Thiamin-Binding Protein from Sunflower Seeds

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Summary A thiamin-binding protein was isolated from sunflower seeds. Its molecular mass was estimated to be 230 kDa by gel filtration. The protein was suggested to be composed of six subunits, which consisted of polypeptides linked by disulfide bond(s). The protein contained a large amount of glutamine or glutamic acid (19.9 mol%) and asparagine or aspartic acid (11.1 mol%). The levels of tryptophan and valine in the protein were low. These properties of the thiamin-binding protein were similar to those of helianthinin. Optimum pH for the thiamin-binding activity of the protein was 8.0 to 9.0. The thiamin-binding activity was not inhibited by thiamin monophosphate, thiamin pyrophosphate, oxy-thiamin, or pyrithiamin. These properties of the thiamin-binding protein from sunflower seeds were similar to those from buckwheat seeds, but not to those from rice seeds and sesame seeds.

Key Words seed, storage protein, sunflower, thiamin, thiamin-binding protein

Plant seeds contain thiamin-binding proteins (TBPs) that specifically bind free thiamin, but not thiamin phosphates (1). The TBPs are assumed to retain thiamin, which is accumulated into dormant seeds for germination, and the proteins are degraded to supply thiamin and a nitrogen source during germination (2). TBPs having such dual functions are novel storage proteins because, in general, storage proteins have no functions except to provide nitrogen sources during germination. However, only TBPs from sesame seeds, rice seeds and buckwheat seeds have been characterized (1–5). The results indicate that these TBPs differ in molecular weight and subunit structure, amino acid composition, optimum pH for thiamin-binding activity, and binding activity for thiamin analogs, although the TBPs are found only in seeds and specifically bind free thiamin, but not thiamin phosphates. The

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TBPs do not share immunological homology with one another (4, 5). On the other hand, TBPs sharing immunological homology with rice germ TBP are found in gramineous plants such as maize, wheat, and sorghum (4). These results suggest that TBPs present in plant seeds might have properties characteristic of individual plant species, although all the TBPs retain free thiamin in dormant seeds.

In this study, we isolated TBP from sunflower seeds, which are oil seeds and used as foodstuffs, and compared the properties of sunflower seed TBP with those of TBPs from other plant seeds.

MATERIALS AND METHODS

Plant materials. Sunflower (*Helianthus annuus* cv. Taiyo) seeds were obtained from Takii Seeds, Kyoto, Japan. The seeds were powdered by using an electric mill and stored at -20°C .

Chemicals. Phenyl Sepharose CL-4B, Sephacryl S-300 superfine, and Q-Sepharose Fast Flow were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Other chemicals were obtained from Wako Pure Chemical Ind. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

Isolation and purification of TBP from sunflower seeds. All procedures were carried out at 4°C . Sunflower seed powder (10 g) was stirred with 100 mL of H_2O for 1 h, then centrifuged at $27,700 \times g$ for 15 min. The precipitate was stirred with 100 mL of 50 mM Na-phosphate buffer (pH 7.5) containing 0.5 M NaCl for 1 h, then centrifuged at $27,700 \times g$ for 15 min. Ammonium sulfate was added to the supernatant to 40% saturation. The suspension was left for 1 h and centrifuged at $27,700 \times g$ for 15 min. The obtained precipitate was dialyzed overnight against the same Na-phosphate buffer. The dialyzed sample was loaded onto a Phenyl Sepharose CL-4B column (1.5 i.d. $\times$ 5.5 cm) equilibrated with 50 mM Na-phosphate buffer (pH 7.5) containing 0.5 M $(\text{NH}_4)_2\text{SO}_4$. The column was washed with 150 mL of the same buffer and eluted with 100 mL of 50 mM Na-phosphate buffer (pH 7.5), then with 60 mL of H_2O . The active fraction eluted from the Phenyl Sepharose CL-4B column was loaded onto a Sephacryl S-300 superfine column (1.8 i.d. $\times$ 81 cm) equilibrated with 50 mM Na-borate buffer (pH 9.0) containing 0.3 M NaCl, then eluted with the same buffer. The active fraction eluted from the Sephacryl S-300 superfine was loaded onto a Q-Sepharose Fast Flow column (1.0 i.d. $\times$ 5.5 cm) equilibrated with 50 mM Na-borate buffer (pH 9.0) containing 0.3 M NaCl. The column was washed with 60 mL of the same buffer and eluted with an increasing linear gradient of NaCl to 0.5 M in the Na-borate buffer, then with 30 mL of the Na-borate buffer containing 1.0 M NaCl.

Protein measurement. Protein was assayed by the method of Bradford (6) with bovine serum albumin as the standard.

