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REVIEWS

Nutritional and Health Benefits of Soy Proteins†

Mendel Friedman* and David L. Brandon

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710

Soy protein is a major component of the diet of food-producing animals and is increasingly important in the human diet. However, soy protein is not an ideal protein because it is deficient in the essential amino acid methionine. Methionine supplementation benefits soy infant formulas, but apparently not food intended for adults with an adequate nitrogen intake. Soy protein content of another essential amino acid, lysine, although higher than that of wheat proteins, is still lower than that of the milk protein casein. Adverse nutritional and other effects following consumption of raw soybean meal have been attributed to the presence of endogenous inhibitors of digestive enzymes and lectins and to poor digestibility. To improve the nutritional quality of soy foods, inhibitors and lectins are generally inactivated by heat treatment or eliminated by fractionation during food processing. Although lectins are heat-labile, the inhibitors are more heat-stable than the lectins. Most commercially heated meals retain up to 20% of the Bowman−Birk (BBI) inhibitor of chymotrypsin and trypsin and the Kunitz inhibitor of trypsin (KTI). To enhance the value of soybeans in human nutrition and health, a better understanding is needed of the factors that impact the nutrition and health-promoting aspects of soy proteins. This paper discusses the composition in relation to properties of soy proteins. Also described are possible beneficial and adverse effects of soy-containing diets. The former include soy-induced lowering of cholesterol, anticarcinogenic effects of BBI, and protective effects against obesity, diabetes, irritants of the digestive tract, bone, and kidney diseases, whereas the latter include poor digestibility and allergy to soy proteins. Approaches to reduce the concentration of soybean inhibitors by rearrangement of protein disulfide bonds, immunoassays of inhibitors in processed soy foods and soybean germplasm, the roles of phytoestrogenic isoflavones and lectins, and research needs in all of these areas are also discussed. This integrated overview of the widely scattered literature emphasizes general concepts based on our own studies as well as recent studies by others. It is intended to stimulate interest in further research to optimize beneficial effects of soy proteins. The payoff will be healthier humans and improved animal feeds.

Keywords: *Anticarcinogenic effects; Bowman−Birk inhibitor; cardiovascular health; disulfide exchange; cholesterol-lowering effects; health benefits; immunoassays; isoflavones; lysine; lysinoalanine; methionine; Kunitz trypsin inhibitor; nutrition; nutritional improvement; soy lectins; sulfitolysis*

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INTRODUCTION

The soybean plant (*Glycine max*) originated in China, and Samuel Bowen introduced the beans to the American colonies in 1765 (*1*). He used soybeans to prepare soy sauce and soy noodles (vermicelli) for export from Georgia to England. Large-scale production of soybeans in the United States appears to have started during the 1850s (*2, 3*). In 1999, farmers planted soybeans on 72 million acres, amounting to 27% of the total crop area in the United States. The resulting harvest yielded ~2.74 billion bushels valued at ~$15 billion (*4*). Soybeans are an economically important crop, which serves as a source of good-quality protein for animals and humans. Nitrogen fixation by root nodule bacteria in soybean plants is the ultimate source of soy proteins (*5*). The seeds contain up to 48% protein and up to 22% oil, which is widely consumed as a cooking oil. The storage soy proteins consist of a mixture of proteins (α-, *β*-, and *γ*-conclycinins, glycinin, and other globulins) ranging in molecular weight from about 140000 to 300000 Da and differing in physicochemical and other properties (*6, 7*). The seeds also contain bioactive proteins including *β*-amylase, cytochrome *c*, lectin, lipoxygenase, urease, the Kunitz inhibitor of trypsin (KTI) (*8, 9*), and the Bowman−Birk inhibitor of chymotrypsin and trypsin (*10, 11*) (Figure 1), as well as secondary metabolites including isoflavones (*12*−*14*) (Figure 2), saponins, phytic acid, flatus-producing oligosaccharides, and goitrogens (*15*). Processing of soy meal improves its digestibility and destroys much of the inhibitors, but may also result in the formation of unnatural amino acids including heat-induced fructosyllysine derived from protein−carbohydrate browning reactions (*16*−*20*), as well as lysinoalanine (*21*−*23*) and D-amino acids (*24, 25*) formed at high pH.

Soybean proteins are used in human foods in a variety of forms, including infant formulas, flours, protein isolates and concentrates, and textured fibers. Soy foods include cheese, drinks, miso, tempeh, tofu, salami, and vegetarian meat substitutes. New soy foods are continually being developed (*26, 27*). Consumption of soy foods is increasing because of reported beneficial effects on nutrition and health. These effects include lowering of plasma cholesterol, prevention of cancer, diabetes, and obesity, and protection against bowel and kidney disease. This paper (a) describes studies delineating the nutritional quality, safety, and health benefits of soy proteins; (b) discusses improved food-processing techniques to inactivate soybean inhibitors of digestive enzymes; (c) outlines the application of immunoassays to measure inhibitors in processed foods and in germplasm; and (d) suggests research needs to enhance nutrition and health benefits of soy proteins.

GENERAL ASPECTS OF SOY PROTEIN NUTRITION

Proteins are an essential component of the diet needed for the survival of animals and humans. Their function in nutrition is to supply adequate amounts of needed amino acids. The protein nutritional quality of a food depends on content, digestion, absorption, and utilization of amino acids. Availability of amino acids

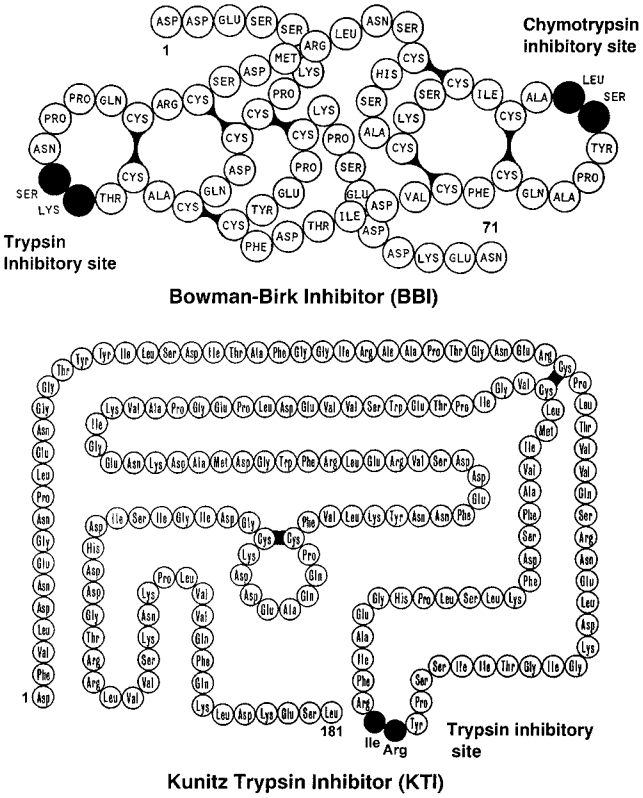

Figure 1. Structures of the two major protease inhibitors of soybeans. BBI has seven disulfide bonds per mole and KTI two (*8*−*11*).

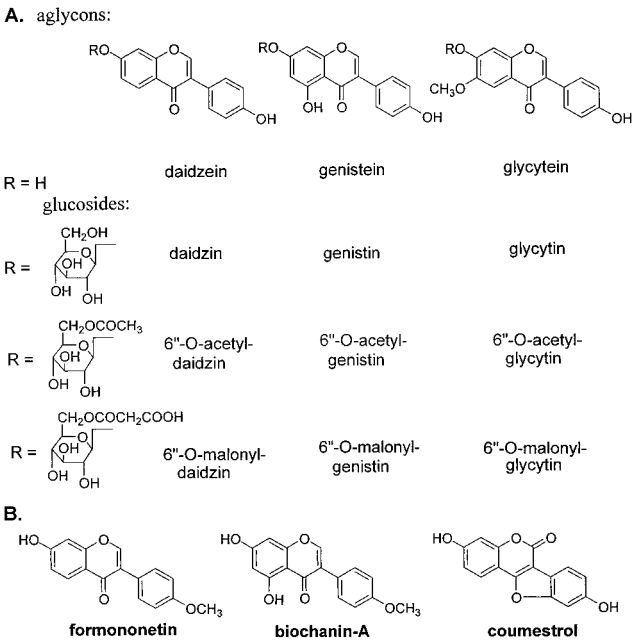

Figure 2. Isoflavones (phytoestrogens) present in (A) soybeans and (B) other legumes (*12*−*14*).

varies with protein source, processing treatment, and interaction with other components of the diet. Proteins that are deficient in one or more amino acids are of poor quality. For example, tryptophan and lysine are nutritionally limiting in corn, lysine in wheat and other cereals, and methionine in soybeans and other legumes.

Table 1 shows that humans in developing countries consume more low-quality proteins derived from cereals than those living in developed countries, who consume more animal proteins (*28*). Table 2 shows the amino acid

* Author to whom correspondence should be addressed [e-mail mfried@pw.usda.gov; fax (510) 559-5777].

