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A Simple Method for Detection of Trypsin Inhibitors in Soybean (*Glycine max*)

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Abstracts

The specific reaction of trypsin inhibitors with trypsin to form stable complexes was successfully applied for detection of trypsin inhibitors in soybean. Soybean extract was treated with Ca^{++} to remove globulin fraction, followed by digestion with trypsin and fractionated by chromatography on Sephadex G-50. The void volume fraction contained the trypsin-trypsin inhibitor complexes as well as trypsin. The trypsin inhibitors were then detected by their molecular weight differences on SDS-polyacrylamide gel electrophoresis, in which the complexes dissociate into trypsin and its inhibitors. With the method proposed, trypsin inhibitors were indentified by their ability forming the stable complexes with trypsin and their anti-tryptic moiety. The formation of the complexes with trypsin was further confirmed by two dimensional electrophoresis and DEAE-Sephadex A-25 chromatography. Employing the proposed method, it was found that soybean (*Glycine max* cv. Hill) contained 7 trypsin inhibitors.

Introduction

Trypsin inhibitors are widely distributed among plants and animals.[1] They are a group of proteins binding trypsin to form inactive complexes. The function of the inhibitors is not yet clear. One possibility is that they might control the proteolytic enzyme action *in vivo* by inhibiting endogeneous proteases.[2] Another suggestion is the inhibition of the proteases from soil fungi and other microorganisms, and thus helping estibilish the young seedlings.[3] In animals, trypsin inhibitors retard their growth and also cause pancreatic hypertrophy.[4,5]

In some of the cultivars of soybean, the inhibitors account up to 6% of total seed proteins.[7] Mumerous trypsin inhibitors have been purified and chracterized by many investigators: Kunitz and Bowman-Birk inhibitors have been isolated and fully characterized.[8,9] Rackis and Anderson[10] isolated $SBTIA_1$, $SBTIA_2$ and SBTI (5x). Yamamoto and Ikenaka[11] isolated 1.9S inhibitor. Frattali and Steiner[12] also purified four inhibitors designated as F_1, F_2, F_3 and STI. Yamamoto and Ikenaka,[11] however, reported that $SBTIA_2$ and F_2 inhibitors were indeed identical to Kunitz inhibitor. Some of the other isolated inhibitors was supposed to be identical one another. Different separation and characterization techniques employed by the investigators make it difficult to compare one inhibitor to the others.

In many cases, such as nutritional evaluations and plant breedings, it is of importance to develop a simple method to investigate the distribution of inhibitors in a sample. Although the several methods to isolate trypsin inhibitors from different sources have been estabilished, it is not an easy task to detect all inhibitors contained in a sample. This paper presents a simple method to detect simultaneously all trypsin inhibitors in soybean samples.

Materials and Methods

Soybean sample

Soybean seed (*Glycine max* cv. Hill) was dehulled and pulverized with moltar and pestle (100 mesh). The flour was defatted with diethyl ether and stored at -20°C before the use.

Extraction and digestion of soybean protein

Total soybean protein was extracted by shaking the defatted flour with 10 vol of 50*mM* Tris-HCl (pH 7.8) containing 0.1*M* NaCl for 2 hrs at room temperature and centrifuged at 6,000 × *g* for 15 min. Globulin fraction of the extract was precipitated by adding one tenth vol of

0.2 *M* $CaCl_2$ solution to the supernatant and then incubated for 30 min in a refrigerator.[13] The clear supernatant after centrifugation at 10,000 × *g* for 20 min was diluted to give a protein concentration of 20 *mg* per *ml* and digested with a trypsin stock solution (20*mg*/*ml*) at 37°C for 3 hrs.

To test digestibility of the soybean extract, varied amounts of trypsin stock solution (20*mg* trypsin per *ml* of 50 *mM* Tris-HCl, pH 7.8, and 0.1 *M* NaCl) were added into the extract to make an appropriate trypsin concentration. After the mixture was incubated at 37°C for 2 hrs, the undigested protein was precipitated with one fifth vol of 30% trichloroacetic acid, followed by centrifugation at 10,000 × *g* for 20 min and the absorbance of the supernatant was measured at 280 *nm*.

