A Comprehensive Scheme for the Isolation of Trypsin Inhibitors and the Agglutinin from Soybean Seeds

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A comprehensive scheme is described for the isolation of the major protein antinutrients present in soybean seeds, including the two classes of protease inhibitors, SBPI [Kunitz (KSTI) and Bowman-Birk (BBI)] and the seed agglutinin (SBA). A main feature is that no intermediate freeze-drying is necessary during the stepwise recovery of the antinutrients. All interfering saccharides are first removed by extraction with aqueous ethanol, during which a part of BBI is also obtained. The remaining antinutrients are selectively extracted at pH 5.8. SBA and SBPI are isolated by affinity chromatography on cross-linked guar gum and anhydrotrypsin-Sepharose 4B, respectively. Final separation of SBPI is done by HPLC on a TSK SP-5W cation exchanger. By the application of this purification scheme, the concerted effects of the protein (and other) antinutrients and possible synergisms in their action on the digestive system will be open for exploration.

INTRODUCTION

Soybeans have long been known to contain a number of major protein antinutrients whose presence imposes limitations on the nutritional utilization of diets based on soybean proteins or raw seed meals (Liener, 1981; Grant, 1989). The most important and extensively studied of the protein antinutrients are the protease inhibitors (SBPI) and the main seed agglutinin (SBA). Other deleterious factors present in the soybean meal, such as the mouse growth inhibitor reported by Schingoethe et al. (1970) and the muscle loss factor described by Grant et al. (1986), may also contribute to the low nutritional value of the seed meal. However, these factors have as yet only been tentatively characterized.

There are two major types of SBPI (Liener and Kakade, 1980), the Kunitz inhibitor (KSTI) and the Bowman-Birk inhibitor (BBI). However, in reality the situation is more complex as, in addition to these two major types, several derivatives and (proteolytically) modified inhibitor components are also present in the seed (Tan-Wilson and Wilson, 1986).

Our understanding of the factors controlling the stability to processing or to passage in the gastrointestinal tract of the two main types of SBPI and their possible modes of biological action has advanced appreciably in the past two decades (Liener and Kakade, 1980; Rackis and Gumbmann, 1981; DiPietro and Liener, 1989; Grant, 1989). Moreover, recent studies have revealed that, by including major changes in the ultrastructure of the gut epithelium, dietary SBA exerts considerable antinutrient effects on and depresses the growth of young animals (Pusztai, 1989; Pusztai et al., 1990). There are also indications of possible synergisms in the effects of the different soybean antinutrients when they act in a concerted fashion. Accordingly, there is a need for a comprehensive fractionation scheme to allow us to isolate all the major protein antinutrients from the seed and test them in different combinations. The details of one such scheme are presented in this paper.

MATERIALS AND METHODS

Whole soybean seeds were purchased locally, ground in a Wiley laboratory mill fitted with a 1-mm mesh screen, and defatted by extraction with petroleum ether at room temperature. The air-dried, defatted meal was the starting material for the purification of the antinutrients.

Analytical Methods. Protease inhibitor assays (Pusztai et al., 1988) and hemagglutination tests (Grant et al., 1983) were done as described before.

SDS-polyacrylamide gel electrophoresis was carried out by a slight modification (Pusztai et al., 1981) of the method originally described by Laemmli (1970).

Isoelectric focusing was performed by a method similar to that given before (Pusztai et al., 1988). However, the composition of the ampholyte solutions was slightly different. For the runs with SBA, the total ampholyte concentration was 3% (1.5% of pH 5-7; 1.0% of pH 6-8, and 0.5% of pH 3-10), while for SBPI the medium contained 2.5% ampholyte (1.75% of pH 3.5-5.0 and 0.75% of pH 3-10).

Rocket immunoelectrophoresis was carried out at 4 V/cm for 16 h in agarose gels (1% w/v; Pharmacia, minimum endosmosis grade) in 0.07 M Tris-glycine buffer, pH 8.6, containing 0.1 M galactose. Antibody concentration was selected to provide optimum size rockets in the concentration range 50-500 ng of SBA per application hole.

Protein Fractionation Methods. Affinity chromatography of SBPI was done on anhydrotrypsin-Sepharose 4B (AT-Seph) as previously described (Pusztai et al., 1988). Further fractionation of the affinity absorbed and eluted fraction was carried out by HPLC cation-exchange chromatography. Samples (400-1000 mg) were separated on a preparative sulfopropyl cation exchanger (TSK SP-5W, Anachem; 21.5 mm × 150 mm) by programmed gradient elution with buffers based on 0.005 M sodium acetate-acetic acid, pH 3.70. Buffers A and B also contained 0.1 and 0.5 M NaCl, respectively.