† We dedicate this paper to Prof. Irvin E. Liener of the University of Minnesota in appreciation of his lifelong contributions to all aspects of soy protein nutrition.

Table 1. Major Sources of Protein in the Diet in Developing and Developed Countries (*28*, *33*)

source	developing countries (%)	developed countries (%)
cereals	58.8	29.1
meat	8.6	26.4
legumes	7.4	1.7
milk and dairy	5.6	16.7
fish, seafood	4.1	7.3
oil crops	3.8	1.9
vegetables	3.5	3.5
starchy roots	3.1	3.2
eggs	1.6	4.3
offals	1.2	2.2
fruit	1.0	1.1

Table 2. Amino Acid Composition of Soy Meal from a Standard Variety (Williams 82) and from a Strain Lacking KTI (L81-4590) (*32*)

amino acid	Williams 82		KTI-free		FAO,[a]
	g/100 g	g/16 g of N	g/100 g	g/16 g of N	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	0.65	1.39	0.68	1.37	
Met	0.65	1.39	0.66	1.32	3.5[b]
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[c]
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	0.33	0.71	0.38	0.76	
total	45.42	97.68	49.48	99.70	

[a] Scoring pattern for an ideal protein (*29*, *33*). [b] Cys + Met. [c] Tyr + Phe.

composition of soy flours from two soybean varieties and the corresponding amounts for essential amino acids recommended by the Food and Agricultural Organization of the United Nations for an ideal protein (*29*). Table 3 lists the amino acid composition of several food sources and suggested amino acid requirements of humans. Although the adult amino acid requirements can be met readily from intakes of high-quality proteins, the data in Tables 1−3 imply that it is challenging to meet adult requirements with low-quality protein. Although processed soy proteins are well utilized by humans, their nutritional value is lower than that of milk protein (*30*). Germination enhances the nutritional quality of soybean seeds, presumably due to increased protein content and decreased content of nondigestible oligosacharides (*31*). Amino acid patterns by themselves are insufficient to predict utilization of a protein. In soybeans and other legumes, antinutritional factors such as inhibitors of digestive enzymes and lectins, as well as poor digestibility, have all been reported to lower nutritional value. For example, raw Williams 82 soy flour produced weight loss; heat treatment improved the nutritional quality of the product (Table 4).

Inhibitors of digestive enzymes adversely affect the nutritional quality of soy protein (Figure 3B). To improve nutritional quality, inhibitors are generally inactivated by heat treatment during food processing or are partially removed by fractionation. However, most commercially heated flours retain 5−20% of the original trypsin and chymotrypsin inhibitor activity. The more protracted heating required to destroy all inhibitor activity would damage the nutritive value of soy proteins. Below we describe efforts to overcome this problem.

METHIONINE IN SOY PROTEIN NUTRITION

The low content of the essential amino acid L-methionine in soy protein limits its nutritive value. For example, we found that the methionine content of soy flour was only 0.65 g/100 g of dry matter, which corresponded to 1.39 g/16 g of N (*32*) (Table 2**)**, much lower than that of cereal and meat proteins (*33*−*37*) (Table 3). Although cystine has a sparing effect on methionine, it does not make up for low methionine levels. The problem is further compounded for two reasons. First, during food processing and storage, L-methionine and other amino acids are chemically modified, further reducing nutritional quality. In the case of methionine, such modifications include oxidation to methionine sulfoxide and methionine sulfone, racemization to D-methionine, and degradation to compounds with undesirable flavors. Second, protein-bound methionine in some plant foods is poorly utilized, presumably because of poor digestibility (*38*−*41*). To overcome these problems, efforts are being made to develop soybean lines that overexpress methionine-rich proteins (*42*, *43*).

A general consensus appears to have evolved on the following aspects of sulfur amino acid nutrition (*44*): (a) The lower nutritional availability of both cyst(e)ine and methionine in raw soybean meals compared to the utilization of these amino acids from heated meals results from poor digestibility. (b) Fortification with methionine significantly improves nutritional quality (Figure 3A). Although the addition of methionine to soy protein consumed by adults had no significant effect on the nutritional value when the intake of nitrogen was adequate, addition of methionine to a soy milk formula increased N retention of malnourished children (*45*−*48*).

Table 3. Essential Amino Acids of Different Protein Sources and Suggested Human Amino Acid Requirements (*33*)

amino acid	amino acid content (mg/g of protein)					FAO/WHO suggested requirements			
	ANRC casein	beef	egg white	soy protein	wheat flour	1 year old	2−5 years old	10−12 years old	adult
Thr	46.4	42.1	46.8	38.4	29.3	43	34	28	9
Cys + Met	34.9	32.7	66.4	68.1	38.7	42	25	22	17
Val	68.5	45.4	67.8	49.1	42.7	55	35	25	13
Ile	53.6	41.8	52.8	47.1	33.4	46	28	28	13
Leu	101.6	77.5	87.6	85.1	68.5	93	66	44	19
Tyr + Phe	125.4	70.2	90.8	96.6	77.8	72	63	22	19
His	29.7	32.0	22.5	25.4	21.9	26	19	19	16
Lys	84.4	79.4	69.8	63.4	26.6	66	58	44	16
Trp	13.1	9.9	14.6	11.4	11.2	17	11	9	5

Table 4. Effect of Autoclave Heat on the PER[a] and Pancreas Weights of Rats Fed a Standard Soybean Variety (Williams 82) and One Lacking KTI (L81-4590) (*32*)

diet	PER	pancreas wt (% of body wt)
Williams 82 flour		
unheated	−0.14	0.806
heated for 10 min	1.41	0.572
heated for 20 min	2.13	0.427
heated for 30 min	2.22	0.446
L81-4590 flour		
unheated	0.46	0.721
heated for 10 min	1.63	0.503
heated for 20 min	2.25	0.430
heated for 30 min	2.28	0.431
casein control	3.27	0.466

[a] PER = weight gain of rats (g)/protein intake (g).

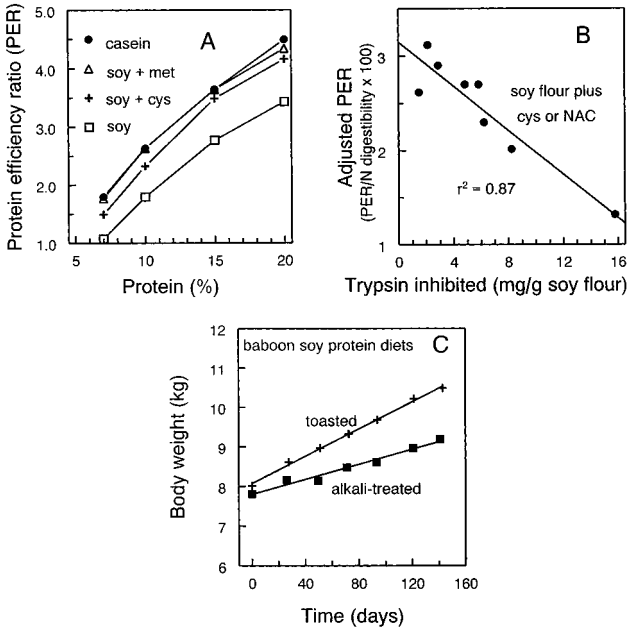

Figure 3. (A) Improvement in the PER of raw soy flour after fortification with L-cystine or L-methionine (*20*). (B) Improvement in the nutritional quality of soy flour as a function of inactivation of trypsin inhibitors by SH-amino acids (*104*). (C) Growth of baboons fed toasted and alkali-treated soy proteins (*21*).

Sulfur amino acids also may play an important role in the feedback mechanism that has been proposed to explain the adverse effects of inhibitors on the pancreas. Complexation between proteolytic enzymes (trypsin and chymotrypsin) and enzyme inhibitors creates a deficiency of proteolytic enzymes in the intestinal tract. An endocrine sensing mechanism then induces increased protein synthesis in the pancreas. Un-denatured trypsin inhibitors and proteolytic enzymes (both rich in sulfur amino acids) are excreted in the form of enzyme−inhibitor complexes. This loss of sulfur amino acids is predicted to be an important factor for the pancreas, which is under hormonal regulation to increase synthesis of sulfur-rich proteolytic enzymes (*49*). These results may reflect the circumstance that the pancreas has a greater ability than other body organs to mobilize sulfur amino acids needed for its function.

