Genetics of the Kunitz Trypsin Inhibitor: An Antinutritional Factor in Soybeans

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ABSTRACT

The major trypsin inhibitor present in soybean seed [*Glycine max* (L.) Merrill] is the Kunitz trypsin inhibitor or soybean trypsin inhibitor A_2 (SBTI-A_2). Four types of SBTI-A_2 9 have been identified in the U.S. soybean germplasm collection. Three of the types designated Ti^a, Ti^b, and Ti^c are electrophoretically distinguishable from one another by their different Rf values of 0.79, 0.75, and 0.83, respectively. The three types are inherited as codominant alleles in a multiple allelic system at a single locus. The fourth type which is the absence of SBTI-A_2 is found in P.I. 157440 and P.I. 196168. The gene for the lack of SBTI-A_2 is designated *ti* and is inherited as a recessive allele to the other three SBTI-A_2 9 types. Ti^a is the most common SBTI-A_2 type in the germplasm collection. Linkage studies revealed that the SBTI-A_2 protein is inherited independently of certain morphological characters and chemical components of seed. Potential applications of the SBTI-A_2 types are discussed.

INTRODUCTION

Over 60 years ago, Osborne and Mendel (1) reported that unheated soybean (*Glycine max* [L.] Merrill) meal was inferior in nutritional quality to soybean meal that had been properly heated. Physiologically, the ingestion of unheated soybean meal causes pancreatic hypertrophy (2-5) in addition to growth inhibition. These effects may be due to the upset in the balance of methionine and cystine in the pancreas (4,6). Among the factors generally attributed to the poor nutritive value of unheated soybeans are a group of proteins known as the trypsin inhibitors (7,8). Although the trypsin inhibitors make up only ca. 6% of the total protein in soybeans (9), it has been estimated that they may be responsible for 30-50% of the growth inhibitory effect and much of the pancreatic hypertrophy which results when monogastric animals ingest unheated soybeans (10,11).

Several different trypsin inhibitors have been reported in soybeans (9,12-17). However, much of the soybean trypsin inhibitor (SBTI) activity is thought to be due to the soybean trypsin inhibitor A_2 (SBTI-A_2) (18), which was first crystallized by Kunitz (16) and is commonly known as the Kunitz trypsin inhibitor. Jirgensons (19) established that the Kunitz inhibitor was a globular protein. The primary structure of this protein was reported by Koide and Ikenada (20) to consist of 181 amino acid residues and to have a molecular weight of 21,384 daltons. The active center of trypsin binding in the Kunitz inhibitor is the arginine 63-isoleucine 64 bond (21). Wolf (21) has reviewed the physical and chemical properties of the Kunitz trypsin inhibitor.

EXPERIMENTAL PROCEDURES

The soybean accessions used in the screening investigations were obtained from R.L. Bernard, USDA, Curator of the Northern Soybean Germplasm Collection, Urbana, Illinois, and from E.E. Hartwig, USDA, Curator of the Southern Soybean Germplasm Collection, Stoneville, Mississippi. The experimental populations were developed and multiplied out at the Agronomy South Farm, Urbana, and/or the Turner Hall Greenhouse, Urbana, Illinois, under the direction of T. Hymowitz.

Following is the procedure developed for extracting SBTI-A_2 and determining the electrophoretic banding patterns.

A single whole seed was crushed in a small coin envelope with a hammer. A 0.1 g portion of the meal was weighed out and placed in a mortar. Three ml of the seed extraction buffer (0.092 M Tris brought to pH 8.1 with HCl and 0.023 M $CaCl_2 \cdot 2H_2O$ in a 13% sucrose solution) was added to the crushed meal. The meal was ground in the mortar with a pestle until well mixed and suspended in the buffer. The suspension was poured into a centrifuge tube and centrifuged for 10 min at 2000 x g. The supernatant was decanted and stored at 0 C until electrophoresis.