Thiamin-binding assay. Protein solution was dialyzed against 50 mM Na-phosphate buffer (pH 7.5) containing 0.5 M NaCl and $0.1 \mu\text{M}$ thiamin for 24 h at 4°C . After equilibration, the thiamin concentrations of the inner and outer so-

Table 1. Summary of purification of thiamin-binding protein from sunflower seeds.

Procedure	Total protein (mg)	Total activity (μ g)	Specific activity (μ g/mg)	Purification (fold)
Crude extract	361.0	4.34	0.012	1.0
Ammonium sulfate fractionation	145.0	3.27	0.023	1.9
Phenyl-Sepharose CL-4B	69.8	2.98	0.043	3.6
Sephacryl S-300 superfine	35.8	1.35	0.038	3.2
Q-Sepharose Fast Flow	12.5	1.03	0.082	6.8

lutions were measured by using a thiochrome fluorescence method (7). Thiamin-binding activity was determined by the difference in the thiamin concentration between the two solutions.

Polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was performed on a 12.5% gel according to the method of Laemmli (8). Native-PAGE was performed on a 10% gel. After electrophoresis, gels were stained with Coomassie brilliant blue R-250.

Estimation of molecular weight. Molecular weight was estimated by gel filtration on a Sephacryl S-300 superfine column (1.8 i.d. $\times$ 81 cm) with 50 mM Na-phosphate buffer (pH 7.5) containing 0.5 M NaCl as the eluant.

Determination of optimum pH. The effect of pH on thiamin-binding activity was investigated by using the same procedure as described in the thiamin-binding assay, except for the use of Britton-Robinson's buffer (9) (pH 5.0–10.0).

Effect of thiamin derivatives and analogs on thiamin-binding activity. Each thiamin derivative and analog was added to the dialysis buffer simultaneously with 0.1 μ M thiamin at a concentration of 1 μ M, then the thiamin-binding activity was measured as described above.

Amino acid analysis. A protein sample was hydrolyzed under reduced pressure in 6 N HCl at 110°C for 24, 48, and 72 h and analyzed with a Hitachi 850 amino acid analyzer. Tryptophan was measured spectrophotometrically in 0.1 M NaOH (10).

RESULTS

Purification of TBP from sunflower seeds

The results of the purification of thiamin-binding protein from sunflower seeds are summarized in Table 1. Thiamin-binding activity was found in the fraction precipitated by ammonium sulfate at 40% saturation. The elution profile from a Q-Sepharose Fast Flow column is shown in Fig. 1. Thiamin-binding activity was detected in the second peak eluted with the increasing linear gradient of NaCl. The peak containing the activity gave a single protein band when the gel of native-PAGE

Fig. 1. Ion-exchange chromatography of TBP from sunflower seeds on a Q-Sepharose Fast Flow column. The protein was eluted with a linear NaCl gradient (0.3–0.5 M). One fraction is 3.0 mL. —, optical density at 280 nm; -----, thiamin-binding activity; ---, NaCl concentration gradient. The marked active fractions (|—|) were pooled, concentrated, and used for further study.

Fig. 2. Native-PAGE of TBP from sunflower seeds.

was stained with Coomassie brilliant blue (Fig. 2). The TBP was purified 6.8-fold, with a recovery of 23.7%.

Molecular mass of TBP from sunflower seeds

The molecular mass of TBP from sunflower seeds was estimated to be 230 kDa by gel filtration (Fig. 3). SDS-PAGE gave 46-kDa, 39-kDa, and 33-kDa protein bands in the absence of 2-mercaptoethanol (Fig. 4a), and 29-kDa, 25-kDa, 24-kDa, 22-kDa, and 21-kDa protein bands in the presence of 2-mercaptoethanol

Fig. 3. Estimation of molecular weight of TBP from sunflower seeds. Marker proteins: 1, ferritin (Mr 440,000); 2, aldolase (Mr 158,000); 3, bovine serum albumin (Mr 67,000); 4, ovalbumin (Mr 43,000). The filled circle represents the TBP.

Fig. 4. SDS-PAGE of TBP from sunflower seeds. The protein sample was treated with 1% SDS in the absence (a) and presence of 5% 2-mercaptoethanol (b).

(Fig. 4b). These results suggest that the TBP of sunflower seeds is composed of six subunits that consist of polypeptides linked by disulfide bond(s), if all the 46-kDa, 39-kDa, 33-kDa proteins consist of polypeptides linked by disulfide bond(s).

Amino acid composition of TBP from sunflower seeds

Table 2 shows the amino acid composition of TBP from sunflower seeds. The TBP contained large amounts of glutamine or glutamic acid and asparagine or aspartic acid. Tryptophan and valine were present at low levels in the protein. The amino acid composition was similar to that of TBP from buckwheat seeds (3).

