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ORIGINAL ARTICLE

Proteomic characterization of Kunitz trypsin inhibitor variants, Tia and Tib, in soybean [*Glycine max* (L.) Merrill]

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Abstract The soybean Kunitz trypsin inhibitor (KTi) has several polymorphic variants. Of these, Tia and Tib, which differ by nine amino acids, are the two main types. In this study, differences in KTi proteome between Tia and Tib were investigated using three soybean cultivars and three mutant lines. Two cultivars, Baekwoon (BW) and Paldal (PD), and one mutant line, SW115-24, were Tia type, whereas one soybean cultivar, Suwon115 (SW115), and two mutant lines, BW-7-2 and PD-5-10, were Tib type. Protein from the six soybean lines was extracted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), non-denaturing polyacrylamide gel electrophoresis (non-denaturing PAGE), and two-dimensional polyacrylamide gel electrophoresis (2-DE). By SDS-PAGE, there was no difference between soybean cultivars and mutant lines, except for SW115-24. Western blot analysis revealed that, in comparison with Tia, Tib type accumulated relatively low amounts of KTi. By non-denaturing PAGE, the three soybean lines of Tib type were characterized by slower mobility than the three soybean lines of Tia type. Zymography detected eight distinct zones of trypsin inhibitory activity among which Tia and Tib lacked the fifth and sixth zone, respectively. By two-dimensional native polyacrylamide gel electrophoresis (2-DN), the spots related to trypsin inhibitory activity showed different mobilities, whereas only one KTi (21.5 kDa) spot was resolved by 2-DE. By two-dimensional zymography (2-DZ), Tib showed a broader activity zone (pI 4–7) in comparison with Tia (pI 4–5). The results indicate that the genotypes with a different type of KTi present different proteomic profiles and trypsin inhibitory activities.

Keywords Kunitz trypsin inhibitor · Mutation · Proteome · Soybean

Abbreviations

2-DE	Two-dimensional polyacrylamide gel electrophoresis
2-DN	Two-dimensional native polyacrylamide gel electrophoresis
2-DZ	Two-dimensional zymography
CBB	Coomassie brilliant blue R-250
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
pI	Isoelectric point
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Introduction

Soybean is an important source of protein and oil. Most commercially grown soybean cultivars contain about 40% protein and 20% oil (Krishnan et al. 2000). The total seed protein content of soybean is about 6% proteinase inhibitors (Wang et al. 2004). These inhibitors have been identified as belonging to two major groups, Kunitz trypsin inhibitor (KTi, Kunitz 1945) and Bowman–Birk trypsin inhibitor (BBTi, Bowman 1946; Birk 1961). The effects of trypsin inhibitors become evident in the inhibition of intestinal digestion. As a result, the presence of proteinase inhibitors even in diets consisting of free amino acids leads to decreased growth (Lajolo and Genovese 2002). Additionally, KTi and BBTi were found to induce enlargement of the pancreas (hypertrophy and hyperplasia) and hypersecretion of digestive enzymes in rodents and birds. The loss in sulfur-rich endogenous proteins, trypsin and chymotrypsin, results in growth depression because soy proteins are deficient in sulfur containing amino acids such as methionine (Lajolo and Genovese 2002).

Soybean KTi is a small, monomeric, and non-glycosylated protein containing 181 residues. This 21.5 kDa protein was firstly isolated and crystallized from soybean seeds (Kunitz 1945). KTi belongs to the family of all antiparallel β-sheet proteins that are highly resistant to thermal and chemical denaturation (Roychaudhuri et al. 2004). KTi has been found to possess 12 distinguishable electrophoretic forms: Tia, Tib (Singh et al. 1969), Tic (Hymowitz 1973), Tid (Zhao and Wang 1992), Tie (Wang et al. 1996, 2001), Ti-null type (Orf and Hymowitz 1979), Tif (Wang et al. 2004), Tibi5 (Wang and Li 2005), Tiaa1, Tiaa2, Tiab1, and Tig (Wang et al. 2008). These types are controlled by co-dominant multiple alleles at a single locus (Wang et al. 2008).

Studies of polymorphic variants of KTi at the amino acid and nucleotide level have revealed large variations in their sequences. A difference of nine amino acid residues was reported between Tia and Tib (Song et al. 1993; Wang et al. 2004). The amino acid sequences of Tic, Tid, and Tie differ from Tia by only one amino acid (Kim et al. 1985; Xin et al. 1999; Wang et al. 2001), and those of Tif and Tib also differ by one amino acid (Wang et al. 2004). Many researchers have investigated the occurrence of polymorphisms in KTi by non-denaturing polyacrylamide gel electrophoresis (non-denaturing PAGE) that resolves three KTi types, Tia, Tib, and Tic (Orf and Hymowitz 1979; Wang et al. 1996, 2001, 2008). Singh et al. (1969) observed an electrophoretic polymorphism between Tia and Tib. Additionally, Wang et al. (1996) reported that the electrophoretic mobility of Tib was slower than that of Tia.

Two-dimensional polyacrylamide gel electrophoresis (2-DE) is one of the most powerful proteomics tools for the separation and quantification of proteins (Herbert 1999; Gore et al. 2000). For functional characterization of proteins; however, runs under non-denaturing conditions, both as 1- and 2-DE, are especially useful. Zymographic techniques may be used to detect proteolytic enzymes in nanogram quantities following electrophoretic separation in gels (Kim et al. 1998). These methods are based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which co-polymerizes with the protein substrate that is to be degraded by the proteases. The enzymes are renatured during incubation in enzyme reaction buffer after the electrophoretic separation (Choi et al. 2004); enzyme activities are visualized as clear bands that contain proteolytically degraded substrate.

Comprehensive and comparative analyses of KTi proteins and of the genetic variation among different soybean cultivars are important in order to better understand allergenicity. In this study, for the proteomic characterization of the KTi activities of Tia and Tib variants, we conducted a detailed comparative analysis of the KTi profiles of three soybean cultivars and of three mutant lines derived from them by SDS-PAGE, non-denaturing PAGE, 2-DE, two-dimensional double native polyacrylamide gel electrophoresis (2-DN), zymography, and two-dimensional zymography (2-DZ).