LYSINE IN SOY PROTEIN NUTRITION

Table 3 shows that the lysine content of soy proteins falls between that of cereal proteins such as wheat

Table 5. Effect of Glucose on Lysine and Arginine Content (in Mole Percent) of Soy Flour (*17*)

amino acid	soy flour control	soy flour + glucose, 37 °C, 10 days	soy flour + glucose, 95 °C, 4 h
Lys	5.67	4.15	2.65
Arg	5.74	5.50	2.51

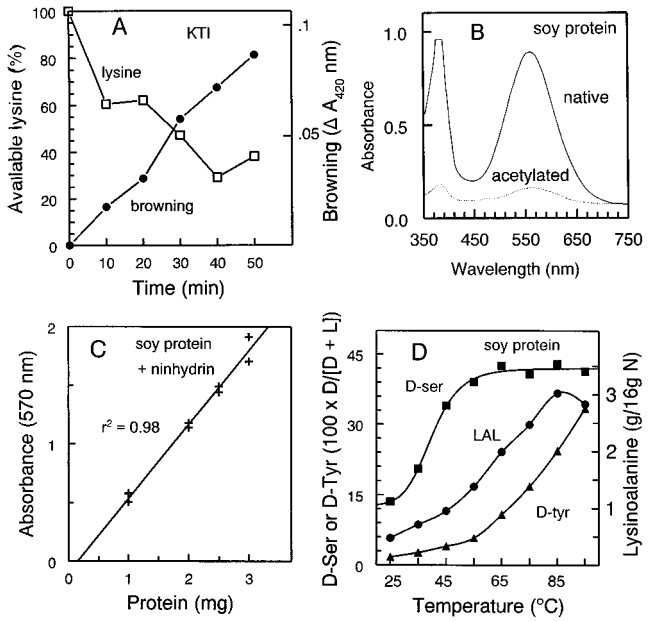

Figure 4. (A) Inverse relationship between the extent of browning and available lysine in KTI (*142*). (B) Absorbance of the ninhydrin chromophore of native and acetylated soy proteins (adapted from refs *56* and *57*). The acetylated protein NH_2 groups do not react with ninhydrin and are protected against lysinoalanine formation and browning reactions. Native and acetylated soy proteins had the same nutritional value in rats (*23*). (C) Linear relationship between the concentration of NH_2 groups in soy flour and absorbance of the ninhydrin chromophore (*56*, *57*). A ninhydrin assay for tryptophan in soy proteins is described in ref *62*. (D) Temperature dependence of the formation of D-serine, LAL, and D-tyrosine in soy proteins at pH 12 (*24*).

gluten and that of animal proteins such as casein (*50*, *51*). Although the lysine content of soy protein is sufficient to support normal growth and development of mammals including humans, efforts are underway to develop soybean varieties with increased lysine content (*52*). This is because lysine can be lost during processing and storage. These losses arise from Maillard browning reactions of ϵ-NH_2 of lysine with carbohydrates to produce fructosyl-lysine and, at high pH, cross-linking of lysine to form lysinoalanine and conversion of L- to D-lysine. Neither lysinoalanine nor D-lysine is utilized to any extent as a source of lysine. The lysine content of soy proteins exposed to carbohydrates can decrease by as much as 85% (*17*, *18*) (Table 5; Figure 4A). Smith and Friedman (*19*) made similar observations with casein. Loss of nutritional quality appears to be related to nitrogen digestibility rather than to destruction of amino acids (*20*).

In contrast to rodent and human nutrition, the utilization of food proteins by ruminants is often enhanced by chemical modification and heat treatment (*53*). Heat-processed proteins resist breakdown by bacteria in the rumen. To optimize such heat treatments for soybeans, Faldet et al. (*54*, *55*) measured available lysine in vitro and in vivo. Heat treatments that generated a loss of 15−22% of chemically available

Table 6. Digestibility and NPU of Toasted and Alkali-Treated Soy Proteins in Rats (*21*)

diet[a]	digestibility (%)	NPU[b]
casein	98.3 ± 0.5	80.6
toasted soy	97.0 ± 0.6	62.4
alkali-treated soy	83.2 ± 0.1	28.3

[a] These diets were also fed to baboons. See Figure 3C. [b] NPU = (N retained/N intake) × 100.

lysine also produced optimal postruminal protection of proteins from bacterial degradation.

To better understand the loss of lysine in foods, improved methods for available lysine are needed. One such simple, rapid, and inexpensive method we developed is a modified ninhydrin assay, which uses a lithium acetate−DMSO ninhydrin reagent (*56*, *57*). This reagent (a) diffuses into complex food matrices, (b) rapidly extracts the proteins, (c) increases the rate of reaction between amino acids and ninhydrin, and (d) stabilizes the ninhydrin chromophore. Figure 4B shows the absorbance of the chromophore in native and acetylated soy proteins, and Figure 4C illustrates a linear relationship between the concentration of protein NH_2 groups and the absorbance.

LYSINOALANINE IN SOY PROTEIN NUTRITION

Changes in composition and nutrition result from exposure of food proteins to pH >9 (*21*, *58*). A process that exposes soy protein to pH 12, called acid spinning, makes it possible to manufacture fibrous proteins resembling meat fibers. An alkaline protein solution (or dope) is injected through a punctured plate (the spinneret) into an acid bath, where the pH is reduced to the isoelectric point of the protein. The precipitated coagulated fibers are then washed and used to manufacture vegetable meat analogues. Exposure of proteins to alkali induces the formation of the unnatural amino acid lysinoalanine **(**Figure 4D).

Friedman et al. (*22*, *23*) carried out detailed studies on the factors governing lysinoalanine formation in soy proteins during exposure to alkaline conditions (pH 8−14) for various time periods (10−480 min) and temperatures (25−95 °C at 10 °C intervals). At the higher temperatures and pH values, all of the cystine and part of the arginine, lysine, serine, and threonine residues were destroyed. These losses were accompanied by the appearance of lysinoalanine. Its formation was suppressed by protein acylation and by the addition of SH-containing compounds (cysteine, *N*-acetylcysteine, and reduced glutathione), copper salts, or glucose. Lysinoalanine formation was accompanied by concurrent racemization of L-amino acid residues to D-isomers (*24*).

Alkali treatment of proteins can either reduce or enhance the nutritional quality of the treated proteins in different animal species. The treatment of soy protein reduced digestibility in rats (Table 6) and lowered body weight gain in baboons (Figure 3C). In contrast, the lower digestibility of lysinoalanine-containing soy proteins reduced the rate of degradation of the modified proteins in the rumen of cattle by bacterial enzymes (*59*). As mentioned, the enhanced N retention results in an improvement in the nutritional value of the proteins consumed by cattle and sheep.

Possible causes for the reduction in digestibility and nutritional quality following treatment in alkali include destruction of arginine, cystine, and lysine, isomerization of L-amino acids to less digestible D-isomers, formation of inter- and intramolecular cross-links, and inhibition of proteolytic enzymes. These changes eliminate and/or hinder access of proteolytic enzymes to substrates.

Generally, the extent of nutritional damage of alkali treatment associated with conversion of lysine may depend on the original lysine content of a protein. For example, a decrease in lysine due to lysinoalanine formation in a high-lysine protein such as soy protein or casein may have a less adverse effect than in a low-lysine protein such as wheat gluten, in which lysine is a nutritionally limiting amino acid.

Feeding alkali-treated soy proteins to rats induces changes in rat kidney cells (*60*). These changes are characterized by enlargement of the nucleus and cytoplasm and disturbances in DNA synthesis and mitosis. Because such changes were not observed after long-term feeding of alkali-treated soy proteins to baboons, consumption of food proteins containing low amounts of lysinoalanine is probably safe for humans (*21*). However, because soy infant formulas are often the sole source of protein for some infants, Pfaff (*61*) recommends that the lysinoalanine content of infant formulas be kept at <200 ppm. It is not known if growing infants and children are more sensitive to the adverse effects of lysinoalanine than are adult humans.

It is also worth noting that tryptophan residues of soy protein are also susceptible to modification, especially under oxidative conditions of food processing (*62*). However, tryptophan losses during processing appear to be less important when compared to the adverse effects on nutrition associated with damage to cystine, methionine, and lysine.

ALLERGY TO SOY PROTEINS

Food allergies occur in 4−6% of children and seem to be increasing in prevalence (*63*). Soy-based infant formulas are widely used for feeding children suffering from allergy to cow's milk and for the prevention of diseases when breast milk is not available (*64*). The soy formulas are inexpensive and nutritionally adequate as a replacement of milk-based formulas and rarely elicit allergic reactions (*65*). However, the high phytic acid content of the formulas could affect mineral absorption and utilization (*66*). From a review of some recent studies, summarized below, it is becoming apparent that some infants may not tolerate soy-based diets. Efforts to eliminate food constituents with potential for intolerance are described by Kitts et al. (*67*). It is also relevant to note that the development of a high-methionine soybean line containing a Brazil nut food allergen has been discontinued (*68*).

A 1993−1996 survey of 61 cases of severe reactions by children to food in Sweden revealed that several youngsters who were allergic to peanuts also experienced soy anaphylaxis (*69*). Evidently, some young people with severe asthma and peanut allergy may also be allergic to soy. However, there appears to be no obvious relationship between soy and milk allergies. A study designed to establish the prevalence of soy allergy in IgE-associated cow's milk allergy among 93 American children <3.5 years old showed that soy allergy occurred in only a small number of these children (*70*). Food allergy can be transitory in children. For example, Hill et al. (*71*) studied 18 children with multiple protein intolerances. They found that most of the children lost

Table 7. Incidence of Pancreatic Hyperplasia and Adenoma in Rats Fed Raw and Heated Soy Protein for 2 Years (*77*)

soy protein isolate	trypsin inhibitor (mg/g of protein)	lesion incidence[a] nodular hyperplasia	lesion incidence[a] acinar adenoma
raw	20.4	27/28 (96.4)	14/28 (50)
heated	6.1	20/33 (60.6)	6/33 (18.2)

[a] Number of rats with lesion/number observed (% incidence).

their intolerance by about the age of three. IgE and IgG binding methods for measuring native and modified soy protein allergens are described by Burks et al. (*72*) and Djurtoft et al. (*73*).