Polyacrylamide disc electrophoresis was run utilizing 10% small pore acrylamide gels with a pH 8.3 Tris-glycine buffer, in an anodic system (22). A 25-50 μl volume of the supernatant (protein extract) was layered on top of the gel columns. A current of 1 mg per tube was used for 5 min and then increased to 3.5 ma per tube for ca. 50 min, or until the bromophenol blue tracking dye almost reached the bottom of the gel columns. The gels were then removed and stained for one hour in an acetic acid-naphthol blue

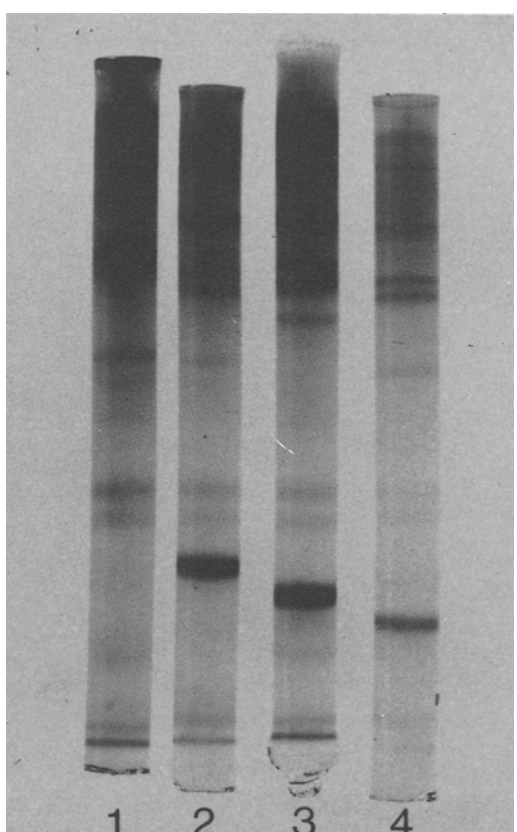

FIG. 1. Polyacrylamide gels of seed extracts from seeds showing SBTI-A_2 bands. From left to right: 1. no SBTI-A_2 band (*ti*); 2. Rf 0.75 (Ti^b); 3. Rf 0.79 (Ti^a); 4. Rf 0.83 (Ti^c).

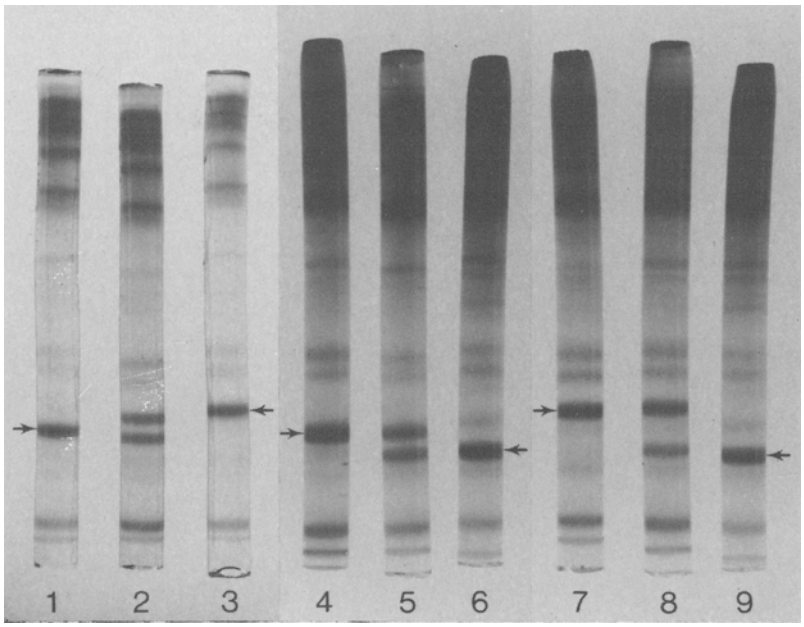

FIG. 2. Polyacrylamide gels of parents and F_1 seeds of soybean lines having different forms of the SBTI-A_2 protein. From left to right: 1. Ti^a (Rf 0.79) arrow points to the SBTI-A_2 band; 2. F_1 cross between Ti^a and Ti^b; 3. Ti^b (Rf 0.75) arrow points to the SBTI-A_2 band; 4. Ti^a (Rf 0.79) arrow points to the SBTI-A_2 band; 5. F_1 of cross between Ti^a and Ti^c; 6. Ti^c (Rf 0.83) arrow points to the SBTI-A_2 band; 7. Ti^b (Rf 0.75) arrow points to the SBTI-A_2 band; 8. F_1 cross between Ti^b and Ti^c; 9. Ti^c (Rf 0.83) arrow points to the SBTI-A_2 band.

