fication reactions is quite similar with the exception of those prepared by esterification of fatty acids with alcohols, both from *S. alba* (Table IV).

It can also be seen from the data given in Table IV that the wax esters prepared by alcoholysis or esterification contain an array of homologues in which the esters having carbon numbers C_{36}, C_{40}, C_{42}, C_{44}, and C_{46} predominate. Composition of some of these wax ester mixtures, especially of those prepared from triacylglycerols of *S. alba* as the only starting material, is intermediary between the composition of wax esters from jojoba and orange roughy (Table IV). A striking feature of the wax esters prepared from triacylglycerols of *L. annua* as one of the starting materials lies in the presence of substantial proportions of C_{46} esters in such products; in contrast, most natural waxes of commercial interest contain very little esters having 46 or more carbon atoms (Table IV).

Finally, Table V shows the composition of lipid classes in the products obtained by alcoholysis and esterification reactions. The wax esters constitute about 80% and 65%, respectively, of the products formed by alcoholysis from triacylglycerols of *L. annua* or *S. alba*. These products contain, in addition to unreacted triacylglycerols and alcohols, the monoacylglycerols, diacylglycerols, and fatty acids, which are formed by hydrolysis of triacylglycerols (Table V).

In contrast to alcoholysis, esterification of fatty acids from both *L. annua* and *S. alba* with the corresponding alcohols yields products almost entirely composed of wax esters (Table V). These products contain, in addition to minor proportions of unreacted fatty acids and alcohols, small amounts of monoacylglycerols and diacylglycerols present as contaminants in the fatty acids used as reaction partners.

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LITERATURE CITED

Brown, W. G. "Reductions by Lithium Aluminum Hydride". *Org. React.* **1951**, *6*, 469–509.

Buisson, D. H. "The Future for Fish Oils in New Zealand". In *Fats for the Future*; Brooker, S. G., Renwick, A., Hannan, S. F., Eyres, L., Eds.; Duromark: Auckland, New Zealand, 1983.

Chalvardjian, A. M. "Fatty Acids of Yellow and Brown Fat". *Biochem. J.* **1964**, *90*, 518–521.

Christie, W. W.; Noble, R. C.; Moore, J. H. "Determination of Lipid Classes by a Gas Chromatographic Procedure". *Analyst* **1970**, *95*, 940–944.

Kates, M. "Simplified Procedures for Hydrolysis and Methanolysis of Lipids". *J. Lipid Res.* **1964**, *5*, 132–135.

Macrae, A. R. "Microbial Lipases as Catalysts for the Interesterification of Fats and Oils". In *Biotechnology for the Oils and Fats Industry*; Ratledge, C., Dawson, P., Rattray, J., Eds.; American Oil Chemists' Society: Champaign, IL, 1984.

Mukherjee, K. D.; Kiewitt, I. "Changes in Fatty Acid Composition of Lipid Classes in Developing Mustard Seed". *Phytochemistry* **1984**, *23*, 349–352.

Mukherjee, K. D.; Kiewitt, I. "Lipids Containing Very Long Chain Monounsaturated Acyl Moieties in Seeds of *Lunaria annua*". *Phytochemistry* **1986**, *25*, 401–404.

Schuch, R.; Mukherjee, K. D. "Interesterification of Lipids Using an Immobilized *sn*-1,3-Specific Triacylglycerol Lipase". *J. Agric. Food Chem.* **1987a**, *35*, 1005–1008.

Schuch, R.; Mukherjee, K. D. "Radiochemical Methods for Studying Lipase-Catalyzed Interesterification of Lipids". *Z. Naturforsch.* **1987b**, *42C*, 1285–1290.

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Definition of Functional and Antibody-Binding Sites on Kunitz Soybean Trypsin Inhibitor Isoforms Using Monoclonal Antibodies

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The interaction of monoclonal antibodies with the three Kunitz trypsin inhibitor isoforms designated Ti^a, Ti^b, and Ti^c was studied by ELISA. Antigenic differences among the isoforms and their complexes with bovine trypsin were observed. Some antibodies were selective for one or two of the isoforms, but others bound comparably to all three isoforms. All but one of the antibodies were able to bind equivalently to Ti^a and its complex with trypsin. The results of this study define six epitopes, one of which is either blocked or altered when trypsin binds to the inhibitor. A combination of antibodies can be used to determine which isoforms of Kunitz trypsin inhibitor are present in a sample and whether the inhibitor reactive site is free or occupied. Consideration of the isoform composition of soy products would improve the accuracy of assays for Kunitz trypsin inhibitor in foods. These immunochemical tools could also be used to study the developmental and environmental regulation of KTI expression and its function in the plant.