EFFECT OF RAW SOY AND SOYBEAN INHIBITORS ON DIGESTIVE ENZYMES OF THE PANCREAS

In lifetime feeding studies of soybean-derived inhibitors, rats developed pancreatic lesions leading to neoplasia or tumor formation. This raises the question of whether it is safe to consume such inhibitors, which are present in all legumes and to lesser extents in cereals and vegetables such as potatoes and tomatoes and in processed soy products (*74*, *75*). An examination of the reported effects of inhibitors on the pancreases of different animal species outlined below could help provide an answer to this question.

McGuiness et al. (*76*) showed that long-term feeding of raw soy flour to rats results in the development of pancreatic adenomas. To help elucidate the significance of these findings, a related long-term (95-week) feeding study was carried out in this laboratory. Soy flour or diets supplemented with soy or potato trypsin inhibitors caused dose-dependent pancreatic pathologies consisting of nodular hyperplasia and acinar lesions (*77*, *78*) (Table 7). However, the lesions described in rats were not observed with mice following long-term feeding. Liener and Hasdai (*79*) also did not observe tumor formation in the mouse pancreas following the long-term feeding of raw soy flour. In related studies it was shown that rats that consumed a soybean trypsin inhibitor concentrate for 14 weeks developed pancreatic hyperplasia and/or hypertrophy (*80*); Myers et al. (*81*) and Roebuck et al. (*82*) found that (a) soybean trypsin inhibitor concentrate promoted the growth of azaserine-induced lesions of the rat pancreas to a greater extent than did corn oil; (b) heat-treated soy protein isolate neither increased azaserine-induced carcinogenesis nor raised plasma cholecystokinin (CCK) levels; (c) the concentrate alone without azaserine did not initiate pancreatic lesions; (d) formation of inhibitor-induced rat pancreatic lesions appears to be mediated by CCK; and (e) the high protein and fat content in some of the diets used in long-term rat feeding studies could be partly or fully responsible for the observed pancreatic lesions (*83*).

Studies with hamsters revealed that feeding raw soy-based diets induced short-term trophic effects on the pancreas as evidenced by increases in pancreatic weights, DNA, RNA, and protein content (*84*). However, the same diets did not potentiate pancreatic cancer in hamsters treated with the cancer initiator *N*-nitroso-bis(2-oxopropyl)amine (BOP) (*85*).

In contrast to the results with rats, no adverse effects on the pancreas were observed in monkeys fed diets containing low levels of trypsin inhibitor for up to 4 years (*86*, *87*). Specifically, neither pancreatic hypertrophy nor hyperplasia was evident, as measured by RNA/DNA and protein/DNA ratios. This was also the case with three baboons that were fed raw soy flour for ~6 months as part of the alkali-treated soy protein study mentioned earlier. Histological examination did not reveal any differences in pancreatic and kidney tissues associated with two diets (M. Friedman, J. J. Dreyer, and W. L. Spangler, unpublished results).

Soy and other protein inhibitors also have a strong affinity for human digestive enzymes (*88*). Studies by Liener et al. (*89*) revealed that in humans, BBI induced 2−3-fold stimulation in the production of the enzymes trypsin, chymotrypsin, elastase, and amylase. Evidently, BBI caused a negative feedback inhibition of pancreatic exocrine secretion in humans (*90*). Holm et al. (*91*) and Toskes (*92*) made similar observations.

Pancreatic enlargement resulting from consumption of inhibitors is also reported to occur in chickens and growing guinea pigs, but not in dogs, pigs, calves, and monkeys (*93*, *94*). In view of the increasing worldwide consumption of soy-based foods, additional epidemiological studies with humans are warranted to rule out possible long-term adverse effects on the pancreas (*95*−*97*).

INACTIVATION OF SOYBEAN INHIBITORS OF DIGESTIVE ENZYMES

Inhibitors of enzymes such as trypsin, chymotrypsin, carboxypeptidase, elastase, and α-amylase appear in many agricultural products including legumes, cereals, potatoes, and tomatoes (*74*). BBI and KTI appear to act as transition-state analogue inhibitors of chymotrypsin and trypsin (*98*−*102*). Soy-based diets containing active protease inhibitors depress growth in rats compared to analogous diets utilizing inhibitor-free soybeans. Growth inhibition and the accompanying pancreatic hypersecretion of trypsin and chymotrypsin, pancreatic hypertrophy, and pancreatic adenoma are presumably partly due to the antitryptic and antichymotryptic activities of the inhibitors. Toasting soy flour largely prevents pancreatic enlargement and related changes. Although the possible significance of trypsin inhibitors for human health is yet to be resolved, any concern could be avoided by eliminating the inhibitors from the diet.

Heat alone does not completely inactivate all inhibitory activity. Whether there are adverse or beneficial effects of residual inhibitors in food is not known. In addition, heat used to inactivate inhibitors may also destroy certain essential amino acids, such as cystine, methionine, and lysine. These considerations prompted us to examine a possible synergistic effect of heat and disulfide bond modification on the activity of soybean inhibitors both in pure form and in soy flour. The following section summarizes some of our findings with two types of disulfide bond modification: sulfhydryl−disulfide interchange and sulfitolysis of disulfide bonds.

Sulfhydryl—Disulfide Interchange. The action of thiols such as cysteine in inhibiting the activity of disulfide-containing enzyme inhibitors is postulated to involve the formation of mixed disulfides among added thiols, enzyme inhibitors, and storage proteins. One of the sulfur atoms of the mixed disulfide originates from the protein and the other from the added sulfhydryl compound. The added compound, therefore, becomes part of the protein structure. Because of structural alterations due to the formation of such mixed disulfides, the modified inhibitors lose their ability to complex with the active sites of trypsin and other proteolytic

Scheme 1. Sulfhydryl−Disulfide Interchange and Oxidation of SH Groups in Soy Proteins Resulting in the Formation of New Disulfide Bonds and Altered Protein Structure

R−SH + In−S−S−In	→	R−S−S−In + HS−In
R−SH + Pr−S−S−Pr	→	R−S−S−Pr + HS−Pr
In−SH + Pr−S−S−Pr	→	In−S−S−Pr + HS−Pr
In−SH + HS−Pr + $^1/_2O_2$	→	In−S−S−Pr + H_2O
In−SH + HS−In + $^1/_2O_2$	→	In−S−S−In + H_2O
R−SH:		L-cysteine, NAC, reduced glutathione
In−S−S−In:		inhibitor (In) disulfide bonds
Pr−S−S−Pr:		storage protein (Pr) disulfide bonds

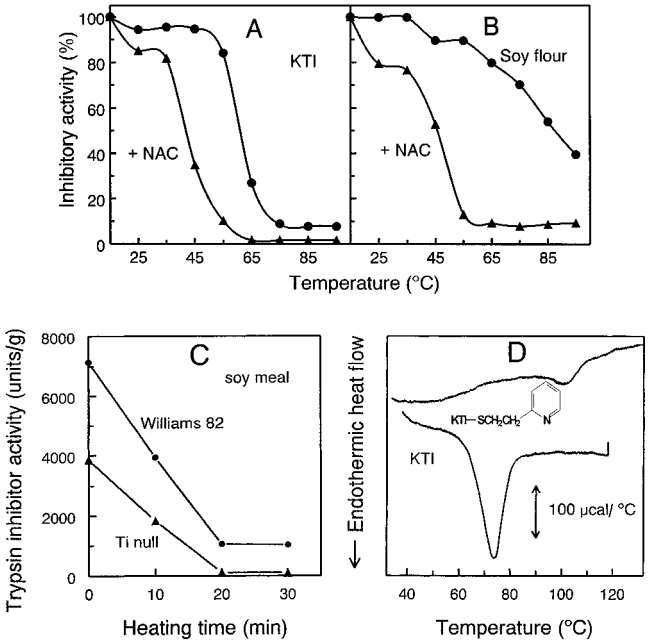

Figure 5. Effect of heat and NAC on trypsin inhibiting activity of (A) KTI and (B) soy meal. (C) Susceptibility of enzyme inhibitors of a standard soybean variety (Williams 82) and one lacking KTI (L81-4590) to heat inactivation (*32*). (D) Differential scanning calorimetry of native KTI and disulfide-modified KTI. The disulfide (S−S) bonds were reduced with tributylphosphine to SH groups, which were alkylated with 2-vinylpyridine to pyridylethylcysteine (2-PEC) residues. The reductive S-pyridylethylation reaction destroyed the native conformation of the inhibitor as well as its ability to inhibit trypsin (*105*, *106*).

