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Identification of the Precursor for the Soybean Kunitz Trypsin Inhibitor

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대두 Kunitz Trypsin Inhibitor 전구체의 동정

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초 록

대두에 존재하는 단백질 분해 효소 저해제로는 Kunitz trypsin inhibitor (SKTI), Bowman-Birk proteinase inhibitor, 그리고 그의 isoinhibitor들이 알려져 있다. SKTI는 분자량이 20K이며 181개의 아미노산으로 구성되어 있다. SKTI의 분자 구조 및 발현 특이성을 밝히기 위하여, 콩에서 순수 분리한 SKTI를 항원으로 사용하여 항체를 제조하였다. 이 항체는 항원으로 사용한 SKTI에 대하여 특이적으로 반응할 뿐만 아니라 기내에서 합성된 그의 전구체와도 특이적으로 반응하였다. 기내에서의 단백질 합성은 미성숙 대두 종자에서 mRNA를 분리한 후, wheat germ extract를 이용하여 실시하였다. 합성된 단백질 중에서 대두에 존재하는 SKTI는 검출되지 않는 반면에 면역침전법에 의해 분자량이 24K인 전구체가 존재하고 있음을 확인하였다. 이와 같이 SKTI는 mRNA로부터 전구체가 합성된 후 post-translational modification에 의해 완전한 SKTI로 변환됨을 알 수 있었다. 또한 SKTI는 대두 종자의 성숙과 함께 발현이 되는 것으로 보아 조직 및 분화 특이성 발현형태를 나타내는 것을 알 수 있었다.

Introduction

Proteinase inhibitors are widely distributed in animals, plants and microorganisms(Richardson 1977). In plants, proteinase inhibitors are well studied especially in the Leguminosae, Gramineae and Solanaceae because the species which belong to these families are the important food resources(Laskowski et al. 1980; Ryan 1973; Richardson 1977). They often form an important part of the protein found in the tissues.

In soybean (*Glycine max*), at least three different classes of proteinase inhibitors have been identified. These include the Kunitz trypsin inhibitor (SKTI), the Bowman-Birk proteinase inhibitor(BBPI) and its isoinhibitors. The SKTI which inhibits both trypsin and chymotrypsin is a single polypeptide chain of 181 amino acids (MW 20K) including two disulfide bonds(Kunitz 1946, 1947a, b). Three kinds of SKTI variants, Ti^a, Ti^b and Ti^c, which shows minor amino acid variation, are known(Kim, 1985). The BBPI is a 71 amino acid single chain polypeptide including 7 disulfide bonds. Its MW is about 8,000. The BBPI is a double headed structure which have both a trypsin and a chymotrypsin inhibitory site (Odani et al. 1972, 1977, 1978; Ikenaka and Norioka 1986). Although isoinhibitors and the BBPI are about 70% homologous in amino acid

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sequences, they are slightly different in size(66 ~76 amino acids), amino acid composition and inhibition specificity.

The function of proteinase inhibitors in plants, however, is poorly understood. In soybean and potato, proteinase inhibitors are stored in storage organ during maturation of seed or tuber. Therefore, the proteinase inhibitor may be part of a storage protein (Goldberg et al. 1981b). In the Leguminosae and Gramineae the proteinase inhibitors are associated particularly with the resting stages of the storage organ such as seeds and tend to decrease during germination. Therefore, these proteinase inhibitors may regulate the endogeneous proteinase activity(Richardson 1977). As the digestive proteinases from a number of insect genera, are inhibited by plant proteinase inhibitors, the proteinase inhibitor found in plant may constitute a defense mechanism against insect(Green et al. 1972; Laskowski et al. 1980; Ryan 1968, 1973).

To study the molecular structure and expression characteristics of the SKTI, biosynthesis and post-translational modification of the SKTI was examined with antibody in this paper. The antibody made against the SKTI purified from seeds makes it possible to track down the post-translational modification of the molecule. A primary translation product is modified post-translationally to yield mature SKTI. SKTI is expressed with the maturation of the seeds in tissue-specific and developmental stage-specific manner.

