodium sulfite at 75 °C lowered the trypsin inhibitor level to zero.

Although many reducing agents can effectively cleave the disulfide bonds in soy proteins, most of them are not practically useful to treat soy proteins for animal feed or potentially for human food applications due to their toxicity and/or cost. For safety and feasibility, sulfites are probably the most practical reducing agent. Sulfites have been used in numerous food products as conditioners, antioxidants, antimicrobial agents, and/or color stabilizers. FDA does not regulate their application limit in food but requires label declaration if the residual sulfite level is 10 ppm or higher in food. There is no regulation on sulfite application in livestock feeds [11]. Therefore, sodium sulfite and sodium metabisulfite were selected to treat soy proteins for improving in vitro and in vivo digestibility in our study.

A few studies have been published on the effectiveness of thiol and non-thiol reducing agents on inactivation of soy antinutritional factors. One investigation showed that 40 min was needed to achieve maximum chicken feed performance when full-fat soybean meal was autoclaved at 121 °C, but only half of the time (20 min) was needed at the same temperature when sodium metabisulfite (SMBS) was added [8]. There is little research on improving overall digestibility of soy proteins by sulfite or SMBS under ambient or mild heating conditions. Such conditions are typical for soybean aqueous processing, thus, are desirable for further protein treatment to improve quality.

The hypothesis for this study was that the digestibility of soy proteins will increase after chemical treatments by sulfite and metabisulfite under milder heating conditions than those used in previously published results. The ultimate goal for this research was to identify a practical method to treat the native protein fraction from AEP process to improve its feed quality. Ideally, such treatment will be a simple mixing of a chemical with the protein stream before the protein fraction is fed to livestock animals without further dewatering or drying.

Experimental Procedures

Materials

A soy white flour with protein dispersibility index (PDI) $\geq$ 85 from hexane-extraction was obtained from Cargill (Minneapolis, MN). It was used as a model for aqueous-extracted proteins since the hexane-extraction process itself causes little heat damage to the soy proteins similarly to the aqueous-extraction process. Bovine serum albumin (BSA), cysteine, 5,5′-dithiobis (2-nitrobenzoate) (DTNB), dithiothreitol (DTT), guanidine thiocyanate, SMBS, and trypsin were purchased from Sigma–Aldrich (St. Louis, MO). All other reagents and supplies were purchased from Fisher Scientific (Waltham, MA).

Sulfhydryl Quantification and Protein Analysis

The sulfhydryl content of soy white flour was determined using a modified procedure initially described by Ellman [12] and Robyt et al. [13]. Detailed procedure and sample pretreatment were discussed in our previous publication [14]. The crude protein content of soy white flour and SMBS-treated soy flour for animal feeding was determined by the micro Kjeldahl method with protein conversion factor of 6.25 [15].

In Vitro Digestibility Assay by Trypsin

The in vitro digestibility of soy protein samples was measured as the degree of protein hydrolysis (DH) by trypsin using a 718 Stat Titrino meter (Metrohm, Herisau, Switzerland). DH is defined as the percentage of peptide bonds cleaved by an enzyme under specific conditions. It is used to quantify the susceptibility of soy proteins to digestive proteases. The measurement conditions were described in our earlier publication [14]. The DH was calculated using the following formula:

$$\mathrm{DH}\,(\%) = B \times N_{\mathrm{B}} \times \frac{1}{\alpha} \times \frac{1}{M_{\mathrm{P}}} \times \frac{1}{h_{\mathrm{tot}}} \times 100$$

where B is the base consumption in milliliters, N_{B} is the normality of the base, α is the average dissociation of the α-amino group, M_{P} is the mass of the protein in grams, 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, for soy protein [16].

Effect of Sulfite and Metabisulfite Treatments on Sulfhydryl Content and Digestibility of Soy Proteins

Soy white flour (2 g) was dispersed in 20 mL of 10 mM phosphate-buffered 0.85% saline (pH 7.5) and incubated at ambient temperature (25 °C) for 1 h (as w/o heat treatment) or heated for 1 h at 55 °C (as with heat treatment). This temperature was chosen according to the mild heating used in our current soybean AEP process. These treatments were used as controls because no reducing agents were used. Two reducing agents, sodium sulfite and SMBS, were tested, each at two addition levels (0.2 and 1.0 mmol/2 g white flour) with heat (incubation at 55 °C). A treatment with 1.0 mmol of sodium sulfite without incubation at 55 °C was also used. After incubation and reaction, all samples were dialyzed against water for 3 days and lyophilized. All treatments were repeated three times. The free sulfhydryl content and digestibility by trypsin were measured as aforementioned.