Table 8. Enhanced Inactivation of Trypsin Inhibitors in Three Legume Flours with Increasing Concentration of NAC[a] (*107*, *108*)

	% inhibitory activity remaining		
NAC (g)	soybean	Great Northern bean	lima bean
0	44.2	50.0	75.6
1	11.4	15.1	25.7
2	4.6	8.5	19.6
3	0.0	2.5	13.0

[a] 100 g of flour in 500 mL of pH 8.5 Tris buffer; 65 °C; 1 h.

enzymes illustrated in Scheme 1 (*103*−*109*). The experimental data in Figure 5 show that the soy trypsin inhibitors are inactivated more readily by heat in the presence of cysteine or *N*-acetylcysteine.

Evidence for the postulated reaction scheme was obtained by measuring the cystine content of soybean flours. Table 8 shows the facilitation of heat inactivation of inhibitors in three legume flours by *N*-acetylcysteine. Table 9 shows that cysteine treatment increased the half-cystine content, as would be expected if the added thiols participated in the postulated sulfhydryl−disulfide exchanges. This result implies that the added

Table 9. Half-Cystine Content of Untreated and NAC-Treated Soy Flours (*107*, *108*)

material	treatment	half-cystine (nmol/mg)
soy flour	dialyzed	67.0
soy flour	+ NAC	139.0

Table 10. Effect of Heating, without or with L-Cysteine, on the Nutritional Value of Soy Flour (*109*)

temp (°C)	PER	digestibility (%)
45	0.95	73.7
45 + cysteine	2.01	81.7
65	1.61	79.9
65+ cysteine	2.43	82.9
75	2.14	79.0
75 + cysteine	2.53	82.7
casein control	3.12	94.5

cysteine or acetylcysteine (a) participated in the indicated sulfhydryl−disulfide interchange and oxidation reactions and (b) became attached to soybean protein chains in the form of mixed disulfides. The cysteine treatments also resulted in improvement in nutritional value as measured by the protein efficiency ratio (Figure 3B; Table 10).

Related kinetic studies revealed that during heat treatment of soy flour, a two-phase inactivation of inhibitors takes place initiated by heat and by SH−SS exchanges (*110*). Sulfhydryl−disulfide rearrangement also appears to facilitate heat-induced denaturation of glycinin, a major storage protein of soybeans (*111*). However, these transformations cannot occur with inhibitors lacking disulfide bonds. This is illustrated by our observation that *N*-acetylcysteine inactivated lima bean lectin, a disulfide-containing protein (*112*), but failed to inactivate soybean lectin, which lacks disulfide bonds (*113*).

Our studies also suggest that controlled disulfide exchange may be useful for inactivation of disulfide-containing toxic proteins including bacterial *exo*-toxins such as botulinum toxin; the castor bean toxin ricin; and soybean, peanut, and wheat gluten protein allergens. With respect to allergens, the rearranged proteins may be more digestible, producing a pattern of peptides in the digestive tract, which differs from the native ones. The different mixture of peptides, which lack the allergenic peptide(s), may lower or prevent intolerance. Studies utilizing thioredoxin to reduce the disulfide bonds of proteins, including BBI and KTI, suggest that disulfide-modifying enzymes may be a promising approach to reduce the toxic potential of the diet (*114*−*117*).

Sulfitolysis of Disulfide Bonds. Heat and sulfite may act synergistically in two ways to improve the nutritional quality and safety of soy flour, as reflected in the PER values and pancreatic weights. Sulfite ions can, in principle, cleave protein disulfide bonds to form a thiol anion ($P-S^-$) and an *S*-sulfocysteine derivative ($PS-SO_3^-$) by the following equations:

$$P-S-S-P + SO_3^{2-} \rightarrow P-S^- + P-S-SO_3^-$$

$$P-S-SO_3^- + P-S^- \rightarrow P-S-S-P + SO_3^{2-}$$

The *S*-sulfocysteine can interact further with the generated $P-S^-$ to form a new disulfide bond and SO_3^-. The net effect is a rearrangement of the protein disulfide bonds catalyzed by SO_3^{2-}. Exposure of disulfide-containing trypsin inhibitors to sulfite ions should

Table 11. PER, Nitrogen Digestibilities and Pancreas Weights of Rats Fed Raw, Heated, and Sulfite-Treated Soy Flour (*118*)

diet	PER	digestibility (%)	pancreas wt (% of body wt)
soy flour, raw	1.55	78.3	0.51
soy flour, heated	2.11	81.9	0.43
soy flour, raw + 0.03 M sulfite, heated	2.49	84.0	0.40
casein control	3.44	93.0	0.40

therefore alter their structures and their inhibitory properties in analogy with the cysteine treatments described earlier. This hypothesis was tested by treating soy flour with sodium sulfite and feeding the material to rats (*118*). The treated soy flour contained no measurable amounts of residual sodium sulfite, and its trypsin inhibitor level was zero. This value is difficult to achieve even at high temperatures without marked deleterious effects on protein quality. The sodium sulfite treatment also enhanced in vivo nitrogen digestibility and the nutritional quality of soy flour (Table 11). The table also shows that pancreas weights were elevated in rats fed raw soy flour but not in those fed heated soy flour, with or without sodium sulfite. These findings show that the inexpensive sulfite treatment can facilitate the inactivation of enzyme inhibitors and enhance the nutritional quality of soy flour (*118*, *119*).

In summary, treatment of raw soy flour with L-cysteine, *N*-acetyl-L-cysteine, or reduced glutathione results in the introduction of new cystine residues into soy proteins, with corresponding improvement in nutritional quality and possibly safety. The proteins are modified through formation of mixed disulfide bonds via sulfhydryl−disulfide interchange among added sulfur amino acids, protease inhibitors, and storage proteins. Except for an increase in cystine content, the modified proteins retain the original amino acid composition. This transformation facilitates the heat inactivation of BBI, KTI, and lima bean lectin, leading to increased nutritive value. Exposure of soy flour to sodium sulfite was also highly effective in inactivating inhibitors and was nutritionally beneficial.

IMMUNOASSAYS OF KUNITZ AND BOWMAN−BIRK INHIBITORS OF DIGESTIVE ENZYMES

General Aspects. The following discussion on the development and applications of enzyme-linked immunosorbent assays (ELISA) is based largely on our studies (*120*−*128*). Trypsin inhibitors constitute ∼6% of the protein of soybeans. The inhibitory activity is largely inactivated by conventionally applied heat treatments of soy flour, but 10−20% of the residual activity remains. The standard methods of measuring protease inhibitors in foods by enzyme assays often give inaccurate results with processed samples having low residual activity (*129*, *130*). Moreover, these low activities must be assessed in the presence of nonspecific inhibitors of proteases.

A further complexity is the existence of multiple protease inhibitors: (a) There are three closely related isoforms of KTI, encoded by alleles in a multiple allelic system at one locus (*131*, *132*). The primary structures of the isoforms were compared by Kim et al. (*133*). KTI isoform c (Ti^c) differs from Ti^a in only one amino acid residue, a change from glycine to glutamic acid at residue 55. Although Ti^b retains glycine at position 55, it differs at eight other positions from Ti^a. (b) Several

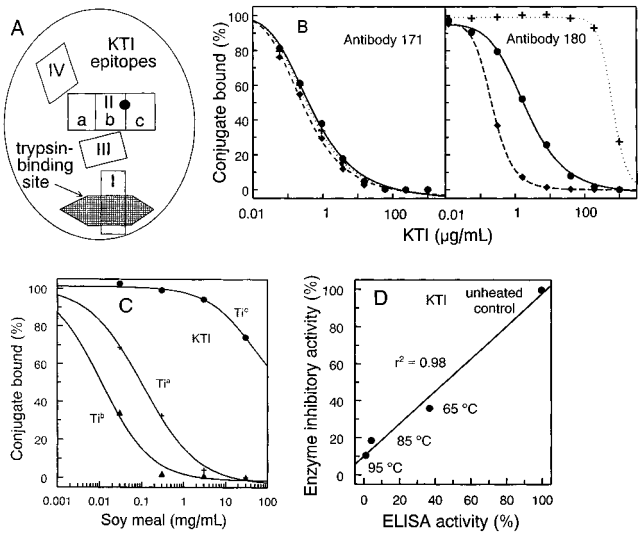

Figure 6. (A) Antibody-binding epitopes in relation to the trypsin-binding site (shaded area). I−IV refer to epitopes, with epitope II further divided into sites a−c. The black dot represents the region of the molecular surface of KTI altered in isoform c due to the substitution of glutamic acid for glycine at residue 55. (B) Competitive binding of KTI isoforms to solid-phase monoclonal antibodies: Ti^a (●); Ti^b (◆); Ti^c (+). (C) ELISA of soy meals from soybean lines expressing each isoform of KTI. (D) Correlation of enzyme inhibitory activity and ELISA of heat-treated KTI (*120*, *123*).

double-headed trypsin inhibitors related to BBI exist in some soybean lines. These as well as KTI may be modified upon germination of the soybean. (c) A glycine-rich inhibitor was identified by Tan-Wilson et al. (*134*, *135*). It is therefore impossible to establish the exact protease inhibitor composition of a sample through enzymatic assay, especially in samples with low residual inhibitor activity, such as toasted soy flours or soy protein isolates. We have found that immunoassays using monoclonal antibodies offer the specificity and sensitivity necessary to analyze complex, processed food samples. These methods could be used to develop improved food-processing strategies for optimizing the content of protease inhibitors in soy foods. In addition, the immunoassays were useful for screening soybean germplasm (*32*, *136*). Below we offer some of our experimental findings in support of these observations.