black staining solution. Destaining was carried out using a gel destainer. Identification of the SBTI-A_2 band type was then made on the destained gels. All sample preparations and electrophoresis operations were carried out at room temperature.

For the inheritance studies, a small portion of seed was cut from the seed oppostie the embryo. The small portion of seed was ground in 1 ml of buffer solution. The extraction and electrophoretic procedure used was the same as the whole seed procedure indicated in the previous paragraphs. After determining the genotype of the seed in the small portion of seed, the rest of the seed was planted to produce the next generation.

Identification of the Kunitz trypsin inhibitor band in the gel or the lack of the band was made by assaying the bands for trypsin activity by a spectrophotometric procedure (23) and by comparing the results with those for commercially available Kunitz trypsin inhibitor.

GENETIC STUDIES

Two electrophoretic types of the ABTI-A_2 protein were identified by Singh et al. (23) and a third type was identified by Hymowitz (24). The three electrophoretic types occur at Rf 0.79, Rf 0.75, and Rf 0.83 (Rf = mobility relative to a bromophenol blue dye front in a 10% polyacrylamide gel anodic system using a pH 8.3 Tris-glycine buffer) and are designated Ti^a, Ti^b, and Ti^c, respectively (25). Recently two accessions, P.I. 157440 and P.I. 196168, were identified as lacking the SBTI-A_2 protein band (26,27). Assays of protein bands in acrylamide gels of both accessions revealed there weren't any major trypsin inhibitor bands present in the gels. Assays of the crude seed extract of P.I. 157440 revealed that there was 30-50% less trypsin inhibiting activity per gram protein than in Amsoy 71, a commercial soybean cultivar. The lack of the SBTI-A_2 protein is designated *ti*. The four electrophoretic types of SBTI-A_2 are shown in Figure 1.

When crosses were made between soybean lines having different electrophoretic types of SBTI-A_2, the F_1 seeds exhibited bands found in both parents (Fig. 2). The F_2 seeds segregated in a 1:2:1 ratio for the first parental genotype: the heterozygous genotype (both bands); the second parental genotype. A summary of the results of crosses made to elucidate the mode of inheritance of the three SBTI-A_2 electrphoretic types is shown in Table I. These results along with additional data established that the three electrophoretic types of the Kunitz trypsin inhibitor occurring at Rf 0.79 (Ti^a), Rf 0.75 (Ti^b), and Rf 0.83 (Ti^c) are controlled by a co-dominant multiple allelic system at a single locus (28,29).

When crosses were made between each of the three different SBTI-A_2 types (Ti^a, Ti^b or Ti^c) and P.I. 157440 (a line which lacks the SBTI-A_2 protein band), the F_1 seed

TABLE I

Observed Segregation of F_2 or F_3 Seeds from Selfed F_1 or F_2 Soybean Plants Heterozygous for the Electrophoretic Bands of SBTI-A_2[a]

Cross							
Genotype	Rf[b]	No. of seeds	Homozygote	Heterozygote	Homozygote	χ^2	Probability
$Ti^aTi^a \times Ti^bTi^b$	0.79 x 0.75	100	26	50	24	0.08	0.96
$Ti^aTi^a \times Ti^cTi^c$	0.79 x 0.83	274	63	136	75	1.07	0.59
$Ti^bTi^b \times Ti^cTi^c$	0.75 x 0.83	433	166	216	101	1.04	0.59

[a]Data taken in part from Hymowitz and Hadley (28) and Orf and Hymowitz (29).

