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Immunoassays of Soy Proteins

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Proteins of soybeans (*Glycine max*) are widely used in animal and human nutrition. In addition to the bulk of the seed storage proteins, which are classified as albumins and globulins, ∼6% of soybean proteins are classified as inhibitors of trypsin and chymotrypsin and ∼0.5% are sugar-binding lectins. The two major classes of inhibitors are the Kunitz trypsin inhibitor, which inhibits trypsin, and the Bowman−Birk inhibitor (BBI), which inhibits both trypsin and chymotrypsin. Unless removed or inactivated, these inhibitors and lectins can impair the nutritional quality and safety of soy-based diets. On the other hand, several studies suggest that BBI can also function as an anticarcinogen, possibly through interaction with a cellular serine protease. Good-quality soybean proteins contribute to the nutritional value of many specialty foods including infant soy formulas and milk replacers for calves, and provide texture to many processed foods. However, they may also induce occasional allergic responses in humans. This paper outlines immunoassays developed to analyze for soy proteins in different soybean lines, in processed foods, and in nonsoy foods fortified with soy proteins. An assessment of the current status of immunoassays, especially of enzyme-linked immunosorbent assays for soybean inhibitors of digestive enzymes, soy globulins, and soy lectins, demonstrates the usefulness of these methods in plant and food sciences and in medicine.

KEYWORDS: Bowman−Birk inhibitor; immunoassay; Kunitz trypsin inhibitor; nutritional improvement; soy germplasm; soy protein; soybean agglutinin; soy lectin; glycinin; β-conglycinin

INTRODUCTION

Soy protein is a major component of the diet of food-producing animals and is increasingly important in the human diet. Adverse nutritional and toxicological effects following consumption of raw soybean meal have been attributed to the presence of endogenous inhibitors of digestive enzymes, lectins, and 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. Trypsin inhibitors constitute ∼6% of the protein of soybeans; lectins, ∼0.5%. There are also minor components, including closely related isoforms of the major polypeptides. Most commercially heated meals retain up to 20% of the Bowman−Birk inhibitor (BBI) of chymotrypsin and trypsin and the Kunitz inhibitor of trypsin (KTI). It may be preferable to retain BBI in foods, however, because its anticarcinogenic and anti-inflammatory effects have been demonstrated in a number of systems (*1*, *2*). The standard methods of measuring protease inhibitors in foods by enzyme assays often give inaccurate results with processed samples having low residual activity (*3*, *4*). Moreover, these low activities must be assessed in the presence of other proteases (*5*, *6*) and nonspecific inhibitors of proteases, such as phenolic compounds (*7*) and fats (*8*).

Lectins are sugar-specific proteins or glycoproteins that have cell-agglutinating activity (*9*). They bind to sugar structures of cells with a specificity and affinity similar to those of antibody−antigen reactions. The major lectin (hemagglutinin) of soybeans, often referred to as soybean agglutinin (SBA), was first characterized by Liener and Pallansch (*10*). This pioneering work led ultimately to an explosion of interest in lectins, with major impacts on research in nutrition, plant physiology, cell biology, and medicine. Immunochemical methods have been important in elucidating the physiological role of lectins, thought to involve specific recognition of carbohydrates on the surface of rhizobial symbionts. This methodology is especially appropriate for investigating lectin localization in plant tissues, which is probably crucial to their physiological function, as well as to their impact on animal and human nutrition. The antinutritional effects of lectins were summarized by Liener (*11*). Because SBA is relatively heat-labile, the measurement of residual activity in thermally processed foods is not a significant issue.

The major soybean albumins and globulins account for ∼90% of soy protein and therefore the vast majority of the amino acid nutrients. However, some of the globulins are poorly digestible and have been associated with soy intolerance and allergy, particularly in human infants and calves (*12*, *13*). Recent immunochemical studies of the major globulins (e.g., ref *14*) have elucidated their structural features and the time course of their digestion in vivo and in vitro.

