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Detection of Two New Variants of Soybean Kunitz Trypsin Inhibitor through Electrophoresis

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Summary

In order to detect mobility variants of soybean Kunitz trypsin inhibitor (SKTI) proteins, we developed a large slab gel method (18.5 cm long X 14.5 cm wide) of electrophoresis, and analyzed 173 cultivars of soybeans (*Glycine max*) from Japan and China, and 62 populations of wild soybeans (*G. soja*) collected from all over Iwate Prefecture, Japan. As a result of this electrophoretic survey, two new mobility variants were discovered: a variant showing a slightly slower mobility than the *Tia* type (tentatively designated as *Tia-s*) in a wild soybean line and another variant with a slightly faster mobility than the *Tib* type (tentatively designated as *Tib-f*) in a cultivated soybean variety. Transmissibility of this variation to consecutive generations was confirmed by parent-offspring tests and hybridization tests using the variants and/or the common types. Isoelectric focusing (IEF) test applied to these two variants revealed differences in the location of the isoelectric points in the gel. Significance of these discoveries was discussed.

Key words : soybean (*Glycine max*), wild soybean (*Glycine soja*), soybean Kunitz trypsin inhibitor (SKTI), electrophoretic mobility variant, frequency distribution of the alleles.

Introduction

Although soybean (*Glycine max*) is an important crop with about 40 % of protein in seeds, the seeds contain trypsin inhibitors accounting for about 6 % of the total protein (Rackis and Anderson, 1964). They can inhibit the growth of some animals and cause pancreatic enlargement to a certain degree (Struthers *et al.* 1983). Therefore, the trypsin inhibitors have been considered to be one of the factors responsible for the low nutritive value of unheated soybeans (Westfall and Hauge 1948). The trypsin inhibitors can be roughly divided into two major groups, Kunitz type (Kunitz 1945) and Bowman-Birk type (Bowman, 1946, Birk, 1961, Birk *et al.* 1963). Since Singh *et al.* (1969) observed a polymorphism of the soybean Kunitz trypsin inhibitor (SKTI), corresponding to *Tia* and *Tib,* SKTI polymorphism has been investigated by some researchers.

In a series of studies on the polymorphism of SKTI, five forms have been electrophoretically identified. *Tia, Tib* (Singh *et al.* 1969), *Tic* (Hymowitz 1973) and the slowest mobility form (Zhao and Wang 1992) were distinguished based on their own mobility, and another form (*ti*) lacking the SKTI was reported by Orf and Hymowitz (1979). The polymorphism of SKTI was often used as an index for analyzing the botanical origin, dissemination (Hymowitz and Kaizuma 1979, Kaizuma *et al.* 1980, Wang *et al.* 1986), geographical diversification (Kiang *et al.* 1992, Yu and Kiang 1993, Hu and Wang 1985) and phylogenetic relationship among the related species of soybeans (Nakamura *et al.* 1984).

Kim *et al.* (1985) revealed that there is a large sequence difference in eight amino acid residues between *Tia* and *Tib* proteins and only one small sequence difference in one amino acid residue between *Tia* and *Tic.* Usually, the *Tia* type has been considered to be the prototype from which the other types were derived. It is difficult to conceive that such a large substitution involving eight amino acid residues, like between *Tia* and *Tib* occurred as a single mutation. Such a large substitution of eight amino acid residues is considered to be brought through the accumulation of several mutations from *Tia.* Therefore, some intermediate or transitional forms with differences in two to seven amino acid residues may occur among cultivated soybeans, especially among wild soybeans, because the differentiation of *Tia* and *Tib* may have occurred before domestication of cultivated soybeans from wild soybeans (Kaizuma *et al.* 1980).

Up to now, there have been no reports on intermediate types among the known types. To detect such SKTI types, we used a large slab gel electrophoretic analysis. The objective of this paper was to identify new mobility variants of SKTI and describe some of their chemical and genetic properties.

