Comparison of a Commercial Soybean Cultivar and an Isoline Lacking the Kunitz Trypsin Inhibitor: Composition, Nutritional Value, and Effects of Heating

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The content and heat stability of protease inhibitors of a standard cultivar (Williams 82) and an isoline (L81-4590) lacking the Kunitz trypsin inhibitor (KTI) were measured by using enzyme inhibition and enzyme-linked immunosorbent assays (ELISA). The KTI content of the isoline was less than 0.2% compared to Williams 82, with the exact content depending on the extent of cross-pollination of the soybeans. Steam heating of the isoline flour (121 °C, 20 min) resulted in a near-zero level of trypsin inhibitory activity, while 20% remained in the Williams 82 sample. The raw soy flour prepared from the isoline was nutritionally superior to the raw flour prepared from the standard variety, as measured by PER and pancreatic weights. The increased PER was likely due to the lower level of trypsin inhibitory activity in the isoline. Steam heating the flours for up to 30 min at 121 °C progressively increased the PER for both strains. Screening of several accessions from the USDA Soybean Germplasm Collection showed variation in the content of trypsin inhibitor, sulfur amino acids, and lectins, indicating that further screening studies could lead to the discovery of soybeans which yield flour that is safe and nutritious, with minimal heating.

INTRODUCTION

Adverse effects following short- and long-term ingestion of raw soybean meal by mammals and birds have been attributed to the presence of soybean protease inhibitors and lectins (Belitz and Weder, 1990; Grant, 1989; Gumbmann et al., 1986; Liener, 1989). These inhibitors include the Kunitz trypsin inhibitor (KTI) (Rackis et al., 1986), the double-headed Bowman–Birk inhibitor (BBI) of trypsin and chymotrypsin (Birk, 1985), a glycine-rich trypsin inhibitor structurally unrelated to either KTI or BBI, and other minor inhibitors and proteolytically modified forms (Tan-Wilson et al., 1987). To minimize possible human health hazards and to improve the nutritional quality of soy foods, inhibitors are generally inactivated by heat treatment during food processing or removed by fractionation (Wolf and Cowan, 1975). Most commercially heated soy flours retain 5–20% of the original trypsin and chymotrypsin inhibitory activity (Rackis et al., 1986). The more protracted heating required to destroy all inhibitor activity would damage the nutritive value of soy proteins.

Another approach to producing low trypsin and chymotrypsin inhibitor foods is to develop soybean cultivars lacking major inhibitors. Orf et al. (1977) used polyacrylamide gel electrophoresis under nondenaturing conditions to determine the phenotype of soybean seeds with respect to KTI. Hymowitz (1986) discusses the genetics of KTI expression in soybeans and the development of KTI-null isolines on three genetic backgrounds. These isolines lack a functioning *Ti* allele, and are denoted *ti*/*ti*.

Earlier studies indicated that the KTI-lacking soybeans had lower trypsin inhibitor levels (Hymowitz, 1986) and supported the growth of pigs better than isolines expressing KTI (Cook et al., 1988). In this study, we further analyzed the protease inhibitor and lectin content of normal and KTI-lacking isolines and studied the nutritional properties of soy flour derived from these strains. Since soybeans contain multiple protease inhibitors and because measurements of enzyme inhibition can be inaccurate at low levels, we used ELISAs specific for KTI and BBI (Brandon et al., 1987, 1988, 1989, 1990, 1991; Oste et al., 1990) to characterize the inhibitor content in raw and heated flours. For comparison we also evaluated the sulfur amino acid, inhibitor, and lectin content of soybean flours derived from seeds in the USDA Soybean Germplasm Collection.

MATERIALS AND METHODS

Materials. Trypsin, chymotrypsin, N^{α}-tosylarginine methyl ester (TAME), *N*-benzoyl-L-tyrosine ethyl ester (BTEE), other reagents, and soybean Kunitz trypsin inhibitor were obtained from Sigma Chemical Co., St. Louis, MO. The Bowman–Birk soybean inhibitor was kindly provided by Prof. Y. Birk (Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel).

Commercial soybeans (Williams 82) and an isoline with highly reduced Kunitz trypsin inhibitor activity (L81-4590) were grown by Illinois Foundation Seeds, Inc., Champaign, IL, under the sponsorship of the Department of Agronomy, University of Illinois. The experimental line was grown in the field or isolated in a greenhouse to minimize cross-pollination.

Sample Preparation. Soybean seeds were ground in a Udy mill and sieved through a No. 60 mesh sieve. The meal was then defatted by extraction with ether in a Soxhlet apparatus for 16 h (AOAC, 1980) and air-dried. Samples (600 g) in porcelain dishes covered with aluminum foil were heated at 121 °C in an autoclave for 10, 20, or 30 min.

Soybean samples from the USDA Soybean Germplasm Collection were selected for analysis of trypsin and chymotrypsin inhibitor, lectin, and amino acid content. Samples were milled but not defatted. Soybean meals were extracted by stirring the meal for 1 h in 0.5 M Tris-HCl buffer, pH 8.5 (500 mg of meal/15 mL).