[b]Rf = mobility relative to a bromophenol blue dye front in a 10% polyacrylamide gel anodic system using a pH 8.3 Tris-glycine buffer.

TABLE II

Observed Segregation of F_2 Seed from Selfed F_1 Soybean Plants for the Presence (+) or Absence (–) of the SBTI-A_2 Band[a]

Cross Genotype	Rf[b]	No. of seeds	+SBTI-A_2	-SBTI-A_2	χ^2	Probability
Ti^a*Ti*^{a} x *ti ti*	0.79	320	239	81	0.017	0.90
Ti^b*Ti*^{b} x *ti ti*	0.75	160	119	41	0.033	0.86
Ti^c*Ti*^{c} x *ti ti*	0.83	320	241	79	0.017	0.90

[a]Data taken in part from Orf et al. (26) and Orf and Hymowitz (25).

[b]Rf = mobility relative to a bromophenol blue dye front in a 10% polyacrylamide gel anodic system using a pH 8.3 Tris-glycine buffer.

TABLE III

Distribution of the Kunitz Trypsin Inhibitor Types in the USDA Soybean Germplasm Collection[a]

Collection	*Ti*^{a}	*Ti*^{b}	*Ti*^{c}	*ti*	Total
Asia					
Japan	284	187	6		477
Korea	366	48	1	2	417
China	794	9			803
Other	345	37	1		383
Europe	405	29			434
Africa	56				56
Other					
Cultivars	320	15			335
Genetic Types	89	5			94

[a]Data taken in part from Hymowitz et al. (27), Clark et al. (30), Hymowitz et al. (31), Orf (32), and Skorupska and Hymowitz (33).

exhibited only one SBTI-A_2 band, the band of the parent containing either *Ti*^{a}, *Ti*^{b} or *Ti*^{c}. The F_2 seed segregated in a 3:1 ratio for the presence or absence of the SBTI-A_2 protein (Table II). These data established that the lack of the SBTI-A_2 protein in soybean seed is inherited as a simple recessive allele to *Ti*^{a}, *Ti*^{b} or *Ti*^{c} (25,26).

SCREENING STUDIES

Virtually the entire U.S. soybean germplasm collection was screened to determine the frequency of the SBTI-A_2 types within the collection and to determine if the SBTI-A_2 types were associated with a particular country or geographical area (24,27,30-33). As shown in Table III, of the 2999 soybean accessions tested, 2659 accessions or 88.7% had the *Ti*^{a} allele. All of the important commercially grown soybean cultivars in the U.S. have the *Ti*^{a} allele. The *Ti*^{b} allele was found in 11% of the population and appears to be associated with the soybean accessions from Japan and Korea. The *Ti*^{c} allele was found 0.3% of the population (8 accessions) and is geographically associated with the Tohoku District of Japan. The *ti* allele occurred in 2 accessions (0.06% of the population), both of which came from Korea (27).

LINKAGE STUDIES

Investigations conducted to determine whether the *Ti* locus was linked to several other loci revealed that the following loci are inherited independently of the *Ti* locus: a) the Dt_1 locus (stem termination); 2) the W_1 locus (flower color); 3) the *Ep* locus (seed coat peroxidase); 4) the Sp_1 locus (a seed protein band) (34); and 5) the *Le* locus (a seed lectin) (35).

GERMINATION STUDIES

Changes in three types of the Kunitz trypsin inhibitor (*Ti*^{a}, *Ti*^{b} and *Ti*^{c}) during germination were detected using polyacrylamide gel electrophoresis (36). In each population there was a shift in the mobility of the SBTI-A_2 band that appeared in the mature dry seed. The changes in the electrophoretic mobilities of the bands began about day 4 into germination and were completed by about day 6 (Figs. 3, 4, 5). Only part of the SBTI-A_2 band found in mature dry seed was changed to the new type during germination. These were no differences in the Rf distances between bands of the three types of SBTI-A_2 found in mature seed and the Rf distances between the original and new bands found during germination. The new types of SBTI-A_2 noted during germination could be due to a modification of the original SBTI-A_2 protein by a protease. Curiously, no changes or modification of the SBTI-A_2 protein take place during seed development. The SBTI-A_2 electrophoretic band that appeared in the developing seed was the same band that was found in the mature seed.