Effect of Temperature, Heating Time, and Reducing Agent SMBS on the In Vitro Digestibility of Soy Proteins by Trypsin

Soy white flour (1 g) was dispersed in 20 mL 0.1 M phosphate buffer (pH 7.0) and either not heated, heated at 80 °C for 15 min, or heated at 100 °C for 15 or 30 min. Samples contained either 0 or 5% SMBS (equivalent to 0.5 mmol/2 g flour). All treatments were repeated three times. This experiment was designed to test the susceptibility of trypsin inhibitor to more severe heating with the presence of SMBS.

Preparation of Soy Protein Samples for In Vivo Feeding Test

Two types of soy proteins were prepared; one was the control (without SMBS treatment) for which the white soy flour (1.8 kg) was dispersed in 9 L of 10 mM phosphate-buffered 0.85% saline (pH 7.5) and incubated for 1 h at 55 °C. The other was treated with the addition of 0.9 mol SMBS (equivalent to 1 mmol/2 g soy flour) under the same conditions. All treated samples were dialyzed against water for 3 days and lyophilized. In addition, a soy flour sample was autoclaved at 121 °C for 40 min to represent extreme heat treatment for comparison with the SMBS-treated soy flour for in vitro digestibility by trypsin.

In Vivo Digestibility by Animal Feeding Trial

To determine the digestibility and bioavailability of the treated proteins, 5-day-old chicks were used to determine the protein efficiency ratio (PER) under formulated diets with low protein content. PER was calculated as described by Trevino et al. [17] as:

$$\mathrm{PER} = (\text{Body weight gain, g})/(\text{Protein consumed, g})$$

Forty-eight five-day-old male chicks (Ross × Ross 308) were weighed and blocked on initial body weight into 16

Table 1 The 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
Corn	51.77	69.02	75.22	74.64
Commercial toasted soy flour	37.11	22.49	–	–
Raw soy flour (untreated)	–	–	17.18	–
SMBS soy flour	–	–	–	17.67
Soybean oil	6.69	4.05	3.12	3.21
Di-calcium phosphate	1.98	2.07	2.10	2.10
Limestone	1.15	1.22	1.24	1.24
Vitamin mix[a]	0.50	0.50	0.50	0.50
Mineral mix	0.10	0.10	0.10	0.10
Salt	0.40	0.40	0.40	0.40
DL-Methionine	0.24	0.10	0.09	0.09
Selenium	0.05	0.05	0.05	0.05
Total	100.00	100.00	100.00	100.00
Calculated nutrient content				
Metabolizable energy (lcal/kg)	3,200.00	3,200.00	3,200.00	3,200.00
Crude fat (%)	9.88	6.64	6.64	6.72
Crude protein (%)	23.00	17.25	17.25	17.25

[a] Vitamin mix donated by DSM Nutritional Products, Ames, IA 50010

pens consisting of 4 pens per treatments (3 birds/pen). The treatments consisted of (1) high protein control (CONT): 23% crude protein (CP) diet containing commercial toasted soy flour, (2) low protein control (LPC): 17.25% CP diet containing commercial toasted soy flour, (3) low protein soy control (LPSC): 17.25% CP diet containing untreated soy flour, and (4) low protein SMBS soy (SMBS): 17.25% CP diet containing SMBS-treated soy flour. All diets were formulated (Table 1) to meet or exceed NRC (1998) standards for vitamins and minerals, and the low protein diets were 75% of the NRC CP recommendations [18]. The diets were fed for 10 days; pen body weights were measured at the start and end of this period, and feed intake was recorded daily. At the end of the 10 days, all birds were euthanized by carbon dioxide asphyxiation and their pancreases removed and weighed to assess pancreatic hypertrophy. All aspects of this research protocol were approved by the Iowa State University Animal Care and Use Committee.

Statistical Analysis

Statistical analyses were performed using SAS JMP [19]. Means were compared and considered different when $P \leq 0.05$. Growth performance, PER, and pancreas weights were subjected to ANOVA by the PROC Mix procedure in SAS (Cary, NC), with the experimental unit being pen of three male chicks.