Trypsin Assays. The following conditions were used: buffer, 46 mM Tris-HCl containing 11.5 mM $CaCl_2$, pH 8.1; substrate, 10 mM TAME (37.9 mg/10 mL of H_2O); enzyme, 1 mg/mL, 1 mM HCl. The enzyme solution was diluted to 10–20 μg/mL. In the absence of inhibitor, 2.6 mL of buffer and 0.3 mL of TAME

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were added to a 3-mL cuvette followed by 0.1 mL of diluted enzyme solution. The absorbance was then recorded at 247 nm (A_{247}) for 3 min on a Perkin-Elmer Lambda 6 spectrophotometer. The increase in absorbance was then determined from the initial linear portion of the curve. In the presence of inhibitors, 2.6 mL of buffer, 0.1 mL of enzyme solution, and 20 μL of inhibitor solution (prepared to give 50% inhibition) were preincubated for 6 min. The reaction was started by adding 0.3 mL of TAME, and A_{247} was recorded for 3 min. Values were based on sample dilutions yielding 40–60% inhibition (*Worthington Biochemical Products*, 1982):

$$\text{units/mg} = (\Delta A_{247}/\text{min} \times 1000 \times 3)/540 \times \text{mg of enzyme used}$$

Chymotrypsin Assays. The following conditions were used: buffer, 80 mM Tris-HCl containing 100 mM $CaCl_2$, pH 7.8; substrate, 1.07 mM BTEE (8.4 mg/25 mL of 50% methanol); enzyme, 1 mg/mL, 1 mM HCl. The enzyme solution was diluted to a concentration of 10–20 μg/mL. In the absence of inhibitor, 1.5 mL of buffer, 1.4 mL of BTEE, and 0.1 mL of enzyme solution were added, and the increase in absorbance at 256 nm (A_{256}) was recorded for 3 min. The ΔA_{256}/minute was then calculated from the initial linear portion of the curve. In the presence of inhibitor, 2.6 mL of buffer, 0.1 mL of enzyme solution, and 20 μL of inhibitor were incubated for 6 min before 1.4 mL of BTEE was added and the absorbance was recorded as above. Values were based on sample dilutions yielding 40–60% inhibition (*Worthington Biochemical Products*, 1982):

$$\text{units/mg} = (\Delta A_{256}/\text{min} \times 1000 \times 3)/964 \times \text{mg of enzyme used}$$

Buffer plus substrate served as controls for all measurements (Friedman and Gumbmann, 1986; Friedman et al., 1984).

A trypsin unit (TU) is defined as the amount of trypsin that catalyzes the hydrolysis of 1 μmol of substrate/min. A trypsin inhibitor unit (TIU) is the reduction in activity of trypsin by 1 TU.

One chymotrypsin unit (CU) is defined as the amount of chymotrypsin that catalyzes the hydrolysis of 1 μmol of substrate/min. A chymotrypsin inhibitor unit (CIU) is the reduction in activity of chymotrypsin by 1 CU.

Pure KTI or BBI was used as a standard with each assay of the soy flours. The calculated values, which are averages from two to three separate determinations, are based on the individual control values.

ELISA Methods. Inhibition ELISA for KTI was performed by using monoclonal antibodies as described by Brandon et al. (1987, 1988) or by competition ELISA with a KTI conjugate of horseradish peroxidase (HRP) as described by Brandon et al. (1988). Antibodies used (129, 142, 171, and 180) bind to four distinct epitopes of KTI (Brandon and Bates, 1988). ELISA for BBI was performed by using monoclonal antibody 238 and BBI-conjugated HRP as described previously (Brandon et al., 1989).

Concentrations were determined by reference to a standard curve or by computation using samples spiked with an internal standard as

$$\frac{\text{KTI (sample)} + \text{KTI (spike)}}{\text{KTI (sample)}} = \text{antilog}\,[I_{50}(\text{sample}) - I_{50}(\text{sample} + \text{spike})]$$

where I_{50} is the concentration of the unknown sample yielding 50% inhibition of maximal binding of labeled ligand, KTI (sample) is the concentration of KTI in the sample, and KTI (spike) is the KTI added as an internal standard. The relative concentration of two samples was computed as follows:

$$\frac{\text{KTI (sample 2)}}{\text{KTI (sample 1)}} = \text{antilog}\,[I_{50}(\text{sample 1}) - I_{50}(\text{sample 2})]$$

Hemagglutination Assay. The sample (soy flour, 150 mg) was mixed with 1.5 mL of phosphate-buffered saline, pH 7.2. Lectin was extracted by stirring for 1 h at room temperature. After extraction, the resulting slurry was immediately chilled and centrifuged at 9000*g* for 5 min in a Beckman microfuge (Beckman Instruments, Palo Alto, CA). When necessary, the extracts were diluted with isotonic phosphate buffer (0.05 M NaH_2PO_4 and 0.15 M NaCl, pH 7.2) before plating so that incipient activity would fall midrange in the plated series.

Fifty-microliter aliquots of glutaraldehyde-stabilized human group A red blood cells diluted with a buffer to 3.3% hematocrit were added to equal volumes of serially diluted extracts and a buffer blank. Agglutination was observed visually after 1 h (Liener, 1974, 1989; Wallace and Friedman, 1985).

Activity is calculated as the reciprocal of the minimum amount of soy flour required to cause agglutination of blood cells under these test conditions. This value is derived from the minimum experimental value (μg/50 mL) that produces hemagglutination. The results of four separate assays conducted on each sample were averaged.

