ABSTRACT: Because the consumption of soybean inhibitors of digestive enzymes in processed foods may have both beneficial and adverse health-related effects, reliable and rapid analytical methods for these inhibitors are needed. Monoclonal antibody–based sandwich enzyme-linked immunosorbent assays (ELISAs) were developed for the 2 major soybean protease inhibitors, the Kunitz trypsin inhibitor (KTI) and Bowman–Birk inhibitor (BBI) of trypsin and chymotrypsin. The ELISAs had limits of quantification of approximately 1 and 3 ng/mL for BBI and KTI, respectively, and were used to measure active inhibitors in soy infant formula. Results were compared with enzymatic analyses and demonstrated that most of the trypsin- and chymotrypsin-inhibitory activities of infant formula were due to constituents other than KTI and BBI. The sandwich ELISA for BBI was also effective in detecting soybean germplasm with atypically low levels of BBI.

Keywords: trypsin inhibitor, soybean, soy infant formula, ELISA, Bowman–Birk inhibitor

Introduction

Soybeans contain 3 major protease inhibitors: the Kunitz soybean trypsin inhibitor (Kunitz 1947), the low-molecular-weight Bowman–Birk inhibitor (BBI) (Birk 1985), and the glycine-rich trypsin inhibitor (Tan-Wilson and others 1989). The changes in the pancreas may occur, with le-
Antibodies to KTI and BBI
Preparation and purification of rabbit polyclonal antibody to KTI (Brandon and others 1988) and mouse MAb to both inhibitors were described previously (Brandon and others 1987, 1989). The IgG fractions were isolated by ammonium sulfate fractionation and ion exchange chromatography using Diethylaminoethyl (DEAE)-cellulose, dialyzed against PBS containing 0.01% sodium azide, and stored at 4 °C at 1 to 3 mg/mL, based on A280, 1 mg/mL = 1.4.

Preparation of labeled antibodies and ELISA materials
HRP conjugates. HRP conjugates were prepared according to the procedure of Nakane and Kawaoi (1974), purified by gel filtration, and analyzed spectrophotometrically (Johnson and others 1978).

HRP-MAB 217. Five milligrams of HRP (Sigma) were reacted with 2,4,6-trinitrobenzenesulfonic acid to block free amino groups, and then the carbohydrate moieties were oxidized with sodium periodate. After the reaction was quenched with ethylene-glycol, HRP was dialyzed against 0.01 M NaHCO3, pH 9.5, and 5 mg MAB 217 was added. After 3 h at room temperature, Schiff’s base linkages were stabilized by reduction with NaBH4 and then dialyzed at 4 °C against PBS. The conjugate was separated from unbound HRP by gel filtration on Sepharose 6B (Pharmacia, Piscataway, N.J., U.S.A.) and analyzed by UV-visible spectroscopy using the following absorbances for HRP: A280, 1 mg/mL = 0.74; A403, 1 mg/mL = 2.4. Protein concentration was determined using bicinchoninic acid (Smith and others 1985). The amount of IgG present was calculated by subtracting the HRP content from the total protein. The resulting conjugate had 2.5 mol HRP/mol IgG and was stored at 4 °C in PBS containing 10 mg/mL bovine serum albumin (BSA) and 0.01% thimerosal.

HRP-inhibitor conjugates. KTI and BBI were conjugated, analyzed, and stored as described previously. The reaction mixtures contained (1) 3 mg KTI, 4 mg HRP and (2) 3 mg BBI, 11 mg HRP. Unconjugated KTI and BBI were removed from conjugates by chromatography on Sephadex G-100 (Pharmacia) and had 1 to 2 mol HRP/mol inhibitor. Biotinylation. MABs were biotinylated using N-hydroxysuccinimidobiotin, as described by Wofsy (1983). Briefly, 0.4 mg of biotin ester (1 mg/mL in dimethylsulfoxide) was added to 2 mg of IgG in 2 mL 0.01 M sodium citrate, pH 4.2. After 4 h, the reaction mixture was dialyzed against PBS + 0.01% NaN3 and then stored at 4 °C.