POTENTIAL APPLICATIONS

Since the inheritance of the SBTI-A_2 is known, the types can be used as genetic markers for determining F_1 hybrids. The different types can be used in cultivar identification or cultivar certification programs by crop improvement associations (37). The SBTI-A_2 types also can be utilized in biosystematic studies of the species in the genus *Glycine* (38).

Clark and Hymowitz (39) found that lines with the *Ti*^{b} allele had less trypsin inhibiting activity than lines with the *Ti*^{a} allel. Subsequent investigations revealed that unheated defatted meal from a *Ti*^{b} near isoline was nutritionally superior to unheated defatted meal from the *Ti*^{a} recurrent parent (40-43). Preliminary studies with chicks suggest that unheated defatted meal of P.I. 157440 (*ti*) gave greater feed efficiency and less pancreatic hypertrophy than unheated defatted meal from Amsoy 71 (*Ti*^{a}) (44). Perhaps soybean seed without the Kunitz trypsin inhibitor does not need to be processed as extensively with moist heat as currently grown soybean seed. Therefore, a savings in energy and

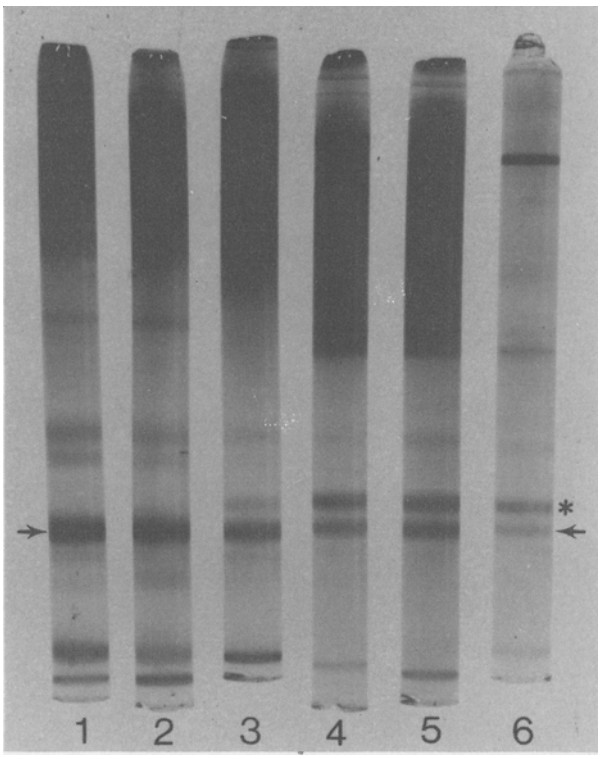

FIG. 3. Polyacrylamide gels of seed extracts from germinated seed having the Ti^a SBTI-A_2 band. From left to right: 1. day 0, Rf 0.79; 2. day 2, Rf 0.79; 3. day 4, Rf 0.79 and Rf 0.75; 4. day 5, Rf 0.79 and Rf 0.75; 5. day 6, Rf 0.79 and Rf 0.75; 6. day 11, Rf 0.79 and Rf 0.75. Arrow points to the original SBTI-A_2 band, and the asterisk indicates the new SBTI-A_2 band.