Immunochemistry of KTI. Antibody specificities were determined by ELISA, using one of several formats: Studies were performed using purified KTI isoforms as well as isolines of soybeans, which express only a single isoform. The results from these studies led us to define the immunochemistry of KTI as shown in Figure 6A.

There are six epitopes, denoted by Roman numerals and lower case letters, corresponding to antibody groups as follows. Group 1 (Epitope I): These antibodies bind poorly to the KTI−trypsin complex. Thus, epitope I overlaps the trypsin-binding site or is affected by allosteric changes, which occur when KTI binds trypsin. Group 2 (Epitopes IIa−c): These antibodies bind to several closely associated sites, denoted by the subdivision of epitope II into three sites. Epitopes IIa and IIc are altered when the glycine at position 55 of isoform a is replaced by glutamic acid (as in isoform c). Epitope IIc is further distinguished from epitopes IIa and IIb by its sensitivity to heat and its proximity to epitope I. Group 3 (Epitope III): These antibodies bind to a site distinct from epitope IIc, but close to epitopes IIa and

IIb. Epitope III is moderately sensitive to heat and to substitution at residue 55, but not as sensitive as epitope IIc. Group 4 (Epitope IV): These antibodies bind to a site that is highly conserved among the three isoforms of KTI. Epitope IV is unaffected by the binding of trypsin and is topographically close to epitope IIb. Examples of the data upon which this model is based are shown in Figure 6B, which illustrates standard curves for the competitive ELISA of the three KTI isoforms using two different monoclonal antibodies. Antibody 171 binds equally to the different isoforms, as illustrated by nearly identical assay curves. Antibody 180 binds better to isoform b than to isoform a, but does not bind isoform c.

The isoform specificity of antibody 180 demonstrated with purified KTI preparations was also obtained using soy meal prepared from soybeans expressing each of the isoforms (Figure 6C). Antibody 180 binds to epitope III, which is labile to mild heat treatment. The immunoassay correlates with enzymatic assays of trypsin inhibition over a wide range of activities (Figure 6D). Antibody 180 can be used to measure *active* KTI isoforms a and b in soy foods containing partially heat-denatured forms. Commercial samples contain mostly isoform a, with some isoform b.

Immunochemistry of BBI. The second major protease inhibitor in soybeans is the low molecular weight BBI (*9*). Native BBI is sufficiently immunogenic in mice to generate high-affinity monoclonal antibodies. The high affinity of antibody 238 resulted in an ELISA sensitivity 100-fold greater than could be obtained with polyclonal antibodies. Our results indicate the presence of two epitopes of BBI. Antibody 238 binds to one epitope, which is altered by heat in parallel to the protease-reactive sites. Antibody 217 binds to a second epitope and is altered even under mild heat conditions not affecting the two BBI reactive sites. An additional monoclonal antibody to BBI, which defines a third epitope, was described by Frokaier et al. (*137*).

The specificity of antibody 238 is illustrated in Figure 7A. The affinity for BBI denatured by treatment with sodium sulfite at 85 °C for 2 h was only 3% relative to native BBI, and cross-reactivity with KTI was ~0.1%. The antibody did not bind to inhibitors from lima beans or chickpeas. Figure 7B shows that there is excellent agreement among the ELISA results and the enzymatic assays, especially in the area of low residual activity. The antibody appears to recognize the native structure of BBI, which is most affected by disruption of disulfides. Monoclonal antibodies were also prepared to BBI with partially reduced disulfide bonds (*138*). These antibodies detected BBI metabolites in the urine of human volunteers. Evidently, after transiting the gut, BBI exists in a partially reduced state. Whether these BBI metabolites are active remains to be shown, but Hogle and Liener (*139*), Friedman et al. (*105*), and Zahnley and Friedman (*106*) described partially reduced BBI and KTI, which retain enzyme inhibitory activity. Immunochemical methods for BBI complement those for KTI and for soy proteins in meat (*140*).

APPLICATIONS OF IMMUNOASSAYS

The inhibition, competition, and sandwich ELISAs we developed provide convenient assays of soy protease inhibitors in plant seeds and foods. The versatility of the assays is illustrated below with processed foods and with seeds of the soybean germplasm collection.

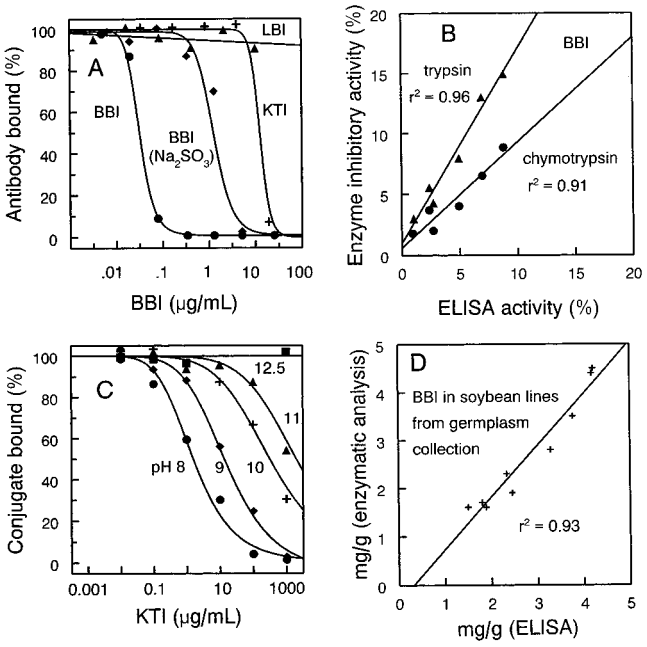

Figure 7. (A) Inhibition ELISA to determine specificity of antibody 238. The reactivity of antibody with BBI was compared with its binding to BBI treated with sodium sulfite at 85 °C, KTI, and lima bean inhibitor (LBI). (B) Correlation of BBI antigenic activity determined by ELISA and enzymatic activity determined by inhibition of chymotrypsin and trypsin. (C) Effects of pH on KTI antigenicity determined by ELISA. (D) Relationship between ELISA and enzyme assays for selected soybean varieties from the germplasm collection (*120*, *122*−*124*).

Processed Soy Products. To assess the protease inhibitor content of toasted flours and to distinguish between KTI and other trypsin inhibitors, we studied soy meal prepared from two isolines of soybeans. The L81-4590 line lacks KTI, whereas the Williams 82 cultivar contains the Ti^a isoform (*32*). After Williams 82 soy meal had been heated for 30 min, the ELISA indicated that only ~1% of the BBI activity remained, whereas 24% of KTI activity could still be measured. The relative stability of KTI under these processing conditions was surprising and was not apparent from consideration of the enzymatic data alone. These results confirmed and extended the findings of Liener and Tomlinson (*141*). Some of the residual inhibitory activity is probably due to other minor protease inhibitors (*134*, *135*) and to nonspecific inhibitors such as phytate and fat. The use of specific monoclonal antibody-based assays of soy protease inhibitors revealed that the matrix in which protease inhibitors are found appears to influence their stability (*32*, *129*).

Chemical changes that may accompany processing of food proteins include cross-linking and browning reactions. In our immunochemical studies, we investigated the effects of heat, high pH, and browning reactions. Figure 7C shows the loss of activity as KTI is treated at progressively higher pH values at 65 °C (*124*). Over 90% of the antigenicity was lost under conditions that have been shown to induce one lysinoalanine cross-link per molecule. In related studies it was shown that heating KTI as a dry powder in the presence of reducing carbohydrates for 50 min at 120° C reduced the antigenicity of KTI by up to 90% compared to control samples lacking carbohydrate (*142*). Nonreducing carbohydrate had a lesser effect. These experiments suggest that food-processing strategies might be developed

Table 12. KTI Content of Commercial Foods Assayed by ELISA (*128*)

product	concn	mg/g of protein	mg/serving[b]
infant formulas			
Prosobee	12.7	0.31	1.2
Soyalac	5.0	0.12	0.5
Isomil	7.5	<0.003	<0.01
Similac	<0.1	<0.003	<0.01
tofu	4.8	0.06	0.54
soy sauce	1.3	0.013	0.02
soy flour (raw)	7750	19	78

[a] KTI concentration is expressed as μg/mL for liquid samples, μg/g for flour and tofu. [b] Serving size was 200 mL of reconstituted formula, 112 g of tofu, 18 g of soy sauce, and 10 g of flour.

Table 13. BBI and KTI Concentrations of Soy Infant Formula[a] by ELISA (*128*)[a]

assay method	BBI (μg/mL)	KTI (μg/mL)
sandwich ELISA	6.3 ± 0.5 ($n = 4$)	6.6 ± 1.9 ($n = 5$)
competition ELISA	7.4 ± 1.7 ($n = 3$)	7.2 ± 1.6 ($n = 3$)

[a] Isomil concentrated liquid (Ross Laboratories, Columbus, OH).

to exploit the beneficial effects of mild nonenzymatic browning or exposure to alkali to inactivate inhibitors selectively.

