Interaction of Monoclonal Antibodies with Soybean Trypsin Inhibitors

David L. Brandon,* Sakhina Haque, and Mendel Friedman

The antigenicity of the Kunitz trypsin inhibitor was studied by use of polyclonal antibodies prepared by inoculating rabbits with native, heat-denatured, and *N*-acetylcysteine-treated Kunitz soybean trypsin inhibitors as well as monoclonal antibodies prepared in mice. Antibodies elicited with the denatured inhibitor were specific for the denatured conformation of the protein. In contrast, native inhibitor elicited antibodies that selectively recognized determinants in both native and heat-treated proteins, but that did not bind trypsin inhibitors treated with *N*-acetylcysteine. These results imply that the disulfide bonds must be intact to maintain the native antigenic conformation and that the inhibitor has at least two distinct antigenic sites (epitopes), one of which is retained under denaturing conditions. They also suggest that monoclonal antibodies can be used to measure processing-induced structural changes in food proteins that may alter allergic reactions.

Soybeans are widely used as a human food, as a component of animal feeds, and as a major export commodity (Wolf, 1977). Soybeans contain inhibitors of proteolytic enzymes that adversely affect nutritional quality and safety (Liener and Kakade, 1980). Since heat treatments used commercially only partially inactivate inhibitor activity (Rackis et al., 1986), a need exists to characterize the inhibitor activity remaining after processing.

The objectives of this study were to define the antigenic sites (epitopes) of Kunitz soybean trypsin inhibitor (KTI), to identify the epitopes that are lost upon denaturation of the inhibitor, and to determine the feasibility of using immunoassays to quantitate the inhibitor and monitor its inactivation. Previous investigators developed immunoassays for KTI (Catsimpoolas and Leuthner, 1969; Catsimpoolas et al., 1969) and noted immunochemical differences between native and heat-treated molecules (Rossebo and Nordal, 1971). However, the epitopes of KTI have not been defined, nor has the additional resolving power of monoclonal antibodies been brought to bear on the problem. The epitope mapping technique, recently applied to the study of α_1-antitrypsin (Herion et al., 1984), seemed an appropriate tool to use for this study. Since the role of disulfides in maintaining the activity of KTI has been well documented (Friedman et al., 1982), we focused on the changes in antigenicity upon heating KTI in the presence and absence of *N*-acetylcysteine. Conditions were selected to produce either partial or complete inactivation of KTI, while avoiding changes that might accompany more severe treatment of the protein.

MATERIALS AND METHODS

Kunitz soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) was characterized by SDS–polyacrylamide gel electrophoresis and by inhibition of trypsin (Friedman et al., 1982). It was used without further purification. Heat-treated and *N*-acetyl-L-cysteine- (NAC-) modified forms of KTI were prepared as described by Friedman and collaborators (Friedman et al., 1982, 1984). Soy flour was a gift from Central Soya, Ft. Wayne, IN. Other chemicals were obtained from commercial sources.

Treatment with Heat and *N*-Acetylcysteine. A sample containing 70 mg of commercial Kunitz trypsin inhibitor and 70 mg of NAC and one containing KTI alone were each dissolved in 10 mL of 0.5 M Tris buffer, pH 8.5. The solutions were heated in a water bath at 65 or 93 °C for 1 h. The contents were then dialyzed in the cold room for 3 days with frequent changes of deionized water and lyophilized. The trypsin inhibitor content, expressed in trypsin inhibitor units (TIU)/milligram (Friedman et al., 1982), was 2.29 for the untreated material, 0.09 for the heat plus NAC-treated sample, 0.84 for the sample treated at 65 °C, and 0.07 for the sample treated at 93 °C. Conditions were selected to produce either partial or complete inactivation of KTI, while avoiding changes that might accompany shorter, more severe treatment of the protein. Samples of soy flour, untreated or inactivated by treatment with sodium sulfite, were prepared as described by Friedman and Gumbmann (1986).