Kunitz trypsin inhibitor (KTI) from soybeans was the first plant protease inhibitor to be extensively characterized. Its structure and mechanism of action [reviewed by Laskowski and Kato (1980)] and its significance in human and animal health and nutrition [reviewed by Rackis and Gumbmann (1981)] have been extensively studied. We have previously prepared monoclonal antibodies that bind to different epitopes on the Kunitz trypsin inhibitor of soybeans (Brandon et al., 1986, and 1987) and have developed an enzyme-linked immunosorbent assay (ELISA) for KTI in soy products, including infant formulas (Brandon et al., 1988). There are three closely related

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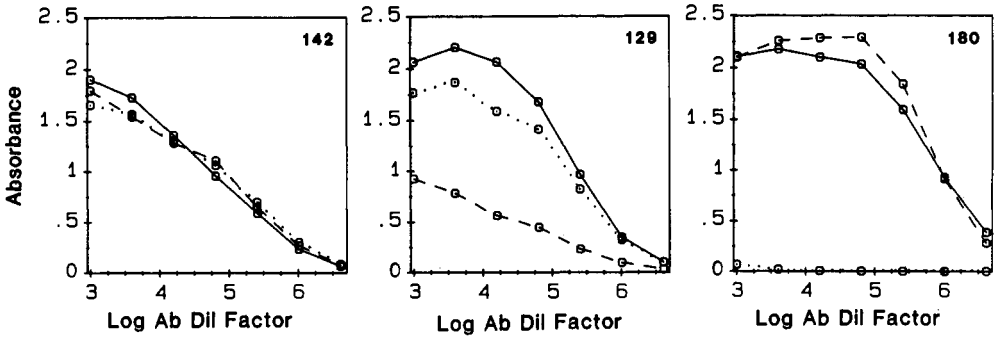

Figure 1. Direct binding of monoclonal antibodies 142, 129, and 180 to KTI isoforms: —, Ti^a; - - -, Ti^b; ···, Ti^c.

isoforms of KTI, encoded by codominant alleles in a multiple allelic system at one locus (Hymowitz and Hadley, 1972; Orf and Hymowitz, 1977). In the course of our work using ELISA, we were struck by the absence of cross-reactive antigenic activity in extracts of soybean isolines expressing the *Ti*^{c} allele coding for KTI, as determined with antibody 180. The *Ti*^{c} gene product, KTI isoform c (Ti^c), differs from the *Ti*^{a} product (Ti^a) in only one amino acid residue (Kim et al., 1985): a change from glycine to glutamic acid at residue 55. On the other hand, an isoline expressing the *Ti*^{b} allele had even more antigenic activity than the *Ti*^{a} isoline. Although Ti^b retains glycine at position 55, it differs at eight other positions from Ti^a (Kim et al., 1985).

To elucidate the antigenic differences among the isoforms of KTI, we examined the interaction of purified isoinhibitors with several monoclonal antibodies. In addition, we determined the antigenicity of complexes of KTI with bovine trypsin. The results support our earlier conclusions about the distinct epitopes on KTI and also demonstrate that Ti^a and Ti^b differ antigenically in a region at or near the reactive (trypsin-binding) site of KTI.

MATERIALS AND METHODS

Kunitz Trypsin Inhibitors. Ti^a (lots 86F-8070, 109C-8085, and 45F-8005) was obtained from Sigma Chemical Co. (St. Louis, MO) and was judged to be at least 90% isoform a with the polyacrylamide gel system of Singh et al. (1969). Ti^b and Ti^c (Kim et al., 1985) were generously provided by Prof. T. Ikenaka (Osaka University). KTI samples were dissolved in phosphate-buffer saline (PBS, 5 mM sodium phosphate, 150 mM sodium chloride, pH 7.0), the concentrations were determined spectrophotometrically ($E_{280,1\ mg/ml}$ = 1.0; Kassell, 1970), and samples were then stored as aliquots at −20 °C.

Other Chemicals. Bovine pancreatic trypsin (type III) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma. Trypsin solutions were prepared immediately before use in PBS. PMSF was stored as a 100 mM solution in 95% ethanol at −20 °C.

Monoclonal Antibodies. Antibodies were derived from mice previously inoculated with KTI isoform a. Antibodies 129–142 were described previously (Brandon et al., 1987), and antibodies 171 and 180 were derived from a second, independent cell fusion (Brandon et al., 1988). Antibodies were purified by ammonium sulfate precipitation and chromatography on diethylaminoethylcellulose (Good et al., 1980) from spent tissue culture medium or ascitic fluids obtained from BALB/c mice inoculated with hybridomas as described previously (Brandon et al., 1987, 1988). The procedures for ascitic fluid production were described by Oi and Herzenberg (1980).

Assays. *1. Preparation of Assay Plates.* Polystyrene assay plates from Costar (Cambridge, MA) were used in these assays. Polyvinyl chloride plates from the same manufacturer were found to have relatively low affinity for KTI, with extreme lot to lot variability. Assay wells were coated with protein solutions (100 μL/well) at a concentration of 10 μg/mL for 4 h at room temperature. Bovine serum albumin (10 mg/mL) in PBS containing 0.05% Tween-20 (BSA–PBS–Tween) was used to block remaining sticky sites after coating with proteins. PBS–Tween was used as washing solution between steps of the assays.