SBA was separated from other proteins of the seed by affinity chromatography on cross-linked guar gum. The absorbent was prepared as described by Appukuttan et al. (1977). Columns of suitable size, depending on the SBA content of extracts, were equilibrated and run with 0.2 M sodium acetate-acetic acid buffer, pH 5.8. Elution of the absorbed lectin from the columns was with the same buffer containing 0.1 M galactose.

RESULTS

The purification scheme is given in Figure 1.

Extraction with Aqueous Ethanol Solutions. Samples of the seed meal were stirred with 60% v/v aqueous ethanol (meal to solvent ratio of 1:5) at 0-4 °C for 30 min and then filtered through a glass filter (no. 3; maximum pore diameter, 16-40 μm). The press cake was re-extracted under the same conditions and filtered again. The combined supernatants were treated with 2 volumes of

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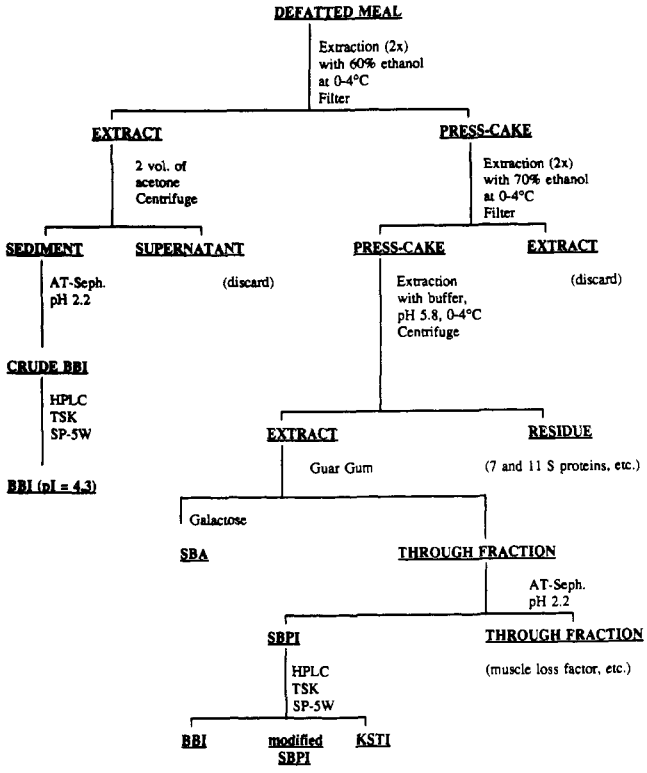

Figure 1. Scheme for the purification of soybean antinutrients.

acetone and left to stand at 0–4 °C overnight. The precipitate was collected by centrifugation and, after short dialysis against distilled water (about 4 h in the cold with one change), was recovered by freeze-drying. The supernatant contained no SBPI and was discarded.

The press cake obtained after two extractions with 60% ethanol was re-extracted twice with 70% aqueous ethanol in the cold. No further SBPI was extracted with this procedure, but the removal of all oligosaccharides and glycosides of the seed was completed. The total amount of material removed in the combined ethanol extracts represents 19.4–20.0% of the original seed meal.

For the preparation of BBI, only the precipitate obtained with acetone from the 60% aqueous ethanol extracts was used. This was dissolved in 0.2 M sodium acetate–acetic acid buffer, pH 5.8, and applied to the AT-Seph column. The absorbed BBI was eluted with 0.05 M glycine hydrochloride buffer, containing 0.2 M NaCl, pH 2.2, and recovered after dialysis and freeze-drying: yield, 160–180 mg of crude BBI/100 g of defatted seed meal. Although this preparation was shown by SDS gel electrophoresis (Figure 2) to contain several bands, all of which had been absorbed by AT-Seph, the doublet characteristic for KSTI was nearly absent. Isoelectric focusing patterns (Figure 3a) also confirmed that this crude BBI preparation contained negligible amounts of KSTI. Finally, the main BBI component (pI = 4.3) was separated from the small amounts of BBI components of higher pI values by HPLC on a TSK SP-5W cation exchanger at pH 3.70 (Figures 4 and 5). The patterns of their reactivities with trypsin or chymotrypsin were characteristic for double-headed inhibitors of the BBI class. The yield of the main BBI component (peaks 1 and 2 in Figure 4a) was about 140 mg/100 g of seed meal.