Table I. Comparison of Amino Acid Composition of Flour from a Standard Variety of Soy (Williams 82) and of a New Experimental Strain Lacking the Kunitz Trypsin Inhibitor (L81-4590)

	Williams 82		KTI-free		
amino acid	g/100 g	g/16 g of N[d]	g/100 g	g/16 g of N[e]	FAO[f] g/16 g of N
Asp	5.19	11.16	5.67	11.42	
Thr	1.86	3.99	1.99	4.01	4.0
Ser	2.45	5.28	2.70	5.45	
Glu	8.77	18.87	9.72	19.58	
Pro	2.61	5.61	2.80	5.64	
Gly	1.93	4.15	2.09	4.22	
Ala	1.94	4.17	2.12	4.27	
Cys[a]	0.65	1.39	0.68	1.37	
Met[b]	0.65	1.39	0.66	1.32	3.5[g]
Val	2.02	4.35	2.15	4.34	5.0
Ile	2.05	4.42	2.13	4.30	4.0
Leu	3.65	7.84	3.91	7.87	7.0
Tyr	1.75	3.76	1.91	3.86	6.0[h]
Phe	2.19	4.70	2.38	4.80	
His	1.18	2.53	1.31	2.63	
Lys	2.88	6.20	3.15	6.35	5.5
Arg	3.33	7.16	3.72	7.49	
Trp[c]	0.33	0.71	0.38	0.76	
total	45.42	97.68	49.48	99.70	

[a] Determined as cysteic acid after performic acid oxidation. [b] Determined as methionine sulfone after performic acid oxidation. [c] Determined in a separate analysis after hydrolysis by barium hydroxide. [d] N (nitrogen) = 7.44. [e] N = 7.94. [f] Provisional amino acid scoring pattern for an ideal protein (FAO, 1973). [g] Cys + Met. [h] Tyr + Phe.

Amino Acid Composition. Three analyses with flour containing about 5 mg of protein (N × 6.25) were used to establish the amino acid composition of the soybean protein (Friedman et al., 1979): (a) standard hydrolysis with 6 N HCl for 24 h in evacuated sealed tubes; (b) hydrolysis with 6 N HCl after performic acid oxidation to measure half-cystine and methionine content as cysteic acid and methionine sulfone, respectively; and (c) basic hydrolysis by barium hydroxide to measure tryptophan content (Friedman and Cuq, 1988). The reproducibility of these analyses is estimated to be ±3% on the basis of past experience (Friedman et al., 1979).

Fatty Acid Composition. Fatty acids were methylated by using boron trifluoride–methanol and subsequently analyzed by GLC analysis utilizing a Varian 5050A gas chromatograph modified for capillary injection using the following conditions: A fused silica capillary column from J&W Scientific Inc., Rancho Cordova, CA; liquid phase, DB Wax + 0.25-μm thickness, 30 m × 0.245 mm; initial temperature 150 °C, final temperature 220 °C, program 2 °C/min for 20 min, total time 55 min, injector temperature 220 °C, FID 300 °C, helium carrier gas flow as 30 cm/s, split ratio 25:1. The estimated reproducibility of these analyses is ±10% (Crawford, 1989).

Animal Feeding Studies. Protein efficiency ratios (PER) were determined by the official method using weanling, male Sprague-Dawley derived rats from Simonsen Laboratories, Inc., Gilroy, CA. Each assay ran 28 days (AOAC, 1980). Food consumption and PER (weight gain/protein intake) were evaluated. Pancreata were excised at the end of the feeding period and weighed (wet weight).

Table II. Effect of Heating in an Autoclave at 121 °C on the Amino Acid Composition of a Standard Soybean Variety (Williams 82) and of an Isoline Lacking KTI (L81-4590) (Listed Values Are g/16 g of N)

	Williams 82 heated for (in min)				KTI-free heated for (in min)			
amino acid[a]	0[b]	10[c]	20[d]	30[e]	0[f]	10[g]	20[h]	30[i]
Asp	11.16	11.79	11.19	11.48	11.42	11.15	11.24	11.52
Thr	3.99	4.26	4.01	4.10	4.01	3.96	3.97	4.01
Ser	5.28	5.60	5.27	5.38	5.45	5.34	5.38	5.40
Glu	18.87	20.13	18.87	19.39	19.58	19.54	19.35	19.69
Pro	5.61	4.81	5.18	5.98	5.64	5.36	5.65	5.87
Gly	4.15	4.40	4.18	4.30	4.22	4.18	4.17	4.19
Ala	4.17	4.42	4.20	4.32	4.27	4.24	4.21	4.27
Cys	1.39	1.42	1.32	1.39	1.37	1.36	1.30	1.41
Val	4.34	4.63	4.36	4.50	4.34	4.39	4.28	4.42
Met	1.39	1.40	1.34	1.43	1.32	1.33	1.32	1.36
Ile	4.42	4.55	4.21	4.31	4.30	4.24	4.16	4.28
Leu	7.84	8.22	7.69	7.89	7.87	7.83	7.76	7.91
Tyr	3.76	4.16	3.74	3.91	3.86	3.85	3.87	3.93
Phe	4.70	5.05	4.75	4.92	4.80	4.80	4.81	4.87
His	2.53	2.68	2.54	2.62	2.63	2.57	2.55	2.62
Lys	6.20	6.67	6.19	6.37	6.35	6.27	6.04	6.26
Arg	7.16	7.67	7.11	7.43	7.49	7.43	7.26	7.53
Trp	0.71	1.06	0.69	0.42	0.76	0.87	0.98	0.858
total	97.68	102.92	96.83	100.14	99.70	98.84	98.34	100.39

[a] See footnotes a–c in previous table. [b] N = 7.44. [c] N = 7.47. [d] N = 7.66. [e] N = 7.48. [f] N = 7.94. [g] N = 7.96. [h] N = 8.09. [i] N = 7.86.