Results and Discussion

Effect of Sulfite and Metabisulfite Treatments on Sulfhydryl Content and In Vitro Digestibility of Soy Proteins

Contrary to our expectation that the proteins would have increased sulfhydryl content after treating with reducing agents, all treatments had lower sulfhydryl content compared to the control, which was without heat treatment (Fig. 1). This is probably due to the reoxidation of sulfhydryl groups. The newly produced sulfhydryl groups from the cleavage of disulfide bonds by the reducing agents might have been reoxidized during the 3-day dialysis process to remove the excess chemicals. Wolf [4] had a similar observation that when dialysis was used to remove the excess reductants, the resulting sulfhydryl levels in glycinin were unusually low.

The in vitro digestibility test by trypsin showed all the treated samples had higher digestibility compared to the control (Fig. 2). Mild heat (55 °C) alone slightly increased the digestibility, probably due to a partial inactivation of trypsin inhibitors. More sodium sulfite or SMBS addition led to higher digestibility. At the same molar concentration, SMBS was more effective than sodium sulfite in increasing digestibility. This is because SMBS can produce two sulfite ions while sodium sulfite can only produce one. After addition of 1 mmol sulfite, the digestibility of the unheated

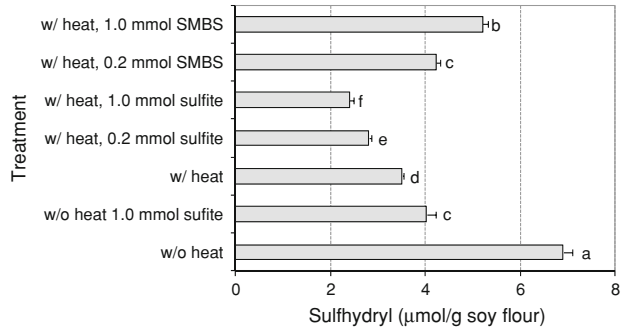

Fig. 1 Effect of mild heat (55 °C) and reducing agents on sulfhydryl content of soy white flour. *SMBS* Sodium metabisulfite. *Different letters* represent significant differences ($P \leq 0.05$)

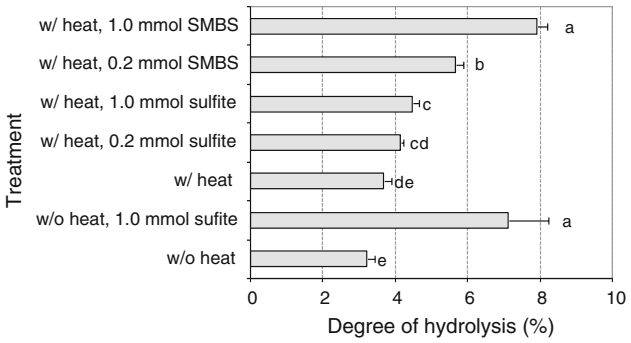

Fig. 2 Effect of mild heat (55 °C) and reducing agents on trypsin hydrolysis of soy white flour. The amounts of sulfite and SMBS are shown in mmol per 2 g soy flour. *SMBS* Sodium metabisulfite. *Different letters* represent significant differences ($P \leq 0.05$)

protein increased more than expected. The reason for this is unknown, and it will be examined further in our future experiments. This is likely a mistake because similar treatment with SMBS at 25 °C as described below did not show any increased digestibility.

These data imply that the digestibility of chemically reduced soy protein may not directly correspond to the detectable free sulfhydryl content. This may be because the newly generated sulfhydryl groups may have formed new disulfide bonds with the net result of disulfide bond rearrangement [4, 20]. Although the exact fate of the newly produced free sulfhydryl groups is unknown, chemical reduction by sulfite and SMBS did alter the soy protein structure as shown by the increased susceptibility to the digestive enzymes.

Although the reductions by sodium sulfite and SMBS are all through the production of sulfite ions in solution, the two chemicals have slightly different chemical properties, for example, SMBS sulfite acts as a weak acid while sodium sulfite acts a weak base.