Materials and Methods

Plant materials

The plant materials used in this investigation are listed in Table 1. One hundred and seventy-three soybean (*G. max*) cultivars from China (25 cvs.) and Japan (148 cvs.) were used. Chinese cultivars were part of the germplasm collection preserved at the Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Sciences, Beijing, which are representative of the cultivars grown in the central area of China. Japanese cultivars originated from the germplasm collection preserved at Iwate University, which were collected from all over Japan. Several seeds of each cultivar were used

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for preparation of a sample solution for SKTI type identification by polyacrylamide gel electrophoresis.

Sixty-two wild soybean populations collected from 20 areas in 1991 and 1992 were directly used in this investigation without growing to the next generation. The areas for collection are shown in Table 1, which covered whole Iwate Prefecture. The materials of each population consisted of 10 to 20 samples. Many samples were composed of the seeds collected from one plant, and a part of samples were composed of mixed seeds from some plants growing nearby. A single seed taken from each sample was used to prepare SKTI extract for electrophoretic analysis.

Polyacrylamide slab gel electrophoresis (PAGE) for detection of new SKTI variants

Extraction of SKTI proteins from seeds was carried out according to Hymowitz and Hadley's procedure (1972). In order to detect slight differences in SKTI band mobility from the well-known types (*Tia, Tib* and *Tic*), we used a large slab gel (18.5 cm long) in place of the common slab gel (7.5 cm long) to the Davis system of the PAGE commonly used for the SKTI type identification.

Table 1. Materials used

Materials	Origin	No. of cvs. orlines
Cultivated soybeans (*G. max*)	China	25
	Japan	148
Total		173

Materials	Area*	No. of population	No. of samples investigated
Wild soybeans (*G. soja*)	1. Tamayama	3	37
	2. Shizukuishi	5	66
	3. Miyako	5	92
	4. Niizato	1	17
	5. Morioka	4	47
	6. Yahaba	1	10
	7. Shiwa	9	102
	8. Ishidoriya	10	129
	9. Kitakami	1	20
	10. Waga	2	27
	11. Hanamaki	4	66
	12. Tohwa	2	28
	13. Tohno	1	20
	14. Ohtsuchi	1	17
	15. Kamaishi	1	20
	16. Ichnoseki	1	20
	17. Hanaizumi	5	84
	18. Fujisawa	1	17
	19. Rikuzentakata	3	55
	20. Kawasaki	1	17
Total		62	890

* The collection areas of the wild soybeans extended from the northern to southern part of Iwate Prefecture, Japan. They covered whole Iwate Prefecture.

In the large slab gel system the polyacrylamide concentration was 13.5 % (2.7 % N, N'-methylenbisacrylamide (Bis)). The electrophoresis was run with Tris-glycine buffer (pH 9.0) at a constant current of 20 mA for 15 hours. After the electrophoresis, SKTI bands were stained with Coomassie Brilliant Blue and then their mobilities were carefully checked against the three standards, namely *Tia* (a soybean cultivar, Meshidou Gong 503 or a commercial SKTI), *Tib* (Norin No. 2) and *Tic* (Raiden). The sample solutions that seemed to be variants of the SKTI band were selected and tested again.

In the case of wild soybean, when a variant of the SKTI band was detected, one to six pods with more than two seeds set in each sample containing the SKTI variant were selected to analyze the stability of the variant. And, the other remaining seeds in the pod were used for obtaining the next generation.

Electrophoretic isolation of SKTI proteins

For confirmation of the newly detected variants, isolation of the SKTI proteins was conducted on a disc preparative electrophoresis instrument, NA-1800, Nihon Eido Co. Ltd., as indicated below.

A separating disc polyacrylamide gel (37 mm diameter X 40 mm height) was set in the cylinder part of the instrument by pouring 45 m*l* of the 10 % polyacrylamide solution (2.7 % Bis) to prepare the separating gel, and by overlaying 10 ml of the 3.5 % polyacrylamide solution on the separating gel for making a stacking gel. The bottom surface of the separating gel was covered with a dialytic membrane to prevent the eluted solution from being dispersed into the Tris-glycine buffer (pH 9.0) in the bottom part of the instrument.