Isolation of SBA. The final wet press cake after extraction with ethanol solutions was extracted with 0.2 M sodium acetate–acetic acid buffer, pH 5.8 (meal to solvent ratio of 1:8), with continuous stirring in a cold room overnight. The slurry was then centrifuged (30000 g; 1 h; 0–4 °C; Sorvall RC-2B) and the sediment re-extracted (meal to solvent ratio of 1:2) and centrifuged again. The combined supernatants (about 800 mL/100 g of meal) were applied to the guar gum column (6 cm × 22 cm) at a flow rate of 160–180 mL/h. The combined through-fraction and the column washings (about 1200 mL) were kept in the cold until they could be used for the separation of SBPI on the AT-Seph column. When the absorption at 280 nm of the column effluents fell below 0.1, the elution buffer was changed to the same pH 5.8 buffer but also containing 0.1 M galactose. The eluted SBA was dialyzed against water and recovered by freeze-drying: yield, 220 mg/100 g of defatted meal. This lectin preparation, which was fully soluble at neutral pH, was shown to contain two closely spaced bands of 30 kDa by SDS gel electrophoresis (Lotan et al., 1975) and several isolectin components by isoelectric focusing (Catsimpoolas and Meyer, 1969) (Figures 2 and 3) similar to those described before.

Isolation of SBPI (Mainly KSTI). The combined through-fractions from the guar gum column were passed through AT-Seph (total SBPI absorptive capacity of about 400 mg) in pH 5.8 buffer. After the collection of the through-fraction, the absorbed SBPI was displaced from the column with glycine hydrochloride buffer, pH 2.2. This process was repeated three more times by reapplying the through-fractions to the AT-Seph column, after which the through-fraction was free of SBPI. This final protease- and lectin-free through-fraction containing the so-called muscle loss factor (Grant et al., 1986) was recovered by freeze-drying: yield, 2.04 g/100 g of defatted seed meal. The total amount of SBPI recovered from the four successive runs on AT-Seph was 0.92 g/100 g of seed meal. Although the preparation contained no protein components other than SBPI and was composed mainly of KSTI, the presence of BBI could be clearly seen, particularly by isoelectric focusing (Figures 2 and 3).

Finally, the main KSTI component (pI = 4.5) was separated from the smaller amounts of BBI and other components of different pI values by HPLC on a TSK SP-5W cation exchanger (Figures 4 and 5). Yield of pure KSTI (peaks 6 and 7 in Figure 4b) was 0.45 g/100 g of seed meal. In addition, 182 mg of BBI (peaks 1 and 2 in Figure 4b) was also obtained from this fraction.

DISCUSSION

One of the main advantages of this purification scheme is that the isolation of the antinutrient factors is carried out in a stepwise manner and without the need for intermediate freeze-drying. SBA is notoriously prone to aggregation on freeze-drying, particularly in an impure state (Schechter et al., 1976). Below pH 5, it may lose calcium and manganese ions, leading to instability of the lectin protomer. Above pH 8, there is an increase in charge heterogeneity, as shown by isoelectric focusing (results not given). In the present method, the pH is kept at 5.8 before the lectin is separated from other proteins of the seed. Indeed, it is important to isolate SBA first and not SBPI, because the elution of the protease inhibitors from AT-Seph is done at a pH (2.2) that would damage the lectin.

Extraction of the seed meal with aqueous ethanol is an essential preliminary step of the purification scheme. Indeed, since BBI is soluble in 60% aqueous ethanol, unlike other proteins of the seed, this extraction step has long been incorporated in BBI isolation procedures (Birk et al., 1963). However, the extraction of the seed BBI with 60% ethanol is clearly not complete (Figures 3–5), and further BBI is recovered together with KSTI from the