Materials and methods

Plant materials

Soybean seeds of cvs. Baekwoon (BW), Paldal (PD), and Suwon115 were irradiated with gamma rays generated using a ^{60}Co gamma-irradiator (150 TBq of capacity; ACEL, ON, Canada) at the Korea Atomic Energy Research Institute (KAERI). Irradiated and control seeds were sown at the Breeding Research Farm at the KAERI. The M_1 plants were harvested individually and carried forward to the M_2 generation. We selected genetically fixed mutant lines with excellent agricultural characteristics such as yield, disease resistance, and tolerance of environment stress from 1989 to 2007. For the analysis of KTi characteristics, the three original cultivars, BW, PD, and SW115, along with three mutant lines, BW-7-2 (M_{21}), PD-5-10 (M_{16}), and SW115-24 (M_{16}), were cultivated in 2008.

SDS-PAGE and non-denaturing PAGE

Total crude proteins were extracted from 100 mg of grained dry seeds in 5 ml 100 mM Tris–HCl buffer (pH

8.0) containing 20 mM $CaCl_2$. The suspension was centrifuged at 15,000 rpm for 30 min at 4°C. The clear supernatant was used for SDS-PAGE and non-denaturing PAGE. For SDS-PAGE, 50 µl of the total crude protein were added to an equivalent amount of 5× SDS-sample buffer (10% SDS, 50% glycerol, 1.96% β-mercaptoethanol, 0.002% bromophenol blue and 1 M Tris–HCl, pH 6.8). The samples were held in boiling water for 5 min and then centrifuged for 5 min. Supernatants containing total seed proteins were used for SDS-PAGE and non-denaturing PAGE. Ten microlitres of the supernatant were used for 12% SDS-PAGE with 0.75 mm gel thickness and Tris–glycine buffer containing 1% SDS in a Mini-PROTEAN 3 Cell (Biorad, CA, USA). Electrophoresis was carried out at a constant voltage of 100 V for 120 min. For non-denaturing PAGE, 50 µl of the supernatant was added to an equivalent amount of 5× non-denaturing-sample buffer (50% glycerol, 0.002% bromophenol blue, and 1 M Tris–HCl, pH 6.8). Ten microlitres of the supernatant were used for 12% non-denaturing PAGE with 0.75 mm gel thickness and Tris–glycine buffer without SDS in a Mini-PROTEAN 3 Cell (Biorad). Electrophoresis was carried out at a constant current of 20 mA per gel for 120 min. The protein patterns after SDS-PAGE and non-denaturing PAGE were visualized by staining with Coomassie brilliant blue R-250 (CBB).

Immunoblot analysis

Total crude proteins (50 µg/10 µl) were resolved by 12% SDS-PAGE with 0.75 mm gel thickness using a Mini-PROTEAN 3 Cell (Biorad). Electrophoresis was carried out at a constant current of 20 mA per gel at room temperature. After electrophoresis, the proteins from the polyacrylamide gels were transferred to nitrocellulose membranes with a Mini Trans-Blot Cell (Biorad) following the procedure recommended by the manufacturer. After the transfer, the nitrocellulose membranes were incubated with TBS (10 mM Tris–HCl, pH 7.5 containing 500 mM NaCl) containing 5% (*w/v*) non-fat dairy milk. Membranes were then incubated with 1:1,000,000 diluted anti-trypsin inhibitor antibodies (Abcam, Cambridge, UK) in TBS containing 2% (*w/v*) non-fat dairy milk. Immunoreactive polypeptides were visualized using the ECL reagent provided by the manufacturer.

Gel electrophoretic analysis of KTI activity

Gel electrophoretic analysis of trypsin inhibitory activity was performed according to Krishnan (2001b). After non-denaturing electrophoresis, gels were incubated for 1 h with constant shaking in 100 mM phosphate buffer (pH 7.4), followed by incubation in 100 mM sodium phosphate buffer (pH 7.4 with 0.1 mg/ml of trypsin) for an additional 10 min at 37°C. The substrate was prepared by dissolving 2.5 mg of acetyl-DL-phenylanine β-napthyl ester in 1 ml of dimethyl formamide, followed by mixing with 9 ml of 0.5 mg/ml tetrazotized ortho-dianisidine in 100 mM phosphate buffer (pH 7.4). Gels were briefly washed with deionized water and then stained for trypsin inhibitory activity in the substrate mixture. After staining for 30 min, the gels were rinsed in deionized water. Regions containing trypsin inhibitory activity remained unstained against a pink background.

Two-dimensional polyacrylamide gel electrophoresis (2-DE)

Extraction of seed proteins using 2-DE was performed according to Natarajan et al. (2006). Soybean seed powder (100 mg) was homogenized with 5 ml of a solution containing 10% (*w/v*) trichloroacetic acid (TCA) in acetone with 0.07% (*v/v*) 2-mercaptoethanol. Total protein was precipitated for 1 h or overnight at −20°C. The extract was centrifuged at 20,800*g* for 20 min at 4°C. The pellet was washed two or three times with acetone containing 0.07% (*v/v*) 2-mercaptoethanol. Then the pellet was dried under vacuum for 30 min, and the acetone dry powder was resuspended in 1 ml of lysis buffer [9 M urea, 1% CHAPS, 1% (*w/v*) ampholytes (pH 3–10), 1% DTT], followed by sonication on ice for 30 min. Insoluble material was removed by centrifugation at 20,800*g* for 20 min at 4°C. The extracted proteins (400 µg/50 µl) were separated by 2-DE. An IPGphor apparatus (GE Healthcare, Piscataway, NJ) was used for IEF with immobilized pH gradient (IPG) strips (pH 4–7, linear gradient, 17 cm). The IPG strips were rehydrated 16 h with 340 µl rehydration buffer (8 M urea, 2% CHAPS, 0.5% Pharmalyte, 0.002% bromophenol blue). The voltage setting for IEF was 500 V for 1 h, 1,000 V for 1 h and 8,000 V to a total 36 kVh. Following electrophoresis, the proteins in the strips were denatured with equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT) and then incubated with the same buffer without DTT for 30 min at room temperature. The second dimension electrophoresis was performed on a 12% SDS gel with 1.0 mm thickness using a PROTEIN II XL Cell (Biorad). The gels were stained with CBB.