The seeds of the SKTI variant plants or standards used for SKTI protein isolation were harvested from the next generation plants of the original (previous generation) seeds. Three ml of the crude solution containing SKTI proteins extracted from 160 mg seed powder were placed on the top of the stacking gel. Electrophoretic separation was performed with eluting Tris-glycine buffer (pH 9.0) under a constant voltage of 100 V. Flow rate was adjusted to 60 drops per hour. Every fraction from the 10 drops was collected in a tube of the fraction collector. The SKTI proteins were included in the concentrated fractions no. 10 to 20. The solution of the tubes no. 12 to 15 was used for electrophoretic examination of the SKTI proteins.

Parent-offspring test and hybridization test for determining genetic variability of a newly detected SKTI variant (Tia-s)

For confirming transmissibility of a newly detected SKTI variation (*Tia-s*), the remaining seeds in the variant plants were grown and harvested for the next generation seeds in 1993. The harvested seeds were electrophoretically examined to determine whether the same electrophoretic mobility was transmitted over the two generations.

Furthermore, various combinations of hybridization between the variant and the standard were carried out as shown in Fig. 4. Hybrid seeds (F_1s) were used for electrophoretic examination. Electrophoretic mobility of the variant SKTI was compared with that of the standard SKTI (*Tia*).

Isoelectric focusing (IEF) of the SKTI proteins

IEF slab gel for testing differences in the isolectric point of the SKTI variants was prepared as described below.

The gel solution containing 10 % polyacrylamide (2.7 % Bis), 1.2 % pharmalyte (pH 4.2-4.9), 0.4 % ampholine (pH 3.5-10.0) and 1.2 % glycerol was used because the isoelectric point of the SKTI (*Tia*) is known to correspond to pH 4.65. The SKTI solutions applied to the IEF gel were the same as those used in the above Davis PAGE. The anode and cathode solutions consisted of 10 mM phosphoric acid and 20 mM sodium hydroxide, respectively. The electrophoresis was run at 200 V for 2 hours at the beginning and then at 400 V for 13 hours in the 6 ℃ cabinet.

Results and Discussion

Frequency distribution of the SKTI types detected in the materials investigated

Frequency distribution of the SKTI electrophoretic mobility types among the materials used is listed in Table 2.

Five SKTI types including the two newly detected types (*Tia-s* and *Tib-f*) were differentiated in this investigation. Three already known SKTI types (*Tia, Tib* and *Tic*) are shown in Fig. 2, and the two newly detected variant types, *Tia-s* and *Tib-f* (the electrophoretic mobility of these SKTI bands was slightly slower than that of *Tia* and slightly faster than that of *Tib,* respectively) are depicted in Fig. 1 and Fig. 2, respectively.

Electrophoretic mobility of the two variants was examined using isolated and concentrated SKTI proteins with a disc preparative electrophoresis instrument, NA-1800 (Fig. 3). The slight differences in the electrophoretic mobility between *Tia* and *Tia-s* or *Tib* and *Tib-f* were revealed more clearly than in the crude solutions of the SKTI proteins.

As shown in Table 2, the frequency distribution of these five electrophoretic types was very different among the materials used. The Chinese cultivars of soy-

Table 2. Distribution of the SKTI polymorphic types observed in the materials investigated

Materials	*Tia*	*Tia-s*	*Tib*	*Tib-f*	*Tic*	Total
G. max						
Chinese cv.	25	0	0	0	0	25
Japanese cv.	68	0	75	1	4	148
G. soja						
Population	56[1)]	1[2)]	5[3)]	0	0	62

[1)] Number of the populations containing only *Tia* type.
[2)] The one population containing an identified *Tia-s* line, *G. soja* 1125, is listed in this Table. Some other populations containing unidentified *Tia-s* lines are excluded from this Table, because these lines need to continue further identification.
[3)] Number of the populations containing *Tib*. The three were the composited of *Tia* and *Tib*. The two contained *Tia, Tib* and the heterozygous *Tia, Tib*.