Procedure for sandwich assay
The previously determined epitopes of KTI and BBI (Brandon and Bates 1988; Brandon and Friedman 2002) provided guidance to select candidate antibodies for a sandwich format. The sandwich assays of KTI and BBI were conducted according to the following protocol, with all steps performed at room temperature, unless noted.
1. The capture antibody (5 μg/mL in PBS, 100 μL/well) is coated on microtiter plates by incubation for 4 h at room temperature or 16 h at 4 °C, followed by rinsing 5 times with distilled water.
2. Uncoated “sticky” sites on wells are blocked with 5% nonfat dry milk for 1 h at room temperature or 16 h at 4 °C.
3. The plates are washed 5 times with water and are then ready for use. Plates can be stored with wells filled with PBS + 0.01% NaN3 at 4 °C for up to 4 wk.
4. A dilution series of the test sample is prepared, using PBS-PBS-Tween as diluent. Standard solutions of protease inhibitors are thawed and diluted in PBS-PBS-Tween to cover the range 0.5 to 200 ng inhibitor/mL.
5. The diluted standards and samples are applied to the assay wells (100 μL/well) and incubated with shaking for 1 h.
6. The assay wells are washed 5 times with water and thoroughly drained.
7. Depending on which detection antibody is used, the following steps are done:
   a. Biotinylated detection antibody (for example, MAB 217 for BBI)
   i. Add antibody to each well (4 μg/mL in BSA-PBS-Tween, 100 μL/well). Incubate for 1 h, repeat step 6.
   ii. Add HRP-streptavidin (1:1000 dilution from commercial stock solution, 100 μL/well) and incubate for 1 h. Repeat step 6.
   b. Rabbit (polyclonal) detection antibody (for example, R276 for KTI)
   i. Add antibody (5 μg/mL in BSA-PBS-Tween, 100 μL/well). Incubate for 1 h, repeat step 6.
   ii. Add HRP-goat anti-rabbit IgG (1:1000 dilution from commercial stock solution, 100 μL/well) and incubate for 1 h. Repeat step 6.
   c. HRP-labeled detection antibody (for example, MAB 217 for BBI)
   i. Add HRP-antibody conjugate (0.1 to 0.2 μg/mL, 100 μL/well). Incubate for 1 h.
   ii. Repeat step 6.
8. Add substrate solution (6.7 mM H2O2, 1 mM M2,2’-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, 60 mM sodium citrate, pH 4.2, 100 μL/well) and incubate 20 min with shaking. Stored refrigerated, the substrate is stable for several months.
9. Add stop solution (10 mg/mL sodium dodecylsulfate, 100 μL/well) and read absorbance at 415 nm on a microplate reader.
10. Subtract blanks, compute standard curve using log-logit fit and obtain values for unknowns.

Competitive ELISA
Competitive ELISAs for KTI and BBI (Brandon and others 1988; Brandon and others 1989) were conducted as follows. Polystyrene assay plates were coated with purified IgG anti-KTI (MAb 129) or anti-BBI (MAb 238) at 5 μg/mL in PBS, 100 μL/well and blocked using BSA-PBS-Tween, as described previously for sandwich ELISA. Antibodies and standard samples, prepared as for the sandwich assays, were premixed with an equal volume of KTI-HRP or BBI-HRP (0.3 or 0.15 μg/mL, respectively, in BSA-PBS-Tween) and incubated in the assay wells for 1 h (100 μL/well) with shaking. Unbound HRP conjugate was removed by washing the plates 5 times with water, and bound HRP was visualized as described above.