Two-dimensional native polyacrylamide gel electrophoresis (2-DN) and two-dimensional zymography (2-DZ)

Total crude proteins (400 µg/50 µl) were separated by 2-DN and 2-DZ. IEF gel electrophoresis and rehydration was performed with the same method as above (non-denaturing PAGE). After the first dimension, the strips were equilibrated using equilibration buffer (50 mM Tris–HCl pH 8.8, 0.002% bromophenol blue). The second dimension electrophoresis was performed on a 12% non-denaturing gel with 1.0 mm

thickness using a PROTEIN II XL Cell (Biorad). The gels were stained with CBB. To identify the differences of KTi activity between Tia and Tib by 2-DZ, the same method of zymography described above was performed using 2-DN gels without staining with CBB.

MALDI-TOF-MS/MS analysis

Coomassie brilliant blue R-250-stained protein spots were excised from gels, washed with 15 mM potassium ferricyanide and 50 mM sodium thiosulfate, and destained with 50 mM NH_4HCO_3 in 50% methanol at 40°C for 15 min. Proteins were reduced with 10 mM dithiothreitol in 100 mM NH_4HCO_3 at 50°C for 1 h, followed by incubation with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min. The gel pieces were minced and allowed to dry, and then rehydrated in 10 mM Tris–HCl (pH 8.5) with 1 pM trypsin at 37°C for 10 h. The digested peptides were extracted from the gel slices with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile/water. The peptide solutions were analyzed using a MALDI-TOF/TOF mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Data were analyzed using GPS Explorer (Applied Biosystems) and Mascot softwares (version 2.2.18) (Matrix Science, London, UK).

Results

SDS-PAGE patterns and immunoblot analysis of soybean cultivars and mutant lines

All the three soybean cultivars and three mutant lines show bands with molecular weights ranging from 15 to 100 kDa (Fig. 1). Two cultivars (BW and PD) and two mutant lines (BW-7-2 and PD-5-10) do not show any difference in their protein banding patterns whereas SW115-24 lacks an approximately 66 kDa band in comparison with the original cultivar (SW115) (Fig. 1, Lane 6). An approximately 24 kDa band is present in all lines. To determine whether or not the lower levels of trypsin inhibitory activity in the Tib type lines are related to lower accumulation of KTi, immunoblot analysis was performed. Antibodies raised against gel-purified KTi detect the 24 KDa band in all lines. The two soybean cultivars (BW and PD) and one mutant line (SW115-24) of Tia type show bands of higher intensity in comparison with the three soybean lines (BW-7-2, PD-5-10, and SW115) of Tib type (Fig. 2).

Non-denaturing PAGE and difference in the major isozyme of trypsin inhibitor

By non-denaturing PAGE the three Tia type soybean lines (BW, PD, and SW115-24) show bands with higher mobility in comparison with the Tib type soybean lines (BW-7-2, PD-5-10, and SW115) (Fig. 3). To characterize the residual activity, we performed zymography, which shows eight distinct zones of trypsin inhibitory activity. Among the eight zones, Tia and Tib do not have the fifth and sixth zone, respectively (Fig. 4).

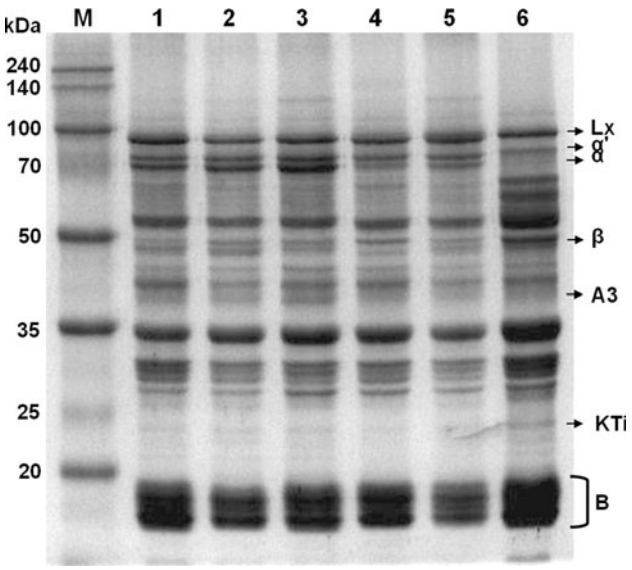

Fig. 1 Protein profiles of three soybean cultivars and three mutant lines by 12% SDS-PAGE: *lane 1* molecular weight marker, *lane 2* BW, *lane 3* BW-7-2, *lane 4* PD, *lane 5* PD-5-10, *lane 6* SW115, *lane 7* SW115-24. Mutants showed similar protein profiles to those of their parent lines. *Lx* lipoxygenase, *α′* α′-subunit, *α* α-subunit, *β* β-subunit, *A3* A3-subunit, *KTi* Kunitz trypsin inhibitor, *B* basic

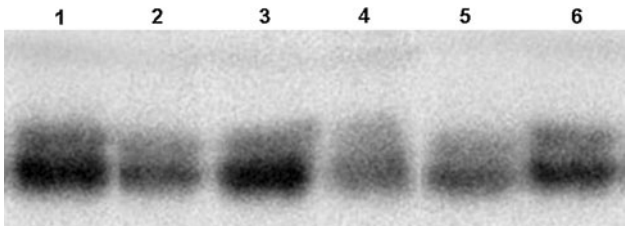

Fig. 2 Western blot of soybean seed proteins. Total seed proteins were resolved by 12.5% SDS-PAGE and then electrophoretically transferred to nitrocellulose for immunological analysis with KTi-specific antibodies. *Lane 1* BW, *lane 2* BW-7-2, *lane 3* PD, *lane 4* PD-5-10, *lane 5* SW115, *lane 6* SW115-24

Two-dimensional polyacrylamide gel electrophoresis (2-DE)

To investigate the differential gene expression of KTi, seed storage proteins were extracted from dry soybean seeds, separated by 2-DE analysis, and stained with CBB (Fig. 5). BW-7-2, PD-5-10, and SW115-24 show different protein