Materials and Methods

1. Materials

Oligo(dT)-cellulose was purchased from Collaborative Research, Inc. and nitrocellulose paper was from Schleicher & Schuell, Inc. Protein A -agarose was purchased from Boehringer Mannheim, GmbH. [^{35}S]methionine was from Amersham. SKTI was from Sigma Chemical Co.

2. Isolation of mRNA

Soybean mRNA was isolated by phenol extraction, LiCl precipitation and chromatography on oligo(dT)-cellulose(Aviv and Leder 1972). About 2g of either seeds or leaves of *Glycine max* cultivar Paldal 30 days after flowering were ground with 10ml extraction buffer(50mM Tris-HCl, pH8.0; 5mM EDTA; 100mM NaCl; 1% β-mercaptoethanol; 2% SDS) and 2g sea sand in pre-chilled mortar for 2min. The supernatant was treated with proteinase K(250μg/ml) for 10min at 65°C and was extracted 3 times with equal volume of saturated phenol and once with chloroform. The aqueous phase was adjusted to 0.1M LiCl, and 2 volumes of EtOH was added. It was placed at −20°C for 24hrs to precipitate nucleic acid. After centrifuge for 10min at 13,000g, the precipitate was resuspended in 2ml of buffer(10mM Tris-HCl, pH 8.0; 1mM EDTA), adjusted to 2M LiCl and RNA was precipitated for 12hrs at 4°C. The precipitate was resuspended in 2ml elution buffer(10mM Tris-HCl, pH8.0; 1mM EDTA; 0.05% SDS), adjusted to 0.5% SDS, heated for 5min at 65°C and quenched. Adjusted to 0.5M LiCl, it was applied to oligo(dT) cellulose column. After washing the column with 10ml binding buffer(10mM Tris-HCl, pH8.0; 1mM EDTA; 0.5% SDS; 0.5M LiCl), the mRNA retained by the oligo(dT)-cellulose was eluted with 1ml elution buffer. Adjusted to 1% SDS, this eluted fraction was heated for 10min at 65°C, quenched and repeated the oligo (dT) cellulose chromatography. The mRNA fraction was precipitated with 200mM NaCl and 2 volumes of EtOH at −20°C overnight. After centrifuge, the mRNA was resuspended in H_2O to 1mg/ml and stored at −20°C.

3. Translation in vitro

In vitro translation was carried out in wheat germ extract. Wheat germ extract was prepared by the method of Roberts and Paterson(1973). In vitro translation was carried out in 25μl

translation mixture containing wheat germ extract, [^{35}S]methionine and the other amino acids for 90min at 30°C. Proteins synthesized in vitro were separated by 12.5% SDS-PAGE. Gel was stained with Coomassie blue and impregnated with 2,5-diphenyloxazole and fluorography was performed with X-ray film at −70°C(Laskey 1975).

4. Preparation of antibodies

Purified SKTI was mixed with equal volume of Freund's adjuvant and injected into rabbits (New Zealand White) 3 times at 2 weeks interval. Immunized rabbits were bled 2 weeks after the 3rd injection and the serum was processed by standard method(Garvey et al. 1980).

5. Immunoprecipitation and immunoblot analysis

Immunoprecipitation was carried out according to the procedure described previously (Choi and Dreyfuss 1984a). Translation mixture was diluted with 200μl immunoprecipitation buffer (50mM Tris-HCl, pH 8.0; 100mM NaCl; 0.5% Triton X-100), 2μl antibody and 25μl of protein A-agarose. After incubation for 2hrs at 4°C, the antigen-antibody and protein A-agarose complex was washed 5 times with immunoprecipitation buffer. This complex was mixed with 25μl of SDS-PAGE sample buffer and analyzed by SDS-PAGE and fluorography with PPO.

Blotting of proteins from SDS-PAGE gels onto nitrocellulose paper was carried out by electrotransfer at 0.15A for 12hrs in 50mM Tris-glycine, pH9.1, containing 20% methanol (Choi and Dreyfuss, 1984b). The nitrocellulose blot was incubated first with anti-SKTI antibody(1 : 50) dilution and then with [^{125}I]-labeled goat anti-rabbit 2nd antibody. It was visualized by autoradiography.