Gel filtration of the digested fraction

To prepare the sample for gel filtration, 4.5*ml* of the extract were mixed with 0.5*ml* of trypsin stock solution and incubated for 3 hrs. The digested solution (5*ml*) was applied on Sephadex G-50 column (2 × 85 *cm*) equilibrated with 50 *mM* Tris-HCl (pH 7.8) containing 0.1 *M* NaCl and 10*mM* $CaCl_2$. The flow rate was 30*ml* per hr and each fraction of 3*ml* was collected. Protein concentration was determined by Folin-Lowry method[14] and some of the fraction was subjected to SDS-gel electrophoresis.

Ion-exchange chromatography

The void volume fraction of the Sephadex G-50 chromatography was applied on DEAE-Sephadex A-25 column (1.5×15*cm*) equilibrated with 10 *mM* sodium phosphate buffer (pH 7.6). The column was washed with 50*ml* of the equilibrium buffer and eluted with the same buffer containing 0.25*M* NaCl. The flow rate was 30 *ml* per hr and 1.5 *ml* fraction was collected.

Protein determination

Protein concentration was assayed by the method of Lowry *et al.*[14]

Polyacrylamide gel electrophoresis

For the native gel electrophoresis, the Davis' procedure[15] was used, where the concentraction of acrylamide in the running gel was 6.5%. For the SDS-gel system, the Laemmli's method[16] was adopted, in which the running gel contained 13% acrylamide. In the both cases, the current was 20*mA* for slab gel (100×130×1*mm*) and 2.5*mA* for rod gel (5*mm* dia.). The gels were stained with Coomassie brilliant blue R-250 and destained with 7% acetic acid. For determination of molecular weight (MW), low MW standard proteins from Sigma (Cat. No. MW-ND-500) were used as a standard.

Two dimentional gel electrophoresis

The method of O'Farrell[17] was slightly modified as follows: For the first dimention, a rod gel (120×2.5 *mm*) of 6.5% acrylamide was prepared and run according to the Davis' procedure.[15] The gel was denatured with the SDS sample buffer for 20 min and loaded on the SDS slab gel containing 12% acrylamide for the second dimention. The gel was run at 20*mA* at 4°C and stained as described above.

Results and Discussion

The association between trypsin and trypsin inhibitors (T-TI) is known to be unusually strong. The dissociation constant for the T-TI complexes is ranged from 10^{-9} to 10^{-14}.[18,19] The stable complexes have been succesfully isolated by chromatographic techniques and crystalline form of the complex was also obtained.[18,20]

Gel electrophoretic behaviours of trypsin, trypsin inhibitor and its complex were tested by both non-dissociating (native) and dissociating (SDS) systems as

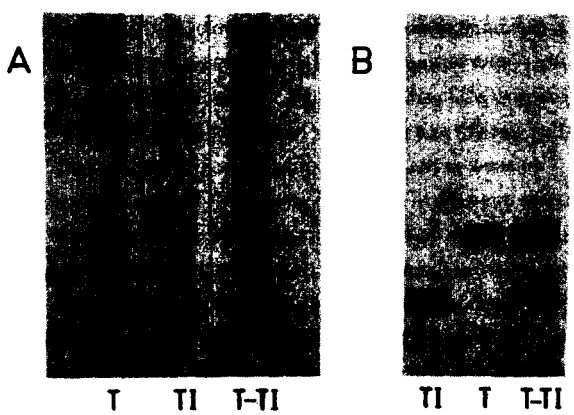

Fig. 1. Gel electrophoretic patterns of trypsin (T), soybean trypsin inhibitor (TI, Sigma type I-S) and this complex (T-TI) in native (A) and SDS (B) gel systems
The native gel electrophoresis was preformed by the procedure of Davis[15] and the SDS-gel electrophoresis by that of Laemmli.[16]

shown in Fig. 1. The commercially available trypsin (porcine pancrease, Sigma type XI) and trypsin inhibitor (soybean, Sigma type I-S) migrated as a single band in the both systems. The T-TI complex formed at a 1:1 molar ratio gave two bands of its component, trypsin and trypsin inhibitor, in the SDS gel electrophoresis.

