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Short Communication

Biosynthesis of thiamin-binding proteins in developing sesame seeds

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Abstract The formation of thiamin-binding proteins (TBPs) from sesame (*Sesamum indicum* L.) seeds (STBP-I, -II and -III) in developing seeds was investigated. Elution profiles of STBPs obtained from seeds at 4 to 6 weeks after flowering from the Q-Sepharose Fast Flow column showed that only one STBP was contained in the seeds at 4 weeks, and two STBPs were contained in the seeds at 5 weeks, and all three STBPs were contained in the seeds at 6 weeks as well as in mature seeds. SDS-PAGE of those STBPs demonstrated that STBP in the seeds at 4 weeks was large STBP termed as STBP-III, the molecular mass of which was higher than STBP-I and -II. In addition, it was demonstrated that small and large STBPs were contained in the seeds at 5 weeks, and STBP-I, -II and -III were contained in the seeds at 6 weeks. On the other hand, Southern blot analysis of digested genomic DNAs from sesame leaves with an *STBP* cDNA probe indicated the presence of only one copy of *STBP* in the sesame genome. These results suggested that STBP-III was first produced from a large proprotein precursor in developing sesame seeds, and STBP-I and -II were produced from the same polypeptide chain by different types of post-translational processing.

Key words: Seed protein, sesame, thiamin, thiamin-binding protein.

Thiamin-binding proteins (TBPs) occur in many kinds of plant seeds. TBPs retain thiamin in dormant seeds and supply it for germ growth during germination. It is assumed that thiamin-TBP complex is a storage form of thiamin in the seeds and thiamin is converted to thiamin pyrophosphate during germination (Mitsunaga et al. 1987). The biochemical, structural and functional properties of the TBPs are well known (Shimizu et al. 1995; Watanabe 1999; Rapala-Kozik and Kozik 1999; Adachi et al. 2000; Adamek-Swierczynska and Kozik 2002; Watanabe et al. 2003, 2004; Golda et al. 2004). Many of TBPs isolated from plant seeds are globulin proteins with molecular masses over 100 kDa; however, TBPs classified into albumin proteins, with smaller molecular masses, have been found in sesame seeds (Shimizu et al. 1995) and pea seeds (Adamek-Swierczynska and Kozik 2002). Pea seeds had two TBPs: globulin TBP with a molecular mass of 150–170 kDa and albumin TBP with a molecular mass of 48 kDa, and sesame seeds had three albumin TBPs, termed STBP-I, -II and -III, with molecular masses of 17–19 kDa, whereas other plant seeds had only globulin TBP with a molecular mass over 100 kDa.

The three STBPs resembled each other in molecular mass, molecular structure, and binding-activity to thiamin and thiamin-related compounds. STBP-I, and -II were composed of two 8.9-kDa subunits, respectively. STBP-III was composed of two 9.3-kDa subunits (Shimizu et al. 1995). STBP-II and -III bound one molecule of thiamin per molecule, and STBP-I bound 0.5 molecule (Shimizu et al. 1995). Moreover, the amino acid sequences of small polypeptides of the three STBPs were the same (Watanabe et al. 1999). The amino acid sequences of the large polypeptides were the same, except for the C-terminus of polypeptide chains resulting in differences in molecular mass between STBP-I, -II and -III. On the other hand, cDNA encoding STBPs was isolated and characterized (Watanabe et al. 2001). These results proposed the formation of STBP-I, -II and -III from the proprotein precursor by post-translational processing. However, it remains unclear whether the three STBPs are produced from the same large proprotein precursor or encoded by a gene family (Watanabe et al. 2001). In this paper, STBPs in developing sesame seeds were analyzed by column chromatography and SDS-PAGE. Furthermore, digested

Abbreviations: Bmax, maximum bound amount; Kd, apparent dissociation constant; STBP, sesame thiamin-binding protein; TBP, thiamin-binding protein.

genomic DNAs from sesame leaves were hybridized with the isolated *STBP* cDNA probe to discuss the biosynthesis mechanism of the three STBPs in developing seeds.

Dry mature sesame (*Sesamum indicum* L.) seeds were purchased from Takii Seeds Co. Ltd (Kyoto, Japan). Sesame was field-grown at the Faculty of Agriculture, Kinki University in Nara, Japan. Developing fruits were harvested at the desired time after flowering. The seeds during maturation were isolated from the fruits and stored at −20°C until use.