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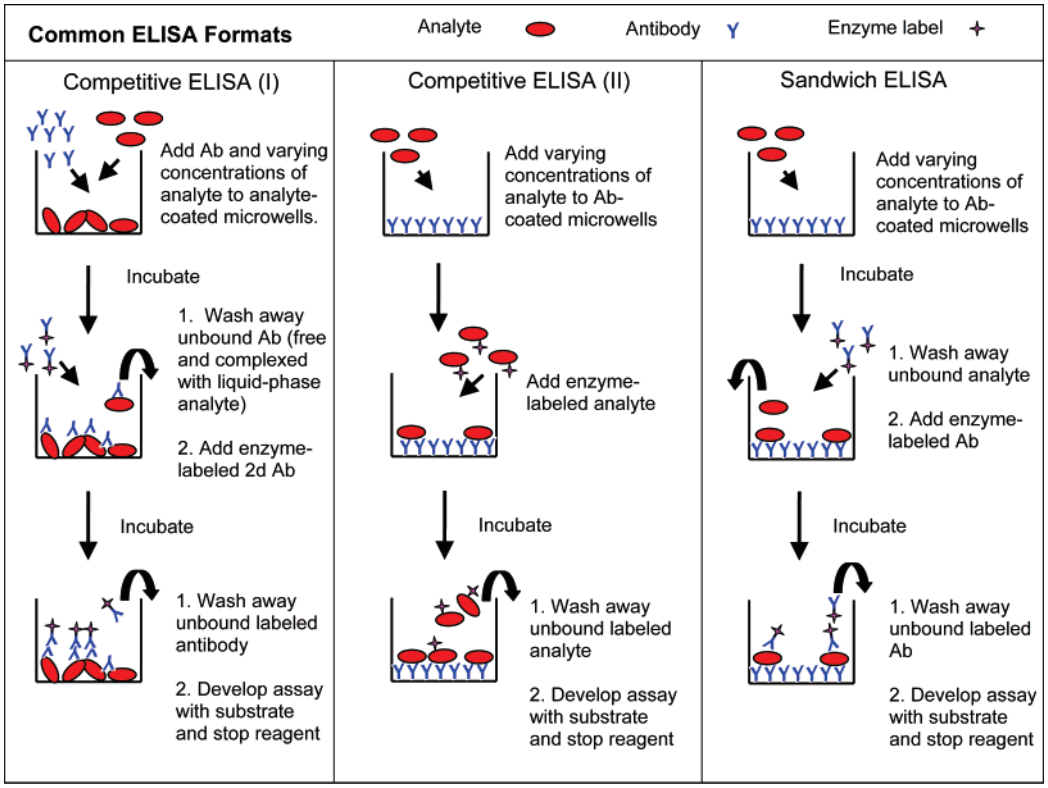

Figure 1. Three common ELISA formats. Proteins for coating plates are typically diluted to 5 μg/mL in phosphate-buffered saline or a more alkaline carbonate buffer. Diluents for other steps usually include detergent and protein (e.g., serum albumin) to reduce nonspecific binding. Washes can be distilled water or a buffered detergent solution. Competitive ELISA format I can be performed using unfractionated antiserum, even with low titer (e.g., diluted 1:100–1:1000). It is most commonly used in screening assays. The assay depends on the availability of purified analyte that can be coated on the plastic wells. It is usually a longer procedure than the other formats because an additional incubation step is added. A labeled second antibody, typically an anti-IgG antibody from a second species, is used to detect the bound first antibody. For example, enzyme-conjugated rabbit anti-mouse IgG can be used to detect mouse antibody. Competitive ELISA format II uses antibody either directly adsorbed onto the plastic wells or bound via a first layer of anti-immunoglobulin. This format is generally very rapid, owing to the single incubation step. However, it is possible for the analytical matrix to affect enzyme activity. If this occurs, an alternative enzyme or format must be used. The sandwich ELISA is useful for analyzing proteins with repeating epitopes or with two different epitopes which can bind two antibody molecules simultaneously. Sandwich ELISA is conducted on plates prepared as for competitive ELISA format II. The immobilized antibody (the "capture" antibody) is often a polyclonal, and the detection antibody is often a purified monoclonal antibody, conjugated to an enzyme.