Table 2 Effect of mild heat and reducing agent (SMBS) on in vitro digestibility (degree of hydrolysis, DH) by trypsin of the soy proteins for animal feeding trial

Sample	DH (%)
Original	1.2d
55 °C, 1-h, without SMBS	1.6c
55 °C, 1-h, with SMBS	8.5a
Autoclaved (121 °C, 40 min)	7.9b

$N = 3$. *Different letters* represent significant differences ($P \leq 0.05$)

$$Na_2S_2O_5 + H_2O \longrightarrow 2Na^+ + SO_3^{2-} + HSO_3^- + H^+$$

$$2Na_2SO_3 + H_2O \longrightarrow 4Na^+ + SO_3^{2-} + HSO_3^- + OH^-$$

The optimum pH for cleavage of disulfide bonds is 7 [21], however, the buffering capacity during the treatments may not have been enough to maintain the pH due to the nature of sodium sulfite and SMBS, giving another variable to the treatment.

The treated sample for the animal feeding trial showed similar in vitro digestibility results (Table 2). The in vitro digestibility by trypsin after SMBS treatment at 55 °C was increased to a value even higher than that of the autoclaved sample. Sulfite ion modified both storage proteins and trypsin inhibitors that contain disulfide bonds, and it is difficult to separate the effect on trypsin inhibitors from that of the storage proteins. It is likely that the in vitro digestibility test using pH-Stat method is highly sensitive to the inhibition assay for trypsin inhibitors [8, 20, 22].

Effect of Temperature, Heating Time, and SMBS on In Vitro Digestibility by Trypsin

Treatment of soy flour at 80 °C for 15 min with 5% SMBS resulted in a much higher degree of protein hydrolysis than treatment of soy flour under the same conditions but without SMBS (Fig. 3). The soy sample treated at 100 °C had the highest in vitro digestibility. Heating time (within 30 min) or SMBS addition at this high temperature had no significant effect on digestibility. Heating alone (without SMBS) had a profound effect on the digestibility of soy proteins by trypsin. The digestibility of soy protein heated at 100 °C alone for 15 min was more than three times higher than the digestibility of soy protein heated at 80 °C alone for 15 min. This demonstrated that severe heating is needed to inactivate the soybean trypsin inhibitors. Adding SMBS could achieve the same degree of improved digestibility when soy protein was heated at the lower temperature, i.e., 80 °C. Digestibility of unheated soy flour with 5% SMBS was not different from the unheated soy flour without SMBS, implying that mild heating is necessary for the reducing agent to work.

The results of this study are similar to the work by Friedman et al. [10] who claimed that heating soy flour with sodium sulfite at 75 °C lowered trypsin inhibitor to zero. In our study, the autoclaved (121 °C and 40 min) soybean sample had a protein hydrolysis of 7.9% (Table 2) compared to an average of about 8.0% (Fig. 3) for the 80–100 °C treated samples. However, our practical goal was to treat the proteins at even lower temperature. Therefore, twice the concentration of SMBS was used for the treatment at 55 °C to produce material for the feeding trial.

In Vivo Digestibility Evaluated by Animal Feeding Test

Chicks fed with treated soy protein (SMBS) and control chicks (LPSC, untreated) both showed poorer performance compared to the LPC and CONT chicks (Table 3). However, chicks fed the SMBS diet had higher gain:feed ratio and PER than chicks fed the control (LPSC), which contained raw soy flour. The increases were 27 and 57%, respectively, for gain:feed and PER (Table 3). The pancreas from chicks fed SMBS had significantly lower weight than that from chicks fed raw soy flour (LPSC), and the SMBS-fed chicks had similar pancreas weight as those fed commercial toasted soy flour controls (CONT and LPC). Diets containing trypsin inhibitors, such as raw soybean diets, are associated with elevations in pancreatic protease, trypsin, chymotrypsin, and pancreatic enlargement. These data suggest that SMBS treatment increased the digestibility of raw soy flour by modifying the disulfide bonds in the soy proteins, including trypsin inhibitors.