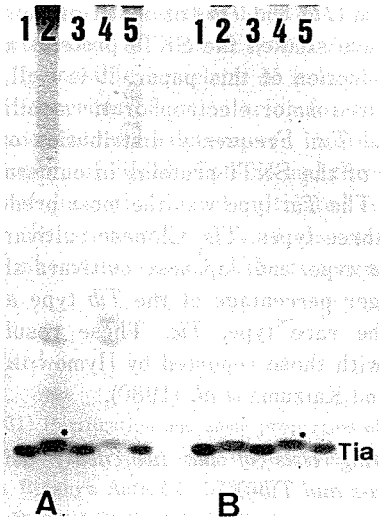

Fig. 1. A new SKTI variant (*Tia-s*) showing a slightly slower electrophoretic mobility than the ordinary one (*Tia*). This characteristic was transmitted to the succeeding generation (B) of the collected seeds (A). Lanes 1 and 3: Commercial SKTI (*Tia*). Lane 5: *G. max* Meshidou Gong 503 (*Tia*). Lanes 2 and 4: *G. soja* 1205 and 1125 (*Tia-s*), respectively.

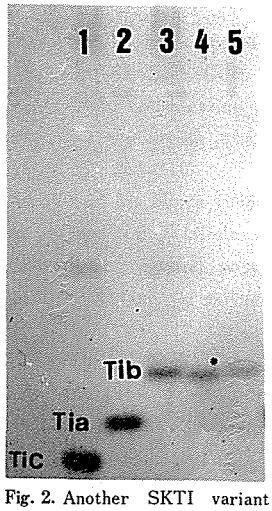

Fig. 2. Another SKTI variant showing a slightly faster electrophoretic mobility than the ordinary one (*Tib*). Lanes 1, 2, 3, and 5: *G. max* cvs. Raiden (*Tic*), Meshidou Gong 503 (*Tia*), Norin No. 2 (*Tib*) and Ohdate No. 1 (*Tib*), respectively. Lane 4: *G. max* cv. Fuki (*Tib-f*).

beans consisted only of one type (*Tia*), while the Japanese ones contained the four types except for the *Tia-s* type. The newly detected variant *Tib-f* was found in a Japanese cultivar, Fuki. Wild soybeans collected from various areas in Iwate Prefecture (Table 1) contained another newly detected variant, *Tia-s,* in a sample (No. 1125) collected at Tamayama village. This was designated as *G. soja* 1125 line.

Since Singh *et al.* (1969) observed electrophoretic polymorphism (*Tia* and *Tib*) of SKTI proteins, many researchers have studied the SKTI proteins as described in the introduction of this paper. It is well known that there are three major electrophoretic mobility variants, *Tia, Tib* and *Tic.* Frequency distribution of the major three types of the SKTI proteins in our study is listed in Table 2. The *Tia* type was the most predominant one among the three types. The Chinese cultivars contained only the *Tia* type, and Japanese cultivars showed a relatively larger percentage of the *Tib* type and the presence of the rare type, *Tic.* These results were in agreement with those reported by Hymowitz and Kaizuma (1979) and Kaizuma *et al.* (1980).

Parent-offspring tests of the two newly detected SKTI variants (Tia-s and Tib-f)

Results of the parent-offspring test in *G. soja* 1125 (*Tia-s*) are shown in Fig. 1. The collected seeds of *G. soja* 1125 line in 1992 and the newly harvested seeds from the same line grown in the following year 1993 were examined in relation to the SKTI electrophoretic mobility. They showed the same mobility as indicated in Fig. 1.

Transmissibility of the *Tia-s* band was tested through hybridization experiments. The SKTI bands of the F_1 seeds derived from a cross between *Tia* and *Tia-s* were composed of two different bands, *Tia* and *Tia-s* as shown in Fig. 4A. Likewise, the transmissibility was also observed in F_1 seeds of the cross between *Tib* and *Tia-s* (Fig. 4 B). Those of the cross between *Tic* and *Tia-s* have the same results as above (data not shown). These results suggest that *Tia-s* may be a co-dominant allele against the known types (*Tia, Tib* and *Tic*). Preliminary results on F_2 segregations of *Tia-s* X *Tib* supported this consideration (data not shown).