Antibody Preparation. Rabbit antisera were elicited in New Zealand white rabbits by a course of subcutaneous injections of KTI (native or treated) emulsified in complete Freund's adjuvant. Sera were characterized initially by immunodiffusion and, later, by solid-phase enzyme immunoassay (EIA). Mouse antibodies were elicited in Balb/c mice by two intraperitoneal inoculations at 3-week intervals with native KTI emulsified in complete Freund's adjuvant. Antibody production was assessed by solid-phase EIA. Monoclonal antibodies were produced essentially according to Oi and Herzenberg (1980). In brief, an antibody-producing mouse was inoculated intravenously with 50 μg of KTI 3 days prior to cell fusion. Equal numbers of immune spleen cells and NS-1 myeloma cells were fused by treatment with 50% poly(ethylene glycol) 1450 (Bethesda Research Laboratories, Bethesda, MD), and the fusion suspension was dispersed into 96-well tissue culture plates, 0.2 mL/well, at a cell density of 1.5×10^6 cells/mL. Hybridomas secreting KTI-specific antibody were identified by solid-phase EIA. Following transfer and expansion of the cultures, hybridomas were cloned by limiting dilution. Clones were expanded in culture, permitting harvest of supernatant, freezing of cell lines, and preparation of ascites fluid. Antibodies are denoted by the number assigned to the cloned hybridomas, e.g., clone 129 produces antibody 129. Monoclonal antibodies were assayed for isotype and light-chain composition by a solid-phase assay similar to the direct-binding assay, but using isotype- and light-chain-specific antibodies conjugated to β-galactosidase (Southern Biotechnology, Birmingham, AL). Myeloma proteins (Litton Bionetics, Kensington, MD) were used to assess the specificity of these antibodies.

Antibody Purification. Balb/c mice were injected with Pristane (Aldrich Chemical Co., St. Louis, MO) 1 week prior to intraperitoneal inoculation with 10^6 viable hybridomas. High-titer ascitic fluid and sera were obtained from four of six mice within 3 weeks of inoculation. For use in assays, the IgG fraction was purified from ascitic

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Table I. Epitope Mapping Procedure

1. coat assay plate with KTI
2. incubate assay wells with excess unlabeled monoclonal anti-KTI
3. wash, drain plate
4. add biotinylated monoclonal antibody to assay wells (in triplicate), incubate
5. wash, drain plate
6. add peroxidase-labeled streptavidin to all assay wells, incubate
7. wash, drain plate
8. add substrate solution to all assay wells, incubate
9. read absorbance at 415 nm (binding of biotinylated antibody revealed by green color development)
10. calculate binding in presence of unlabeled monoclonal antibody as percent of control performed in absence of unlabeled antibody

fluid or spent tissue culture medium by ammonium sulfate precipitation and chromatography on [(diethylamino)-ethyl]cellulose (DE-52, Whatman, Ltd., Maidstone, U.K.).

Preparation of Biotinylated Derivatives. The *N*-hydroxysuccinimide ester of biotin (Calbiochem, La Jolla, CA) was used to label the partially purified monoclonal antibodies (Wofsy, 1983). The antibodies were purified by dialysis against phosphate-buffered saline (PBS, 5 mM sodium phosphate and 150 mM sodium chloride, pH 7.0) containing 0.01% sodium azide and characterized by titration in a solid-phase enzyme immunoassay using horseradish peroxidase conjugates of rabbit anti-mouse Ig (Zymed Laboratories, South San Francisco, CA) and streptavidin (Amersham Corp., Arlington Heights, IL).

Immunoassays. *Plate Preparation.* Polyvinylchloride assay plates (Costar, Cambridge, MA) were coated with Kunitz trypsin inhibitor (10 μg/mL, 50 μL/well). The plates were incubated for 4 h and then washed with PBS containing 0.05% Tween-20 (Sigma). Remaining sites were blocked by incubating for 1 h with 100 μL/well of 1% bovine serum albumin (BSA, Miles Laboratories, Elkhart, IN) in PBS–Tween solution. Plates were then rewashed and used immediately or stored for up to 1 week in PBS with azide. Assay plates stored longer were less efficient in binding assays.

Direct-Binding Assays. Antibody samples to be assayed were diluted in PBS. The resulting samples (50 μL/well) were applied to KTI-coated plates, generally in triplicate, and incubated with shaking for 2 h. Plates were emptied, washed, rinsed with distilled water, and drained. Labeled reagent (50 μL/well of peroxidase-labeled rabbit anti-mouse IgG or streptavidin), at appropriate dilution in PBS–Tween–BSA, was added and incubated for 1 h with shaking. The wells were again emptied, washed, rinsed, and drained. Next, 50 μL of substrate [1 mM 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) and 6.7 mM H_2O_2 in 60 mM sodium citrate buffer, pH 7.3] was added, and the absorbance at 415 nm was determined on an EIA reader (Model 308, Bio-Tek, Burlington, VT). Assays were generally read after 15 min or terminated by addition of 50 μL/well of 10% sodium dodecyl sulfate.