IMMUNOASSAY METHODS

To set the following discussion in perspective, we will briefly outline the characteristics of the most widely used immunoassays, enzyme-linked immunosorbent assays (ELISAs). ELISAs were first reported by Engvall and Perlmann (*15*), who adapted the radioimmunoassay (RIA) procedure of Yalow and Berson (*16*) by utilizing an enzyme label and creatively exploiting apparent nonspecific binding of antibodies to test tubes. The simple acronym, ELISA, belies the multitude of formats and variations that exist. Three of the most common formats using plastic microwells, usually in an 8 $\times$ 12 array, are illustrated in **Figure 1**. Horseradish peroxidase (HRP), alkaline phosphatase, and β-galactosidase are the most commonly used enzyme labels. Generally, an ELISA can be completed in 1−3 h, with a sensitivity in the parts per billion range. Experimental details for ELISAs and related immunochemical methods are described in Crowther (*17*).

STRUCTURE AND IMMUNOCHEMISTRY OF THE KUNITZ SOYBEAN TRYPSIN INHIBITOR

There are three closely related isoforms of KTI, encoded by alleles in a multiple allelic system at one locus (*18*). The primary structures of the isoforms were compared by Kim et al. (*19*). KTI isoform c differs from isoform a in only one amino acid residue, a change from glycine to glutamic acid at residue 55. Although isoform b retains glycine at position 55, it differs at eight other positions from isoform a.

Monoclonal antibodies (MAbs) were developed and specificities were determined by ELISA, using one of several formats (*20*−*22*): (a) direct binding of antibody to solid-phase KTI; (b) competitive ELISA format I, in which liquid-phase sample inhibits binding of antibody to the solid-phase KTI; (c) competitive ELISA format II, in which liquid-phase sample and enzyme-labeled KTI compete for solid-phase antibody-binding sites; and (d) epitope mapping, in which labeled and unlabeled monoclonal antibodies compete for solid-phase KTI epitopes. Studies were performed using purified KTI isoforms as well as isolines of soybeans that express only a single isoform. All of the results were reproduced in several ELISA formats. The results from these studies led us to define KTI immunochemically, as shown in **Figure 2**.

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 is assumed to overlap the trypsin-binding site or be affected by allosteric changes that 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

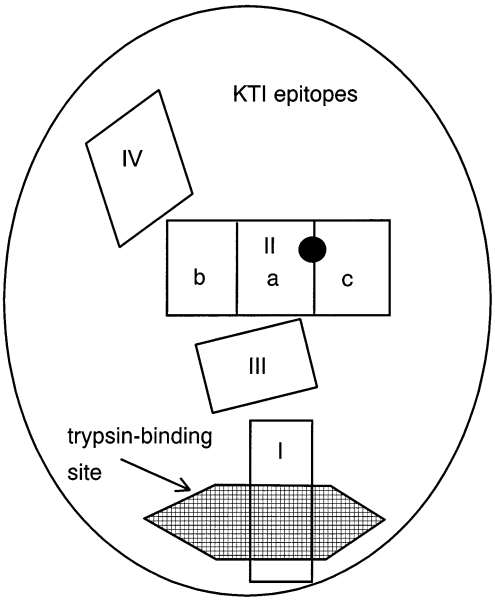

Figure 2. Antibody-binding epitopes in relation to the trypsin-binding site (shaded area). Numerals I–IV refer to epitopes, with epitope II further divided into sites a–c. The black dot represents the region of the molecular surface of KTI altered in isoform c due to the substitution of glutamic acid for glycine at residue 55. (Adapted from ref *22*.)

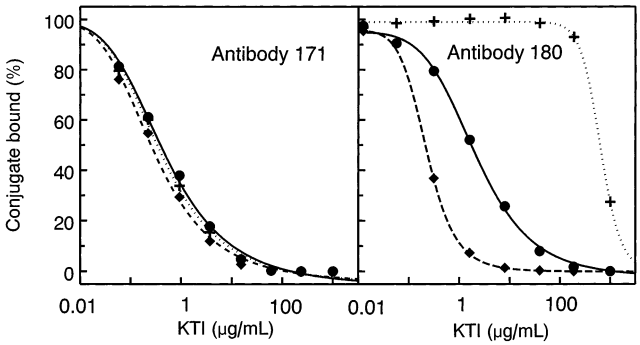

Figure 3. Competitive binding of KTI isoforms to solid-phase monoclonal antibodies: –, isoform a; - - -, isoform b; • • •, isoform c. (Reprinted with permission from ref *22*. Copyright 1986 American Chemical Society.)