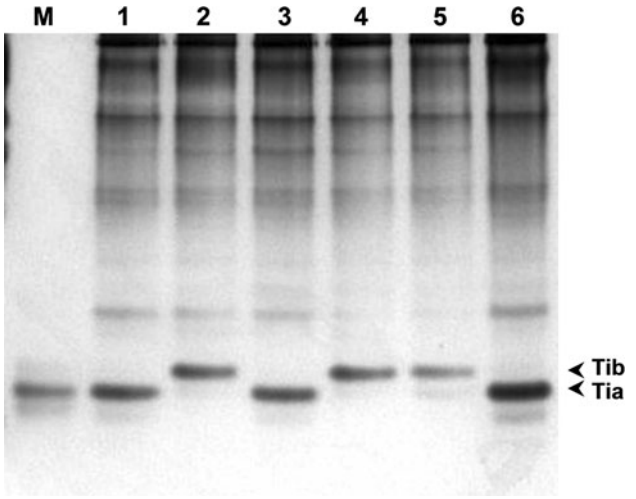

Fig. 3 Electrophoretic profiles of three soybean cultivars and three mutant lines as determined by 12% non-denaturing PAGE. *Lane 1* Tia type marker, *lane 2* BW, *lane 3* BW-7-2, *lane 4* PD, *lane 5* PD-5-10, *lane 6* SW115, *lane 7* SW115-24. Tib type was slower mobility than Tia type

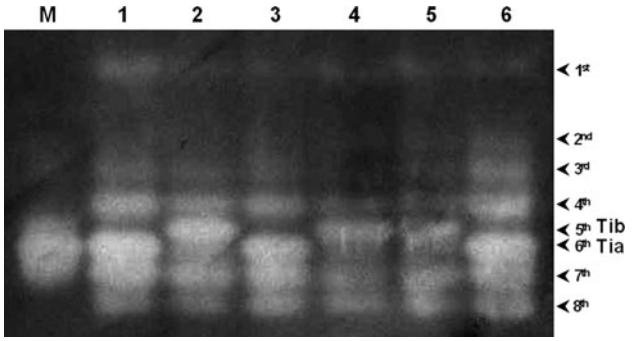

Fig. 4 Gel electrophoretic analysis of trypsin inhibitor activity. Samples were resolved by non-denaturing PAGE and then stained for trypsin inhibitor activity. *Lane 1* molecular weight marker, *lane 2* BW, *lane 3* BW-7-2, *lane 4* PD, *lane 5* PD-5-10, *lane 6* SW115, *lane 7* SW115-24

spots in comparison with their original cultivars. Mutant-specific proteins in BW-7-2, PD-5-10, and SW115-24 correspond to 10, 4, and 19 spots, respectively. The numbers of different proteins in BW-7-2 and PD-5-10 compared to original cultivars, BW and PD are 10 and 3 spots, respectively (Fig. 5a, b), whereas SW115-24 has no new spots (Fig. 5c). Immunoblot analysis detects a positive spot with pI 4 (data not shown).

Characterization of KTi by two-dimensional native polyacrylamide gel electrophoresis (2-DN) and two-dimensional zymography (2-DZ)

To further characterize the differences between Tia and Tib, we separated the proteins by IEF on precasted gel strips with a linear IPG (pH 4–7) followed by 12% non-denaturing PAGE. The gels were then stained with CBB, or processed for zymographic analysis.

As shown in Fig. 6, BW and BW-7-2 (panel A) display more protein spots (4) than the other soybean lines, which have two each (PD and PD-5-10 are shown in panel B, SW115 and SW115-24 in panel C). Taking the other spots as reference, B3 and B4, P2 and P3, and S2 and S3 show different mobilities, with the spots of Tib type slower than those of Tia type.

In Fig. 7, a zymogram of the Tib type lines shows a broader activity pattern in comparison with the Tia type lines. The enzymatic forms of Tia are isoelectric under acid conditions (pI 4–5). Instead, the Tib types have pI in the range 4–5 as well as 5–7.

Identification of amino acid sequences

To elucidate the structures of these KTi proteins, the protein spots with different mobilities were excised from the non-denaturing polyacrylamide gel, and sequence analysis was performed using Mascot search tools for protein identification (Table 1). Among the 11 protein spots, 9 were identified as trypsin inhibitor or proteins related to trypsin inhibitor. The protein spots with different mobilities between Tia and Tib, B3 and B4, P2 and P3, and S2 and S3, were identified as soybean trypsin inhibitor chain A (B3, P3, and S2), trypsin inhibitor subtype B (*Glycine max*) (P2 and S3), or KTi C (B4). The protein spots B1, P1, and S1 were identified as Kunitz-type trypsin inhibitor precursor. The remaining two spots (B2 and B5) were identified as beta-conglycinin alpha chain precursor and unknown protein, respectively.

Discussion

Kaizuma et al. (1980) demonstrated that differentiation of Tia and Tib was very ancient and probably occurred before domestication of cultivated soybeans from wild soybeans. Although there is no other evidence to enforce this finding, it is supported by the high frequency of Tia in wild soybeans of China and other Asian countries reported by Hymowitz and Kaizuma (1981) and Li (1993). In our previous study, we identified changes in KTi types from Tia to Tib or vice versa induced by gamma-rays in eight soybean mutant lines and occurrence of point mutations at specific places (Kim et al. 2010). In the present study, the proteomic differences between Tia and Tib were analyzed using three soybean cultivars and their mutant lines. Tia and Tib showed different band mobilities in non-denaturing electrophoresis as a result of the net charge shift connected with their differing by nine amino acid residues.