Several other lines with bands whose mobilities are similar to *Tia-s* were also observed in this investigation, but they were not identified in detail through hybridization tests and isoelectric points.

Another new SKTI variant, which migrated slightly faster than *Tib,* was detected among the Japanese soybean cultivars. It was found in only one cultivar Fuki, which is one of the well-known Japanese vegetable soybeans presently grown in Japan. This property was stably transmitted to the following generation as shown by the parent-offspring test (Fig. 2).

Parent-offspring test showed that both *Tia-s* and *Tib-f* were stably transmitted from generation to generation. Also, based on hybridization experiments the *Tia-s* variant was found to transmit this characteristic to the

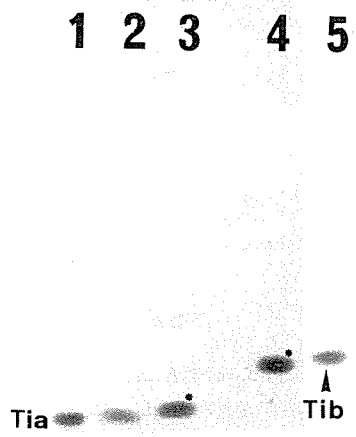

Fig. 3. The two SKTI variants with slightly slower (*Tia-s*) and faster (*Tib-f*) electrophoretic mobilities, confirmed based on electrophoretically isolated SKTI proteins from the seeds harvested in the year 1993 (by growing the 1992 year's original seeds). Lane 1: *G. max* Meshidou Gong 503 (*Tia*). Lane 2: *G. soja* 1368 (*Tia*). Lane 3: *G. soja* 1125 (*Tia-s*). Lane 4: *G. max* Fuki (*Tib-f*). Lane 5: *G. max* Norin No. 2 (*Tib*).

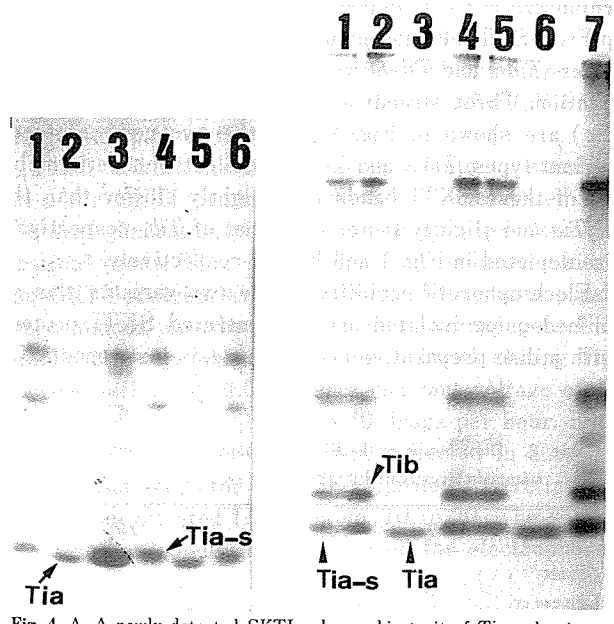

Fig. 4. A. A newly detected SKTI polymorphic trait of *Tia-s* showing a different mobility from that of *Tia,* revealed by duplicated SKTI bands of F1 seed between *G. max* Meshidou Gong 503 (*Tia*) and *G. soja* 1125 (*Tia-s*). Lanes 1, 4, and 6: Paternal *G. soja* 1125 (*Tia-s*), Lanes 2 and 5: Maternal *G. max* Meshidou Gong 503 (*Tia*). Lane 3: F1. B. *Tia-s* polymorphism revealed by double bands of F1 seeds between *Tib* and *Tia-s,* and simultaneously by comparing the electrophoretic mobilities of *Tia* and *Tia-s.* Lanes 1 and 2: F1 seed between *G. max* Tachisuzunari (*Tib*) and *G. soja* 866 (*Tia-s*). Lanes 4 and 5: F1 seed between Tachisuzunari (*Tib*) and *G. soja* 926 (*Tia-s*). Lane 7: F1 seed between Tachisuzunari and *G. soja* 1205 (*Tia-s*). Lanes 3 and 6: *G. max* Meshidou Gong 503 (*Tia*).

next generation. These newly detected variants are considered to be intermediate types between *Tia* and *Tib*.