RESULTS

Amino Acid Composition. Table I compares the amino acid composition of a standard variety of defatted soy flour and of the isoline lacking KTI. This analysis is compared to the values of the scoring pattern of the essential amino acids for an ideal protein, as defined by the Food and Agricultural Organization of the United Nations (FAO, 1973). Neither strain meets the provisional requirements for the sulfur amino acids (Cys + Met). Table II compares the amino acid composition of the unheated and heated flours. There was no significant difference between unheated samples (g/16 g of N) as evidenced by the standard normal test. Linear regression showed no significant linear change in the amino acid composition between unheated and heated samples (Madansky, 1959).

ELISA Analysis of L81-4590. ELISA was performed in both the inhibition and competition formats as described by Brandon et al. (1988). Initial results using the inhibition format indicated that isoline L81-4590 expressed KTI at less than 2% of the Williams 82 isoline levels. To ascertain the nature of this low level of expression, additional samples were analyzed. These samples were grown on two experimental plots (Swine Farm and South Farm) or in a greenhouse and were analyzed by using antibody 180, with specificity for native KTI. The results, shown in Figure 1, analyzed as described under Methods and Materials, indicated that the Swine Farm samples contained 1.8% of control levels of KTI and the South Farm sample, 0.97%, compared to Williams 82 isoline. Assay of the greenhouse-grown sample at very high concentration produced 25% inhibition of antibody binding. Although the KTI level is too low to assess accurately by ELISA using this antibody, extrapolation of the steepest section of the assay curve leads to an upper estimate of the KTI content as about 3 μg/g, or less than 0.1% of the level in Williams 82 cultivar expressing KTI. Direct reading from the assay curve (82% inhibition at a log concentration value of 3.9) indicates a shift of 4.0 log units from the control (Williams 82) sample, equivalent to a 10 000-fold reduction of KTI concentration.

Antibody 180 binds to only one epitope of KTI and is highly specific for the native molecule. Therefore, to examine whether the L81-4590 experimental line expressed a truncated form of the KTI molecule with altered electrophoretic mobility, extracts of soybeans were examined

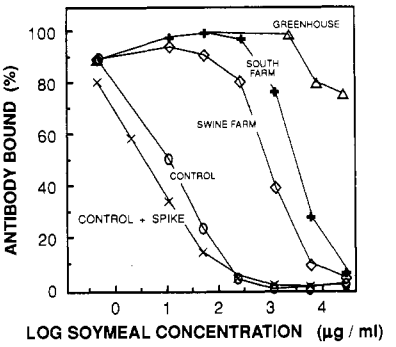

Figure 1. Analysis of KTI in soybean meal by inhibition ELISA using antibody 180. Williams 82 (control, with or without addition of an authentic KTI spike of 10 mg/g) was analyzed, along with L81-4590 grown on two different experimental plots or isolated in a greenhouse. The standard curve is given in Brandon et al. (1988).

for the presence of each of the major epitopes of KTI (Brandon and Bates, 1988) by using competition ELISA and three other antibodies. The results of these assays agreed qualitatively with the low level (<5 μg/g) detected by using antibody 180: antibody 129, 11 μg/g; antibody 142, 5 μg/g; antibody 171, <10 μg/g. Thus, the amount of protein possessing the structural or functional properties of KTI is less than 0.2% of the level found in Williams 82.

Analysis of Protease Inhibitors in Raw and Heated Flours. Table III and Figure 2 presents the protease inhibitor content derived from enzymatic measurements. Flour from L81-4590 had 54% of the trypsin inhibitory activity and 79% of the chymotrypsin inhibitory activity of flour from Williams 82. Less heat was needed to inactivate trypsin inhibitory activity in the L81-4590 experimental line than in the standard variety. In contrast, the chymotrypsin inhibitory activity of both strains was equally susceptible to inactivation by heat, reaching zero after 20 min. Table IV shows the ELISA analysis of the two major inhibitors, KTI and BBI. KTI was present at a very low, but measurable, level in L81-4590. In Williams 82, the KTI content decreased during the first 20 min of heating to about 20% of the initial value. No further reduction was observed at 30 min. BBI levels were identical in the two strains. The time course for heat inactivation of BBI was similar for both flours.

Hemagglutinating Activity. Table V shows that the

Table III. Trypsin and Chymotrypsin Inhibitor Content Determined by Enzyme Assays of Unheated and Heated Soy Flours[a,b]

	trypsin inhibitor			chymotrypsin inhibitor		
		trypsin inhibited			chymotrypsin inhibited	
sample	TIU/g	mg/g	% remaining	CIU/g	mg/g	% remaining
Williams 82 flour						
unheated	7136 ± 96	36.0 ± 0.5	100	144 ± 6.4	4.2 ± 0.2	100
heated for 10 min	3933 ± 52	25.0 ± 0.3	69	28 ± 6.0	0.8 ± 0.2	19
heated for 20 min	1058 ± 39	7.0 ± 0.2	19	0	0	0
heated for 30 min	1030 ± 16	6.0 ± 0.0	17	0	0	0
L81-4590 flour						
unheated	3858 ± 53	20.0 ± 0.4	100	113.0 ± 2.8	3.3 ± 0.1	100
heated for 10 min	1838 ± 59	12.0 ± 0.4	60	24.0 ± 0	0.7 ± 0	21
heated for 20 min	111 ± 13	0.8 ± 0.1	4	0	0	0
heated for 30 min	100 ± 0	0.6 ± 0	3	0	0	0

[a] Samples were heated in an autoclave for the indicated time periods at 121 °C. [b] Listed numbers are averages from two separate determinations ± standard deviations.