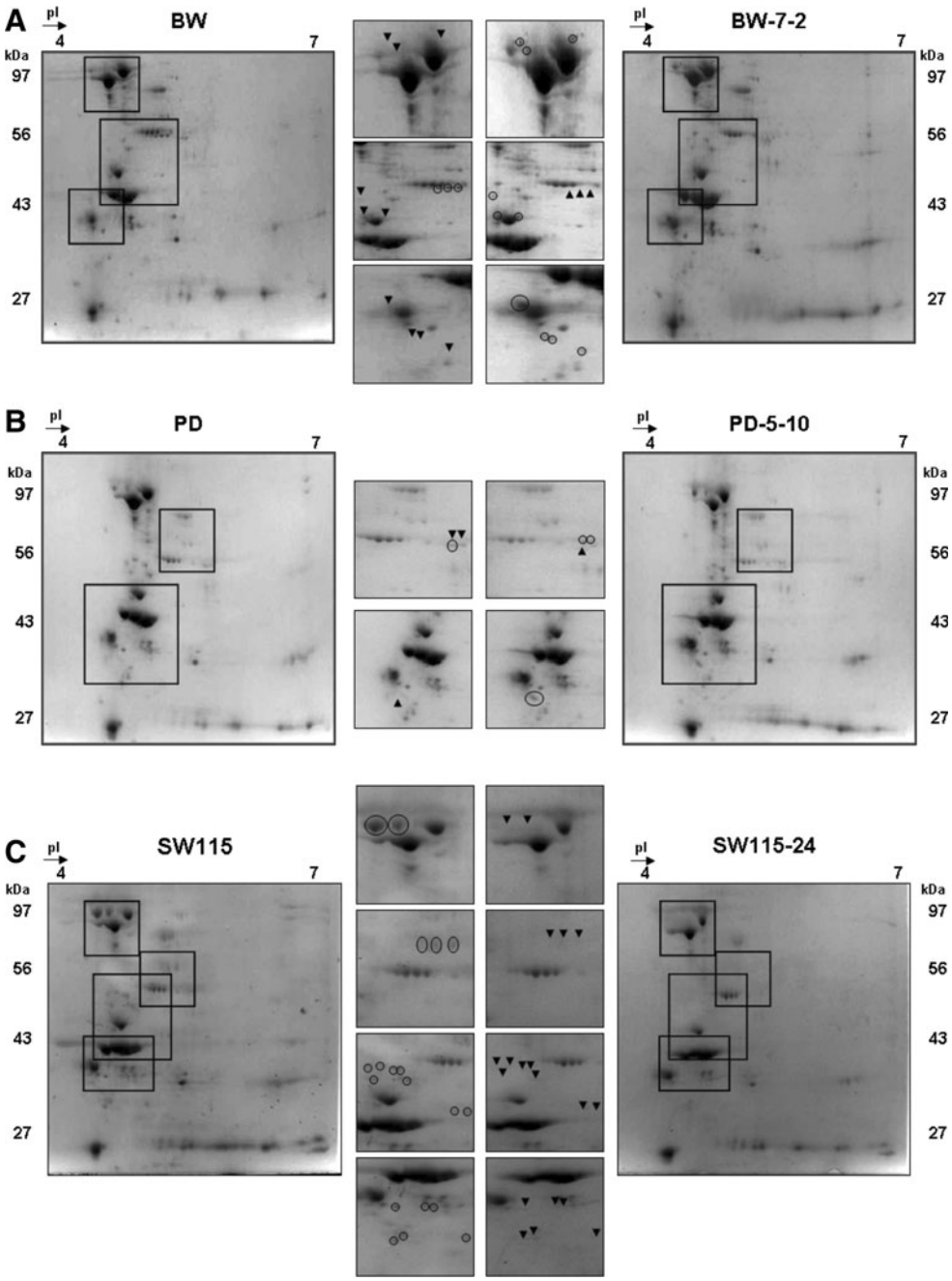

Fig. 5 Protein expression pattern in soybean cultivars and mutant lines. The proteins were extracted from dry seeds, separated by 2-DE, and assessed by CBB staining. IEF was used for first dimension 2-DE. *Circles* indicate protein spots on the gels. *Black triangles* indicate absent spots compared to the other KTi type

Yu and King (1988) suggested that the different electrophoretic mobility in *tsf* mutant proteins is due to 15 amino acid substitutions and to the resulting changes in net charge. Martz et al. (2002) reported that the exchange of two Lys for two Leu in UDP-glucose pyrophosphorylase led to a modification in the net charge and to a decrease in pI, reflected by higher protein mobility during non-denaturing PAGE.

Our previous studies have shown that typical trypsin inhibitory activity ranges from 16.8 to 20.5 trypsin inhibitor unit (TIU) and that Tia type lines show higher activity than those of Tib type (Kim et al. 2010). In the present study, Tib type lines showed by immunoblotting lower band than those of Tia type (Fig. 2). Also, by predicting the protein structure of Tia and Tib, we envisaged a different orientation of the trypsin binding site in Tia and in Tib (data not shown) and suggested that this could influence the interaction of KTi with trypsin.

Non-denaturing electrophoresis is an often overlooked technique for determining native size, subunit structure, and for planning optimal protein separation conditions. Since mobility depends on size, shape, and intrinsic charge of the protein, non-denaturing electrophoresis provides a set of separation parameters that are distinctly different from mainly size-dependent denaturing SDS-PAGE and