when the glycine at position 55 of isoform a is replaced by glutamic acid (as in isoform c). Epitope IIc is further distinguished from 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, as revealed by competitive binding studies. Examples of the data upon which this model is based are shown in **Figure 3**, 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 observed using soymeal prepared from soybeans expressing each of the isoforms (*22*). Antibody 180 binds to epitope III, which is labile to mild heat

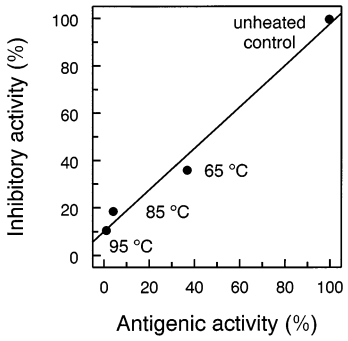

Figure 4. Correlation of enzyme inhibitory activity and ELISA of heat-treated KTI ($r^2 = 0.98$). (Reprinted with permission from ref *21*. Copyright 1988 Institute of Food Technologists.)

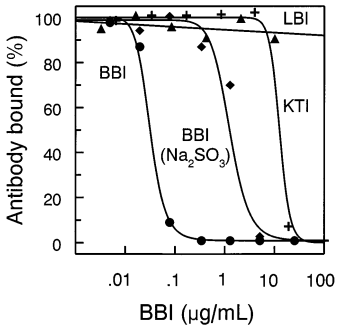

Figure 5. ELISA to determine specificity of antibody 238. The reactivity of antibody with BBI was compared with its binding to lima bean inhibitor, KTI, and BBI denatured by treatment with sodium sulfite at 85 °C. (Adapted from ref *24*.)

treatment and produces an assay that correlates with enzymatic estimation of trypsin inhibition over a wide range of activities (**Figure 4**). We have used antibody 180 to measure *active* KTI isoforms a and b in soy foods containing significant amounts of partially heat-denatured forms. Commercial samples contain mostly isoform a, with some isoform b.

IMMUNOCHEMISTRY OF THE BOWMAN–BIRK INHIBITOR

The second major protease inhibitor in soybeans is the low molecular weight BBI, reviewed by Birk (*23*). Despite earlier reports that BBI must be heat-modified to enhance immunogenicity, unmodified BBI proved to be sufficiently immunogenic in mice to generate high-affinity monoclonal antibodies (*24*–*26*). 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 epitope I, which is altered by heat in parallel to the protease-reactive sites. A second heat-labile BBI-specific monoclonal antibody, antibody 217, binds to epitope II and is altered even under relatively mild conditions that do not affect the two BBI protease-reactive sites. A third epitope can be deduced from the results of Frokiaer et al. (*27*).

The specificity of antibody 238 is illustrated in **Figure 5**. 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. There is excellent agreement among the ELISA results and the enzymatic assays, especially in the area of low residual activity (*24*). The antibody appears to recognize the native structure of BBI, which is readily inactivated by disruption of disulfides. The antibody is also

useful when immobilized for affinity chromatography and can be used to detect BBI in immunoblots (*28*). Gladysheva et al. (*29*) have recently used immunoaffinity chromatography and ELISA methods to quantify BBI in different cultivars.

Monoclonal antibodies were also prepared to BBI with partially reduced disulfide bonds (*30*). Although these antibodies were derived from cell lines obtained serendipitously when partially reduced BBI was used as a "carrier" in a hapten conjugate (*31*), they proved to be useful for quantifying BBI metabolites in the urine of human subjects after consumption of soy milk containing up to 175 mg of BBI (*32*). Evidently, after transiting the gut, BBI exists in a partially reduced state and could be bioavailable in organs outside the gastrointestinal tract. Whether these BBI metabolites are active remains to be shown, but Friedman et al. (*33*) and Hogle and Liener (*34*) described partially reduced BBI, which retains enzyme inhibitory activity.