The complex also showed two bands, one major and one minor, in the native gel electrophoresis. The major band was the complex and the minor was belived to be a mixture of trypsin and trypsin inhibitor, which were dissociated from the complex during electrophoresis. The equilibrium of a complex formation is a dynamic state;

$$T + TI \rightleftarrows T - TI$$

(T: trypsin, TI: trypsin inhibitor, T-TI: trypsin-trypsin inhibitor complex)

When current was applied to the equilibrium mixture during electrophoresis, trypsin and trypsin inhibitor were removed from the mixture, since they migrated faster than the complex (Fig. 1). A new equilibrium, then, should be estabilished in the direction that dissociated the complex into trypsin and trypsin inhibitor, which in turn formed a new band. This notion was evidenced by the observation that a longer electrophoresis time yielded a more diffused and intense minor band (compare Fig. 1 with Fig. 8).

Calcium ion not only stabilize the T-TI complexes but also precipitate major soybean storage globulins.[13,21] Soybean extract was digested with trypsin after removing storage globulins by treating the extract with Ca^{++}. The digestibility was determined by absorbance at 280 *nm* after removing the undigested proteins by precipitation with 5% TCA.

The optimal concentration of trypsin for digestion of protein was estimated by incubating the extract with varied concentrations of the enzyme at 37°C for 2 hrs. The digesion increased with increasing concentrations of trypsin up to 1:10 weight rato of trypsin to soybean protein. At a fixed trypsin concentration of 10% with respect to soybean protein, digestion increased until 2 hrs of incubation in terms of absorbance changes at 280 *nm*. In parallel changes with the absorbance, the changes in protein patterns were also monitored by SDS-gel electrophoresis. As shown in Fig. 2, the high MW proteins disappeared rapidly within 30 min of incubation transforming into smaller proteins, which thereafter

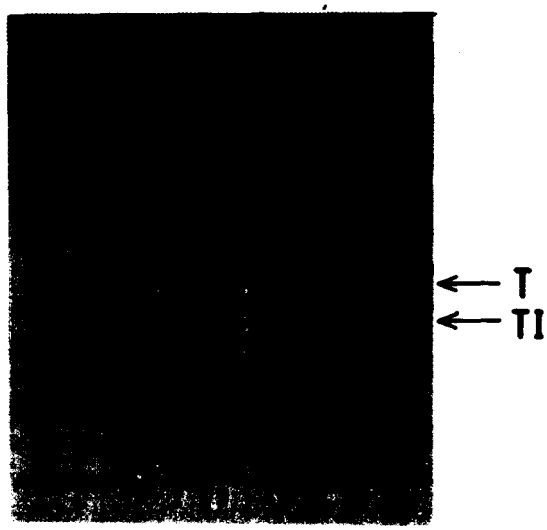

Fig. 2. SDS-gel electrophoretic paterns of soybean protein digested with trypsin at different periods of time
Soybean protein extract (20 *mg/ml*) was incubated with 0.1 vol of trypsin stock solution (20*mg/ml*), at 37°C. T; trypsin, TI; soybean trypsin inhibitor from Sigma (type I-S). C; undigested soybean extract (0 hr), 1; 0.5hr, 2; 1.0 hr, 3; 1.4 hrs, 4; 2.0 hrs, 5; 2.5 hrs, and 6; 3.0 hrs.

degraded slowly. After 3 hrs of incubation, no further changes of the remaining bands were observed in the SDS-gel electrophoresis.