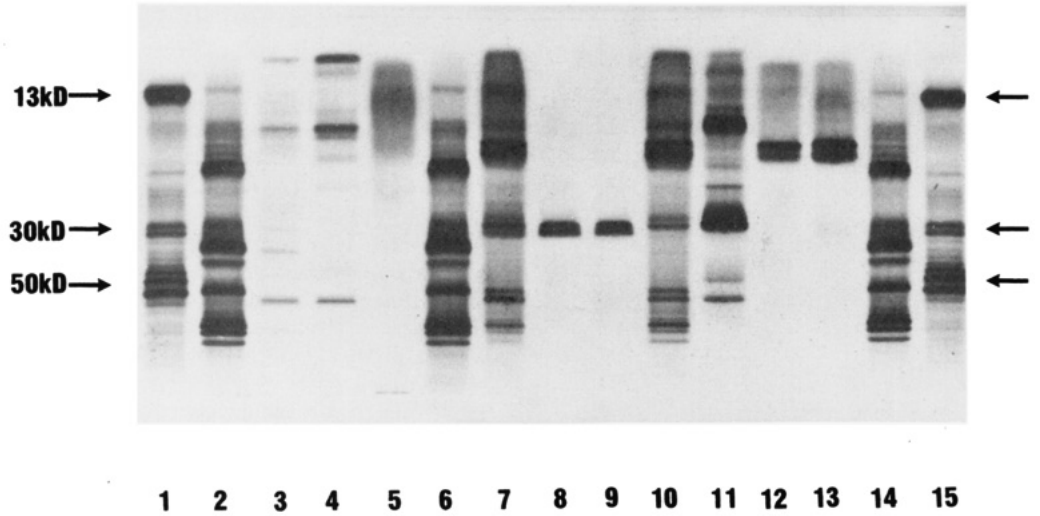

Figure 2. SDS–polyacrylamide gel electrophoretic patterns of fractions recovered from raw defatted soybean meal in the course of the purification scheme given in Figure 1. The following materials were run: (lane 1) standards (13 kDa, cytochrome *c*; 30–31 kDa *Phaseolus vulgaris* lectin E and L subunits; 50 kDa, second lowest subunit of phaseolin); (lane 2) soya meal; (lane 3) acetone precipitate of ethanol extract; (lane 4) crude BBI (pH 2.2 fraction from AT-Seph); (lane 5) nonabsorbed by AT-Seph; (lane 6) soya meal; (lane 7) soluble at pH 5.8; (lane 8) SBA; (lane 9) SBA standard; (lane 10) through-fraction from guar gum; (lane 11) nonabsorbed by guar gum and AT-Seph; (lane 12) KSTI (first pH 2.2. fraction); (lane 13) KSTI (second pH 2.2 fraction); (lane 14) soya meal; (lane 15) standards.

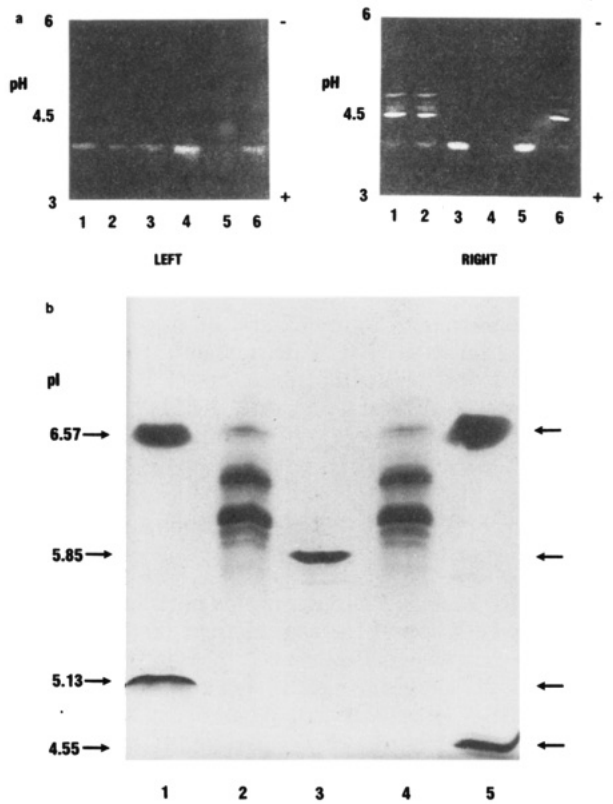

Figure 3. Isoelectric focusing patterns of (a) soybean inhibitor fractions visualized after reaction with the appropriate proteases. Chymotrypsin (left side): (lanes 1) SBPI (mixed BBI and KSTI); (lanes 2–5) four successive pH 2.2 eluted fractions from AT-Seph of pH 5.8 seed extract; (lane 6) acetone precipitate of 60% ethanol extract of the seed. Trypsin (right side): (lanes 1–4) four successive pH 2.2 eluted fractions from AT-Seph; (lane 5) acetone precipitate of 60% ethanol extract; (lane 6) SBPI. (b) Patterns of the agglutinin from soybean (SBA) isolated by affinity chromatography on guar gum. (Lane 1) Markers, human carbonic anhydrase B, pI = 6.57, and β-lactoglobulin A, pI = 5.13; (lane 2) SBA; (lane 3) bovine carbonic anhydrase B, pI = 5.85; (lane 4) SBA; (lane 5) human carbonic anhydrase B, pI = 6.57, and KSTI (Sigma pI = 4.55).