Procedure for analyzing infant formula
Isomil concentrated liquid (Ross Laboratories, Columbus, Ohio, U.S.A.) was purchased locally and was diluted serially (2- or 5-fold dilution series) in BSA-PBS-Tween for ELISA, using spikes of 30 μg/mL of KTI or BBI for determination of recovery. ELISAs of KTI used MAB 129 in the competitive ELISA (n = 5) and combinations of antibod-
Trypsin inhibitors in soy infant formula

ies in the sandwich ELISA (n = 5; MAb 129/biotinylated MAb 130, n = 2; n = 1 for the other formats; MAb 130/biotinylated MAb 129, MAb 129/R276, MAb 130/R276). Assays of BBI utilized MAb 238 in the competitive ELISA (n = 4) and as capture antibody in the sandwich assay (n = 4), with HRP-labeled or biotinylated MAb 217 used as detection antibody in the sandwich assay (MAb 238/biotinylated MAb 217, n = 2; n = 1 for the other formats; MAb 238/HRP-MAb 217, MAb 217/biotinylated MAb 238). Inhibitor content was determined by calculating values in the working range of the assay (20% to 80% antibody bound). To determine the recovery of protease inhibitor activity by enzymatic assay, infant formula samples were spiked with KTI at 16, 32, and 64 μg/mL or with BBI at 21.5, 43, and 86 μg/mL. Calculations used the following equation: Recovery (%) = (inhibitor concentration in spiked sample × 100)/(endogenous inhibitor concentration + spike concentration).

Procedure for analyzing soybeans for BBI

Perennial Glycine species, some of which had been identified as presumptive BBI nulls by competitive ELISA (Domagalski and others 1992), were provided by Prof. T. L. Hymowitz (Univ. Illinois, Urbana-Champaign, Ill., U.S.A.). In 2 replicate experiments, several seeds of each species were crushed and 20 to 50 mg of meal was weighed into small vials. Tris-Cl buffer, 0.1 M, pH 8.5 (20 μL/mg) was added and the mixtures were stirred for 1 h and clarified by centrifugation (5000 × g, 10 min). BBI standards (100, 100, 20, 4, 0.8, and 0.16 ng/mL) and seed extracts (an initial 1:100 dilution, and 3-fold dilutions from 1:300 to 1:8100) were applied to MAb 238-coated assay plates as duplicates, incubated for 1 h, then washed. MAb 217-HRP conjugate (0.1 μg/mL, 100 μL/well) was used to complete the sandwich. After 1 h incubation, plates were washed and developed with substrate as described above.

Enzyme assays

Assays were conducted as follows (Friedman and others 1991). All rates were computed using the initial, linear portion of absorbance curves, with buffer plus substrate used as control. Pure KTI or BBI was used as a standard in each assay. Values are based on sample dilutions yielding 40% to 60% inhibition of enzyme activity.

Trypsin assays. The reaction mixtures consisted of 2.6 mL buffer (46 mM Tris-Cl, 11.5 mM CaCl₂, pH 8.1), 0.1 mL of trypsin (10 to 20 μg/mL), ± 20 μL inhibitor. After a preincubation (6 min), the reaction was started by adding 0.3 mL of 10 mM Nα-tosyl-l-arginine methyl ester (Sigma). A₄₁₇ was recorded for 3 min.

Chymotrypsin assays. The reaction mixtures consisted of 1.5 mL buffer (80 mM Tris-Cl, 100 mM CaCl₂, pH 7.8, 0.1 mL of chymotrypsin (10 to 20 μg/mL), ± 20 μL inhibitor. After preincubation (6 min), the reaction was started by adding 1.4 mL of 1.07 mM N-Benzoyl-l-tyrosine ethyl ester (Sigma) in 50% methanol. A₂₅₆ was recorded for 3 min.