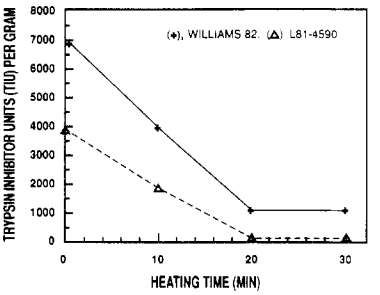

Figure 2. Effect of autoclaving time on trypsin inhibitory activity of soybean meals from Williams 82 and L81-4590 determined by enzyme assay.

hemagglutinating activities of the raw flours derived from Williams 82 and L81-4590 were within a factor of 2. Since a visual endpoint was used to assess the agglutination in a 2-fold dilution series, further analysis is needed to assess whether this difference is significant. Autoclave treatment resulted in rapid and parallel decreases in activities in both varieties.

Fatty Acid Content. Gas chromatography showed identical distribution of fatty acid isomers in the two soybean strains (Table VI).

Nutritional Quality and Effects on the Pancreas. Table VII shows the results of feeding studies in rats of the raw and heated soy flours. Raw Williams 82 flour had a negative PER (−0.14), while the isoline had a PER of 0.46. Total food consumption, body weight gain, and PER increased with heating time. By 20 min, both flours had identical PER. However, for samples heated for 10 min, the isoline had a significantly higher PER than Williams 82.

The improvement in nutritional quality following heat treatment was accompanied by a decrease in pancreatic weights. However, the KTI-free soy flour required only 10 min of heating for the pancreatic hypertrophy to be normalized, compared to 20 min for the Williams 82 flour.

Screening for Cultivars with Modified Protease Inhibitor Content. Since the KTI-free soybean cultivars still contain inhibitors of the Bowman–Birk type, it would be useful to develop soybean cultivars that lack both KTI and BBI. We carried out a feasibility study by screening 13 of the approximately 14 000 soybean cultivars from the USDA Soybean Germplasm Collection for BBI content by enzymatic methods. All of the samples inhibited both trypsin and chymotrypsin (Table VIII). The values ranged from 4.7 to 13.2 mg of chymotrypsin and from 37.2 to 61.4 mg of trypsin inhibited per gram, about a 2-fold range in inhibitory activity. Table VIII and Figure 3 also show that there was good agreement between the BBI content of nine of these samples calculated from the chymotrypsin inhibition assay and ELISA. Error-in-variables regression assuming an error variance ratio of 1 was used to compare the two assays (Madansky, 1959). The calculated regression equation is BBI (enzyme assay) = −0.44 + 1.11 (BBI ELISA assay), with a 95% confidence interval of upper and lower limits on the slope of (0.87, 1.42). The fact that the confidence interval includes the value of 1 implies that the same variable is being measured by both assays.

These results show that other varieties contain more or less KTI or BBI than Williams 82. These samples were also evaluated for their content of sulfur-containing amino acids and lectins (Table IX). The half-cystine content ranged from 0.60 to 0.90 g/100 g and the methionine content from 0.51 to 0.73 g/100 g. There was not a strong correlation between the sulfur-containing amino acid levels and the content of BBI. The hemagglutinating activity varied over a 6-fold range.

DISCUSSION

Amino Acid Analysis. The fact that the two strains have identical amino acid compositions is surprising, in view of the fact that they differ genetically at the locus of a major protein. At this time we do not know whether the amino acids otherwise used in the synthesis of KTI are free or whether they are incorporated into other proteins. However, the result suggests that the Ti-null isoline maintains normal amino acid distribution despite reduced expression of a significant structural gene.

Protease Inhibitors. In agreement with electrophoretic, enzymatic, and genetic data (Orf and Hymowitz, 1979; Hymowitz, 1986; Jofuku et al., 1989), ELISA shows that isoline L81-4590 produces a low level of KTI. Jofuku et al. (1989) show that the reduction in the KTI is a consequence of three mutations resulting in a frameshift in the *ti* allele. This frameshift causes lower mRNA levels and the premature termination of the mRNA translation, producing low levels of a truncated protein. We found a residual activity of less than 0.2% of the wild type, which may reflect the low level of expression of truncated KTI or the product of a related KTI gene. Another inference from the ELISA studies is that KTI can increase when field-grown samples are cross-pollinated by KTI-expressing cultivars. This may explain the significantly higher KTI content of the samples from Swine Farm and South Farm. If the KTI-null isolines are to be used commercially, the ELISA method could be an important quality control test.

Table IV. Kunitz (KTI) and Bowman-Birk (BBI) Content of Unheated and Heated Soy Flours by ELISA

soy flour	KTI, mg/g	% of control	BBI, mg/g	% of control
Williams 82				
unheated	7.6 ± 0.65 (3)[a]	100	3.31 ± 0.34 (3)	100
heated for 10 min	4.6 ± 0.55 (2)	59	0.725 ± 0.19(3)	22
heated for 20 min	1.5 ± 0.24 (3)	19	0.04 ± 0.025(3)	1.2
heated for 30 min	1.9 ● 0.065 (2)	25	0.04 ± 0.03 (3)	1.2
KTI-free (L81-4590)				
unheated	0.008 ± 0.0025 (3)		3.31 ± 0.185(3)	100
heated for 10 min	nd[b]		1.165 ± 0.30(3)	35
heated for 20 min	<0.005 (3)		0.02 ± 0.005(3)	0.6
heated for 30 min	nd[b]		0.02 ± 0.005(3)	0.6

[a] Values in parentheses are number of separate determinations. [b] Not done.