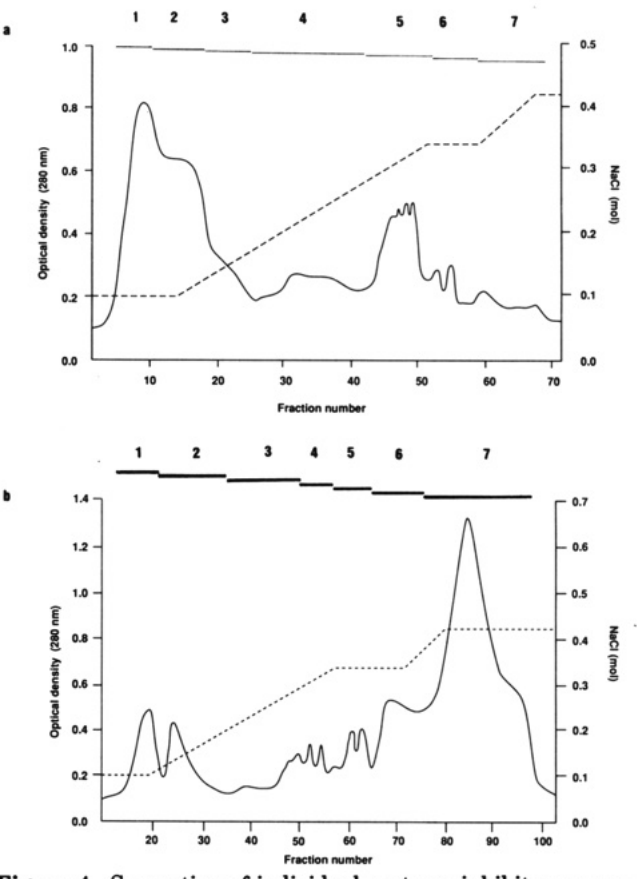

Figure 4. Separation of individual protease inhibitor components, BBI, KSTI, or other modified inhibitors by HPLC on a TSK SP-5W cationic exchanger from (a) the AT-Seph-purified protease inhibitor of the 60% aqueous ethanol extract and (b) the SBPI fraction obtained from the lectin-free whey fraction (fraction soluble at pH 5.8) after AT-Seph affinity chromatography. The poolings (fractions 1–7) are as indicated in the diagrams.

residue which is left behind after ethanol extraction. However, further extractions with 60% ethanol would be counterproductive as small amounts of other seed proteins are progressively solubilized by such treatment. Furthermore, although BBI (or other proteins) is not soluble in 70% ethanol, this step completes the removal of the glycosides and oligosaccharides of the seed meal. The