2. Labeled Reagents. Rabbit IgG anti-mouse IgG, conjugated with horseradish peroxidase (HRP), was obtained from Zymed Laboratories (South San Francisco, CA). The conjugate of KTI with HRP (KTI–HRP) was described previously (Brandon et al., 1988). Streptavidin–HRP conjugate was obtained from Amersham Corp. (Arlington Heights, IL). The presence of HRP bound to assay plates was detected with substrate solution consisting of 1 mM 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid) and 6.7 mM H_2O_2 in 60 mM sodium citrate buffer, pH 4.2. The absorbance at 415 nm was determined on a plate reader (Model 308; Bio-Tek Instruments, Winooski, VT), generally after 15-min incubation at room temperature.

3. Direct Binding Studies. In these assays, antibodies (stock solutions of 1–3 mg/mL IgG) were serially diluted in BSA–PBS–Tween from an initial 1:1000 dilution, and the samples were applied to KTI-coated assay plates. Following incubation (1–2 h) at room temperature with shaking, the wells were washed with PBS–Tween and rinsed with distilled water. HRP–anti-mouse IgG in BSA–PBS–Tween was used as labeled reagent, with an incubation period of 1 h.

4. Competitive Binding Studies. KTI samples were diluted serially in BSA–PBS–Tween at concentrations ranging from 10 ng/mL to 1 mg/mL. Samples were then analyzed in one of two assays.

Inhibition ELISA was conducted by addition of antibody to the KTI solutions, with preincubation of the mixtures (1–2 h) prior to application of aliquots of the mixture to assay wells coated with Ti^a. The assay was then continued as described for direct binding studies.

Competition ELISA utilized HRP–KTI and antibody-coated plates and was described in detail previously (Brandon et al., 1988). An equal volume of KTI–HRP (0.6 μg/mL) was added to each dilution, and aliquots of the mixture were added to antibody-coated assay wells. After an incubation of 1–2 h, the plates were washed and drained, substrate was added, and the absorbance was determined after 15 min. Some assays were terminated by the addition of 100 μL of 10% sodium dodecyl sulfate, prior to determining the absorbance.

5. Epitope Mapping. This procedure was based on the method of Wagener et al. (1984) and consisted of competitive binding studies using unlabeled KTI-specific monoclonal antibodies and a panel of biotinylated monoclonal antibodies. The procedures were described in detail pre-

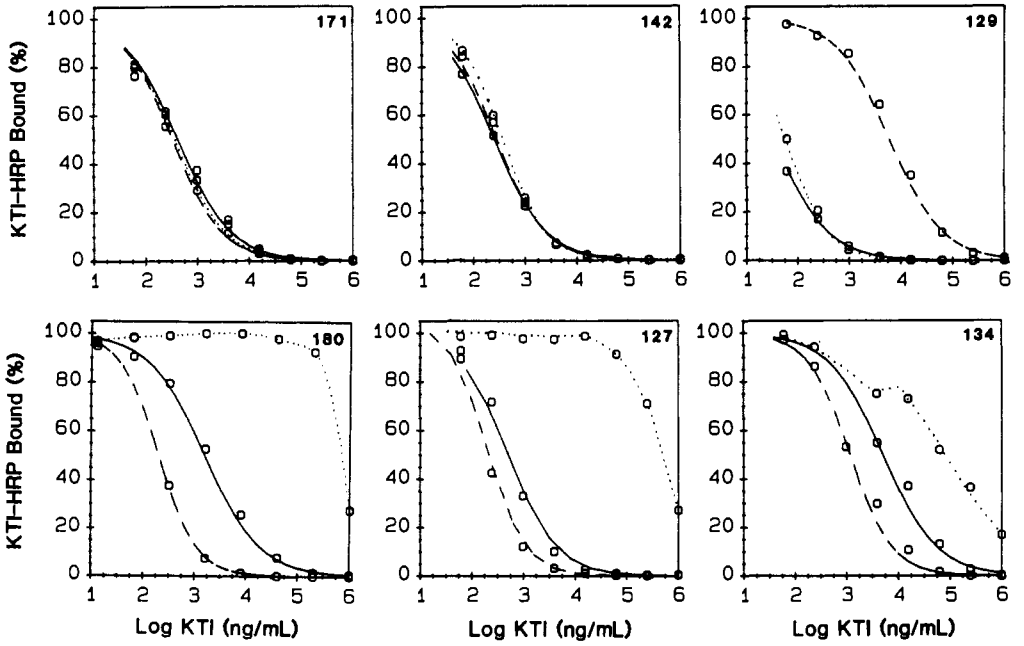

Figure 2. Competitive binding of KTI isoforms to solid-phase monoclonal antibodies: —, Ti[a]; - - -, Ti[b]; ···, Ti[c].

viously (Brandon et al., 1987).

6. Studies of KTI–Trypsin Complexes Formed on a Solid Phase. Assays were conducted by adaptation of the methods described above. The effect of trypsin on the interaction of KTI with antibodies was determined by modification of the direct binding procedure as follows. KTI-coated assay wells were preincubated with trypsin (200 μL/well, 10 mg/mL) or BSA—PBS–Tween blocking solution as a control, washed, and then incubated with 1 mM PMSF, prior to conducting incubations with antibodies, as in the direct binding studies.