Based on the above observations, the soybean extract was hydrolyzed at 37°C for 3 hrs at a 10% trypsin to soybean protein ratio, prior to gel filtration. A chromatographic profile of the trypsin-digested extract

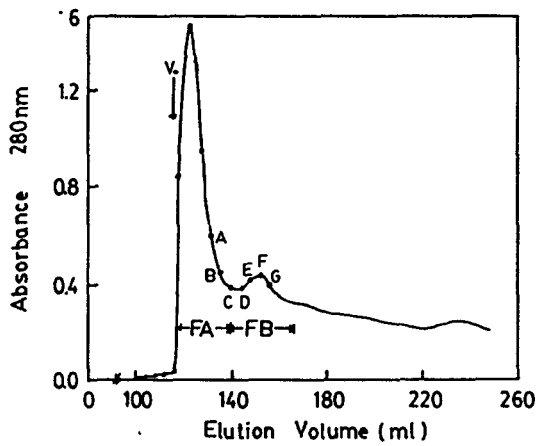

Fig. 3. Chromatographic profile of the trypsin-digested soybean extract on Sephadex G-50 column (2×85 *cm*)
The flow rate was 30*ml* per hr. Vo represent the void volume of the column. A, B,..... G indicate the fractions subjected to the SDS-gel electrophoresis.

on Sephadex G-50 is shown in Fig. 3. The main peak at the void volume followed by a small peak was obtained. All fractions after the void volume also had 280 *nm* absorbance of more than 0.3.

The SDS-gel electrophoresis of each fraction indicated that the void volume contained a wide range of different MW proteins (52 kD to 10 kD) along with trypsin and the later fractions only smaller peptides than trypsin (Fig. 4). Since Sephadex G-50 excludes globular proteins of more than 30 kD molecular weight in its void volume, the proteins contained in this fraction, whose MW are less than 30 kD, can be eluted only as complexed forms with trypsin. The peptides smaller than trypsin in this fraction were tentatively identified as trypsin inhibitors on the basis that these had anti-tryptic activity and formed complexes with trypsin. Most of the fractions after main peak contained small MW peptides that were digested globulins by trypsin.

The SDS-gel electrophresis indicated that this

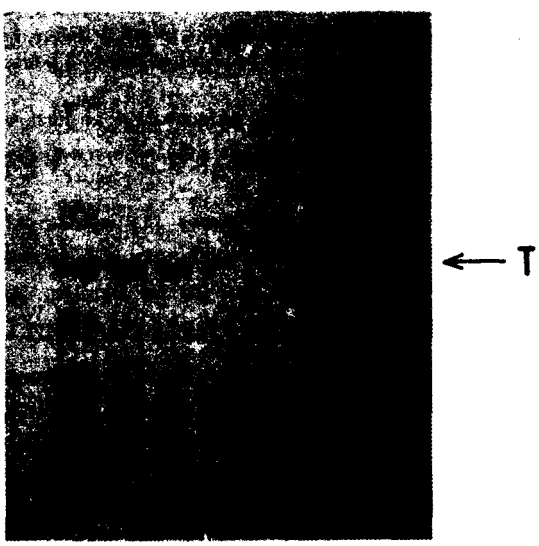

Fig. 4. SDS-gel electrophoreti patterns of the column fractions from Sephadex G-50 chromatography
T; trypsin. The far-left column; sample before applying to the column, A, B,..... G are the column fractions as indicated in Fig. 3.