Inhibition Assays. Antibody and antigen (Kunitz trypsin inhibitor) were mixed and preincubated in microtitration dishes for 2 h at room temperature prior to assay. To maximize sensitivity of the assay, antibodies were used at a concentration within the linear range of the direct-binding titration curve. Aliquots of the preincubation mixture were assayed on KTI-coated plates as described above, except that the incubation with antibody was for 1 h only.

Epitope Mapping Assays. Monoclonal antibodies were tested for their ability to inhibit the binding of other antibodies as outlined in Table I. Unlabeled antibodies were incubated in KTI-coated wells at a standard concentration based on their titers (reciprocal of the dilution giving 0.8 absorbance unit after 15 min under standard assay conditions). Following a 3-h incubation at room temperature, the assay plates were washed, rinsed, and drained and the binding of various biotinylated antibodies was determined. Mutual inhibition of antibodies suggested that the two antibodies bound to the same or nearby sites.

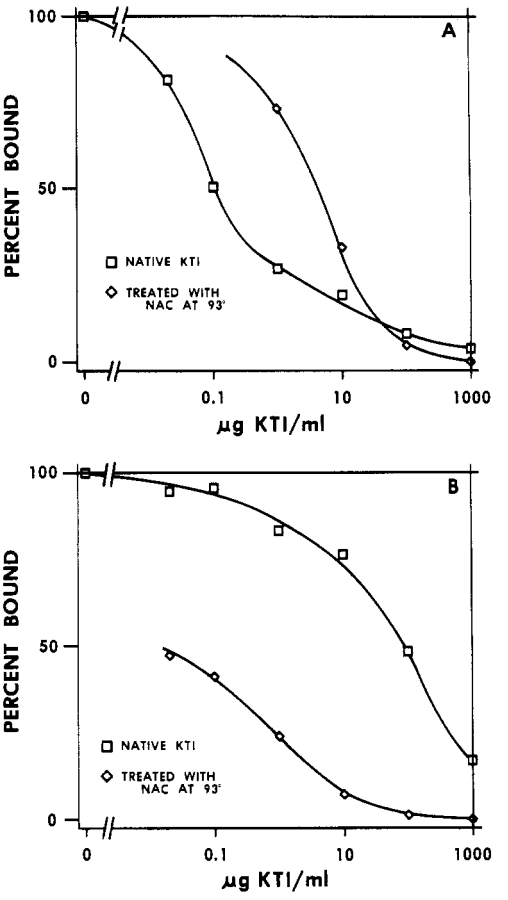

Figure 1. Analysis of native and modified Kunitz trypsin inhibitor with rabbit antibodies: (A) antibodies elicited with native KTI, NAC-treated KTI bound about 50-fold more weakly than native KTI; (B) antibodies elicited with heat-inactivated KTI (10% residual activity), native KTI bound more weakly by 2–3 orders of magnitude compared to KTI heated in the presence of NAC. Competitive-binding assays were performed on KTI-coated assay plates. Trypsin inhibitor was preincubated with antibodies before they were applied to the assay plate. Peroxidase-conjugated goat anti-rabbit IgG was used to detect rabbit anti-KTI bound to the plate.

Immunoassay of Kunitz Trypsin Inhibitor. The assay was conducted in an assay plate coated with antibody 136. Biotinylated KTI was used as the labeled ligand and was visualized with peroxidase–streptavidin. The assay was developed with substrate for 30 min. The amount of biotinylated KTI used yielded an absorbance of 0.5 in the absence of additional unlabeled KTI. Solutions of KTI or suspensions of soy flour in phosphate-buffered saline were mixed with an equal volume of biotinylated KTI, dissolved in buffered saline containing 1 mg/mL bovine serum albumin. Aliquots of 50 μL were applied to the assay plates, which were then incubated for 1 h and treated as in steps 5–9 of Table I.

RESULTS

Rabbit Antibodies. Figure 1 illustrates the results obtained for rabbit antisera elicited with (A) native or (B) heat-denatured KTI. There were two distinct patterns of