IMMUNOCHEMICAL STUDIES OF SOYBEAN LECTINS

Pueppke et al. (*35*) used several analytical techniques, including radioimmunoassay (RIA), to quantify SBA in growing soybean plants. They observed that the lectin level became undetectable by 12−15 days after planting, a time when nodulation could still occur. Su et al. (*36*) also demonstrated the rapid decline of lectin in the developing plant as well as the poor correlation between nodulation capability with *Rhizobium* and the apparent content of the major soybean lectin. Although the role of SBA and other lectins in rhizobial symbiosis remains poorly understood, Gibson et al. (*37*) used RIA to demonstrate an additional possible role of SBA in the resistance to pathogens. Vodkin and Raikhel (*38*) used immunocytochemistry, immunoblotting, and heterologous sandwich ELISA (i.e., using two different antibodies) to study the distribution of soybean lectin in seeds and roots. These experiments also included immunochemical localizations of 11S glycinin and KTI. Interestingly, KTI was detected immunochemically in roots, but it was not determined whether roots also contain BBI. When the localization of SBA was determined in varieties lacking the seed lectin, the lectin was found in roots, but not cotyledons. Kishinevsky et al. (*39*) used homologous sandwich ELISAs (i.e., utilizing the same antibody for both capture and detection), with sensitivity over 100-fold greater than hemagglutination assays to detect lectins from pea and soybean. Miller et al. (*40*) reported a sandwich ELISA for SBA, using a monoclonal to detect the captured analyte, with a detection limit of ~0.3 ng/mL.

Following the above-mentioned studies designed to discover the distribution of lectins in plant tissue, studies began to appear on the applications of SBA in other biological systems. Those that used immunoassays are briefly outlined below. Hajos et al. (*41*) used ELISA and rocket immunoelectrophoresis to quantify SBA in washings of stomach and intestinal mucosa following treatment of rats by intragastric intubation. In agreement with earlier results, these investigators found that >90% of SBA is bound to the intestinal mucosa but could be recovered by washing the mucosa with saline containing galactose. It should be noted that these authors also studied the distribution of protease inhibitors in these washings, exploiting the ability of their rabbit antibodies to bind to inhibitors even when complexed with proteases. In our studies using monoclonal antibodies, we also had found that some antibodies can bind KTI or BBI even in complexes with proteases (*22*, *24*). It can be expected that the specificity of monoclonal antibodies for SBA and the enhanced sensitivity of immunochemical methods compared to agglutination techniques will help answer remaining questions about the distribution and function of lectins in legume tissues. In view of the possible beneficial effects of lectins in cancer prevention, immunochemical techniques should prove useful in elucidating lectin distribution and metabolism in animal and human tissues and fluids.

IMMUNOASSAYS FOR THE MAJOR GLOBULINS

Marcone (*42*) has recently reviewed the structural features of plant storage proteins, with particular emphasis on the 11S globulins. Preparation of highly specific antibodies to the soy globulins has opened up new opportunities for characterizing these proteins and their functional properties, digestibility, and involvement in allergy (*43*, *44*).

Immunoassays, including those available as commercial kits, have been developed to detect the presence of soy proteins in various foods (*45*−*48*). ELISA techniques had been able to detect trace quantities of soy protein in soy lecithin, margarine, and soy oil (*49*). A more recent report of the immunochemical detection of soy proteins in ostensibly nonprotein fractions employed polyclonal and monoclonal antibodies to determine that some soybean lecithin products contain allergenic components, such as the acid subunit of glycinin (*40*).

Using MAbs to glycinin and β-conglycinin, Huang et al. (*14*) defined two epitopes of these proteins and studied the thermal stability of the epitopes. Both epitopes appeared to be "continuous" (ability to bind antibody not destroyed by loss of secondary and tertiary structure). In fact, MAb 0025 bound more strongly to thermally denatured glycinin that had a disrupted intersubunit disulfide bond, perhaps because of the greater accessibility of the 19 amino acid epitope in the denatured molecule. The heat stability of soy globulin antigens, in addition to the survival of partially digested polypeptides in the intestinal tract, are consistent with frequent observations of soy allergenicity. Earlier investigators (e.g., ref *50*) postulated that food proteins which resist digestion tend to provoke food allergies, an intriguing but as yet unproven concept. Tukur et al. (*51*) reported that in vitro tests using polyclonal antibodies were more predictive than monoclonal antibody tests for in vivo conditions such as poor digestibility and soy-specific antibody responses. It may be that the best use of immunochemical methods, therefore, would be to produce a battery of MAbs to food proteins of interest. Studies could then be performed with individual antibodies or a defined cocktail of antibodies.