The leaves were frozen in liquid nitrogen and stored at −80°C to isolate genomic DNA.

The seeds were ground with a pestle and mortar in 0.05 M sodium-phosphate buffer (pH 7.0) containing 1% NaCl at 4°C. The slurry was stirred with a five-fold volume of the same buffer for 1 h at 4°C, then centrifuged at 28,000×*g* for 15 min at 4°C. From the resulting supernatant, STBPs were purified by fractionation by the addition of ammoniun sulfate and then continuous chromatography on columns of SP-Sepharose Fast Flow, Sephadex G-50 Fine and Q-Sepharose Fast Flow, as reported previously (Shimizu et al. 1995).

The thiamin-binding activity of the isolated STBPs was measured by the equilibrium dialysis method as described previously (Shimizu et al. 1995).

SDS-PAGE was performed according to the method of Schagger and Von Jagow (1987) with a 16.5% gel. After running, the gel was stained with Coomassie brilliant blue R-250.

Genomic DNA was isolated according to the urea-phenol method (Shure et al. 1983; Liu et al. 1995). Sesame leaves were ground in liquid nitrogen with a pestle and mortar. The ground leaves were mixed with isolation buffer (0.3 M NaCl, 50 mM Tris-HCl (pH 7.5), 25 mM EDTA, 0.5% SDS, 5 M urea, 10 mM 2-mercaptoethanol and 5% (w/v) phenol) and extracted with phenol/chloroform/isoamyl alcohol (25 : 24 : 1). DNA was collected by ethanol precipitation and dissolved in TE buffer. The isolated DNA (20 μg) was digested with *Eco*R I or *Hin*d III. The resulting DNA fragments were subjected to electrophoresis in a 1.0% agarose gel, transferred to a nylon membrane, and subjected to hybridization with *STBP* cDNA (accession no. AJ310131) as a probe. Labelling of the probe, hybridization and detection of the signal were performed with an AlkPhos Direct Labelling and Detection System kit (Amersham Bioscience, Buckinghamshire, UK) according to the manufacturer's protocol.

STBPs were isolated from the seeds at 4, 5, 6 weeks after flowering. The elution profile of STBPs from the Q-Sepharose Fast Flow column is shown in Figure 1. Only one protein peak (peak a) with thiamin-binding activity was obtained in the seeds at 4 weeks after flowering by Q-Sepharose Fast Flow column chromatography (Figure