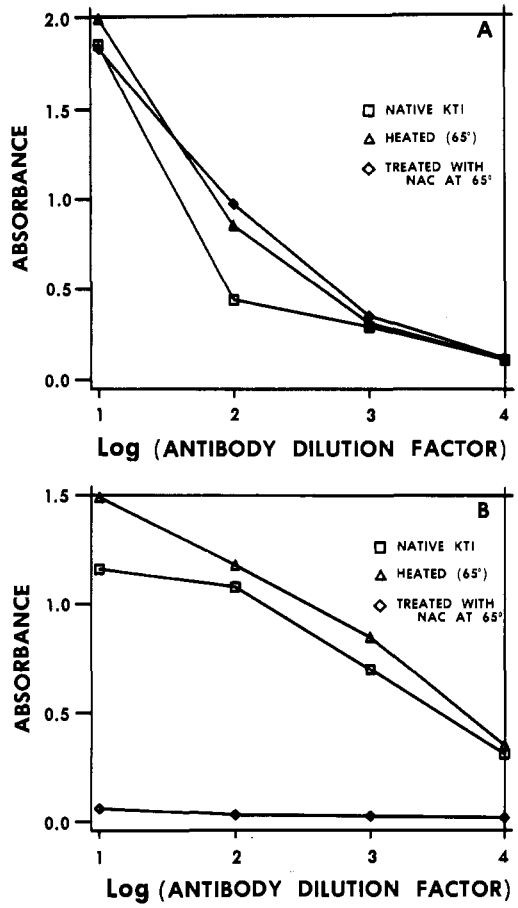

Figure 2. Titration of KTI-specific antibodies: (A) antibody 129; (B) antibody 136. The graphs illustrate the binding of antibodies to assay plates coated with native KTI, heat-treated KTI, or heat plus NAC-treated KTI. Antibody dilutions are expressed in arbitrary units.

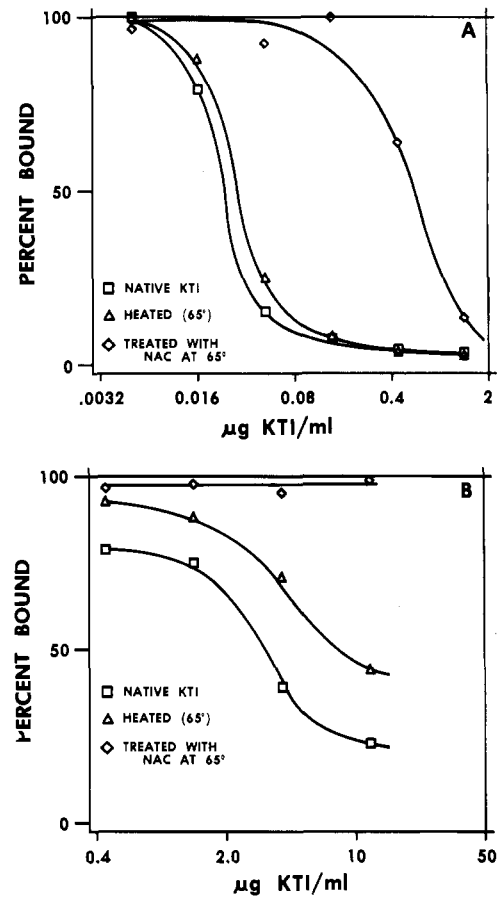

Figure 3. Competitive inhibition assays. The graphs illustrate that the binding of antibodies to assay plates depends on the concentration of KTI and the processing conditions. Native KTI, heat-treated KTI, or heat plus NAC-treated KTI differentially inhibited the binding of (A) antibody 129 or (B) antibody 136. The average coefficient of variation in these assays was 8%.

reactivity. Antibodies elicited with native KTI were inhibited at low concentrations of native KTI, but only at higher concentrations of NAC-modified KTI. The treatment with NAC destroyed or greatly altered the antigenic determinants. Antibodies elicited with heat-inactivated inhibitor, however, selectively recognized determinants in denatured KTI, confirming that distinct antigenic changes accompany thermal inactivation of KTI.

Studies with Monoclonal Antibodies. These antigenic differences were explored with monoclonal antibodies derived from mice inoculated with native KTI. Fusion cultures were screened for antibody production with use of assay plates coated with native KTI. All of the antibodies obtained were of the $IgG_1(\kappa)$ isotype.

The antibodies were tested by direct binding to assay plates coated with native KTI or samples of KTI treated with heat or NAC plus heat and having low residual trypsin inhibitor activity (Friedman et al., 1982). Representative results are illustrated in Figure 2. These assays demonstrated that most of the monoclonals had similar binding patterns, with poor discrimination between native and modified KTI. Antibody 136 was exceptional due to its low binding to denatured KTI. The most likely explanation for the relatively poor discrimination is the nature of the assay itself. The plates are coated with a saturating concentration of KTI, so that even the residual active molecules of KTI, approximately 5% in the most denatured samples, would be sufficient to permit substantial binding by antibodies specific for native KTI as well as antibodies that recognize determinants on the modified inhibitor.