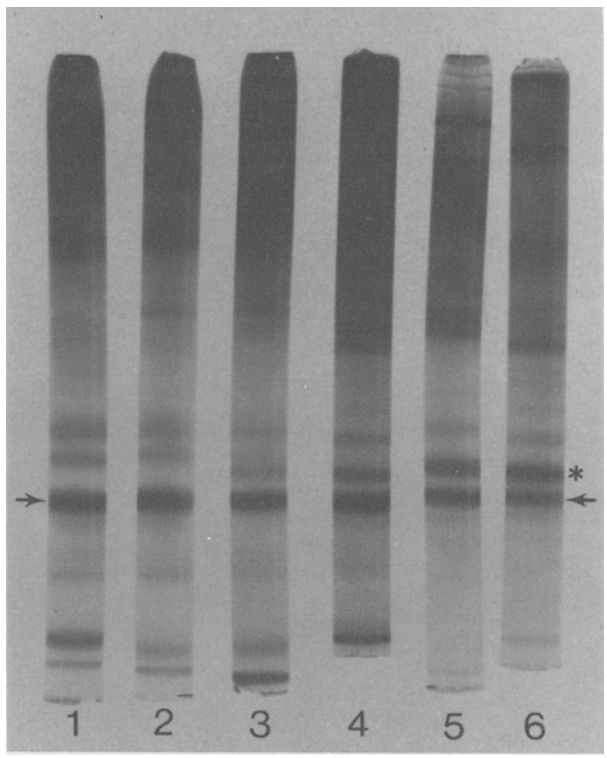

FIG. 4. Polyacrylamide gels of seed extracts from germinated seed having the Ti^b SBTI-A_2 band. From left to right: 1. day 0, Rf 0.75; 2. day 2, Rf 0.75; 3. day 4, Rf 0.75 and Rf 0.71; 4. day 5, Rf 0.75 and Rf 0.71; 5. day 6, Rf 0.75 and Rf 0.71; 6. day 11, Rf 0.75 and Rf 0.71. Arrow points to the original SBTI-A_2 band, and the asterisk indicates the new SBTI-A_2 band.

processing costs might be realized by the processors of soybeans for the feed and food industry.

Obviously, the soybean does not need the Kunitz trypsin inhibitor for its survival. What exactly is the role of the protein in soybean seed, and what is the selective advantage of soybeans having the Kunitz trypsin inhibitor? The availability of soybean lines without the Kunitz trypsin inhibitor would appear to be useful to plant physiologists and biochemists studying the role of trypsin inhibitors in soybean seed.

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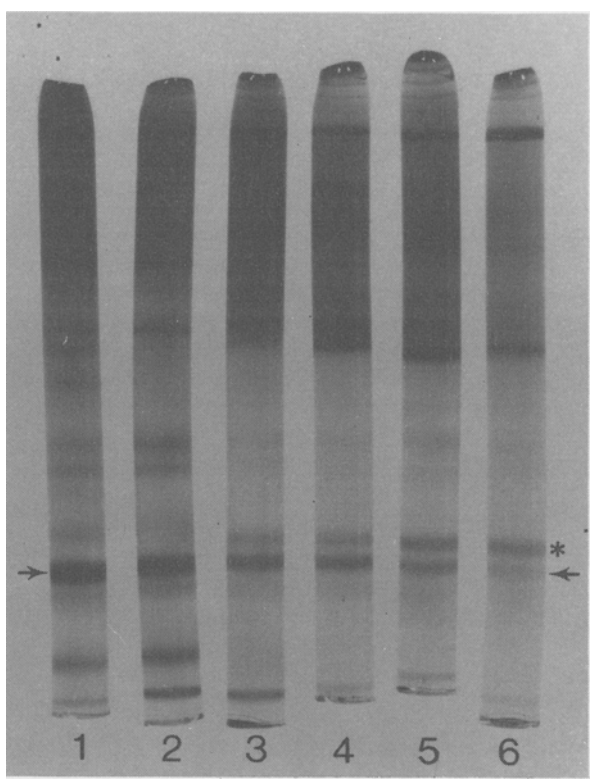

FIG. 5. Polyacrylamide gels of seed extracts from germinated seed having the Ti^c SBTI-A_2 band. From left to right: 1. day 0, Rf 0.83; 2. day 2, Rf 0.83; 3. day 4, Rf 0.83 and Rf 0.79; 4. day 5, Rf 0.83 and Rf 0.79; 5. day 6, Rf 0.83 and Rf 0.79; 6. day 11, Rf 0.83 and Rf 0.79. Arrow points to the original SBTI-A_2 band, and the asterisk indicates the new SBTI-A_2 band.

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