Tables 12 and 13 list values for inhibitor content of commercial infant formulas and other soy foods determined by ELISA. We conclude that soy formula contains active KTI and BBI at ~0.1% of the levels found in raw soy flour. An infant obtaining 100% nutrition from soy formula would consume ~10 mg of active KTI plus BBI per day. The impacts of soy protease inhibitors are likely to be most pronounced on infants receiving soy-based formula because of the quantity consumed and because the infant's lower gastric acidity and increased intestinal permeability could affect the fate of dietary protease inhibitors in the digestive tract. The health significance of these concentrations remains to be determined (*75*).

Screening of the Genus *Glycine*. An immunochemical study indicated considerable variation of BBI content among varieties of soybeans (Figure 7D). Soybeans with reduced amounts of protease inhibitors could have enhanced value, especially for animal feed, because much of the heat processing could be eliminated. As a first step toward developing soybean cultivars lacking both major protease inhibitors, Domagalski et al. (*136*) screened each accession from the USDA Northern and Southern Soybean Germplasm Collections and additional lines of wild perennial species. A competitive ELISA with antibody 238 was used to identify lines lacking BBI, with confirmation by immunoblotting and sandwich ELISA. To prepare extracts for immunoassay while maintaining seed viability, a 30−40 mg seed chip from each accession was crushed, homogenized in buffer, and clarified by centrifugation. Thus, BBI variants identified by ELISA could be propagated.

All 12370 accessions of the USDA collections were positive for BBI. However, 126 BBI nulls were identified among the 260 samples from wild perennial species. Assays of trypsin and chymotrypsin inhibitory activities were also performed on some of the accessions. All samples, including the nulls, had considerable enzymatic inhibitory activities, which could possibly be attributed to other inhibitors (KTI, the glycine-rich inhibitor, and isoforms of BBI) and to nonprotein inhibitors such as phytate. The immunochemical data show that the germplasm collection provides a resource for the development of new high-BBI as well as low-BBI soybean lines.

Conclusions about Immunoassays. Monoclonal antibody-based immunoassays were developed that can measure low levels of the KTI and BBI in processed foods and could also be used to study the expression and regulation of the genes, which encode these protease inhibitors. These assays could be provided as a kit for use with modest laboratory facilities. In addition to the analysis of foods, the assays could be used to screen soybean germplasm or to monitor the fate of inhibitors in nutritional or other studies. The methods have already proven to be effective in determining the level of BBI in soybean varieties and in identifying varieties lacking BBI. Monoclonal anti-BBI also proved to be effective for immunoaffinity isolation of BBI from extracts of soybeans.

BENEFICIAL EFFECTS OF SOY-CONTAINING DIETS

Cholesterol-Lowering Effects of Soy Diets. Elevated plasma levels of low-density lipoproteins (LDL) and triglycerides present a risk for cardiovascular disease. By contrast, high-density lipoproteins (HDL) are beneficial. A low ratio of LDL to HDL and low plasma triglyceride levels decrease the risk of cardiovascular disease. It has been recognized for some time that consumption of plant proteins often results in significant lowering of LDL and total cholesterol levels. Thus, Mokady and Liener (*143*) found that rats fed soy- and wheat-based diets had lower serum cholesterol concentrations than those on a control casein diet. Total cholesterol was significantly decreased in rats fed five plant proteins (alfalfa, fava, gluten, pea, and soy) compared to those fed three animal proteins (casein, lactalbumin, and ovalbumin) (*144*). It has also been recognized that vegetarians generally suffer less from cardiovascular diseases than do nonvegetarians (*145*). A possible mechanism of the cholesterol-lowering effect of soy protein is its ability to modulate LDL receptor levels in the liver (*146*).

The animal data on hypocholesterolemic effects of soy proteins have been confirmed by numerous human studies. Although statistically significant, the decreases are only ~6−12% of total cholesterol. Some of the recent human studies are summarized below.

On the basis of a food-frequency questionaire given to 4800 Japanese men and women, Nagata et al. (*147*) noted decreasing total plasma cholesterol concentrations with increasing consumption of soy products. In a related study it was found that consumption of soy-based diets induced a significant decrease in plasma levels of LDL cholesterol of both normocholesterolemic and hypercholesterolemic men (*148*). The extent of LDL cholesterol-lowering by soy-based diets was dependent on the concentrations of naturally occurring estrogenic isoflavones (*149*). Potter et al. (*150*) and Washburn et al. (*151*) investigated the role of soy isoflavones on the risk of cardiovascular disease and menopausal symptoms in women consuming soy-based diets. They noted significant improvement in lipid and lipoprotein levels, blood pressure, and vasomotor symptoms. A double-blind study suggests that even partial replacement of animal protein with a soy protein was effective in lowering of plasma cholesterol in 21 severely hypercholesterolemic patients (*152a*). Soy protein 7S subunits seem to activate LDL receptors in the human liver by a mechanism for plasma cholesterol reduction different

from that proposed for other diets and hypolipidemic drugs (*146*, *152b*).

Role of Isoflavones. The presence of soybean isoflavones in soy-based diets can confound interpretations of the effects of soy protein because it has been suggested that the isoflavones themselves could have a role in coronary heart disease prevention (*153*, *154*). However, this aspect appears to be unresolved because the addition of an isoflavone-rich soy extract to casein did not have a lipid-lowering effect in primates (*155*). By contrast, consumption of a high-isoflavone soy diet (128.7 mg/day) by premenopasual women for three menstrual cycles lowered LDL cholesterol by 7.6−10% (*156*). The situation is even more puzzling in view of the observations that consumption by 10 premenopausal Asian women of a diet supplemented with traditional soy foods (32 mg of isoflavones/day) for 7 months resulted in a significant 17.4% decrease in serum luteal estradiol (*157a*). By contrast, the decrease was only 1.2% among 10 non-Asian women consuming the same diet. The reason for this difference in soy-induced changes of hormone levels is not known. It is also noteworthy that although decreases in plasma cholesterol in elderly men consuming soy beverages correlated with total isoflavone concentration, the diets had no effect on elevated levels of prostate-specific antigen (PSA) (*157b*).

The variable results ascribed to isoflavones may be due to the fact that different diets may contain different total amounts as well as different ratios of the 12 isoflavones (3 aglycones, 3 glucosides, 3 acetyl glucosides, and 3 malonyl glucosides; Figure 2). This suggestion is reinforced by the reported range of total isoflavone content between 1161 and 2743 μg/g for 210 soybean cultivars (*158*), a 2.5-fold variation from lowest to highest values. The individual isoflavones may differ in biological potency, and certain combinations may act synergistically or antagonistically, as described elsewhere for the interaction of potato glycoalkaloids at cell membranes of frog embryos (*159*, *160*). The concurrent consumption of additional phytoestrogens present in other legumes (biochanin A, coumesterol, and formononetin) (Figure 2) could also impact the results, especially if interactions of mixtures of two or more isoflavones occur at cell receptor sites.

The U.S. Food and Drug Administration (FDA) has approved the use of health claims on product labels about the role of soy protein in reducing the risk of coronary heart disease (*161*, *162*).

We observed a large reduction in LDL and triglyceride plasma levels in hamsters fed tomato diets (*163*−*165*). Factors that may explain this observation include fiber, antioxidants, free amino acids, the similarity of the amino acid composition of the tomato to that of soy proteins (*166*), and the presence of noncholesterol sterols such as tomatine, which can form an insoluble complex with cholesterol in vivo. The complex is then eliminated in the feces. These considerations suggest that diets rich in both tomato and soy may be especially effective in lowering cholesterol and triglycerides in humans.

Soybean Bowman—Birk Inhibitor as an Anticarcinogen. Factors in soy foods that may inhibit tumors in rats include phytoestrogens (diadzein and genistein), deficiency of methionine, and enzyme (protein kinase) inhibitors (*167*). Troll et al. (*168*) cite possible pathways by which dietary protease inhibitors might contribute to the prevention of human cancer. They may block the formation of active oxygen species by stimulated neutrophils, inhibit tumor promotion, and prevent the digestion of proteins to amino acids, thus depriving rapidly growing cancer cells of essential amino acids. The pioneering studies by these investigators on the reduction of breast and skin tumors in rodents by dietary protease inhibitors stimulated extensive studies on possible beneficial anticarcinogenic effects of BBI in animals and humans. Some of these are briefly outlined below.

Evidence from case-control studies suggests that consumption of soy-containing products may protect against breast cancer in women (*169*). Whether the protection is due to the interaction of soy isoflavones such as genistein with estrogen receptors (*170*) or to other factors such as BBI and phospholipids (*171*) is not known. The possible involvement of BBI is indicated by in vitro studies showing that the inhibitor suppresses the production of superoxide anion free radicals in HL-60 differentiated cells (*172*), potentiates radiation- and cisplatin-induced killing of human (breast, cervical, head and neck, lung, and ovarian) cancer cells (*173*), and also suppresses the growth of human prostate cancer xenografts in nude mice (*174*). The suppression of colon carcinogenesis in mice by BBI may be due to the observed uptake of the inhibitor by intestinal epithelial cells (*175*). Such internalization of BBI by colonic cells could facilitate the inhibition of intracellular proteases associated with the transformation of normal to malignant cells. Studies in progress have begun to address whether the reported activity of BBI in cell culture and in animals is relevant to human therapy.