Calculations. The following equations were used, based on the above measurements of enzyme inhibitory activities of infant formula samples (8.15 mL/g dry weight) and standards:

KTI (mg/g sample) = (mg trypsin inhibited/g sample) × (mg KTI/mg trypsin inhibited) (1)

BBI (mg/g sample) = (mg chymotrypsin inhibited/g sample) × (mg BBI/mg chymotrypsin inhibited) (2)

Results and Discussion

Analysis of soy infant formula

KTI analysis. Figure 1 shows a standard curve for the KTI sandwich ELISA and analysis of infant formula, using MAb 129 for capture and biotinylated MAb 130 for detection. The working range of the assay was approximately 3 to 20 ng/mL (estimated as the range giving 20% to 80% detection antibody bound). The other sandwich formats gave similar results. The infant formula dilutions and KTI standards produced parallel curves, suggesting there were minimal matrix effects within the working range of the assay. Table 1 summarizes the ELISA analyses. The mean value for KTI in infant formula was 6.4 μg/mL. The average within-assay coefficient of variation (CV) for the sandwich ELISA was 8%, whereas the between-assay CV was 28%. In comparison, the competitive ELISA yielded a value of 6.8 μg/mL, indistinguishable from that obtained with the sandwich formats. For the competitive ELISA, within-assay CV averaged 15%; between-assay, 26%; and the working range

Table 1—Analysis of soy infant formula by ELISA

<table>
<thead>
<tr>
<th>Method</th>
<th>Pro tease inhibitora (μg/mL)</th>
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<tbody>
<tr>
<td>Sandwich ELISA</td>
<td>6.3 ± 0.5 (n = 4)</td>
</tr>
<tr>
<td>Competitive ELISA</td>
<td>8.2 ± 1.4 (n = 4)</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>86 ± 6 (n = 2)</td>
</tr>
</tbody>
</table>

aMean ± SD

Figure 1—Kunitz trypsin inhibitor (KTI) standards and serial dilutions of infant formula analyzed by sandwich ELISA, using immobilized MAb 129 and biotinylated MAb 130 (mean of 3 well replicates; error bars, SD of well replicates).

Figure 2—Bowman-Birk inhibitor (BBI) standards and serial dilutions of soy infant formula analyzed by sandwich ELISA, using immobilized MAb 238 and HRP-labeled MAb 217 (mean of 3 well replicates; error bars, SD of well replicates).
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was 15 to 80 ng/mL. Recovery of KTI by ELISA averaged 74%.

BBI analysis. Figure 2 shows a standard curve for the BBI sandwich ELISA and analysis of infant formula, using MAB 238 for capture and HRP-labeled MAB 217. The working range of the assay was approximately 0.7 to 4 ng/mL. As with the KTI ELISA, the infant formula dilutions and the standards resulted in nearly parallel assay curves. Table 1 shows that the mean value of BBI in soy infant formula was 6.3 μg/mL. The average within-assay CV for the sandwich ELISA was 5%, whereas the between-assay CV was 7%. The results of the analyses using the competitive ELISA are also summarized in Table 2. The mean value was 8.2 μg/mL, not significantly different from the value from sandwich ELISA. For the competitive ELISA, within-assay CV averaged 5%; between-assay, 17%; and the working range was 15 to 100 ng/mL. Recovery of BBI by ELISA averaged 86%.

Enzymatic analysis. Table 2 shows the results of enzymatic titration of trypsin and chymotrypsin-inhibitory activities. The amounts of pure KTI or BBI corresponding to these activities (used for the 100% spike level) are also shown. Recoveries, 81% to 84%, were similar to those obtained using ELISA, and the trypsin inhibitory activity agreed with previously reported results (Peace and others 1992). These data indicate that most of the enzymatically measured activity must be attributable to substances other than immunoreactive KTI and BBI.