Table V. Effect of Heat on Lectin Activity of a Standard Soybean Variety (Williams 82) and a KTI-Free Cultivar (L81-4590)

	activity[a]	
heating time, min	Williams 82	L81-4590
0	31.5	65
10	8.0	8.0
20	3.2	6.5
30	0.85	1.65
60	0.16	0.32

[a] Activity is defined as the reciprocal of the minimum concentration of sample (expressed as mg/mL) that causes agglutination of human red blood cells.

Table VI. Fatty Acid Composition (g/100 g of Oil) of a Standard Soybean Variety (Williams 82) and an Isoline Lacking the Kunitz Trypsin Inhibitor (L81-4590)

fatty acid	Williams 82	L81-4590
C16:0	11.1	11.2
C18:0	4.2	4.0
C18:1ω9	20.9	19.9
C18:1ω7	1.3	1.3
C18:2ω6	55.2	56.2
C18:3ω3	7.3	7.4

L81-4590 still contains 54% of the trypsin inhibitor (TI) activity of Williams 82. On the basis of our experimentally determined values for BBI (81 CIU and 545 TIU/mg), the results in Tables III and IV indicate that 49% of the TI activity of Williams 82 can be ascribed to non-KTI inhibitors, including BBI and other specific and nonspecific inhibitors of trypsin. The residual 54% TI activity of L81-4590 is thus in the range expected for a KTI-lacking isoline. It is, therefore, unlikely that truncated KTI molecules or geneticaly related minor forms contribute significantly to the total TI activity.

In agreement with our previous paper (Brandon et al., 1989), the BBI content of L81-4590 is identical with that of Williams 82, as determined by ELISA. The results permit us to estimate BBI from the chymotrypsin inhibition data as well, on the basis of the assumption that all of the chymotrypsin inhibitory activity is due to BBI. Using the empirically determined relationship that 1 mg of chymotrypsin is inhibited by 0.34 mg of BBI, we calculate the BBI content of the flours as 1.4 mg/g for Williams 82 and 1.1 mg/g for L81-4590. Thus, the ELISA gave a higher estimate of BBI than the enzymatic assay of soybean meal for these varieties. In contrast, the analysis of nondefatted flours from nine other varieties indicated close agreement between enzymatic and ELISA estimates of BBI. These results suggest that some of the BBI in defatted soybean meal is not fully active as a chymotrypsin inhibitor, perhaps due to processing-induced changes in BBI structure or its interaction with other soybean constituents. Since antibody 238 binds to BBI outside the region of the chymotrypsin-reactive site (Brandon et al., 1990), it would still detect BBI that had formed a complex with and was inhibited by another component of the soybean meal.

Effects of Heating. Heat treatments for up to 30 min at 121 °C, though sufficient to inactivate trypsin and chymotrypsin inhibitors, did not appear to affect the amino acid composition of the soy flours. Such heat treatments often damage lysine, arginine, and the sulfur amino acids (Smith and Friedman, 1984). However, when measured as g/16 g of N, the content of these amino acids was unchanged under the experimental conditions of this study.

The ELISA data indicate that KTI is responsible for the heat-stable trypsin inhibitory activity for commercial soy flour, as illustrated in Figure 2, which shows that the trypsin inhibitory activity of L81-4590 can be 100% inactivated without the residual activity found in the Williams 82 sample. This could result from the stability of KTI itself or from an effect of KTI on other inhibitors. However, it appears that the BBI contents of both varieties are similar, with equal susceptibility to inactivation under the moist heating conditions of the study. Previous studies have indicated the sensitivity of KTI to inactivation by moist heat (Friedman et al., 1984, 1989; Sanderson et al., 1982; Brandon et al., 1988). In contrast, BBI was unaltered antigenically by a 1-h treatment at 95 °C and also retained most of its enzyme inhibitory activities (Brandon et al., 1989). These data indicate that KTI in solution is more susceptible than BBI to inactivation by heat. However, pure KTI in the dry state appears highly stable to heat inactivation (Oste et al., 1990).

The microenvironment in the soy flour appears to catalyze heat inactivation of BBI to a greater extent than KTI. Other studies have also found that the matrix has a strong influence on the susceptibilities of inhibitors to heat inactivation (Begbie and Pusztai, 1989; DiPietro and Liener, 1989a,b; Hancock et al., 1989; Liener and Tomlinson, 1981; Paik, 1988; Peters and Czukor, 1989; Tanahashi et al., 1988). A possible explanation of these results is that sulfhydryl groups found in the proteins of soy flour participate in sulfhydryl–disulfide interchange with the disulfide-rich BBI (Friedman et al., 1984). The disruption of disulfide bonding facilitates further denaturation of the BBI. The influence of disulfide-disrupting agents on KTI and BBI has been described previously (Friedman and Gumbmann, 1986; Friedman et al., 1989; Brandon et al., 1988, 1989).

In addition to moisture and the presence of agents that can induce changes in disulfide bonds, interaction with other constituents such as carbohydrates appears to contribute to the denaturation of inhibitors (Oste et al., 1990). It seems likely that the thermal gradients to which the inhibitors are exposed could also influence heat-induced denaturation.