Isoelectric points of the SKTI proteins

The isoelectric points of the SKTI proteins (*Tia, Tib* and *Tic*) were as follows: those of *Tia* and *Tib* were almost the same and showed relatively higher values (more alkaline range), while that of *Tic* showed a comparatively lower value (more acidic range), as shown in Fig. 5. The isoelectric points of the two newly detected variant SKTI proteins (*Tia-s* and *Tib-f*) were located differently in the IEF gel. Namely, the isoelectric point of the variant (*Tia-s*) was slightly lower than that of the SKTI (*Tia*) and slightly higher than that of the SKTI (*Tic*). On the other hand, the isoelectric point of another variant (*Tib-f*) was slightly higher than that of the SKTI (*Tib*) as shown in Fig. 5. Thus, the test of IEF suggested that the two variants may differ in the amino acid residues from *Tia* and *Tib*, respectively.

Evolutionary significance and problems to be solved

As already mentioned in the introduction of this paper, SKTI is one of genetic markers that has been studied in detail for geographical distribution of alleles in soybean. Of the five forms detected previously, *Tia* and *Tib* are most frequently observed in both cultivated and wild soybeans (Hymowitz and Kaizuma 1979, Kaizuma *et al.* 1980, Hu and Wang 1985, Kiang *et al.* 1992, Yu and Kiang 1993). The hypotheses on the origin and dissemination paths of cultivated soybean mostly stem from extensive work on genetic polymorphism of the marker (Hymowitz and Kaizuma 1979, Wang *et al.* 1986). A sequence analysis of amino acids revealed that *Tia* and *Tib* differ in eight amino acid residues to each other (Kim *et al.*, 1985). This leads to an interesting question what mechanisms are involved in an evolutionary step from *Tia* to *Tib* or vice versa, because it is not easy to conceive that such a large substitution may result from a single mutation. In this paper, the genetic variability of SKTI proteins was reevaluated with a newly devised method. As a result, two new mobility variants were detected in a wild soybean population collected in Iwate Prefecture and a vegetable soybean cultivar, respectively, which possessed different isoelectric points from common types as *Tia* and *Tib*. Since the differentiation of mobility variants of SKTI may have occurred before domestication of cultivated soybean (Kaizuma *et al.*, 1980), the analysis should thus be enlarged to the wild soybeans distributed all over Eastern Asia, especially from China, a possible homeland of cultivated soybean. In addition, sequences of amino acids for the newly detected variants should be determined in order to evaluate the phylogeny of SKTI proteins and thereby discuss the process of domestication of cultivated soybean. The analysis for the segregation data is also needed to determine the inheritance mode of the two newly detected variants.

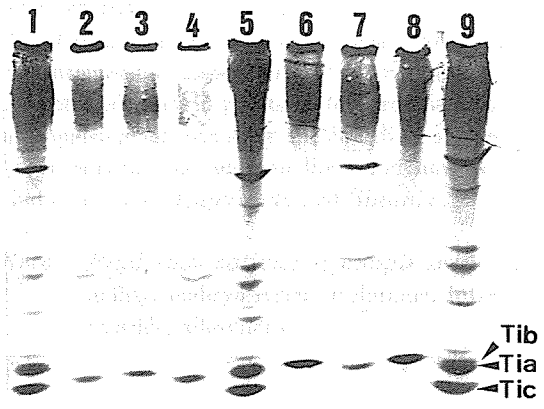

Fig. 5. Differences in isoelectric points differences between SKTI proteins. Lanes 1, 5 and 9: Mixture of *G. max. Meshidou Gong 503 (Tia)*, Norin No.2 (*Tib*) and Raiden (*Tic*). Lanes 2 and 4: *G. soja* 1125 (*Tia-s*). Lane 3: *G. max* Meshidou Gong 503 (*Tia*). Lanes 6 and 8: *G. max* Fuki (*Tib-f*). Lane 7: *G. max* Norin No.2 (*Tib*).

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