6. Gel electrophoresis

Protein samples were analyzed by SDS-containing discontinous polyacrylamide gel electrophoresis system (PAGE)(Choi and Dreyfuss 1984a) or non-denaturing polyacrylamide gel electrophoresis (Lee et al. 1985). The separating gel of SDS-PAGE was prepared from a stock of 33.5% acrylamide and 0.3% N,N′-bisacrylamide to a final concentration of 12.5% acrylamide. The separating gel buffer contained 0.38M Tris-HCl, pH 9.1. The stacking gel was prepared from a stock of 30% acrylamide and 0.44% N,N′-bisacrylamide to a final acrylamide concentration of 4% in 0.125M Tris-HCl, pH 6.8. Both gels contained 0.1% SDS and were polymerized with ammonium persulfate and N,N,N′,N′-tetramethylene diamine. The electrode tank buffer was 25mM Tris-192mM glycine containing 0.1% SDS. Samples were prepared by boiling for 3min in a 0.125M Tris-HCl, pH6.8, buffer containing 1% SDS, 5% mercaptoethanol, 10% glycerol, and bromophenol blue.

Results and Discussion

1. Results

1) Purification of the SKTI polypeptides

To study the molecular structure of the SKTI, it was purified by preparative electrophoresis and band isolation from commercially available SKTI which was partially purified from soybean. It was resolved into Ti^a and Ti^b by non-denaturing PAGE (Fig. 1). The identity of each band was assigned by measuring the inhibitory activity of each band after extraction (Lee et al. 1985). Excised bands from the gel were extracted and purified SKTI was obtained. The purity of the SKTI was shown by SDS-PAGE in Fig. 2. Numerous bands of proteins including major storage proteins are present in total soybean extract (lane Ext). Commercially available SKTI is relatively pure compared to total soybean extract (lane Tc). The Ti^a and Ti^b purified by preparative electrophoresis is pure on the basis of Coomassie Blue staining (lanes Ti^a and Ti^b). SDS-PAGE analysis of purified Ti^a and Ti^b shows that these two SKTI variants have similar molecular weight of 20K.

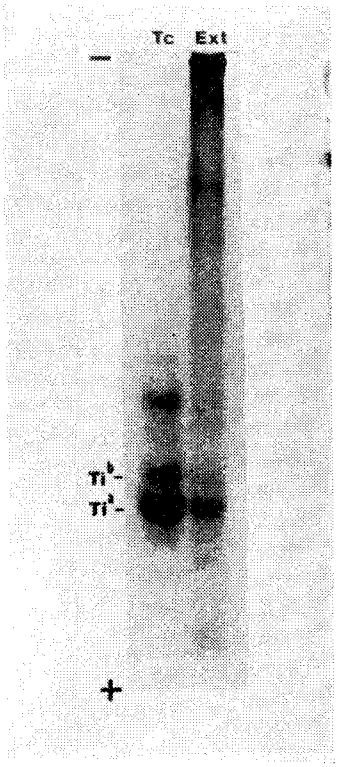

Fig. 1. Non-denaturing PAGE of soybean Kunitz trypsin inhibitor. Protein samples were analyzed by non-denaturing PAGE. The gel was run from the top (negative) to the bottom (positive). Ti^a and Ti^b were assigned according to the procedure described previously(Lee et al. 1985).
Lane Ext : Soybean total extract
Lane Tc : Commercial SKTI, partially purified