Similar results were observed when feeding chicks with SMBS-treated full-fat soybean meal [8], however, that particular study was conducted on proteins treated with SMBS under autoclaving conditions. Another feeding trial showed that PER improved from 2.11 for rats fed soy flour treated with heat at 75 °C alone to 2.49 (38% increase) for rats fed the flour treated with sodium sulfite at the same temperature [23]. Sessa and Nelsen [24] showed that trypsin inhibitors in model systems under 65–90 °C with SMBS treatment can be reduced by 40–85% compared to the original activities. However, our proteins were treated at 55 °C. Chicks fed SMBS-treated soy flour in our study still had lower PER than the controls with roasted soy flour,

Table 3 Effect of diets containing soy protein treated with reducing agent (SMBS) on feed intake, growth performance, protein efficiency ratio, and pancreas weight in chicks

	CONT	LPC	LPSC	SMBS
Average daily feed intake (g/dl)	54.9a	53.4a	37.9b	42.8b
Average daily gain (g/dl)	39.3a	27.2b	12.7d	18.2c
Gain:feed	0.72a	0.51b	0.33d	0.42c
Protein efficiency ratio (PER)	2.2a	1.5b	0.7d	1.1c
Pancreas weight (g/100 g BW)	0.43b	0.45b	0.76a	0.45b

$N = 4$ pens of three chicks per pen

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

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

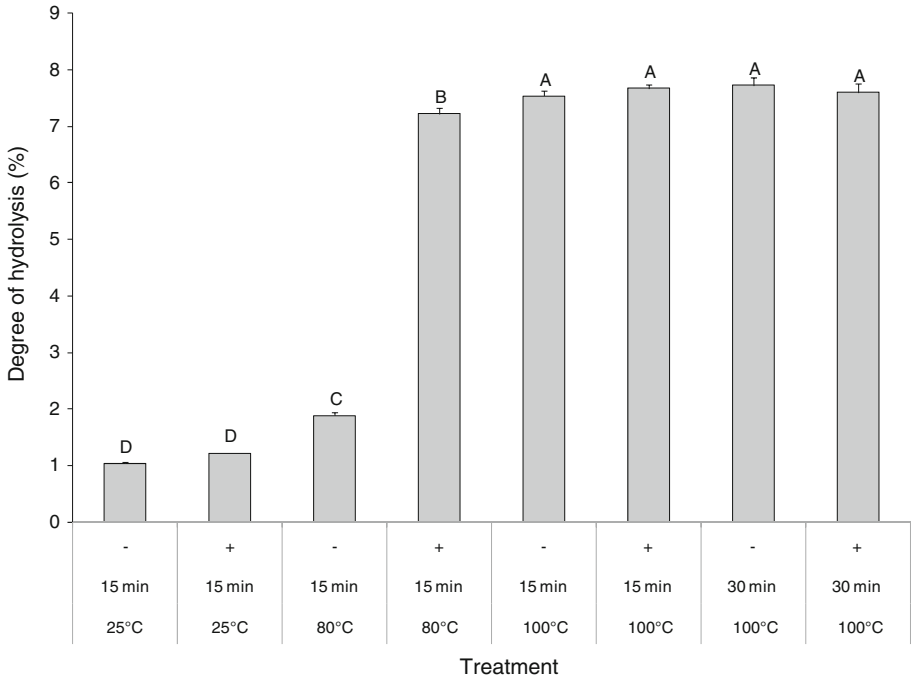

Fig. 3 Effect of temperature, heating time, and either 0% (−) or 5% (+) SMBS addition on degree of hydrolysis by trypsin as measured by pH-Stat method. *Different letters* represent significant differences ($P \leq 0.05$)

indicating there is still potential to further improve the in vivo digestibility of the chemically treated soy flour. Since our treatment was done at 55 °C for 1 h at 0.5 mmol SMBS/g soy flour, it is possible that higher (such as 80 °C) but not extreme temperature, longer treatment time, or higher SMBS dosage may further increase the feeding performance of soy flour to a level of that of toasted sample. It should be noted that the observation that SMBS-treated soy flour had better in vitro digestibility than the autoclaved soy flour while the in vivo feeding test showed less effectiveness indicates that the in vitro digestibility test may only serve as a supplement test to the animal feeding trial.

Conclusion

In summary, metabisulfite and sulfite under mild heating (55 °C) conditions increased in vitro and in vivo digestibilities of soy proteins compared to the untreated sample. The improvement may be due to the rearrangement of disulfide bonds through reduction and reoxidation reactions. The results of this study indicate that it is possible to use safe chemicals and lower temperature than previously reported to improve the nutritional properties of the aqueous-processed and less heat-damaged soybeans. Further optimization of treatment conditions for the best feeding performance is necessary and possible to meet the AEP requirements.

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