Pancreatic Effects. According to the biofeedback

Table VII. Food Consumption, Body Weight Gain, Protein Efficiency Ratio, and Pancreas Weights of Rats Fed Unheated and Heated Soy Flours[a,b]

diet	total food consumption, g	total body weight gain, g	PER	pancreas weights: absolute, g	pancreas weights: relative to body weight, %
Williams 82 flour					
unheated	156.8 ± 9.6[a]	−1.8 ± 1.7[f]	−0.14[f]	0.421[f]	0.806[a]
heated for 10 min	282.4 ± 13.1[c]	40.2 ± 3.2[c]	1.42[d]	0.537[cde]	0.572[c]
heated for 20 min	386.4 ± 10.5[a]	82.4 ± 4.2[b]	2.13[b]	0.581[bcd]	0.427[de]
heated for 30 min	395.8 ± 12.4[a]	88.0 ± 3.4[b]	2.22[b]	0.635[b]	0.446[de]
L81-4590 flour					
unheated	189.0 ± 14.9[d]	9.2 ± 2.6[e]	0.46[e]	0.458[ef]	0.721[b]
heated for 10 min	325.8 ± 6.4[b]	40.6 ± 5.0[c]	1.63[c]	0.525[de]	0.503[d]
heated for 20 min	390.0 ± 11.2[a]	87.4 ± 1.0[b]	2.25[b]	0.609[bcd]	0.430[e]
heated for 30 min	398.0 ± 14.0[a]	90.8 ± 3.4[b]	2.28[b]	0.624[bc]	0.431[e]
casein control	405.0 ± 3.3[a]	132.6 ± 1.0[a]	3.27[a]	0.870[a]	0.466[de]

[a] Defatted soy flours were placed in autoclave and heated at 121 °C for the indicated time periods. [b] Duncan's multiple range test (Duncan, 1955). Means without the same superscript letter for food consumption, weight gain, PER, and pancreatic weight are significantly different, $P < 0.05$; six rats per group fed 28 days. PER (protein efficiency ratio) is defined as grams of body weight gain per gram of soybean protein ingested.

Table VIII. Trypsin and Chymotrypsin Inhibition by Cultivars of Soybeans Evaluated as Milled Flours (Not Defatted)

soybean variety	inhibitor activity, mg/g: trypsin	inhibitor activity, mg/g: chymotrypsin
COB 1983 MISS	37.2 ± 2.2[a]	4.87 ± 0.2
HOL 1983 MISS	63.8 ± 1.1	13.0 ± 0.2
JAC 1983 MISS	47.1 ± 0.7	5.71 ± 0.1
ROA 1983 MISS	44.0 ± 2.8	4.71 ± 0.2
MM610 SF83	58.8 ± 1.0	13.2 ± 0.2
T908 SF83	61.4 ± 0.9	10.3 ± 0.2
BSA1004 SF84	56.2 ± 0.9	8.3 ± 0.2
FUJ822 SF84	44.9 ± 1.6	4.8 ± 0.1
ML1014 SF84	48.6 ± 0.7	6.8 ± 0.01
MLB1015 SF84	58.1 ± 1.0	8.77 ± 0
CAY408 SF85	53.4 ± 0.9	9.03 ± 0.4
POL814 SF85	44.0 ± 2.8	7.02 ± 0
BSC803 SF86	56.2 ± 0.9	11.2 ± 0.2
raw soy flour[b] (control)	49.0 ± 0.6	7.22 ± 0.2

[a] Number of separate determinations is 2. [b] Prepared from soybeans purchased in a local store.

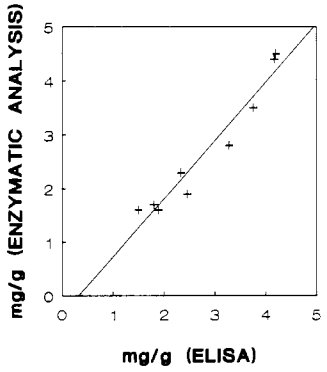

Figure 3. Linear relationship between BBI content of soybean accessions of the USDA Soybean Germplasm Collection, determined by enzyme (chymotrypsin) assays and ELISA.

hypothesis (Gumbmann et al., 1986; Liener et al., 1988), complexation between proteolytic enzymes and enzyme inhibitors such as the Kunitz and Bowman–Birk types in the intestinal tract creates a deficiency of proteolytic enzymes. This deficiency triggers an endocrine sensing mechanism involving cholecystokinin and gastrin. The endocrine system then induces increased protein synthesis in the pancreas, which can result in pancreatic enlargement (hypertrophy) followed by hyperplasia and development of adenoma. The results of the present study are in agreement with this hypothesis. The two soy flours, whether raw or heated, did not differ from each other in amino acid composition and their lectin contents were within a factor or two. Therefore, the differences in both the PER values and pancreatic weights are probably due to differences in inhibitor content.

The nutritional value of the raw KTI-free soy flour, although greater than that of the standard variety, is still too low to sustain normal growth and development. While both samples needed to be heated to achieve this objective, a shorter heating time sufficed for the KTI-free flour. A flour with a trypsin inhibitor content of about 1000 TIU/g supported growth well, without pancreatic weight gain. Our results suggest that this level of trypsin inhibitor could be achieved by a 15-min heat treatment of the KTI-free flour, compared to 20 min for Williams 82 (Figure 2). Table VII shows that a flour with 1800 TIU/g did not produce pancreatic hypertrophy. Figure 2 shows that this level of trypsin inhibitor activity could be achieved by an estimated heating time of 18 min for Williams 82, compared to only 10 min for the KTI-free sample. The precise conditions necessary to achieve these parameters, with minimal use of heat, could readily be determined by using the ELISA for BBI. An important difference between flours from the two cultivars is that the KTI-free flour could be processed to achieve near-zero levels of TI after 20 min, well below the level achievable with standard varieties (Figure 2).