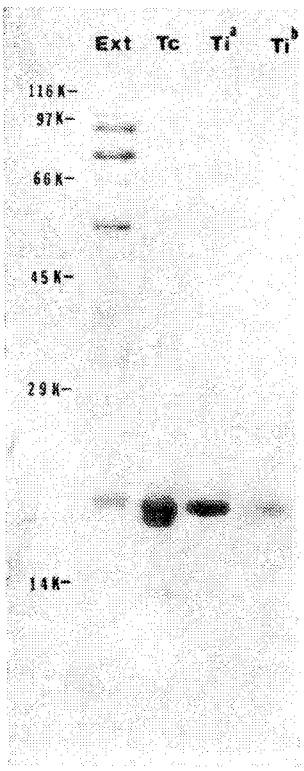

Fig. 2. SDS-PAGE of soybean Kunitz trypsin inhibitor. Protein samples were analyzed by 12.5% SDS-PAGE and stained with Coomassie Blue.
Lane Ext : Soybean total extract
Lane Tc : Commercial SKTI, partially purified
Lane Ti^a : Purified Ti^a
Lane Ti^b : Purified Ti^b

2) Preparation of anti-SKTI antibody

As a molecular probe for the SKTI, anti-SKTI antibody was obtained by immunizing rabbits with purified Ti^a polypeptide of soybean. Specificity of anti-SKTI antibody was demonstrated by immunoblot analysis(Fig. 3). Proteins separated by SDS-PAGE as in Fig. 2 were transferred onto nitrocellulose paper and the blot was probed with anti-SKTI antibody. The antibody reacts specifically with the SKTI of MW 20K present in both commercial SKTI (lane Tc) and soybean seed total extract (lane Ext) out numerous kinds of protein. These bands migrate at the same rate as purified SKTI (lanes Ti^a and Ti^b). They correspond to the major or the only band in Fig. 2. These results demonstrate the specificity of anti-SKTI antibody. The antibody recognized not only Ti^a but also Ti^b (Fig. 3, lanes Ti^a and Ti^b). It suggests that Ti^a and Ti^b are similar in their molecular structure and therefore their antigenic determinants seem to be quite similar.

3) Translation in vitro of soybean mRNA

To pursue the molecular origin of the SKTI, total polyadenylated RNA was isolated from immature soybean seeds by phenol extraction and oligo(dT) chromatography and translation was carried out in wheat germ extract in vitro. In eukaryotic cells including plant and animal cells, mRNA has been known to have about 200 nucleotides of adenylic acid at 3′ end(Birns-

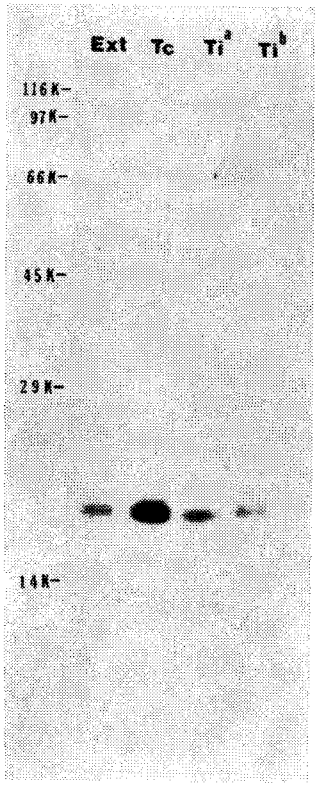

Fig. 3. Immunoblot analysis of anti-soybean Kunitz trypsin inhibitor antibody. SDS-PAGE was carried out with one third amount of sample as in Fig. 2, transferred onto nitrocellulose paper and probed with anti-SKTI antibody.
Lane Ext : Soybean total extract
Lane Tc : Commercial SKTI, partially purified
Lane Ti^a : Purified Ti^a
Lane Ti^b : Purified Ti^b