Lalles et al. (*52*) determined that glycinin peptides survived duodenal passage and could be detected in the ileum of preruminant calves up to 10 h after a meal. On the other hand, β-conglycinin immunoreactivity could not be detected under these circumstances. In a related study, Perez et al. (*53*) used antisera reactive with the heat-stable epitopes of soy globulins to investigate the digestion of soy storage proteins. Immunoreactive soy globulins were found in the gut and bound to gut tissues 3 h after rats had been dosed. Analysis by electrophoresis and immunoblotting revealed substantial amounts of partially proteolyzed peptides for most of the subunits, including intact B (basic) polypeptides of glycinin.

In studies of soybean glycinin G1 acidic chain, Beardslee et al. (*54*) mapped the IgE epitopes (sites recognized by the class of immunoglobulins most frequently associated with allergic reactions). They used ELISA and immunoblotting techniques and identified a reactive sequence that is homologous to a previously identified peanut allergen. Although cross-reacting antibodies to peanut and soy allergens have been reported in human allergy sufferers (*55*), their clinical significance is not clear.

Hessing et al. (*56*) compared the antigenicity of various legume proteins, including the major soybean globulin, comparing the results of parenteral immunization in rabbits to oral immunization in calves. Antibodies to glycinin and β-conglycinin were present in both preparations when analyzed by immunblotting, but the orally induced antibodies bound to several other unidentified proteins as well. β-Conglycinin was identified as a trace component of commercially available KTI preparations, a possible confounding factor in earlier studies of allergenicity.

A significant problem to handlers of soybeans is the potential for the hull dust to provoke asthma. Codina et al. (*57*) demonstrated that heat processing of soybean hulls generates new antigens, as determined by reactivity with sera from soybean asthmatic subjects. Gonzalez et al. (*58*) used MAbs to the major soybean allergen of soybean hulls to develop a heterologous sandwich ELISA that could detect the allergen in samples recovered from air filters. Although both cross-reactive and unique allergens among closely related species have been described, for example, soy and peanut (*55*), there can even be significant cross-reactivity of plant components from diverse sources. For example, Rihs et al. (*59*) defined a new soybean pollen allergen that cross-reacts with profilin from birch pollen. The report of a patient with allergy to KTI (*50*) has been echoed in more recent studies (*60*, *61*). The latter report documented serum IgE, reactive with both KTI and lipoxidase, in bakers who were suffering from workplace-related respiratory symptoms. It had previously been observed that patients with wheat-induced asthma produced antibodies that cross-reacted with components of soy flour (*62*).

APPLICATIONS OF IMMUNOASSAYS

TI Content of Processed Soy Products. To assess the protease inhibitor content of toasted flours and to distinguish between KTI and other trypsin inhibitors, we studied soymeal prepared from two isolines of soybeans. The L81-4590 line lacks KTI, whereas the Williams 82 cultivar contains the Ti^a isoform (*63*). ELISA analysis indicated that in the Williams 82 sample only ~1% of the BBI activity remained, whereas 24% of KTI activity could still be measured after 30 min. 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 by Liener and Tomlinson (*64*). Some of the residual inhibitory activity measured enzymatically is probably due to other minor protease inhibitors (*5*, *6*) and nonspecific inhibitors such as phytate and fat. However, the use of specific monoclonal antibody-based assays of soy protease inhibitors confirmed that the matrix in which protease inhibitors are found appears to influence their stability (*3*, *63*). A related monoclonal antibody-based immunochemical technique for measuring low residual levels of active KTI and BBI in processed foods was reported by van Amerongen et al. (*65*). This method detects inhibitors captured by proteases immobilized in microtitration wells. The remarkable sensitivity of immunochemical techniques, as well as the ability of KTI and other allergenic soy proteins to contaminate nonprotein fractions, was documented by the detection of KTI and other soy proteins in lecithin using IgE antibodies from allergic patients (*66*).