Selected antibodies were then compared by inhibition assays. Representative results are shown in Figure 3. Native KTI inhibited the binding of all the antibodies to the solid-phase coating. Heat-treated KTI was similarly inhibitory, but KTI treated with NAC at an elevated temperature was less effective in this assay, although the relative inhibition varied among the different antibodies. Thus, these results lead to conclusions similar to those drawn from the experiments with polyclonal antibodies. Native and heat-denatured KTI are similar antigenically, whereas disruption of the disulfide bonds to form mixed disulfide forms between protein and added cysteine sulfhydryl groups causes a major change in antigenic determinants.

Epitope Mapping. The results obtained with the direct-binding assays and the inhibition assays motivated us to seek a higher resolution analysis of antibody specificity. This would enable a more detailed definition of the antigenic determinants of KTI and possibly help in selecting antibodies with desired specificity for immunoassays. The results are summarized by the data in Figure 4, which is a composite of three experiments. On the basis of these data, we visualize two major antigenic regions: the first is centered around the binding sites of antibodies 126, 140, and 129; the second is centered around the binding sites of antibodies 127, 130, 138, and 142. Antibodies were grouped as tabulated in Table II.

While further studies are called for, certain hypotheses can be entertained now. Group 1 antibodies may define a single site or a cluster of at least three overlapping de-

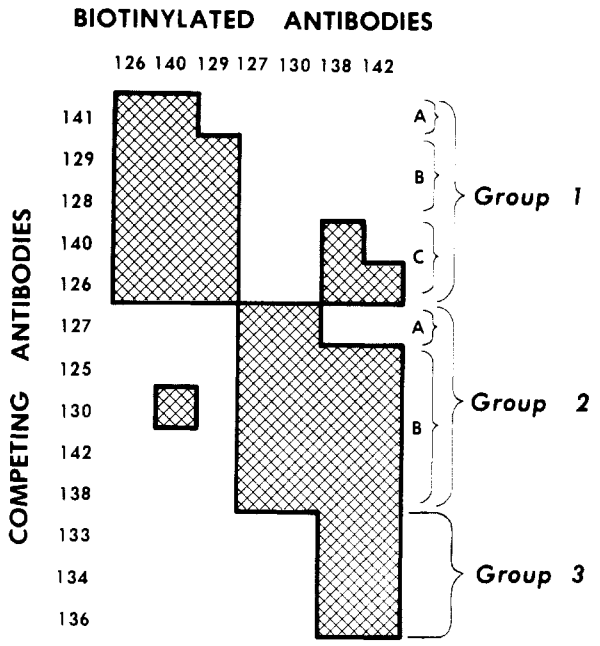

Figure 4. Epitope mapping using mouse monoclonal antibodies. Monoclonal antibodies were purified and derivatized with the *N*-hydroxysuccinimide ester of biotin. In the mapping experiment (see Table I), labeled antibodies (listed horizontally) were tested for binding to KTI-coated wells that had been pretreated with unlabeled antibodies (listed vertically). The results are derived from three separate experiments. Shaded areas indicate inhibition. The criteria for grouping antibodies are described in Table II.

Table II. Classification of Monoclonal Anti-Kunitz Trypsin Inhibitors

antibody group	criterion
group 1	competition with antibody 140
subgroup A	no competition with antibody 129
subgroup B	competition with antibody 129, but not antibody 138
subgroup C	competition with antibodies 129 and 138
group 2	competition with antibody 130
subgroup A	no competition with antibody 138
subgroup B	competition with antibodies 138 and 142
group 3	competition with antibodies 138 and 142, but not antibodies 129 or 130

terminants. The second major antigenic site seems likely to consist of two subsites: site 2A, defined by antibodies 127 and 130; site 2B, defined by 138 and 142. Alternatively, there may be only a single site 2, but different antibodies may bind to the same epitope in different orientations, giving rise to the complex inhibition pattern observed. Group 3 antibodies bind to site 2B, without inhibiting binding to site 2A. The hypothetical site 3 may overlap partially with site 2B or may be identical with it.