Table 2. Amino acid composition of thiamin-binding protein from sunflower seeds.

Amino acid	mol %	Amino acid	mol %
Asx	11.1	Ile	4.1
Thr	4.0	Leu	6.5
Ser	5.4	Tyr	2.2
Glx	19.9	Phe	5.0
Gly	7.6	Lys	2.1
Ala	7.0	His	3.1
Val	1.1	Arg	6.7
Cys	5.3	Pro	5.6
Met	2.5	Trp	0.9

Each value is the mean of duplicate experiments.

Fig. 5. Effect of pH on thiamin-binding activity of TBP from sunflower seeds. The active fraction (—) shown in Fig. 1 was used for the experiments. The mean value of triplicate experiments is presented.

Optimum pH for thiamin-binding activity of TBP from sunflower seeds

The optimum pH for the thiamin-binding activity of TBP from sunflower seeds was 8.0 to 9.0 (Fig. 5). The optimum pH was similar to that of TBPs from rice seeds and buckwheat seeds (1, 3).

Effect of thiamin derivatives and analogs on the binding of thiamin to TBP from sunflower seeds

The effect of thiamin derivatives and analogs on the binding of thiamin to TBP from sunflower seeds is shown in Table 3. A 10-fold molar concentration of thiamin monophosphate or thiamin pyrophosphate did not inhibit the binding of thiamin to TBP from sunflower seeds or to TBPs from other plant seeds (1–3).

Table 3. Effect of thiamin derivatives and analogs on the binding of thiamin to TBP from sunflower seeds.

Addition	Molar ratio to thiamin	Thiamin-binding activity (%)
None	0	100
Thiamin monophosphate	10	103
Thiamin pyrophosphate	10	116
Oxythiamin	10	88
Pyrithiamin	10	83

Each value is the average of triplicate experiments.

Oxythiamin and pyrithiamin also had little inhibitory effect on the binding.

DISCUSSION

TBPs of plant seeds are interesting proteins because they have dual functions: retention of thiamin in dormant seeds and provision of a nitrogen source at germination. The TBPs from rice seeds, sesame seeds, and buckwheat seeds differ in their structural and biochemical properties (1-5), though they all bind only free thiamin and not thiamin phosphate, which is a characteristic property of TBPs from plant seeds. TBP from sunflower seeds was estimated to be a 230-kDa protein by gel filtration (Fig. 3). It was suggested that the TBP was composed of six subunits that consisted of polypeptides linked by disulfide bond(s) (Fig. 4). Glutamine or glutamic acid and asparagine or aspartic acid were present at high levels in the TBP (Table 2). These properties of TBP from sunflower seeds are similar to those of the major storage protein in sunflower seeds, 11S globulin, also termed helianthinin (11, 12). Thus the TBP from sunflower seeds might be an 11S globulin.

TBP from buckwheat seeds is estimated to be a 320-kDa protein and is composed of six subunits consisting of polypeptides linked by disulfide bond(s) (5). This TBP contains a large amount of glutamine or glutamic acid and asparagine or aspartic acid (3). On the other hand, the TBP from rice seeds is a 107-kDa protein that consists of two polypeptides, and glutamine or glutamic acid and glycine are present at high levels in the protein (4). The TBPs from rice seeds and sunflower seeds did not share immunological homology (unpublished data). Three kinds of TBPs from sesame seeds are 17-, 17-, and 19-kDa proteins. These proteins are composed of two subunits consisting of polypeptides linked by disulfide bond(s) (2). Glutamine or glutamic acid and arginine are present at high levels in these TBPs (2). It is assumed that the TBP from sunflower seeds is similar to the TBP from buckwheat seeds in subunit structure, molecular mass, and amino acid composition.

The optimum pH for the thiamin-binding activity of the TBP from sunflower

seeds was pH 8 to 9, where the TBPs from buckwheat seeds and rice seeds also exhibit maximum activity (1, 3). In contrast, the thiamin-binding activity of the TBP from sesame seeds is maximum in the pH 6 to 8 region (2). The thiamin-binding activities of the TBPs from sunflower seeds and other plant seeds were not inhibited by thiamin derivatives or analogs (Table 3). It may be possible to classify TBPs in plant seeds into two groups: (1) high molecular-weight proteins having high thiamin-binding activity at weak basic pH; (2) low molecular-weight proteins having high activity at neutral pH. Moreover, the TBPs of plant seeds have structural and biochemical properties characteristic of individual plant species. An analysis of TBPs present in closely related species, which share immunological homology, is required to clarify the characteristic properties.

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