Genetic Identity of Soybean Seeds. The importance of being able to verify the genetic identity of food ingredients such as soybeans has been highlighted most recently by the controversy surrounding the introduction of "genetically modified organisms" (GMOs) and the demands that foods containing engineered ingredients be identified or, in the extreme, be banned outright. Our work with soy protease inhibitors, although not involving genetically engineered varieties, demonstrated the power of immunoassays to detect selected varieties and to detect the effects of cross-pollination on expression of traits removed in the nulls. As shown in **Figure 6**, soybean meal prepared from a conventional KTI-containing cultivar, Williams 82, can readily be distinguished from the Ti-null cultivar, L81-4590 (*63*). However, it is clear that the amount of KTI present in seeds of the null cultivar depended on where it was grown, presumably due to cross-pollination. The lowest level of KTI was found in soybeans from plants grown in the greenhouse, more isolated than the two farm fields.

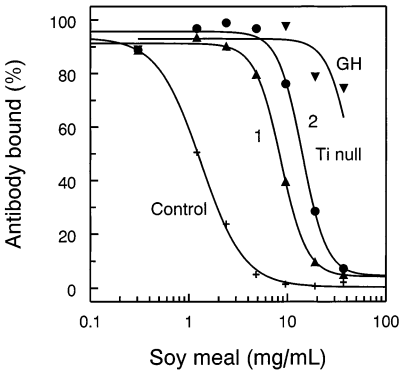

Figure 6. Analysis of KTI in soybean meal by ELISA using antibody 180. Williams 82 was analyzed (control), along with the Ti-null L81-4590 grown on experimental plot 1 or 2 or in a greenhouse (GH). (Adapted from ref *63*.)

Food-Processing Effects. Chemical changes that may accompany the processing of food proteins include cross-linking and browning reactions. In our immunochemical studies, we investigated effects of heat, high pH, and browning reactions on antigenic activity. KTI loses activity as it is treated at progressively higher pH at 65 °C (*21*). We hypothesized that cross-linking contributes to inactivation because >90% of the antigenic change occurred under conditions expected 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 (*67*). Nonreducing carbohydrate had a lesser effect. These experiments suggest that food-processing strategies might be developed to exploit the beneficial effects of mild nonenzymatic browning or exposure to alkali to inactivate inhibitors selectively.

Recent studies by Babiker et al. (*68*) also suggest that antigenic sites of soy proteins can be altered by reaction with carbohydrates or by enzyme-induced cross-linking. Another example of processing-induced antigenic changes was reported by Saitoh et al. (*69*). These workers found that passage of soybean hypocotyl meal through a twin-screw extruder reduced soy globulin antigenicity to 1% of its original activity. This observation appears to conflict with other reports of the stability of these antigens, but it is possible that this particular bovine antiserum may recognize labile epitopes selectively or that enzymes present in the hypocotyl meal may destroy most of the epitopes under the processing conditions.

Trypsin Inhibitor Content of Soy Foods. Table 1 shows the inhibitor content of commercial infant formulas and other soy foods determined by ELISA. KTI and BBI are found in comparable levels in soy-based infant formulas (*25*), at ~0.1% of the levels found in raw soy flour. An infant obtaining 100%

Table 1. Kunitz Trypsin Inhibitor Content of Commercial Foods Assayed by ELISA[a]

product	concn[b]	mg/g of protein	mg/serving[c]
infant formulas[d]			
Prosobee	12.7	0.31	1.2
Soyalac	5.0	0.12	0.5
Isomil	7.5	0.21	0.75
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] Adapted from Friedman et al. (*74*). Competitive ELISA was conducted in assay wells coated with monoclonal antibody 180, using KTI–horseradish peroxidase (HRP) as labeled ligand. [b] KTI concentration is expressed as μg/mL for liquid samples and as μg/g for flour and tofu. [c] Serving size was 200 mL of reconstituted formula, 112 g of tofu, 18 g of soy sauce, and 10 g of flour. [d] Formulas were analyzed as concentrates. Similac is a nonsoy formula, included as a negative control.