As a test of our hypothesis that the three groups of antibodies bind to different antigenic regions of the molecule, we compared the binding of three representative antibodies—129, 127, 136—from groups 1–3, respectively. These antibodies were titrated on solid-phase assay plates coated with either native KTI or KTI reduced and denatured by treatment with sodium dodecyl sulfate and 2-mercaptoethanol. The results are summarized in Table III. It is apparent that antibody 136 has the greatest specificity for native KTI and that antibody 127 has the least. We hypothesize that site 2 is similar in native KTI and denatured KTI and probably consists of a linear sequence of amino acid residues. Site 3 appears to differ most in native and denatured KTI, in both this assay and the inhibition assays. We hypothesize that site 3 is unique to the native protein and depends on amino acid residues adjacent to each other in the tertiary structure of native KTI. These results suggest that group 2 antibodies should be most useful in assaying mixtures of native and denatured KTI for total inhibitor content or for detecting KTI in polyacrylamide gels under denaturing conditions. On the other hand, group 3 antibodies may be most useful in detecting native KTI. The relationship among the subgroups of antibodies requires further study now in progress.

Table III. Binding of Monoclonal Antibodies to Native and SDS-Denatured Kunitz Trypsin Inhibitors (KTI)[a]

gp	antibody	diln	OD_{415} (std dev) native KTI	OD_{415} (std dev) denatured KTI	binding ratio
1	129	1:100	2.21 (0.01)	0.129 (0.011)	17.1
2	127	1:500	2.74 (0.11)	1.49 (0.10)	1.84
3	136	1:500	2.11 (0.5)	0.023 (0.002)	91.7

[a] Culture supernatants titered on assay plates coated with native KTI or KTI denatured with sodium dodecyl sulfate (1%) plus 2-mercaptoethanol (10%) at 100 °C. Binding assessed with peroxidase-conjugated rabbit anti-mouse IgG. Plates read after 15 min of development with substrate.

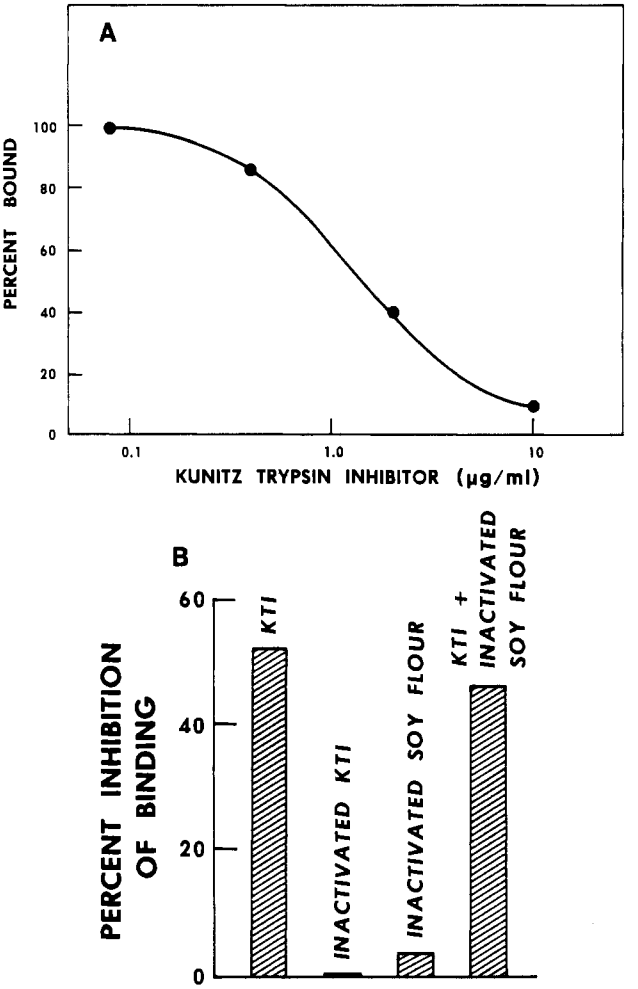

Figure 5. Assay of Kunitz trypsin inhibitor: (A) A solid-phase assay plate coated with antibody 136 bound biotinylated KTI, which was detected with peroxidase–streptavidin conjugate. In the presence of unlabeled KTI, the binding of biotinylated KTI was reduced, resulting in the standard curve. (B) Denatured KTI and heat-treated soy flour lacking KTI do not appear to interfere with the assay of KTI. Addition of soy flour lacking trypsin inhibitor activity results in a small (10%) perturbation of the binding reaction between biotinylated KTI and the solid phase.