7. Studies of KTI–Trypsin Complexes Formed in Solution. KTI–trypsin complexes were formed in solution by adding trypsin to KTI at a 5:1 weight ratio. Samples were incubated at room temperature for 15 min, followed by addition of PMSF (0.5 mM final concentration) to inhibit excess trypsin. Complex formation between trypsin and KTI under these conditions was verified by polyacrylamide gel electrophoresis as described above. Complexes were analyzed in the inhibition and competition ELISA's described above. Samples were incubated on the assay plates for 40 min. Control samples were prepared with addition of BSA instead of trypsin.

The most important antigen–antibody binding reactions in the above assay procedures can be summarized as follows:

Direct binding or titration:

$$KTI_{solid} + Ab_{soln} \xrightarrow[\text{washing}]{\text{incubn}} KTI\text{–}Ab_{solid}$$

Competitive binding assays:

(1) Inhibition ELISA using antibody in solution

$$KTI_{solid} + (Ab + KTI)_{soln} \xrightarrow[\text{washing}]{\text{incubn}} KTI\text{–}Ab_{solid}$$

(2) Competition ELISA using KTI–HRP in solution

$$Ab_{solid} + (KTI, KTI\text{–}HRP) \xrightarrow[\text{washing}]{\text{incubn}} Ab\text{–}KTI\text{–}HRP_{solid} + Ab\text{–}KTI_{solid}$$

RESULTS

Direct Binding Studies on KTI Solid Phases. As a preliminary test for specificity, antibodies were titrated on solid phases coated with each of the three isoforms of KTI. Three patterns of binding were observed, as illustrated in Figure 1. The three patterns are (a) equivalent binding to the three isoforms; (b) preferential binding to isoforms a and c; and (c) strong binding to isoforms a and b, with no binding to isoform c.

Table I. Binding of KTI Isoforms by Monoclonal Antibodies

antibody	isoform a: I_{50}, μg/mL	isoform b (rel I_{50})[a]	isoform c (rel I_{50})	n
129	0.067 ± 0.033[b]	78 ± 13	0.67 ± 0.19	2
127	0.52	0.29	880	1
180	2.40 ± 0.90[c]	0.083 ± 0.014	302 ± 31	5
134	5.5	0.23	16	1
142	0.26	1.1	1.4	1
171	0.43 ± 0.01[c]	0.77 ± 0.16	0.77 ± 0.07	3

[a] Values for isoforms b and c are I_{50} concentrations relative to those observed for isoform a. [b] Average deviation from the mean. [c] Standard deviation.

Competitive Binding Studies with KTI–HRP. To analyze antibody specificity quantitatively, a competitive ELISA was performed using antibody-coated plates, with competition between KTI and KTI–HRP for antibody. The results of these studies are summarized in Figure 2. The binding curves were fit to a logistic model (Finney, 1964), and exponential functions were calculated and plotted. The results agree with the direct binding studies. The three binding patterns observed by direct binding assays using antibodies 142, 129, and 180 (Figure 1) were apparent with the larger panel of antibodies used in the competitive assays. The concentrations of KTI isoforms producing 50% inhibition of binding (I_{50}) were calculated from the exponential functions. These values are summarized in Table I and permit quantitative description of the antibody specificities. Antibody 129 bound selectively to isoforms a and c. Antibodies 127, 180, and 134 each bound the isoforms in order of preference b > a > c, but their selectivity was not identical. For example, antibody 180 had 19-fold greater selectivity for isoform a over c compared to that of antibody 134. Both antibodies 171 and 142 bound the three isoforms equivalently.

Antigenicity of KTI–Trypsin Complexes. To investigate the relationships among the antibody-binding

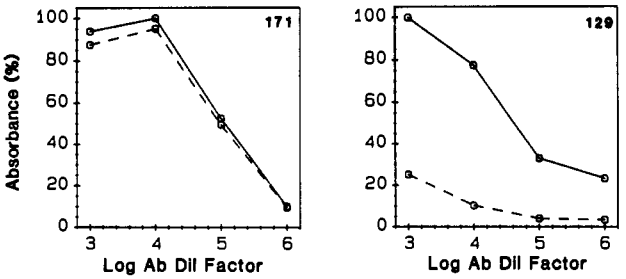

Figure 3. Direct binding of antibodies 171 and 129 to solid-phase KTI (—) or KTI–trypsin (- - -).