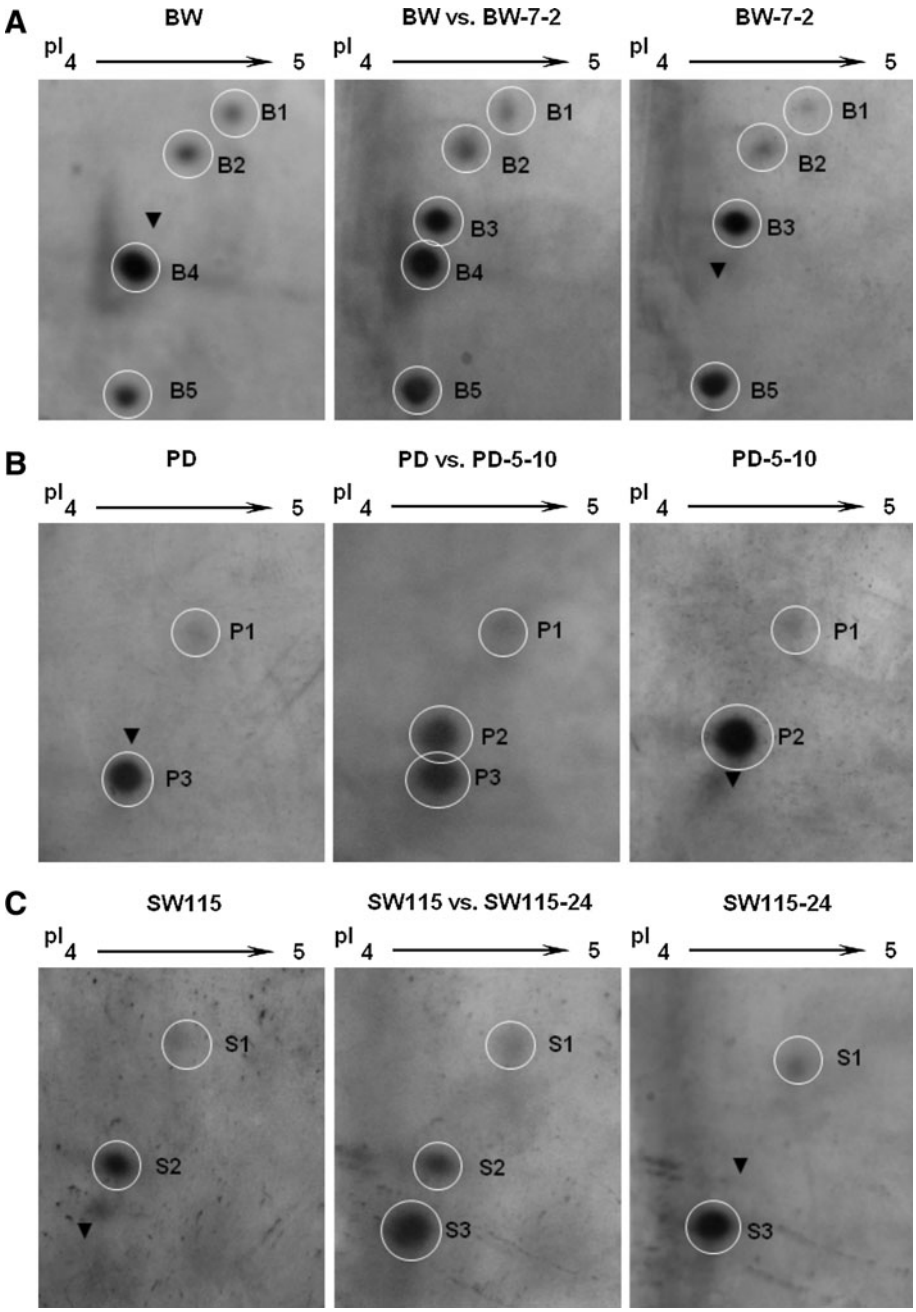

Fig. 6 Proteomic comparison between protein spots of Tia and Tib as determined by non-denaturing 2-DE. The first dimension was run using a pH gradient from 4 to 7 while the second dimension was carried out by 12% non-denaturing PAGE. The middle figures (A2, B2, and C2) overlapped each other in Figs. 1 and 3. *Circles* indicate protein spots on the gels. *Black triangles* indicate absent spots compared to the other KTi type

charge-dependent IEF (Gallagher 1995). Previously, Singh et al. (1969) observed an electrophoretic polymorphism between Tia and Tib in the native PAGE. Additionally, Wang et al. (1996) reported that the electrophoretic mobility of Tib is slower than that of Tia. In our study no difference could be appreciated by SDS-PAGE (Fig. 1). However, by non-denaturing PAGE, the mobility of Tia was found to be faster than that of Tib. 2-DN showed numerous spots indicating different mobility between Tia and Tib. The B4, P3, and S3 spots of the Tia type lines BW, PD, and SW115-24 had faster mobility than the B3, P2, and S2 spots of the Tib type lines BW-7-2, PD-5-10, and SW115 (Fig. 7). According to the MS analysis, the spots B4, P3, and S3 from Tia type soybean lines and B3, P2, and S2 from Tib type soybean lines may be identified as KTi (Table 1).

Visualization of KTi activity in non-denaturing PAGE revealed five distinct bands in Tia and Tib. Krishnan (2001a) reported that P.I. 196168 accumulates low amounts of KTi protein when compared to Amsoy 71, which contains at least five distinct zones of trypsin inhibitory activity, whereas P.I. 196168 contains only four zones of enzyme inhibition. In our study, although Tia and Tib showed significantly different inhibitory activities, no difference could be detected in the number of inhibitory activity