tiel et al. 1985). Though some precursors of mRNA have the poly (A) tail, their relative amounts are very little. The RNA, therefore, isolate in this experiment could be considered as mRNA. The presence and the species of the mRNA encoding the SKTI was examined by translation in vitro in wheat germ extract. Fig. 4 shows polypeptides translated in vitro in wheat germ extract. They show the synthesis of numerous kinds of polypeptides in the range of MW~100K. Background translation by endogeneous mRNA in wheat germ extract was almost none and the translation was quite specific for exogeneous mRNA added. The patterns of proteins synthesized in vitro are quite distinct in two different tissue types. The mRNAs encoding the polypeptides of MW 13.5K, 18K, 24K, 48K, 59K, 65K, 86K and 90K are present only in seeds but not in leaves, and of 14K and 30K in leaves vice versa. This demonstrates that many genes are expressed in tissue specific manner. However, those major polypeptides synthesized in vitro(Fig. 4, lanes SEED and LEAF) may not be the major proteins present in seeds and leaves. The relative intensity of these bands are related with both the content of [^{35}S] methionine in polypeptide used for label during translation in vitro and the relative amount of the mRNAs encoding those proteins. Even the major proteins synthesized in vitro can not be compared to the known proteins in seed directly on the basis of the mobility in SDS-PAGE. In plant, a lot of proteins is known to be modified post-translationally(Argos et al. 1982; Barton et al. 1982; Vodkin 1981). These reactions can not be expected to occur identically in translation in vitro in wheat germ extract. To overcome these problems, anti-SKTI antibody was used to characterize the SKTI polypeptide translated in vitro.

The presence of the mRNA which code for the SKTI was demonstrated by immunoprecipitation of in vitro translates with anti-SKTI antibody. The antibody did not immunoprecipitate the SKTI of MW 20K but the protein of MW 24K (Fig. 4, lane aSKTI). It was not detected by control immunoprecipitation with non-immune serume demonstrating the specificity of the immunoprecipitation (lane NI). It was immunoprecipitated only from seed mRNA translate but not from leaf mRNA translate (data not shown). When the molecular size of them were compared by running side by side in SDS-PAGE, the MW 24K polypeptide is larger than the antigen, SKTI of MW 20K (Fig. 4, lanes aSKTI and SKTI). It suggests that SKTI is synthesized as the precursor polypeptide of MW 24K, which is converted into the SKTI of MW 20K by post-translational modi-

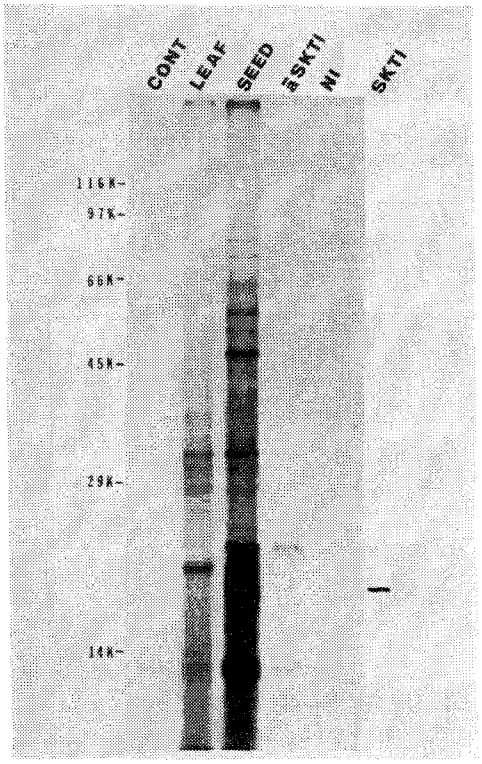

Fig. 4. In vitro translates of mRNAs isolated from soybean. Translation in vitro was carried out in wheat germ extract with [^{35}S]methionine as a label. They were separated by 12.5 % SDS-PAGE and visualized by fluorography. The position of mature SKTI purified from soybean run side by side is shown by arrow head.

Lane CONT : No exogeneous mRNA was added
Lane LEAF : Soybean leaf mRNA was added
Lane SEED : Soybean seed mRNA was added
Lane aSKTI : Immunoprecipitation with anti-SKTI antibody
Lane NI : Immunoprecipitation with non-immune serum
Lane SKTI : The position of mature SKTI from soybean seed stained by Coomassie blue

fication. Post-translational processing, however, did not occur in the wheat germ extract system in vitro.