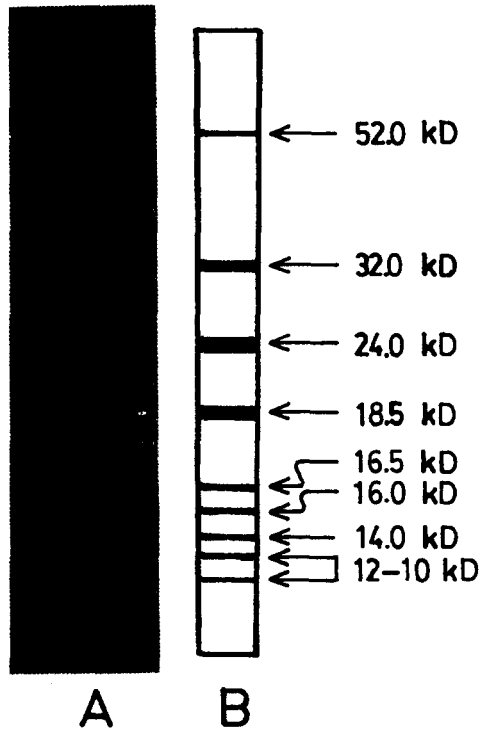

Fig. 5. SDS-gel electrophoretic pattern (A) and its diagram (B) of the void volume fraction from Sephadex G-50 column chromatography
Electrophoresis was performed according to the Laemmli's method[16], in thich the running gel cotained 13% acrylamide.

chromatographic system might spearate T-TI complexes and trypsin from the smaller MW proteins than trypsin. Co-elution of the complexes and trypsin is important to prevent dissociation of the complexes during chromatography, as discussed earlier.

Trypsin inhibitors were detected by SDS-gel electrophoresis in which the T-TI complexes were dissociated into trypsin and its inhibitors. The SDS-gel electrophoresis separated the void volume fraction into 9 bands as given in Fig. 5. None of these bands disappeared when the void volume fraction was re-digested

Table 1. SDS-gel electrophoretic components of soybean extract having anti-tryptic activity

Band No.	1	2	3	4	5	6	7	8 and 9
MW (kD)	52.0	32.0	24.0	18.5	16.5	16.0	14.0	12 - 10
Identified	unknown	TI*	T**	TI	TI	TI	TI	TI

* trypsin inhibitor , ** trypsin

with trypsin. The MW of each band was estimated by co-electrophoresis with standard proteins in the SDS-gel system and summerized in Table 1.

To confirm the ability of formation of T-TI complexes for proteins smaller than trypsin, two dimensional gel electrophoresis was performed. As shown in Fig. 6, electrophoresis of the void volume fraction yielded 4 major bands under the native condition (the first dimension) and 9 bands in the SDS-gel systems (the second dimension). The two dimensional electrophoresis showed that all proteins composing the first, second and third bands on the native gel were associated with trypsin. All trypsin inhibitors whose MW were smaller than trypsin appeared in the second and third bands, indicating that their complexes had similar mobilities in the native gel. The first band in the native system contained only one trypsin inhibitor, whose MW was estimted to be 32 kD. The highest MW protein in the SDS-gel did not form complex with trypsin, although it had anti-tryptic activity. This protein was not identified.

Since trypsin inhibitor of 32 kD molecular weight has not been reported previously, its complex with trypsin was further isolated by DEAE-Sephadex A-25 chromatography. When the void volume fraction of Sephadex G-50 was applied on a DEAE-Sephadex A-25 column, the complex corresponding to the first band in the native gel did not bind to the column under the experimental

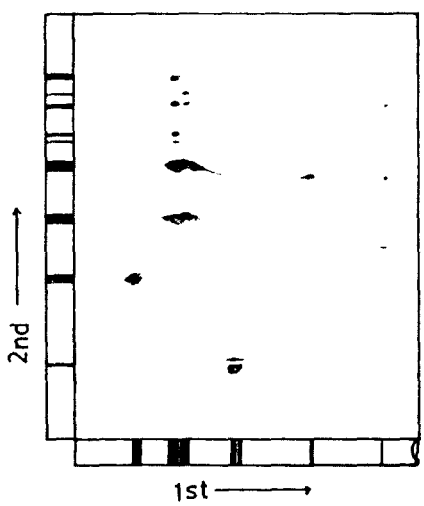

Fig. 6. Two dimensional gel electrophoresis of the void volume fraction of Sephadex G-50
The first dimention was native gel system after the method of Davis[15] and the second dimension was SDS-gel system after the method of Laemmli.[16]