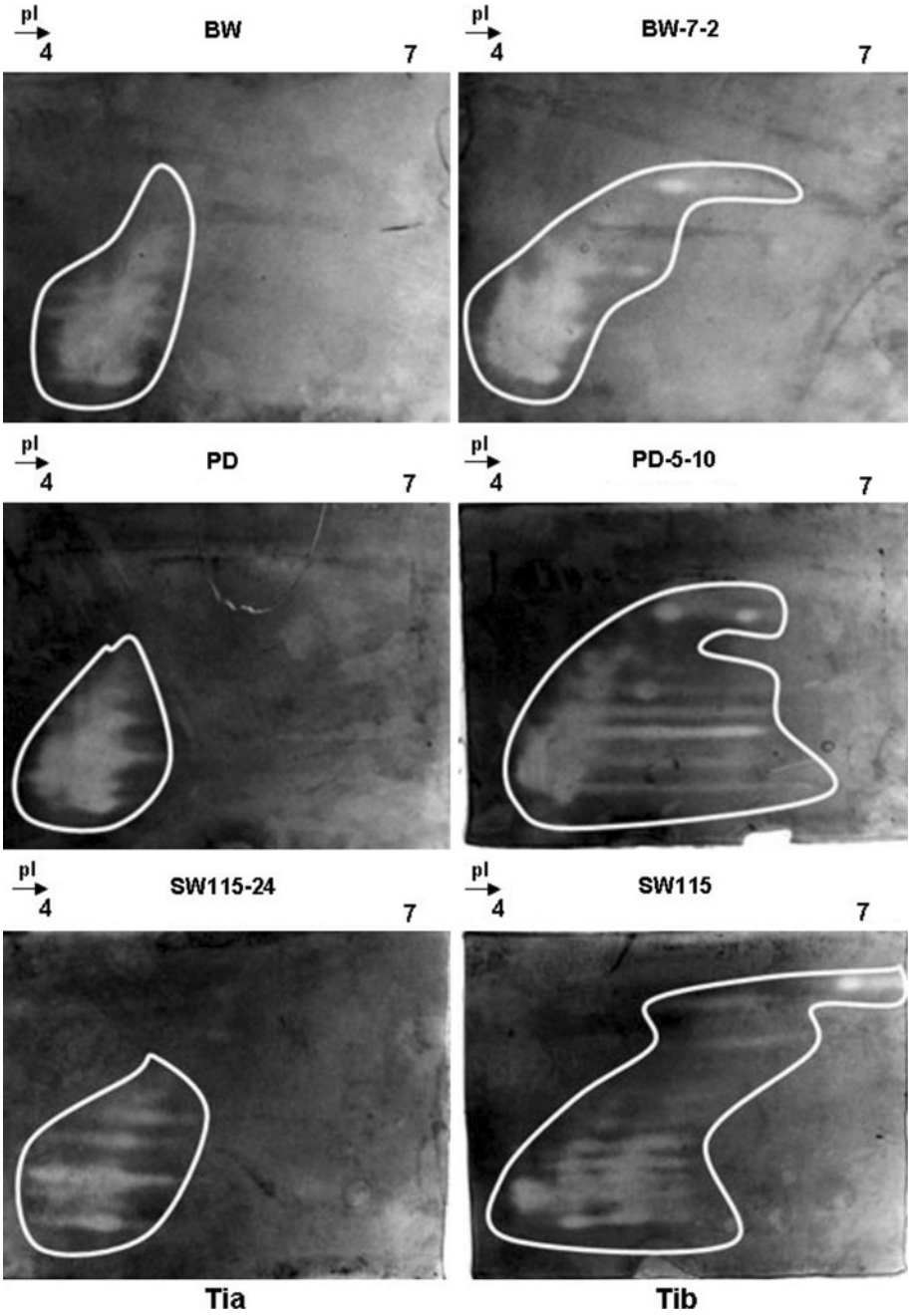

Fig. 7 Detection of KTi activities of Tia and Tib by 2-DE. The soybean lines of Tib type showed a more broad range (pI 4–7) compared to those of Tia type (pI 4–5). *White lines* indicate the activity areas of KTi

zones. However, the fifth and sixth zones were significantly different, as determined by staining with Coomassie blue in non-denaturing PAGE (Fig. 4). We concluded that these corresponded with major zones, interacting with trypsin with the highest affinity. Krishnan (2001) also supposed that since the method used to visualize trypsin inhibitory activity does not differentiate between KTi and BBTi, some of the activity bands could be due to BBTi.

To improve the resolution of the KTi bands, we resorted to the two-dimensional approach to the zymographic assay. When 2-DZ was applied to the seed protein extracts, the latent forms of these proteins revealed distinct patterns of KTi activity in Tia and Tib, whereas the activated forms displayed a similar pattern (Fig. 7). With Tib the area of KTi activity spread over a broader pI range; in the mutants the trypsin inhibitor isozyme, such as BBTi, was modified in net charge and pI as an effect of gamma irradiation.

It is important to know the KTi content of soybean seeds, as active inhibitors can pass through the stomach unaltered due to stability against pepsin and low pH (Weder and Kahley 2003). In this study, the proteomic differences between two major types, Tia and Tib, of KTi

Table 1 Identified proteins are listed according to KTi type

Spot number	Mr	pI	Sequence	Homologous protein (coverage of homology)	Accession number
B1	22.5	4.97	DTVDGWFNIER NKPLVVQFQK	Full = Kunitz-type trypsin inhibitor KTI1; flags: precursor (10%)	GI125722
B2	70.3	5.07	QFPFPRPPHQK NKNPFLFGSNR	Full = Beta-conglycinin, alpha chain; flags: precursor (3%)	GI121281
B3	20.1	4.61	DFVLDNEGNPLENGGTYYILSDITAFGGIR NELDKGIGTIISSPYR IGENKDAMDGWFR DAMDGWFR	Chain A, soybean trypsin inhibitor (32%)	GI3318877
B4	20.2	4.57	DFVLDNEGNPLENGGTYYILSDITAFGGIR NKPLVVQFQK	Trypsin inhibitor C (Kunitz)-soybean (22%)	GI68821
B5	7.3	8.99	DHATVPSLR	Unknown protein (*Oryza sativa* Japonica group) (1%)	GI45680427
P1	22.5	4.97	DTVDGWFNIE NKPLVVQFQ	Full = Kunitz-type trypsin inhibitor KTI1; flags: precursor (10%)	GI125722
P2	24	4.98	GIGTIISSPFR FIAEGNPLR IGENKDAVDGWFR	Trypsin inhibitor subtype B (*Glycine max*) (15%)	GI18772
P3	20.1	4.61	DFVLDNEGNPLENGGTYYILSDITAFGGIR GIGTIISSPYR IGENKDAMDGWFR DAMDGWFR	Chain A, soybean trypsin inhibitor (29%)	GI3318877
S1	22.5	4.97	DTVDGWFNIER NKPLVVQFQK	Full = Kunitz-type trypsin inhibitor KTI1; flags: precursor (10%)	GI125722
S2	20.1	4.61	DFVLDNEGNPLENGGTYYILSDITAFGGIR GIGTIISSPYR IGENKDAMDGWFR NKPLVVQFQK	Chain A, soybean trypsin inhibitor (35%)	GI3318877
S3	24.0	4.98	GIGTIISSPFR IGENKDAVDGWFR DAVDGWFR NKPLVVQFQK	Trypsin inhibitor subtype B (*Glycine max*) (15%)	GI18772

Spot numbers show protein location on the 2D gel in Fig. 6

were identified in soybean cultivars and mutant lines, contributing to their biochemical characterization.