4) The SKTI biosynthesis in soybean seeds

The expression characteristic of the SKTI was examined in leaves and developing soybean seeds. Total soluble seed proteins were extracted with SDS-PAGE sample buffer from immature seeds at different stages of development and analyzed by immunoblotting. Developing soybean seeds were collected and divided into 5 groups by their size assuming that they are at different stages of development. Embryo size was about 5mm (average 23mg per seed) for stage I, 8mm (62mg) for stage II, 11mm(110 mg) for stage III, 13mm(210mg) for stage IV, and 15mm(367mg) for stage V. The seeds at stage V were yellow-green and at their maximum size.

The amount of the SKTI in unit weight of seed increases abruptly with the development of seeds suggesting that the level of the SKTI is controlled in a developmental stage specific manner(Fig. 5). Furthermore, the SKTI shows a tissue-specific expression pattern since no SKTI has been found in leaves (lane L), which

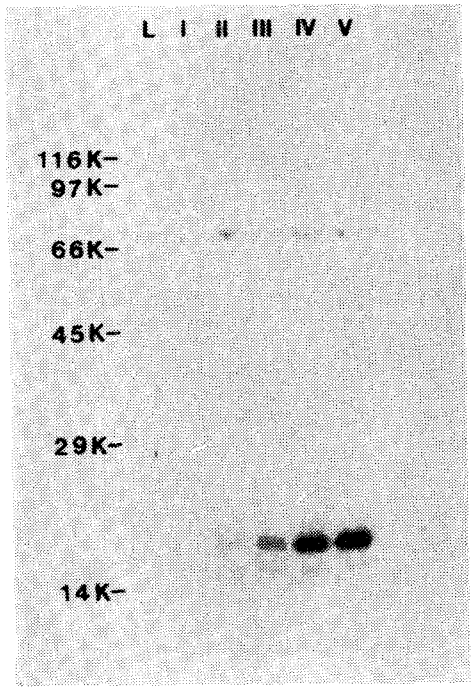

Fig. 5. Immunoblot analysis of soybean Kunitz trypsin inhibitor in developing soybean seed. Immature soybean seeds were collected and divided into 5 groups by the size. Crude extract was analyzed by immunoblot with anti-SKTI antibody.

Lane I : Extract from seed at stage I
Lane II : Extract from seed at stage II
Lane III : Extract from seed at stage III
Lane IV : Extract from seed at stage IV
Lane V : Extract from seed at stage V

is consistent with in vitro translation result. There were unknown band of MW 16K just below the SKTI band detected by anti-SKTI antibody(Fig. 5). It was not detected in mature seed extract(Fig. 2). It might be proteolytic degradation products of the SKTI generated during the extraction of developing immature seed, in which biological activities were more active than in mature dry seeds.

2. Discussion

The SKTI is one of the most extensively studied proteins (Laskowsky and Kato 1980; Read and James 1986). Its physical and chemical properties has been well studied. Its amino acid sequences has been determined(Koide and Ikenaka 1973a, b, c; Kim et al. 1985) and the elucidation of the three-dimensional structure of the trypsin inhibitor complex has been one of the classic achievement of protein chemistry (Sweet et al. 1974). Its molecular biology including biosynthesis mechanism, however, is not well studied. To understand the molecular mechanism of the SKTI biosynthesis in soybean seed, mRNA of developing soybean seeds has been isolated and protein synthesis was carried out in wheat germ extract. Anti-SKTI antibody has been utilized to identify the translation product of the SKTI mRNA in this study.

Antibody has been recognized as a very useful tool in protein chemistry to study the structure of the antigens and the identification of the derivatives. The post-translational modification and the heterogeneity occur in general in plant proteins(Argos et al. 1982; Barton et al. 1982; Vodkin 1981). The heterogeneity appears in various kinds of way such as the size of polypeptide, amino acid composition and charge by post-translational modification such as proteolysis and/or glycosylation. It is not easy, therefore, to identify the primary translation product in vivo and in vitro which are different from the mature protein in cell. Anti-SKTI antibody obtained in this experiment was highly specific for the mature SKTI as well as its precursor. It also recognizes the SKTI variants Ti^a and Ti^b as well reflecting structural similarities each other. In fact, there are only 8 amino acids difference each other(Kim et al. 1985).