Accessions of the USDA Soybean Germplasm Collection. The preliminary screening of 13 soybean cultivars indicated that there is considerable variation in the content of protease inhibitors and sulfur amino acids. We found a linear correlation between the BBI content determined by the enzyme and ELISA assays (Figure 3). The pancreatic response to protease inhibitors is altered by the quality and quantity of the protein, as well as by the sulfur amino acid content of the diet (Gumbmann and Friedman, 1987). Therefore, the amino acid composition of soybean cultivars should be taken into account when they are being evaluated for commercial use. There was no apparent correlation between sulfur and amino acid content and the chymotrypsin inhibitor activity, presumably because the inhibitors contribute only about 15–20% to the total sulfur amino acid content. Therefore, our preliminary data suggest that it may be possible to discover, through screening of the Soybean Germplasm Collection, varieties low in KTI and BBI but with a sulfur amino acid

Table IX. Lectin Activity and Sulfur Amino Acid Content of Different Soybean Cultivars

soybean cultivar	hemagglutinating activity[b]	half-cystine		methionine	
		g/100 g	g/16 g of N	g/100 g	g/16 g of N
Williams 82	36.0 ± 15 (3)[b]	0.65	1.39	0.65	1.39
L81-4590	78.1 ± 13 (4)	0.68	1.37	0.66	1.32
COB 1983 MISS	64.9 ± 7.6 (2)	0.60	1.62	0.52	1.41
HOL 1983 MISS	13.7 ± 2.8 (2)	0.72	1.72	0.60	1.44
JAC 1983 MISS	82.0 ± 16.1 (2)	0.75	1.58	0.63	1.32
ROA 1983 MISS	64.1 ± 30 (2)	0.60	1.58	0.51	1.35
MM 610 SF83	25.0 ± 0 (2)	0.63	1.70	0.52	1.41
T 908 SF83	27.5 ± 5.6 (2)	0.80	1.67	0.70	1.46
BSA 1004 SF84	54.9 ± 11 (2)	0.90	1.89	0.75	1.59
FUJ 822 SF84	27.3 ± 5.6 (2)	0.57	1.55	0.54	1.45
ML 1014 SF84	24.0 ± 0 (2)	0.75	1.49	0.65	1.28
MLB 1015 SF84	13.7 ± 2.8 (2)	0.86	1.86	0.75	1.62
CAY 408 SF85	38.5 ± 10.9 (2)	0.76	1.54	0.67	1.36
POL 814 SF85	48.0 ± 0 (2)	0.76	1.47	0.66	1.28
BSC 803 SF86	24.0 ± 0 (2)	0.80	1.71	0.73	1.56

[a] Activity is defined as the reciprocal of the minimum concentration of sample (expressed as mg/mL) that causes agglutination of human red blood cells. [b] Values in parentheses are number of separate determinations.

content that meets the nutritional requirements for an ideal protein (FAO, 1973; Gumbmann and Friedman, 1987).

FUTURE STUDIES

While this and other studies (Cook et al., 1988) demonstrate that low-KTI soybeans may offer nutritional advantages, it would be desirable to develop varieties with low levels of BBI as well. The genetic diversity of soybeans with respect to the major seed lectin has been studied (Stahlhut et al., 1981), and double nulls for the lectin and KTI have been produced (Prischmann and Hymowitz, 1988). If the low-BBI characteristic can be bred into a lectin–KTI double null, soybean meal from these varieties could possibly be used as animal feed with no processing. In addition, flours from such varieties might be processed into human foods more economically than current commercial flours.

The potential beneficial effects of protease inhibitors should also be taken into account in establishing criteria for improved varieties. There is substantial evidence that wound-induced inhibitors of digestive enzymes may play a role in protecting potato and tomato plants against insects and other phytopathogens (Brown et al., 1986; Johnson et al., 1990). The protection presumably arises directly from inactivation of insect digestive enzymes or indirectly by the overstimulation of trypsin secretion, resulting in depletion of sulfur amino acids (Broadway and Duffus, 1986). It has been suggested that such inhibitors, in combination with other toxicants, could form a battery of defenses in the Leguminosae (Janzen et al., 1986). Evidence presented thus far suggests that KTI is not agronomically important in soybeans, since TI-nulls appear equivalent to their parental strains (Hymowitz, 1986). It is therefore important to determine whether the elimination of additional inhibitors from soybeans by classical or molecular genetic methods such as antisense RNA methodology (Delauney et al., 1988) would have adverse agronomic consequences.

Soy protease inhibitors have been shown to inhibit carcinogenesis, in both in vitro and in vivo systems (Yavelow et al., 1983; Troll et al., 1986; St. Clair et al., 1990), and to stimulate human T cells (Richard et al., 1989). Therefore, more data are also needed on the health effects of protease inhibitors to guide further development of soybean varieties with enhanced health-promoting qualities.

An alternative approach to optimizing the content of protease inhibitors in soybean protein is to use food processing to achieve the desired mix. This study and others have noted that temperature, moisture, and interaction with other nutrients can influence the inactivation of protease inhibitors. A comparison of the present results to those from a previous study (Brandon et al., 1989) suggests that sample size and defatting of the flour may influence the relative susceptibilities of KTI and BBI to heat inactivation. Food processing could be combined with genetic approaches. For example, inhibitors modified by site-directed mutagenesis to produce heat-labile molecules could protect the plants but be eliminated from foods and feeds by mild processing. High-resolution techniques, such as the monoclonal antibody based ELISAs for measuring KTI and BBI, could help optimize production of the most nutritious and healthful soy protein product.

ADDED IN PROOF

The University of Illinois Plant Variety Review Committee has recommended to the Director, Agricultural Experiment Station, that experimental line L81-4590 be released for commercial production under the name "Kunitz".

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