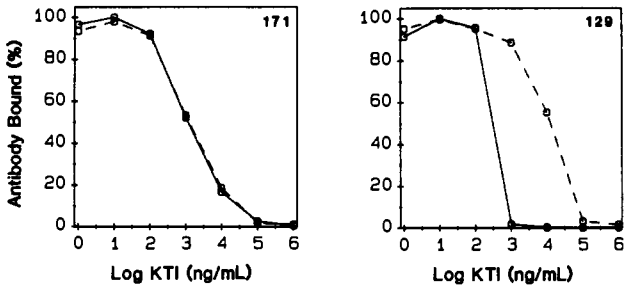

Figure 4. Inhibition ELISA of antibodies 171 and 129 on solid-phase KTI. The samples are KTI (—) and KTI–trypsin (- - -). Preincubation time of sample with antibody is 20 min.

sites and the trypsin-binding reactive site of KTI, the interactions of trypsin and KTI isoform a were studied in three assay formats.

(1) Trypsin–KTI Interactions on a Solid Phase. Eight monoclonal antibodies were assayed by titration on KTI solid phases pretreated with trypsin or with BSA as a control. Two representative titrations, for antibodies 129 and 171, are presented in Figure 3. Prior treatment of KTI with trypsin substantially reduced the binding of antibody 129. Other antibodies (130, 133, 134, 142, 180) bound to the trypsin-treated, KTI-coated assay wells with slightly reduced efficiency (data not shown). The titration curves of antibody 171 on the two solid phases were identical, indicative of unhindered binding of the antibodies to trypsin–KTI complexes.

(2) Trypsin–KTI–Antibody Interactions in Solution Studied by Inhibition ELISA. Assays were performed with two representative antibodies, selected because of their different patterns of binding to trypsin complexes formed on the solid phase. The results are shown in Figure 4. On KTI-coated assay plates, KTI isoform a and the KTI–trypsin complex produced identical inhibition of binding by antibody 171, indicative of equivalent binding to this antibody. In contrast, the inhibition of binding by antibody 129 was not identical. The binding of antibody 129 to KTI–trypsin complexes was reduced 30-fold compared to its binding with uncomplexed KTI. These results indicate that trypsin and antibody 129 were unable to bind simultaneously to Ti^a.

(3) Trypsin–KTI–Antibody Interactions Studied by Competition ELISA. The same two antibodies were coated on assay plates and used to analyze the competitive binding of KTI and KTI–trypsin, using KTI–HRP as labeled ligand. The results, shown in Figure 5, agreed qualitatively with the analysis by inhibition ELISA. The complexation of Ti^a with trypsin diminished its ability to compete with KTI–HRP for binding sites on antibody 129 (8.5- and 10-fold in two experiments), but not antibody 171.

Epitope Mapping. As a further means of relating antibody-binding sites to functional domains, a panel of biotinylated monoclonal antibodies was prepared for epitope mapping. Unlabeled monoclonal antibodies were

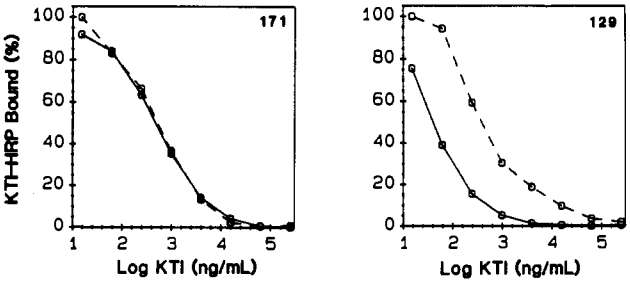

Figure 5. Competition ELISA on solid phases coated with antibody 171 or 129. KTI–HRP is the labeled analyte, with KTI (—) and KTI–trypsin (- - -) as samples.

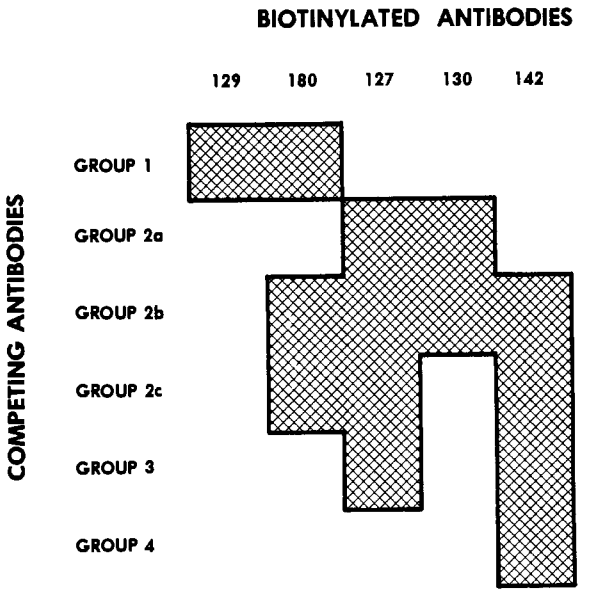

Figure 6. Epitope mapping using unlabeled antibodies listed by groups and a panel of biotinylated monoclonal antibodies. Shaded areas indicate that the competing antibody group prevents binding of the indicated biotinylated antibody. For example, group 1 antibodies inhibit the subsequent binding of biotinylated antibodies 129 and 180. The results are based in part on data presented by Brandon et al. (1987).