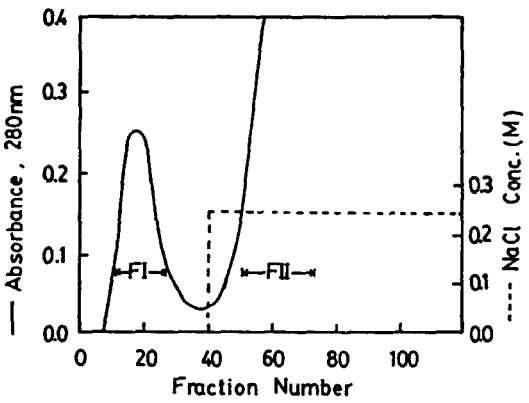

Fig. 7. Ion-exchange chromatography of the void volume fraction from Sephadex G-50 on DEAE-Sephadex A-25 column (1.5 × 15*cm*) equilibrated with 10 *mM* sodium phosphate buffer (pH 7.6)
The column was eluted with the equilibrium buffer, followed by the same buffer containing 0.25 *M* NaCl. The flow rate was 30 *ml* per hr and each fraction of 1.5*ml* was collected.

conditions, while the other complexes bound (Fig. 7). The unbound fraction gave a single band in the native gel and two bands in the SDS-gel, one of which was corresponded to trypsin (Fig. 8). The result indicated that the protein of 32 kD molecular weight may also be a trypsin inhibitor.

The existance of high MW trypsin inhibitor(32 kD) and an unknown substance having anti-tryptic activity is of interest and needs further studies. Trypsin inhibitors

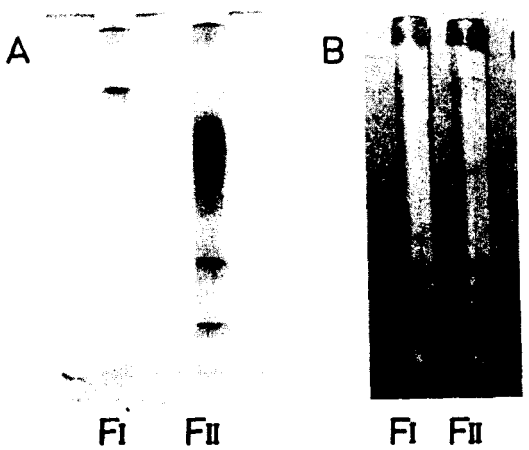

Fig. 8. Polyacrylamide gel electrophoretic patterns of the DEAE-Sephadex A-25 column effluent on the native gel (A) and SDS-gel (B)
F_I; unbound fraction, F_{II}: effluent with 0.25 *M* NaCl.

present in this cultivar of soybean were proven to be 7.

This method is considered to be useful to detect trypsin inhibitors, whose MW are smaller than trypsin. Since most of the naturally occuring trypsin inhibitors are smaller than trypsin[11,12], this simple method make it possible to detect most of the trypsin inhibitors simultaneously. Trypsin inhibitors whose MW are larger than trypsin need to be confirmed by auxillary methods such as two dimensional electrophoresis, ion-exchange chromatography or other isolation techniques. To apply this method to other samples than soybean, it is necessary to examine the optimal digestion condition for a given sample.

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대두 Trypsin Inhibitor의 간이검정법

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Trypsin inhibitor가 trypsin과 안정한 복합체를 형성하고, 동시에 일반 단백질은 trypsin에 의하여 가수분해되는 원리를 이용하여 trypsin inhibitor를 용이하게 검정할 수 있는 방법을 고안하였다. trypsin으로 가수분해시킨 대두추출액을 Sephadex G-50을 이용하여 trypsin-trypsin inhibitor 복합체를 분리시킨 후에 SDS 전기영동으로 trypsin inhibitor를 검정할 수 있었다. 이들 trypsin inhibitor는 trypsin에 의한 2차 가수분해에서도 가수분해 되지 않았으며, 또한 2차원 전기영동과 DEAE-Sephadex A-25 크로마토 그래피를 이용하여 trypsin inhibitor가 trypsin과 복합체를 형성하는 능력을 검정함으로써 본 방법의 유효성을 확인하였다. 본 실험 방법으로 대두(Hill 품종)의 trypsin inhibitor를 검정한 결과 7개의 trypsin inhibitor를 찾아 낼 수 있었다.