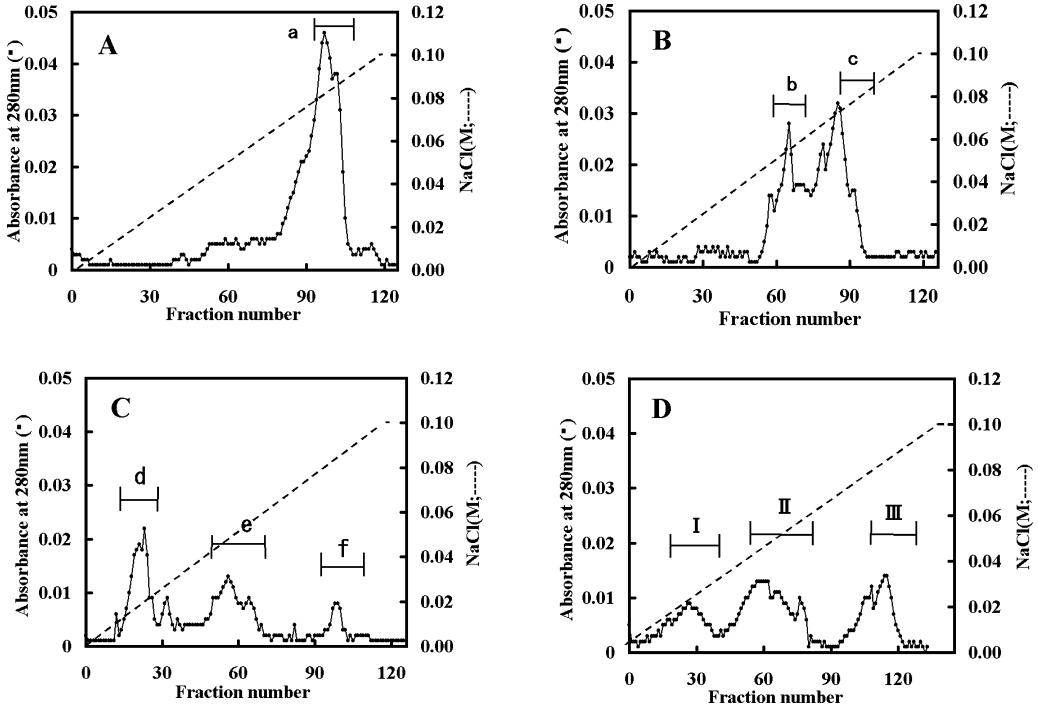

Figure 1. Elution profile of STBPs in sesame seeds during maturation from a Q-Sepharose Fast Flow column. STBPs were put on a Q-Sepharose Fast Flow column (1.0×4.0 cm) equilibrated with 0.05 M borate buffer (pH 9.0), and eluted by the NaCl increasing-gradient from 0 to 0.1 M. The flow rate was 5 ml h^{-1} and the elute was collected in 3-ml fractions. A) seeds at 4 weeks after flowering, B) seeds at 5 weeks after flowering, C) seeds at 6 weeks after flowering, D) dry mature seeds. Letters a to f in the figure indicate a protein peak with thiamin-binding activity. I, II, and III are STBP-I, -II, and -III of mature seeds.

1A). Two protein peaks (b and c) with thiamin-binding activity were obtained from seeds at 5 weeks by column chromatography (Figure 1B). Thiamin-binding activity was not detected in fractions other than those protein peaks. On the other hand, three protein peaks (d, e and f) with thiamin-binding activity were obtained in the seeds at 6 weeks (Figure 1C) as well as dry mature sesame seeds (Figure 1D). Figure 2 shows the SDS-PAGE of proteins obtained from sesame seeds at 4 to 6 weeks after flowering by Q-Sepharose Fast Flow column chromatography shown in Figure 1. The proteins of peaks a, c and f were larger than those of peaks b, d and e in Figure 1. The molecular masses of the proteins of peaks a, c and f were the same as that of the large STBP (STBP-III of mature seeds). On the other hand, the molecular masses of the proteins of peaks b, d and e were the same as that of the small STBP (STBP-I of mature seeds). The molecular masses of STBP-I and -II are the same as described previously (Shimizu et al. 1995). The level of small STBPs increased from 0.14 mg per 100 grains at 5 weeks to 3.78 mg per 100 grains at mature seeds with the seed development. The level of large STBP increased less. The level was 2.66, and 3.41 mg per 100 grains for seeds at 4 weeks, and mature seeds, respectively. These results suggested that the large STBP (STBP-III) was produced first in developing sesame seeds, and then small STBPs (STBP-I and -II) were produced.

Figure 3 shows Southern blot analysis of sesame genomic DNA with the *STBP* cDNA probe. The analysis revealed only one signal band when DNA was digested with *Eco*R I or *Hin*d III. *STBP* cDNA have no cleavage site of *Eco*R I and *Hin*d III (Watanabe et al. 2001); thus, the number of hybridizing signal bands indicated the presence of only one copy of *STBP* in the sesame genome.

Many kinds of plant seeds have globulin TBP (Shimizu et al. 1995; Adamek-Swierczynska and Kozik 2002); however, sesame seeds do not have globulin TBP but three albumin TBPs (STBP-I, -II and -III). On the other hand, only one cDNA encoding STBPs has been isolated (Watanabe et al. 2001); thus, the biosynthesis mechanism of the three STBPs remains unclear. The properties of the three STBPs resemble each other. The apparent dissociation constant (Kd) is 132, and 110 nM, and the maximum bound amount (Bmax) is 58, and 55 nmol mg^{-1} protein for STBP-II, and -III, respectively. Kd and Bmax of STBP-I is 18.0 nM, and 34 nmol mg^{-1} protein, respectively, suggesting that the affinity for thiamin of STBP-I is lower than those of STBP-II, and -III (Shimizu et al. 1995). On the other hand, the molecular masses of STBP-I, and -II are the same. They are composed of two 8.9-kDa subunits and their amino acid sequences are the same (Shimizu et al. 1995; Watanabe et al. 1999). The molecular mass of STBP-III

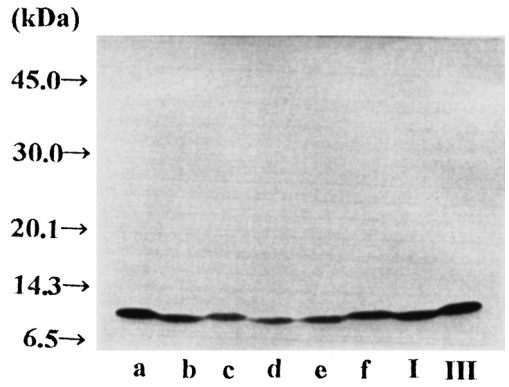

Figure 2. SDS-PAGE of STBPs from sesame seeds during maturation. STBP samples were treated with 1% SDS in the absence of 2-mercaptoethanol. Lanes a to f refer to peaks a to f indicated in Figure 1, respectively. I and III are STBP-I and -III from mature seeds, respectively.