Analysis of Soy Flour Samples. We have performed a pilot experiment to examine whether KTI can be quantitated by monoclonal antibody-based immunoassay. Figure 5A illustrates a standard curve for an inhibition-type, solid-phase immunoassay for KTI. The assay employs biotinylated KTI as the labeled ligand and measures the binding of the ligand to assay wells coated with purified antibody 136. The bound biotinylated KTI is detected with peroxidase-conjugated streptavidin, which binds specifically to biotin and does not adhere to assay wells coated with antibody 136 or with unmodified KTI. Preliminary data on utilization of the assay are illustrated in Figure 5B. The presence of heat-denatured KTI does not interfere with the binding of biotinylated KTI to the solid phase. Addition of a suspension (50 μg/mL) of soy flour lacking trypsin inhibitor activity resulted in small perturbations of negative and positive controls, indicating that active KTI can be assayed in the presence of inactivated KTI and other processed proteins. This assay format is therefore practical, though further work remains to be done on preparation and analysis of complex samples.

DISCUSSION

The experiments described above point the way to understanding the antigenic structure of KTI. Extension of the epitope mapping procedure with more antibodies could give a higher resolution picture of the protein. In addition, more antibodies—derived from independent cell fusions—must be analyzed to establish whether all the antigenic sites have been defined. In characterizing antibody specificities, it will be essential to determine the affinity of the antibodies. It seems likely that antibody 136—and perhaps other group 3 antibodies—are low-affinity antibodies. This conclusion is drawn from the inhibition assay data (Figure 3), which showed that about a 10-fold higher concentration of KTI is needed to inhibit the binding of antibody 136 than of antibody 129. Groups 2 and 3 antibodies may differ in affinity rather than localization of binding. A higher affinity antibody with group 3 specificity should enable a more sensitive assay of KTI. However, if group 3 is really a low-affinity subset of group 2, it may be difficult to find a better combination of affinity and specificity.

These experiments, using antibodies elicited with native and modified Kunitz trypsin inhibitor, demonstrate the importance of disulfide bonding in maintaining the native structure of the protein. Treatment with NAC transformed the disulfide bonds in native KTI to mixed disulfide forms in the antigenically inactive protein. Heat denaturation of the inhibitor, in the absence of a reducing agent, appears to produce a conformation closely resembling the native form. Epitope mapping with monoclonal antibodies indicates the presence of two, or possibly three, antigenic regions of native Kunitz trypsin inhibitor. One of these antigenic determinants appears to be retained, even after denaturation in the presence of sodium dodecyl sulfate and a reducing agent.

The results of the study are also relevant to monitoring and possibly eliminating the allergenicity of food proteins. Kunitz trypsin inhibitor, in addition to its activity as a toxicant and antinutrient, has been established as a human allergen (Moroz and Yang, 1980). Other soy proteins have been implicated in adverse intestinal responses to soy protein, which limit the use of soybean products as economical milk replacers in young calves (Barratt et al., 1978; Seegraber and Morrill, 1982). The digestive disturbances appear to correlate with immunological sensitization of the calf to glycinin and β-conglycinin (Sissons et al., 1982). Processing conditions can influence the concentration of allergens by altering the immunochemical structure of proteins and by influencing the digestibility of antigenic proteins (Barratt et al., 1979; Srihara, 1984). The antigenicity and potential allergenicity of soy-based infant formulas may depend on the processing techniques (Heppell et al., 1985). While we have not studied the major allergenic proteins of soy, our results indicate that protein epitopes are affected differentially by conditions encountered during food processing. In addition, these results suggest that tests for allergic responses to foods should take into account changes in antigenicity that accompany processing food proteins.

Changes in nonsoy proteins during processing and storage have also been documented (Morton and Deutsch, 1961; Kato et al., 1983), but the consequences of enteric encounter with modified proteins represent a gap in scientific knowledge. The potential of food proteins to contribute to chronic systemic disease (Allison, 1982; Brandon, 1984) suggests that new processes be evaluated for their effect on food protein antigenicity. In addition, the loss of antigenicity during some processes such as treatment of KTI with NAC and heat suggests the possibility that allergenicity may be altered by selection of appropriate techniques. The epitope mapping procedure is one method that could be used to monitor the results. Evaluation of changes in the antigenicity of food proteins would permit the development of more reliable immunoassays of processed foods. These methods would also help in the development of strategies for the prevention of allergenic and other immunotoxicological hazards.

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Registry No. Kunitz trypsin inhibitor, 9088-41-9; *N*-acetylcysteine, 616-91-1.