Screening soybean germplasm

Figure 3 illustrates the analysis of perennial soybean seeds by BBI sandwich ELISA. Although quantitative data can be obtained using this assay, the figure shows that useful, semiquantitative data may be obtained quickly, without reading the plate on a colorimeter. Among the samples analyzed were 5 that yielded no visible color when the assays were developed. This result indicated that the BBI content of these soybean extracts (negative at 1:100 dilution) was lower than that of typical, positive samples, such as samples 6 and 8 (strongly positive at 1:8100 dilution), by more than 100-fold. When results were analyzed quantitatively, within the working range of the assay, positive samples contained 0.7 to 5.5 mg BBI/g soy meal; negatives, <3 μg/g.

Conclusions

The results demonstrate that monoclonal antibody-based sandwich ELISAs can be useful in measuring low levels of active protease inhibitors in soy infant formula. The sensitivity of the sandwich ELISAs was about 5-fold greater for KTI and about 10-fold greater for BBI, compared with the competitive ELISAs. The BBI sandwich ELISA was also found to be useful for visual assay of soybean extracts, by permitting the quick identification of low- or null-BBI seeds from a germplasm collection. By manipulating the dilution series used to analyze samples, a quick screening could be developed to flag either low- or high-inhibitor levels in a sample. Because BBI has been shown to be a useful marker for molecular taxonomy in the genus Glycine (Kollipara and others 1995), the sandwich assay could complement other techniques in establishing genomic relationships among soybean species. Appropriate selection of capture and labeled antibodies permits the sandwich ELISAs to be optimized for particular applications. Previous results (Brandon and Friedman 2002) suggested that the MAB 217 epitope is more heat-labile than the epitope for MAB 238, raising the possibility that some BBI in processed foods would escape detection using MAB 217. However, the agreement of the sandwich and competitive ELISAs for BBI suggests that the infant formula samples did not contain modified BBI, which could possibly go undetected by MAB 217.

The sandwich ELISA offers several advantages over other formats. The analyte dilution is less critical because it is applied to the assay wells in a separate step from the application of detection antibody or enzyme conjugate. Interference with the assay is minimized because sample components (other than captured analyte) are washed away before application of the biotinylated or enzyme-labeled 2nd antibody. Positive samples (containing analyte) give a positive signal in the assay, unlike the inverse response obtained with assays dependent on competitive binding. These immunassays could be provided as a kit for use by nonspecialists with modest laboratory facilities. The enzyme conjugates are remarkably stable in antigenic and enzymatic activities and can be stored in the refrigerator for at least 2 y, or indefinitely, with cryoprotectant, in the freezer. Properly desiccated, dried assay plates perform well after storage for over 6 mo in the refrigerator. The sandwich format using HRP-labeled detection antibody has the fewest steps and is therefore the most rapid assay.

Although sandwich assays are well suited for both qualitative and semiquantitative screening tests, the results demonstrate that they also provide sensitive, quantitative methods to food scientists and could help in the development of more nutritious and healthful soy products for human and animal consumption. KTI and BBI were detected in soy-based infant formula in amounts approximately 0.1% of the levels of active KTI and BBI found in raw soy flour.

### Table 2—Trypsin- and chymotrypsin-inhibitory activities in infant formula

<table>
<thead>
<tr>
<th>Activity (μg of enzyme inhibited/mL)</th>
<th>Trypsin-inhibitory activity</th>
<th>Chymotrypsin-inhibitory activity</th>
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<tbody>
<tr>
<td>65 ± 3</td>
<td>KTI</td>
<td>99 ± 7</td>
</tr>
<tr>
<td>84 ± 8</td>
<td>KTI</td>
<td>81 ± 7</td>
</tr>
<tr>
<td>Equivalent amount of pure inhibitor (μg/mL)</td>
<td>32</td>
<td>BBIb —5 0</td>
</tr>
<tr>
<td>15 ± 3</td>
<td>—</td>
<td>99 ± 7</td>
</tr>
</tbody>
</table>

*Mean ± SD; n = 3
bBBI = Bowman-Birk inhibitor; KTI = Kunitz trypsin inhibitor
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