Table II. Binding of Antibodies to KTI Isoforms and Trypsin Complexes

antibody	binding to solid-phase isoform Ti^a	Ti^b	Ti^c	binding to KTI–trypsin complex	group[a]
129	+	–	+	–	1
127	+	+	–	nd[b]	2a
130	+	+	+	+	2b
142	+	+	+	+	2b
134	+	+	–	+	3
180	+	+	–	+	2c
171	+	+	+	+	4

[a] Antibodies 129–142 are grouped as described in Brandon et al. (1987). Antibodies 171 and 180 are assigned new designations on the basis of data in this manuscript. [b] Not determined.

first applied to solid-phase Ti^a, and the biotinylated antibodies were subsequently applied. The pattern of inhibited binding is summarized in Figure 6. The antibodies were grouped under the system presented previously (Brandon et al., 1987) but extended by the additional results with antibodies 171 and 180. There were six groups, but three appeared closely related and are designated as subgroups 2a, 2b, and 2c. The relationship of isoform specificity, effects of trypsin complex formation, and the epitope mapping are summarized in Table II.

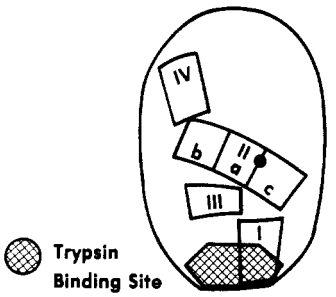

Figure 7. Schematic model of antibody-binding epitopes in relation to the trypsin-binding site (shaded area; surrounding residues 63 and 64). Roman numerals I–IV refer to epitopes, with epitope II further divided into sites a–c. The black dot represents the region of the molecular surface of KTI altered in isoform c due to the substitution of glutamic acid for glycine at residue 55.

DISCUSSION

The salient features of these binding studies are depicted schematically as hypothetical binding sites in Figure 7. The assignment of antibodies to groups corresponding to proposed binding sites is based in part on data presented previously (Brandon et al., 1987), and on additional data in this report. Groups 2c and 4 are specificities not evident in the earlier study. These specificities were confirmed by binding studies using extracts from soybean isolines lacking KTI or expressing only one of the isoforms (Brandon, D. L., Bates, A. H., and Friedman, M., manuscript in preparation) and with Ti^a from three independent lots. ELISA's defining the major specificity patterns for antibody binding to the three isoforms and to the Ti^a–trypsin complex were replicated for estimation of the precision of the measurements (e.g., Table I). All of the results were confirmed by the qualitative agreement of assays performed in alternative ELISA formats (direct binding, inhibition, competition).

The most significant features of the groups are as follows:

Group 1 exhibits impaired binding to KTI–trypsin complexes. Its epitope (site I) may overlap the trypsin-binding site, or allosteric changes in the KTI molecule could be involved.

Group 2 antibodies bind to several closely associated sites (IIa–c). Sites IIa and IIc—but not site IIb—are altered by substitution of glutamic acid for glycine at residue 55. Site IIc is distinguished from IIa and IIb by its sensitivity to heat. Site IIc appears to be located close to site I. Sites IIa–c do not overlap with the trypsin-binding site.

Group 3 antibodies bind to site III, which is topographically distinct from site IIc, but close to sites IIa and b. Site III is moderately altered by heat and by the substitution at residue 55 in isoform c, but not as dramatically as site IIc.

Group 4 antibodies bind to an epitope, site IV, that is highly conserved among the three isoforms of KTI. This site is unaffected by the binding of trypsin and is topographically close to site IIb.

The most striking result of the analysis of the specificity of monoclonal antibodies raised against Ti^a was the effect of a change of a single residue from glycine to glutamic acid at position 55. There was a 300-fold reduction of binding by antibody 180 and an 880-fold reduction of binding by antibody 127. The crystal structure of KTI (Sweet et al., 1974) indicates that residue 55 is centrally located in the molecule. It does appear reasonable that addition of a charged side chain in this position could distort the conformation of the protein at the surface, where it interacts with antibodies. The structural change attendant upon the substitution of glutamic acid at position 55 does not appear to affect the binding of trypsin, however, since Kim et al. (1985) reported that the bovine trypsin inhibition constants for Ti^a and Ti^c are nearly identical. Antibody 129 binds similarly to isoforms a and c. Together with the information concerning the binding of antibody 129 to trypsin complexes, the results imply that the conformation of the region surrounding the trypsin-binding site is similar in Ti^a and Ti^c.

Antibody 129 shows greatly reduced avidity for isoform b and binds poorly to the Ti^a–trypsin complex. The most appealing explanation is that the binding site for this antibody, site I, overlaps with the trypsin-binding site of KTI. This explanation is supported by the crystallographic analysis by Sweet et al. (1974) of the complex of Ti^a with porcine trypsin defining 12 contact residues on KTI involved with trypsin binding. Two of these, at positions 62 and 71, are altered in Ti^b (tyrosine → phenylalanine; histidine → asparagine), and a third, position 13, is adjacent to altered residue 12 (glycine → serine), as reported by Kim et al. (1985).