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Determination of Glycinin and β-Conglycinin in Soybean Proteins by Immunological Methods

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Glycinin and β-conglycinin contents in soybean seeds and in protein fractions were immunologically assayed. Whole extractable proteins (WBE) were composed of 32% glycinin, 23% β-conglycinin, and 45% remainder unassayed by anti-glycinin and anti-β-conglycinin. The acid-precipitable fraction (APP) at pH 4.8 was composed of 34% glycinin, 27% β-conglycinin, and 39% remainder. The protein composition of APP was similar to that of WBE. The crude 7S, precipitating at pH 4.8, was composed of 52% β-conglycinin, 3% glycinin, and 45% remainder. However, the remainder content in the pH 6.4 precipitated fraction (crude 11S) was reduced from 45% to 15%. Thus, the amount of contaminating protein in globulin fraction is proportional to the acidity. Treatment with 10% NaCl and ammonium sulfate fractionation improved the glycinin plus β-conglycinin content. Contaminating proteins were evidently concentrated in a 0–51% saturated ammonium sulfate precipitated fraction.

Isolation of the two major reserve soybean proteins, glycinin (11S globulin) and β-conglycinin (7S globulin), has been examined by many researchers (Wolf, 1972). A method by Thanh and Shibasaki (1976), however, is the only straightforward procedure for simultaneous preparation of the two globulins. Almost all researchers have, during the last 5 years, used this method for preparing both glycinin and β-conglycinin for studies of thermal denaturation (Damodaran and Kinsella, 1982), functional properties (Nakamura et al., 1984; Umeya et al., 1980), and other properties of these proteins (Damodaran and Kinsella, 1981; Honig et al., 1984) since it is a simple and large-scale separation method.

Purity and composition of the various fraction obtained during isolation were usually determined by ultracentrifugation. Thanh and Shibasaki (1976) reported that ultracentrifugal analysis, discontinuous gel electrophoresis, and immunodiffusion indicated very little cross-contamination between the prepared fractions; however, the color of the freeze-dried crude 7S fraction is tan relative to that of the crude 11S fraction. This suggests a contamination by other components. Compared to ultracentrifugal (Wolf, 1970) or densitometric analysis (Sato et al., 1986), an immunological method (Iwabuchi and Shibasaki, 1981; Murphy and Resurreccion, 1984) is well suited for measuring the absolute amounts of glycinin and β-conglycinin in various fractions.

The present study was undertaken to (a) obtain the glycinin and β-conglycinin content in soybean seeds, (b) obtain quantitative information concerning the purity of protein fractions, and (c) study the effect of a reductant on protein fractionation.

EXPERIMENTAL SECTION

Materials. Soybeans (*Glycine max* var. Raiden) were finely ground in a Waring Blendor and defatted with hexane at room temperature. Soybean meals for protein extraction were used without screening. Sepharose CL-6B, DEAE-Sephadex A-50, and Con A-Sepharose 4B were purchased from Pharmacia Co., and 2-mercaptoethanol (2-ME) and all other reagent-grade chemicals were obtained from Nakarai Chemicals.

Methods. *Preparation of Whole-Buffer Extract, Acid-Precipitated Protein, Glycinin, and β-Conglycinin. Step 1. Preparation of the Isoelectric Precipitated Fraction.* A sample (100 g) of defatted meal was extracted once with 2 L of 0.03 M Tris-HCl buffer containing 10 mM 2-ME, at pH 8.0 at room temperature. After centrifugation the supernatant was designated whole-buffer extract (WBE I; Figure 1a). The WBE I was acidified to pH 4.8 with 2 N HCl. After centrifugation, the SUP I was the whey protein fraction and was designated Whey I. The precipitated protein curd was washed twice with pH 4.8 water, dispersed in water, and titrated to pH 8.0 while stirring. By these operations the acid-precipitated curd was completely resolubilized. The clear supernatant was designated acid-precipitated protein (APP; Figure 1a).

Another whole-buffer extract was prepared for preparation of crude 7S and 11S fractions. Following the procedure of Thanh and Shibasaki (1976), the 11S fraction was obtained by isoelectric precipitation at pH 6.4, resolubilized in a pH 7.6 phosphate buffer (2.6 mM KH_2PO_4, 32.5 mM K_2HPO_4, 0.4 M NaCl, 10 mM 2-ME, pH 7.6, ionic strength 0.5), and designated crude 11S fraction (Figure 1b). The SUP II was further separated into 7S and whey fractions by acidification to pH 4.8. The resulting precipitate was centrifuged, completely resolubilized in 0.03 M Tris-HCl buffer by back-titrating to pH 8.0, and designated crude 7S fraction (Figure 1b). The SUP III was designated Whey II. Usually pH 4.5 rather than pH 4.8 has been used to prepare globulins. Here and in Figure 1a we used pH 4.8 according to Thanh and Shibasaki (1976) and Wolf and Briggs (1959).

Step 2. Fractionation with 10% NaCl Extraction. Another resolubilization of the acid-precipitated curds of

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