The protective effect of BBI in human oral cancer (leukoplakia) may be due to the inhibition of serine proteases, which cleave the *neu* oncogene protein, a cell surface biomarker for human cancer (*176*). A possible consequence of protease inhibition is the accumulation of the *neu* protein on the cell surface, leading to enhanced immune recognition of the cancer cells. This in turn permits more efficient destruction of tumors by cytotoxic lymphocytes and natural killer cells. The selective inhibition of serine proteases important for the growth of tumor cells may be a key step in cancer prevention by BBI. The effect of BBI may also be related to its ability to reach intracellular target molecules. A phase I clinical trial with 24 oral leukoplakia patients revealed that a single-dose consumption of BBI equivalent to up to 800 chymotrypsin inhibitor units was well-tolerated (*177*), paving the way for further studies.

Although significant amounts of BBI fed by gavage to mice were absorbed from the digestive tract and distributed into various organs within 3 h (*178*), BBI is probably more stable in the oral cavity than in the digestive tract. Expectations are that BBI is less readily hydrolyzed by saliva than in the digestive tract. Thus, its therapeutic action in the oral cavity may therefore be of longer duration than in the digestive tract or other organs. Results from future studies will undoubtedly better define the potential usefulness of BBI and of soy-based diets in human oncology. However, as mentioned earlier, a word of caution is in order. Possible adverse consequences of the long-term consumption of dietary trypsin and chymotrypsin inhibitors are as yet unresolved (*95*−*97*, *179*−*181*). From the discussion in the cited references, it appears that the susceptibility of the rat pancreas may not be relevant to humans consuming low levels of inhibitors.

Although the biochemical and molecular bases for the beneficial effect of BBI need to be further elucidated, it raises the question of how a protein can prevent cancer. One possibility is that the inhibitor−protease complexes formed in vivo can act as free radical traps whereby the free electrons of hydroxyl or other radicals that can damage DNA are dissipated to sulfur atoms of the cystine-rich inhibitors or complexes (*172*). The high sulfur amino acid content of the BBI is similar to that of human hair and wool (*182*, *183*) with one of every five residues being half-cystine. Another possibility is that the insoluble inhibitor complex acts as an insoluble dietary fiber, physically adsorbing carcinogens during passage through the digestive tract, thus preventing induction of colon cancer.

Other Beneficial Effects. Numerous other benefits are reported to be associated with soy-based diets. Thus, in relevant studies the following conclusions were drawn: (a) Feeding of pasta made of durum wheat supplemented with defatted soy flour or soy flour supplemented with 0.3% methionine significantly lowered both serum cholesterol and glucose levels in hypercholesterolemic diabetic albino rats (*184*). (b) Lowering of fat gain in mice fed soy protein isolate supplemented with cornstarch may be the result of reduction in energy intake and in plasma glucose levels (*185*). (c) Soy protein may be useful in energy-restricted diets for the treatment of obesity (*186*). (d) Soybean inhibitors protected mice against induced pancreatitis (*187*). (e) Soybean, but not casein, diets protected rats against gastrointestinal mucosal injury caused by the widely used drug methotrexate (*188*, *189*). (f) BBI protected *Escherichia coli*-infected rats against nephrotoxicity caused by the antibiotic gentamycin (*190*). (g) A soy-based diet slowed the progression of chronic renal failure in humans (*191*). (h) A phospholipid that copurifies with BBI exhibited antiapoptotic activity; that is, it protected the cells against programmed cell death (*171*). (i) Although phytic acid in soy-based diets could adversely affect mineral utilization, a fermented soybean preparation prevented bone loss in ovariectomized rats (*192*, *193*), presumably by decreasing calcium excretion and/or liberation of phosphorus from phytates during fermentation of the soybean product. (j) Soy isoflavones attenuated bone loss from the spine of premenopausal women (*194a*,*b*). (k) Adding soy flour to hamburger meat reduced the heat-induced formation of carcinogenic heterocyclic amines (*195*). (l) The soy polypeptide, lunasin, was found to inhibit mitosis in mammalian cells (*196*). Whether lunasin potentiates the anticarcinogenic effects of BBI concentrates awaits further study.

ROLE OF SOYBEAN LECTINS IN THE DIET

Liener (*197*−*199*) discovered a class of bioactive glycoproteins in soybeans, which agglutinate red blood cells, the so-called hemagglutinins or lectins. Although the lectins inhibited the growth of rats, removal of the lectins from a soy extract by passage through a column of concanavalin A did not result in significant improvement in the PER. The lectins are largely inactivated by heat treatment of the soybean meal. However, lectins in other legumes such as black beans, kidney beans, and lima beans are not as heat-labile as the soybean lectins (*38*, *200*). They are also more growth-inhibiting in rats than the soybean lectins. As mentioned earlier, unlike lima bean lectins, soybean lectins do not contain disulfide bonds and their hemagglutinating activity was not affected by SH-containing amino acids (*113*).

Because soybean lectins have a strong affinity for carbohydrates on cell surfaces, they are used in biochemistry and medicine in a variety of applications. These include fractionation of bone marrow cells (*201*), treatment of ulcerative colitis (*202*), as a diagnostic reagent for stomach cancer (*203*), and to quantitate *Bacillus anthracis* microorganisms (*204*). These observations suggest that the possible effects of residual levels of lectins that may be present in soy foods merit study.

RESEARCH NEEDS

The relationship between dietary content and disease is a major concern for human health. Epidemiological evidence suggests that dietary factors may have both adverse and beneficial effects in various diseases. The mechanisms of these effects are largely unknown. In view of the increased interest in soy-based foods, these considerations suggest the need to develop plant genetic and food-processing strategies to define the relationship between specific diet components such as soy proteins and human health, especially the mechanisms of beneficial cholesterol-lowering and anticarcinogenic effects of soy proteins and other soybean components. Finding answers to the following questions will benefit the human diet:

- Will isoforms of BBI (*134*, *135*) have different anticarcinogenic potencies?
- Will inhibitors that are structurally similar to BBI, such as the bromelain inhibitor from pineapple stems (*205*), behave as anticarcinogens?
- Will inhibitors present in other foods such as winged beans (*206*) and potatoes (*207*) enhance the anticarcinogenic effects of BBI?
- Will cholesterol-lowering plant foods such as tomatoes (*163*) enhance the cholesterol-lowering effects of soy proteins?
- Will adding antimicrobial spices such as cinnamaldehyde (*208*) to soy-containing foods (*209*, *210*) protect the food or the consumer against infection by human pathogens?
- Will soy proteins protect against gastrointestinal mucosal injury caused by human pathogens and drugs (*188*, *189*)?
- Can new soybean varieties be created with low inhibitor content for use in soy infant formulas and with high content for use in medicinal foods (*132*, *136*)?
- Will the soybean plant compensate for the overexpression of sulfur-rich, high-methionine albumin proteins by synthesizing less of the sulfur-rich BBI (*42*, *43*)?
- Can the inhibitor content of soybeans be altered to protect the plant against insects and human pathogens (*211*)? It is worth noting that the composition and nutritional value of a glyphosphate-tolerant, pest-resistant soybean variety was comparable to that of the parental line (*212*).
- Are soy isoflavones such as genistein stable to food-processing conditions (*213*)?
- What is the dietary significance of a biologically active protein (soyatoxin) present in Brazilian soybean lines (*214*, *215*)?
- Is it possible to compile a comprehensive database of the content of inhibitors and phytoestrogenic compounds in soy and other foods to facilitate epidemiologic and other studies (*14*, *75*, *158*)?

• Is it possible to prevent occasional co-harvesting of soybeans with so-called toxic weed seeds (*216*−*221*)?

Understanding of the overlapping chemical, nutritional, and pharmacological aspects of soy protein nutrition will provide valuable insight into plant physiological, biochemical, immunochemical, nutritional, and medical aspects of soy proteins. This, in turn, can lead to better and safer foods and feeds and improved human health. To achieve this worthwhile goal, there is a need for better interaction and coordination among different disciplines including plant and food scientists, nutritionists, biomedical scientists, and physicians—everyone interested in enhancing the nutritional and medical value of soy-based diets. We are challenged to respond to this need.

ABBREVIATIONS USED

BBI, Bowman−Birk inhibitor; CCK, cholescystokinin; ELISA, enzyme-linked immunosorbent assay; GSH, reduced glutathione; HDL, high-density lipoprotein; IgE and IgG, immunoglobulins E and G; KTI, Kunitz trypsin inhibitor; LAL, lysinoalanine; LBI, lima bean inhibitor; LDL, low-density lipoprotein; NAC, *N*-acetyl-L-cysteine; NPU, net protein utilization; PER, protein efficiency ratio; SBA, soybean agglutinin.

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