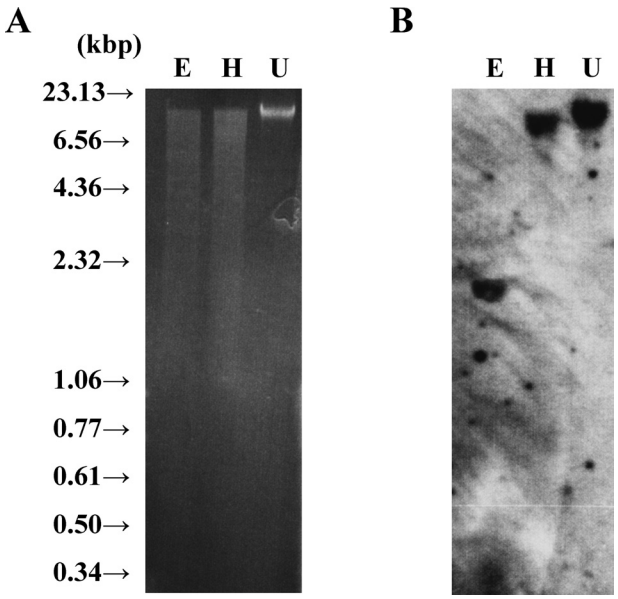

Figure 3. Southern blot analysis of sesame genomic DNA. Genomic DNA was digested with *Eco*R I (E) and *Hin*d III (H), subjected to electrophoresis and hybridized with *STBP* cDNA. A) ethidium bromide-stained gel, B) Southern hybridization. Lane U, undigested genomic DNA.

is higher than that of STBP-I, and -II. STBP-III is composed of two 9.3-kDa subunits and the amino acid sequence is identical to that of STBP-I and -II, except for the C-terminus of large polypeptide resulting in the difference of molecular mass (Shimizu et al. 1995; Watanabe et al. 1999). Consequently, STBP-I and -III differ from one another in Kd, Bmax, and molecular structure.

In this study, it was revealed that the large STBP (STBP-III) was formed first in developing sesame seeds, then the small STBPs (STBP-I, and -II) were formed in the seeds (Figures 1, 2). Based on Southern blot analysis of genomic DNA with *STBP* cDNA, sesame plants were

estimated to possess only a single copy of the *STBP* gene (Figure 3). The amino acid sequences of the three STBPs is the same, except the C-terminus of large polypeptide of STBP-III as mentioned above. The results suggested that the three STBPs were generated from the same large proprotein precursor by different types of post-translational processing. Judging from the elution profile and SDS-PAGE of the proteins and the Kd and Bmax values of STBPs, it is proposed that large STBP-III is first formed in the seeds, then STBP-II, and STBP-I are formed.

The post-translational processing of seed storage proteins has been reported in many kinds of plants (Croy et al. 1980; Tumer et al. 1981; Yamagata et al. 1982). Several small polypeptides were produced from a large precursor protein in plants. A single processing enzyme could convert various types of proprotein precursors into the respective mature forms (Krebbers et al. 1988; Hara-Nishimura et al. 1991). The Kd and Bmax values of STBPs were similar to those of globulin TBPs (Shimizu et al. 1995). However, the affinity of albumin TBP from pea seeds to thiamin was lower than those of other TBPs; thus it was reported that the major thiamin reserve was provided by globulin TBP in pea seeds as well as other plant seeds (Adamek-Swierczynska S, Kozik A 2002). STBP was accumulated and thiamin content increased in sesame seeds during maturation (Watanabe et al. 2003). It suggests that albumin TBPs (STBPs) may work as the major thiamin reserve in sesame seeds. Further studies are needed to clarify why three STBPs are produced from a precursor protein in sesame seeds although other plant seeds have globulin TBP or globulin TBP and albumin TBP.

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