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References

Birk Y (1961) Purification and some properties of a highly activity inhibitor of trypsin and α-chymotrypsin from soybean. Biochim Biophys Acta 54:378–381

Bowman DE (1946) Differentiation of soybean antitryptic factors. Proc Soc Exp Biol Med 63:547–550

Choi NS, Yoo KH, Yoon KS, Maeng PJ, Kim SH (2004) Nano-scale proteomics approach using two-dimensional fibrin zymography combined with fluorescent SYPRO ruby dye. Biochem Mol Biol 37:298–303

Gallagher SR (1995) One-dimensional electrophoresis using non-denaturing conditions. In: Current Protocols in Protein Science. Wiley, New York, pp 10.3.1–10.3.11

Gore A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21: 1037–1053

Herbert B (1999) Advances in protein solubilization for two-dimensional electrophoresis. Electrophoresis 20:660–663

Hymowitz T (1973) Electrophoretic analysis of SBTI-A2 in the USDA soybean germplasm collection. Crop Sci 13:420–421

Hymowitz T, Kaizuma N (1981) Soybean seed protein electrophoresis profiles from 15 Asian countries or regions; hypotheses on paths of dissemination of soybean from China. Econ Bot 35:10–23

Kaizuma N, Oikawa K, Miura M (1980) Consideration on the cause of the differential ti alleles frequency distributions found among some regional populations of soybean (Glycine max (L.) Merrill) land varieties. J Fac Agric Iwate Univ 15:81–96

Krishnan HB (2001a) Characterization of a soybean [*Glycine max* (L.) Merr.] mutant with reduced levels of Kunitz trypsin inhibitor. Plant Sci 160:979–986

Kim DS, Lee KJ, Kim J-B, Kim SH, Song JY, Seo YW, Lee B-M, Kang S-Y (2010) Identification of Kunitzt trypsin inhibitor mutations using SNAP markers in soybean mutant lines. Theor Appl Genet 121:751–760

Kim SH, Choi NS, Lee WY (1998) Fibrin zymography: a direct analysis of fibrinolytic enzyme on gel. Anal Biochem 263: 115–116

Kim SH, Hara S, Hase S, Ikenaka T, Tode H, Kitamura K, Kaizuma N (1985) Comparative study on amino acid sequence of Kunitz-type soybean trypsin inhibitors, Tia, Tib, and Tic. J Biochem 19:435–448

Krishnan HB, Jiang G, Krishnan AH, Wiebold WJ (2000) Seed storage protein composition of non-nodulating soybean [*Glycine max* (L.) Merr.] and its influence on protein quality. Plant Sci 157:191–199

Krishnan HB (2001b) Characterization of a soybean [*Glycine max* (L.) Merr.] mutant with reduced levels of Kunitz trypsin inhibitor. Plant Sci 160:979–986

Kunitz M (1945) Crystallization of a trypsin inhibitor from soybean. Science 101:668–669

Lajolo FM, Genovese MI (2002) Nutritional significance of lectins and enzyme inhibitors from legumes. J Agric Food Chem 50:6592–6598

Li FS (1993) Studies on the ecological and geographical distribution of the Chinese resources of wild soybean (G. Soja). Sci Agri Sin 26:47–55

Natarajan SS, Xu C, Bae H, Caperna TJ, Garrett WM (2006) Characterization of storage proteins in wild (*Glycine soja*) and cultivated (*Glycine max*) soybean seeds using proteomic analysis. J Agric Food Chem 54:3114–3120

Martz F, Wilczynska M, Kleczkowski LA (2002) Oligomerization status, with the monomer as active species, defines catalytic efficiency of UDP-glucose pyrophosphorylase. Biochem J 367:295–300

Orf JH, Hymowitz T (1979) Inheritance of the absence of the Kunitz trypsin inhibitor in seed protein of soybeans. Crop Sci 19:107–109

Roychaudhuri R, Sarath G, Zeece M, Markwell J (2004) Stability of the allergenic soybean Kuntiz trypsin inhibitor. Biochem Biophys Acta 1699:207–212

Singh LC, Wilson M, Hadley HH (1969) Genetic differences in soybean trypsin inhibitor separated by disc electrophoresis. Crop Sci 9:489–491

Song SI, Kim CH, Baek SJ, Choi YD (1993) Nucleotide sequences of cDNA encoding the precursors for soybean (*Glycine max*) trypsin inhibitors (Kunitz type). Plant Physiol 101:1401–1402

Wang KJ, Kaizuma N, Takahata Y, Hatakeyama S (1996) Detection of two new variants of soybean Kunitz trypsin inhibitor through electrophoresis. Breed Sci 46:39–44

Wang KJ, Takahata Y, Ito K, Zhao YP, Tsutsumi KI, Kaizuma N (2001) Genetic characterization of a novel soybean Kunitz trypsin inhibitor. Breed Sci 51:185–190

Wang KJ, Yamashita T, Watanabe M, Takahata Y (2004) Genetic characterization of a novel Tib-derived variant of soybean Kunitz trypsin inhibitor detected in wild soybean (*Glycine soja*). Genome 47:9–14

Wang KJ, Li XH (2005) Tif type of soybean Kunitz trypsin inhibitor exists in wild soybean of northern China. In: Proceedings of the 8th national soybean research conference of China, pp 167–168

Wang KJ, Takahata Y, Kono Y, Kaizuma N (2008) Allelic differentiation of Kunitz trypsin inhibitor in wild soybean (*Glycine soja*). Theor Appl Genet 117:565–573

Weder JKP, Kahley R (2003) Reaction of lentil trypsin-chymotrypsin inhibitors with human and bovine proteinase. J Agric Food Chem 51:8045–8050

Xin H, Xie KF, Dong AW, Uan QY, Gu QM (1999) The amino acid sequence determination of a new variant of Kunitz soybean trypsin inhibitor (SBTi-A2). Soyb Genet Newsl. http://www.soybgenetics.org/aricles/sgn1999004.html. Accessed 24 Mar 1999

Yu M, King J (1988) Surface amino acids as sites of temperature-sensitive folding mutations in the P22 tailspike protein. J Biol Chem 263:1424–1431

Zhao SW, Wang H (1992) A new electrophoretic variant of SBTi-A2 in soybean seed protein. Soyb Genet Newsl 19:22–24