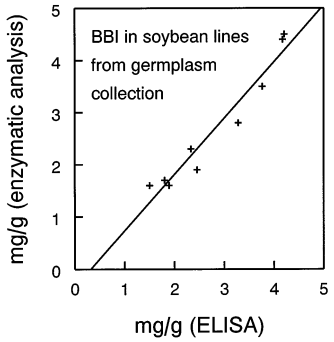

Figure 7. Relationship between ELISA and enzyme assays for selected soybean varieties from the germplasm collection ($r^2 = 0.93$). (Adapted from ref *63*.)

nutriture 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 (*70*).

Screening the Genus *Glycine*. As shown in **Figure 7**, an immunochemical study indicated considerable variation of BBI content among varieties of soybeans. Soybeans with reduced amounts of protease inhibitors could have enhanced value, especially for animal feed, because much of the heat processing could possibly be eliminated. As a first step toward developing soybean cultivars lacking both major protease inhibitors, Domagalski et al. (*28*) screened each accession from the USDA Northern and Southern Soybean Germplasm Collections and additional lines of wild perennial species. The competitive ELISA with antibody 238 was used to identify lines lacking BBI, with confirmation by immunoblotting and sandwich ELISA. To prepare extracts, a 30−40-mg seed chip from each accession was crushed, homogenized in buffer, and clarified by centrifugation. This procedure retained seed viability, so presumptive BBI variants could be propagated.

As shown in **Table 2**, all of the 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 presumptive nulls, had considerable enzymatic inhibitory activities, which could possibly be attributed to other inhibitors (KTI, the glycine-rich inhibitor, 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, depending on need (*18*, *71*).

Table 2. Bowman–Birk Inhibitor in Species of *Glycine* Screened by ELISA[a]

species	no. of accessions tested	BBI-negative	BBI-positive
G. max	11692	0	11692
G. soja	678	0	678
G. argyrea	3	0	3
G. canescens	35	0	35
G. clandestina	47	0	47
G. curvata	1	1	0
G. cyrtoloba	12	11	1
G. falcate	3	0	3
G. latifola	17	17	0
G. latrobeana	1	0	1
G. microphylla	11	10	1
G. tabacina	88	78	10
G. tomentella	102	9	93

[a] Adapted from Domagalski et al. (*28*). A competitive ELISA was performed on extracts (1 mL) prepared from a 30–40-mg seed chip, clarified by centrifugation prior to assay. MAb 238 was coated on assay wells, and BBI–HRP was used as labeled ligand. All presumptive BBI-nulls were confirmed by immunoblotting.

NEW IMMUNOCHEMICAL TECHNIQUES

Two examples of new immunochemical techniques available to the food industry are quantitative immunohistochemistry to detect soy proteins in meat products (*72*) and surface plasmon resonance (SPR) biosensor detection of nonmilk proteins in milk powder (*73*). In the latter study, rabbit polyclonal antibodies were raised against soy protein isolate (and other vegetable proteins), and the IgG fractions were purified by affinity chromatography and immobilized on the proprietary carboxymethylated dextran-coated chips. The binding of soy protein could be detected directly and specifically in the presence of milk protein, with a detection limit of about one part per thousand.

CONCLUSIONS

Immunoassays of soy proteins have been used in plant science, nutritional studies, and clinical investigations into food allergy. These techniques have enabled rapid qualitative and quantitative detection of soy proteins in complex foods. Immunohistochemistry, immunoblotting techniques, and ELISA technology have facilitated investigation into the role of lectins and other soy proteins in the plant itself and have permitted investigators to characterize changes in protein structure during food processing and digestion. Having contributed to our understanding of the potential health impacts of lectins, protease inhibitors, and globulins, immunochemical methods can be expected to facilitate future studies to improve soybean germplasm and develop more efficient feeds and healthful foods.

ABBREVIATIONS USED

Ab, antibody; BBI, Bowman−Birk Inhibitor; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; GMO, genetically modified organism; KTI, Kunitz trypsin inhibitor; MAb, monoclonal antibody; RIA,

radioimmunoassay; SBA, soybean agglutinin; SPR, surface plasmon resonance

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