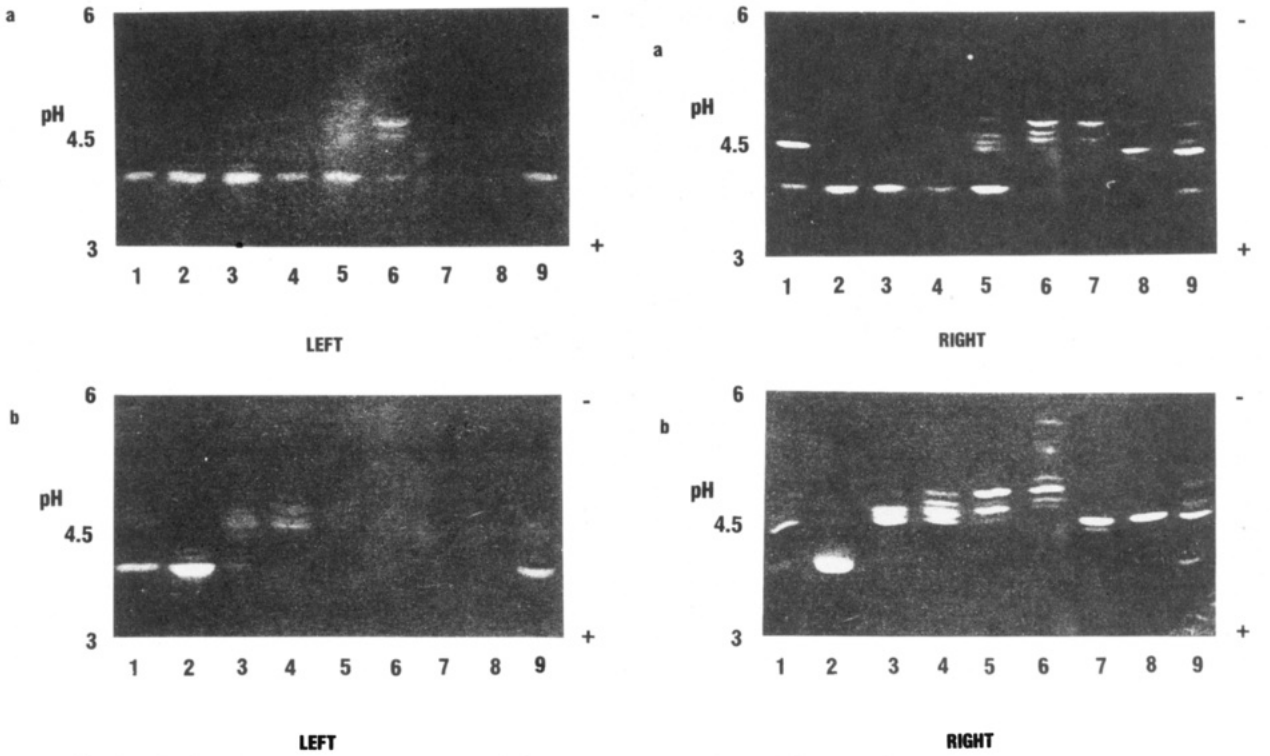

Figure 5. Isoelectric focusing patterns of protease inhibitor components obtained by HPLC from (a) pooled fractions 1–7 (lanes 2–8), from the BBI preparation as given in Figure 4a, and (b) pooled fractions 1–7 (lanes 2–8), from the mainly KSTI containing SBPI fraction as given in Figure 4b. (Lanes 1 and 9) Standard mixed SBPI preparation. For the visualization of the protease inhibitors, their reaction with chymotrypsin (left) and trypsin (right), respectively, was used.

seeds of soybean contain fairly large amounts (6–9% of the defatted meal) of α-galactosidic oligosaccharides, and these are effective inhibitors of binding of SBA to the guar gum column. As the inhibitory capacity of some of these oligosaccharides is about 14% of that of *N*-acetyl-D-galactosamine (Pereira et al., 1974), it is not surprising that binding of SBA to guar gum is negligible when aqueous extracts of the seed meal are applied directly to the column, without the oligosaccharides of the seed being removed first.

Clearly, the aim of any purification scheme is to recover the required component as efficiently as possible. However, the evaluation of the efficiency of overall recovery of the antinutrients of the seed is dependent not only on the amounts of these factors in the seeds, which will vary from one cultivar to another, but also on the method used for the estimation of their concentration in the seed. For example, the concentration of SBA in the seed used for this study was estimated by immunorocket electrophoresis (results not given) to be 0.39% w/w. However, the amount recovered was only equivalent to about 0.22%, even though when reapplied, none of the through-fraction from the first run on the guar gum column was bound to the affinity matrix. The "missing" 0.17% of immunoreactive lectin could, however, still be detected by the immunorockets in the SBA-depleted through fraction. This suggests that the seed may contain protein(s) that are immunochemically cross-reactive with SBA but lack lectin activity or have lectin activity different from that of the bulk of the SBA.

Similar problems may arise with the estimation of the total protease inhibitory content of the seed meal. If this is based on estimation of inhibitory activity, the specific activities with different endopeptidases of the two main components, the double-headed BBI and the mainly trypsin inhibitor KSTI, and those of the proteolytically modified inhibitor components are so different that it is nearly impossible to arrive at a reliable estimate of the total SBPI concentration of the seed. The exhaustive use of affinity columns, with checks that recoveries are as quantitative as possible, probably gives more reliable estimates of the true concentration of an individual antinutrient in the seed meal. Accordingly, the soybean cultivar used in the present study contained 0.22% SBA and at least 0.32% unmodified BBI (p*I* = 4.3) and 0.45% KSTI of p*I* = 4.5. A total of about 0.32–0.42% of protease inhibitors of different p*I* values was also recovered.

The nutritional and physiological properties of these fractions will be described elsewhere.

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