Antibodies such as 171 and 142 bind to all three isoforms and are not hindered by the complexation of KTI with bovine trypsin. The availability of antibodies to site 4 of KTI permits the analysis of complexes of KTI with trypsin, as demonstrated above, and, possibly, with other proteins. For example, KTI constitutes at least 2% of the storage protein of soybeans. KTI and other protease inhibitors of the soy seed undergo both physical changes (Hwang et al., 1978) and proteolytic modification (Freed and Ryan, 1978; Hartl et al., 1986) following germination of the seed. The function of KTI in germination has not been elucidated. It is not known whether KTI functions as a protease inhibitor in the dry seed or whether it serves as a storage protein. The possibility that KTI may be complexed with a specific trypsin-like protease could be tested immunochemically, using antibody 171, which recognizes KTI in a complex with bovine trypsin, and antibody 129, which does not. The state of KTI in other tissues of the soybean and under other physiological conditions may also be analyzed in this manner. For example, protease inhibitors are both developmentally and environmentally regulated and may function as protective molecules in the plant (Brown et al., 1986). The regulation of KTI expression and function could be studied immunochemically as outlined above.

The results of this study were dependent on the nonspecific binding of KTI to plastic microtitration dishes, involving hydrophobic forces (Catt and Tregear, 1967). Altered recognition of solid-phase antigens has been reported (Dierks et al., 1986). The inclusion of the anionic detergent Tween-20 appears to stabilize HRP conjugates when adsorbed onto plastic (Berkowitz and Webert, 1981) and may be essential to obtain consistent results. In the assays described in this paper, we included Tween-20 in buffers used for washing plates and for diluting samples. All the results obtained with KTI adsorbed onto plastic dishes were replicated in other formats, in which KTI was bound via antibody. These results suggest that artifacts were not produced in the attachment of KTI to the plastic and that selective steric hindrance of certain epitopes did not occur when KTI was adsorbed onto the solid phase. In addition, the results obtained with antibody 171, which bound all isoforms of KTI, strongly support the assumption that each of the isoforms bound to the plastic equivalently.

KTI is a food protein and a known allergen in humans (Moroz and Yang, 1980). Soy protease inhibitors have a variety of antinutritional, toxicological, and, possibly, an-

ticarcinogenic effects (Burns, 1987; Roebuck, 1987; Troll et al., 1980). The fate of KTI in the gastrointestinal tract and the possible translocation of KTI to other tissues could be studied immunochemically, with antibodies that can detect the specific epitopes.

The analysis of the antigenicity of this molecule could be extended in two directions. First, the immunogenicity of the molecule via enteric exposure could be studied. Definition of KTI epitopes recognized by B and T cells and the influences of food processing conditions on this immunogenicity would provide information on alterations in allergenicity of KTI attendant upon processing. These studies should include definition of epitopes in relationship to the binding of digestive and tissue proteases from other species, including humans. Second, the study could be extended to other food proteins such as major soy and wheat storage proteins implicated in food intolerance with an immunological basis (Barratt et al., 1979; Goodwin and Rawcliffe, 1983) as well as other food proteins of known allergenicity, such as egg, milk, and peanut proteins. Finally, analysis of the immune response to native and processed food proteins could elucidate the possible link between food proteins and systemic diseases with an immunological basis (Allison, 1982).

LITERATURE CITED

Allison, R., Ed. In *An Evaluation of the Potential for Dietary Protein to Contribute to Systemic Disease*; FASEB: Rockville, MD, 1982; pp 1–53.

Barratt, M. E. J.; Strachan, P. J.; Porter, P. "Immunologically Mediated Nutritional Disturbances Associated with Soya-Protein Antigens". *Proc. Nutr. Soc.* **1979**, *38*, 143–150.

Berkowitz, D. B.; Webert, D. W. "The Inactivation of Horseradish Peroxidase by a Polystyrene Surface". *J. Immunol. Methods* **1981**, *47*, 121–124.

Brandon, D. L.; Haque, S.; Friedman, M. "Antigenicity of Native and Modified Kunitz Soybean Trypsin Inhibitors". In *Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods*; Friedman, M., Ed.; Plenum: New York, 1986; pp 449–467.

Brandon, D. L.; Haque, S.; Friedman, M. "Interaction of Monoclonal Antibodies with Soybean Trypsin Inhibitors". *J. Agric. Food Chem.* **1987**, *35*, 195–200.

Brandon, D. L.; Bates, A. H.; Friedman, M. "Enzyme-linked Immunoassay of Soybean Kunitz Trypsin Inhibitor Using Monoclonal Antibodies". *J. Food Sci.* **1988**, *53*, 97–101.

Brown, W. E.; Graham, J. S.; Jong, S. L.; Ryan, C. A. "Regulation of Protease Inhibitor Genes in Food Plants". In *Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods*; Friedman, M., Ed.; Plenum: New York, 1986; pp 281–291.

Burns, R. A. "Protease Inhibitors in Processed Plant Foods". *J. Food Protect.* **1987**, *50*, 161–166.