The presence of the SKTI precursor was shown in vitro translation products by the immunoprecipitation with anti-SKTI antibody. The antibody recognizes the polypeptide of MW 24K in the translates rather than mature SKTI of MW 20K in soybean seed. It suggests that the SKTI is synthesized as a precursor molecule and subsequent post-translational modification results in the mature protein in seed. The nature of the post-translational modification for the SKTI precursor, however, is not known. Considering the reduction of molecular size, endopeptidic cleavage might be considerable. It is known that many proteins which are synthesized at rough endoplasmic reticulum(rER) and transported to the organelle such as protein body, peroxysome, lysozyme or outside plasma membrane have signal peptide of 10~30 amino acids at NH_2-terminal(Blobel et al. 1975; Heijne 1986). These signal peptides are removed when their NH_2-terminals are translocated to the lumen of rER. This process is explained by the molecular cell biological process of vectorial transport which occurs in cell. In fact, these post-translational modifications are observed in many proteins such as soybean glycinin(Ereken-Tumer 1982), maize zein (Rhighetti et al. 1987) and barley α-amylase(Chrispeels et al. 1982). It is not known whether the SKTI is translated in rER or free ribosome and where it is stored. Considering the post-translational modification by proteolytic cleavage, the SKTI seems to be synthesized in rER like glycinin and conglycinin in soybean. It suggests that such a endopeptidic cleavage need a specific peptidase and/or cellular components such as microsomes which are not present in wheat germ extract. This result also has been observed in translation in vitro using reticulocyte lyzate, in which no post-translational modification was occurred

(Vodkin 1981). It is still possible that there are additional endopeptidic cleavage at C-terminal of the precursor molecule as observed in soybean glycinin. The real nature of the post-translational modification, however, might be understood by studying the structure of the gene and the mRNA.

The polypeptides translated in vitro with mRNA isolated from soybean seed show the seed specific expression pattern (Fig. 4). Immunoblot analysis with the antibody of the tissue extract is consistent with this result (Fig. 5). The SKTI is synthesized predominantly during cell expansion phase of seed development for short period, but not synthesized in other tissues. The tissue specific and/or developmental stage specific expression patterns have been reported for several cases including plant storage proteins. In case of potato and tomato, proteinase inhibitors are known to be expressed at very low level in leaves in normal condition. But when their leaves are wounded by insects or other mechanical forces, the proteinase inhibitors are expressed at high level (Ryan 1968, 1973). Studying the mechanism of the developmental and tissue specific expression of the SKTI might provide an opportunity to understand the regulation of gene expression in eukaryotic cell, especially in plant cell, at molecular level.

Abstract

Three classes of proteinase inhibitors are known in soybean; the Kunitz trypsin inhibitor (SKTI), the Bowman-Birk proteinase inhibitor and its isoinhibitors. To study the molecular structure and expression characteristics of the SKTI, antibody was obtained by immunizing rabbit with the SKTI purified from soybean by preparative electrophoresis. Anti-SKTI antibody was not only specific for mature SKTI in soybean seed but also recognized the precursor which was synthesized in vitro. Translation in vitro was carried out in wheat germ extract with polyadenylated mRNA isolated from developing soybean seeds. One of the seed specific translation products, MW 24K, was identified to be the precursor for the SKTI by immunoprecipitation with anti-SKTI antibody. Mature SKTI of MW 20K, however, was not detected in the translates in vitro. These results suggest that the precursor polypeptide is synthesized from the mRNA and is cleaved to yield mature SKTI in soybean seed. The SKTI gene was expressed with the maturation of soybean seed in a tissue-specific and development stage-specific manner.

Abbreviations used:

SKTI : Soybean Kunitz trypsin inhibitor
BBPI : Bowman-Birk proteinase inhibitor
MW : Molecular weight
K : 1,000 in molecular weight
PAGE : Polyacrylamide gel electrophoresis
SDS : Sodium dodecyl sulfate

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