Catt, K.; Tregear, G. W. "Solid-phase Radioimmunoassay in antibody-coated Tubes." *Science (Washington, D.C.)* **1967**, *158*, 1570–1572.

Dierks, S. E.; Butler, J. E.; Richerson, H. B. "Altered Recognition of Surface Adsorbed Compared to Antigen Bound Antibodies in the ELISA". *Mol. Immunol.* **1986**, *23*, 403–411.

Finney, D. J. In *Statistical Method in Biological Assay*; Hafner: New York, 1964; pp 454–456.

Freed, R. C.; Ryan, D. S. "Changes in Kunitz Trypsin Inhibitor during Germination of Soybeans: An Immunoelectrophoresis Assay System". *J. Food Sci.* **1978**, *43*, 1316–1319.

Good, A. H.; Wofsy, L.; Kimura, J.; Henry, C. "Purification of Immunoglobulins and Their Fragments". In *Selected Methods in Cellular Immunology*; Mishell, B. B., Shiigi, S. M., Eds.; W. H. Freeman: San Franciso, 1980; pp 278–286.

Goodwin, B. F. J.; Rawcliffe, P. M. "Food Allergies Associated with Cereal Products". *Food Chem.* **1983**, *11*, 321–338.

Hartl, P. N.; Tan-Wilson, A. L.; Wilson, K. A. "Proteolysis of Kunitz Soybean Trypsin Inhibitor during Germination". *Phytochemistry* **1986**, *25*, 23–26.

Hwang, D. L.; Yang, W.-K.; Foard, D. E.; Lin, K.-T. D. "Rapid Release of Protease Inhibitors from Soybeans. Immunochemical Quantitation and Parallels with Lectins". *Plant Physiol.* **1978**, *61*, 30–34.

Hymowitz, T.; Hadley, H. H. "Inheritance of a Trypsin Inhibitor Variant in Seed Protein of Soybeans". *Crop Sci.* **1972**, *12*, 197–198.

Kassell, B. "Trypsin and Chymotrypsin Inhibitors from Soybeans". *Methods Enzymol.* **1970**, *19*, 853–862.

Kim, S.; Hara, S.; Hase, S.; Ikenaka, T.; Toda, H.; Kitamura, K.; Kaizuma, N. "Comparative Study on Amino Acid Sequences of Kunitz-type Soybean Trypsin Inhibitors, Ti^a, Ti^b, and Ti^{c-}". *J. Biochem.* **1985**, *98*, 435–448.

Laskowski, M., Jr.; Kato, I. "Protein Inhibitors of Proteinases". *Annu. Rev. Biochem.* **1980**, *49*, 593–626.

Moroz, L. A.; Yang, W. H. "A Specific Allergen in Food Anaphylaxis". *New Engl. J. Med.* **1980**, *302*, 1126–1128.

Oi, V. T.; Herzenberg, L. A. "Immunoglobulin-producing Hybrid Cell Lines." In *Selected Methods in Cellular Immunology*; Mishell, B. B., Shiigi, S. M., Eds.; W. H. Freeman: San Francisco, 1980; pp 351–372.

Orf, J. H.; Hymowitz, T. "Inheritance of a Second Trypsin Inhibitor Variant in Seed Protein of Soybeans". *Crop Sci.* **1977**, *17*, 811–813.

Rackis, J. J.; Gumbmann, M. R. "Protease Inhibitors: Physiological Properties and Nutritional Significance". In *Antinutrients and Natural Toxicants in Foods*; Ory, R. L., Ed.; Food and Nutrition Press: Westport, CT, 1981; pp 203–237.

Roebuck, B. D. "Trypsin Inhibitors: Potential Concern for Humans?" *J. Nutr.* **1987**, *117*, 398–400.

Singh, L.; Wilson, C. M.; Hadley, H. H. "Genetic Differences in Soybean Trypsin Inhibitors Separated by Disc Electrophoresis". *Crop Sci.* **1969**, *9*, 489–491.

Sweet, R. M.; Wright, H. T.; Janin, J.; Chothia, C. H.; Blow, D. M. "Crystal Structure of the Complex of Porcine Trypsin with Soybean Trypsin Inhibitor (Kunitz) at 2.6-Å Resolution". *Biochemistry* **1974**, *13*, 4212–4228.

Tan-Wilson, A. L.; Rightmire, B. R.; Wilson, K. A. "Different Rates of Metabolism of Soybean Proteinase Inhibitors during Germination". *Plant Physiol.* **1982**, *70*, 493–497.

Troll, W.; Wiesner, R.; Shellabarger; Holtzman, S.; Stone, J. P. "Soybean Diet Lowers Breast Tumor Incidence in Irradiated Rats". *Carcinogenesis* **1980**, *1*, 469–472.

Wagener, C.; Fenger, U.; Clark, B.; Shiveley, J. "Use of Biotin-labeled Monoclonal Antibodies and Avidin-peroxidase Conjugates for the Determination of Epitope Specificities in a Solid-phase Competitive Enzyme Immunoassay". *J. Immunol. Methods.* **1984**, *68*, 269–274.

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