Characterization of Thiamin-Binding Protein from Buckwheat Seeds

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Summary A thiamin-binding protein from buckwheat (Fagopyrum esculentum Moench) seeds gave two bands of 56- and 50-kDa in the absence of 2-mercaptoethanol and a single band of 25-kDa in the presence of 2-mercaptoethanol on sodium dodecylsulfate gel electrophoresis. These results indicate that the protein consists of polypeptides linked by disulfide bond(s). The protein isolated from buckwheat seeds did not have immunological homology with the thiamin-binding proteins from rice seeds and sesame seeds. However, the binding of the protein to thiamin was inhibited by the modification of the carboxyl residues in the protein as well as that of the thiamin-binding protein from rice seeds. These results suggest that the thiamin-binding protein from buckwheat seeds differs from those from rice seeds and sesame seeds as to subunit structure or immunological properties, but resembles them in the mechanism of binding thiamin.

Key Words buckwheat, seed protein, thiamin, thiamin-binding activity, thiamin-binding protein

Many kinds of plants have thiamin-binding proteins (TBPs) in their seeds (1–5). The proteins bind free thiamin specifically, but not thiamin phosphates. Thus, they differ structurally and functionally from TBPs in microorganisms and animal tissues. The TBPs in plants are thought to be storage proteins because of their localization in plant seeds. This information leads us to the belief that the nutritional quality of seeds for feed and food could be improved by using genetic engineering techniques with the genes producing TBP.

The properties of the TBPs from rice seeds and sesame seeds have already been studied. The TBP from rice seeds is composed of two 50-kDa subunits which have no disulfide bonds (1, 2). Modification of the carboxyl groups in the TBP from rice seeds results in a loss of its binding activity for thiamin (3). The TBPs

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from sesame seeds are composed of two 17-kDa or 19-kDa subunits, each of which consists of a large polypeptide and a small polypeptide linked by disulfide bond(s) (4). However, the subunit structure and thiamin-binding site of the TBP from buckwheat seeds are still unknown (5). For this reason, we isolated TBP from buckwheat seeds (BTBP) and compared its structural properties with those of the TBPs from other plant seeds.

**MATERIALS AND METHODS**

*Plant materials.* Buckwheat (*Fagopyrum esculentum* Moench) seeds were harvested in Kagoshima, Japan, in 1995. The seeds were ground to powder form in an electric coffee mill and stored at $-20^\circ$C until use.

*Chemicals.* Diethylaminoethyl (DEAE)-Sepharose Fast Flow and Phenyl-Sepharose CL-4B were purchased from Pharmacia LKB Technology. TSK-GEL G3000SW was purchased from Tosoh. All other chemicals were of analytical grade.

*Purification of BTBP.* All procedures were done at 4°C. One hundred milliliters of 0.05 M Na-phosphate buffer (pH 7.0) containing 1% NaCl was added to 10 g of the buckwheat powder. After stirring for 1 h, the mixture was centrifuged at 28,000 × g for 15 min. Solid ammonium sulfate was added to the supernatant to 30% saturation. The suspension was allowed to stand for 1 h, then centrifuged at 28,000 × g for 15 min. The supernatant was brought to 70% saturation by the addition of solid ammonium sulfate. After standing for 1 h, it was centrifuged at 28,000 × g for 30 min. The precipitate was dissolved in the above-mentioned phosphate buffer and dialyzed against the same buffer overnight. The supernatant obtained by centrifugation at 28,000 × g for 15 min was dialyzed against 0.05 M Tris-HCl buffer (pH 7.0) containing 0.1 M NaCl. The resulting suspension was applied to a DEAE-Sepharose Fast Flow column (2.5 × 11 cm) equilibrated with the same buffer. The column was washed with 150 mL of the same buffer, and BTBP was eluted with 100 mL of the buffer containing 0.5 M NaCl. The active fraction containing BTBP was dialyzed against 0.05 M Na-phosphate buffer (pH 7.0) containing 0.7 M ($\text{NH}_4$)$_2$SO$_4$, and then applied to a Phenyl-Sepharose CL-4B column (1.5 × 15 cm) equilibrated with the same buffer. The column was washed with 75 mL of the same buffer, and BTBP was eluted with 75 mL of the buffer excluding ($\text{NH}_4$)$_2$SO$_4$.

*Measurement of thiamin-binding activity.* The thiamin-binding activity was measured as described previously (4).

*Polyacrylamide gel electrophoresis (PAGE).* Native-PAGE and sodium dodecylsulfate (SDS)-PAGE were done in 12.5% gel by the method of Laemmli (6). The gels were stained with Coomassie Brilliant Blue R-250.

*Estimation of molecular weight.* The molecular weight of the purified BTBP was estimated by gel filtration on TSK-GEL G3000SW (0.75 × 60 cm) with a Shimadzu LC-6A Liquid Chromatograph. The conditions of high-pressure liquid chromatography (HPLC) were as follows: solvent system, 0.05 M Na-phosphate
buffer (pH 7.0) containing 1% NaCl at a flow rate of 0.7 mL/min; detector, SPD-10A UV Spectrophotometric Detector (Shimadzu), at 280 nm; and standard proteins, MW-Marker (Oriental Yeast).

Preparation of antiserum and immunodiffusion. Antiserum to BTBP was prepared in the same manner as described previously (2), except that booster shots were given at intervals of 7–12 d. The antibody was generated after the third injection.

Double immunodiffusion (7) was done in 1% agar gel in 50 mM Na-phosphate buffer (pH 7.0) containing 1% NaCl at room temperature for 24 h.

Inactivation of BTBP by a carboxyl-modifying reagent. For the modification of carboxyl residues in BTBP, 5 mM glycine methylester and 0.5 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were added to a BTBP sample. The resulting suspension was adjusted to pH 4.75 with 0.1 N HCl. The reaction was done at room temperature, and then stopped by the addition of 5 mM Na-acetate buffer (pH 4.75) after 0 to 60 min of the reaction. The thiamin-binding activity of the BTBP treated with EDAC was measured as described previously (4).

RESULTS AND DISCUSSION

Properties of BTBP

The purified BTBP gave a single protein band on native PAGE (Fig. 1). BTBP gave two bands of 56 and 50-kDa in the absence of 2-mercaptoethanol (Fig. 2a) and a single 25-kDa band in the presence of 2-mercaptoethanol (Fig. 2b) on SDS-PAGE. These results suggested that BTBP was composed of 50 and 56-kDa subunits, although a combination of these subunits, if any, in the BTBP molecule has not been established yet. Additionally, it was assumed that the 50-
kDa

97.4
66
46
30
21.5

(a)  (b)

Fig. 2. SDS-PAGE of BTBP. BTBP was treated with 1% SDS in the absence (a) and presence (b) of 5% 2-mercaptoethanol.

kDa subunit consisted of two 25-kDa polypeptides linked by disulfide bond(s). Further studies on the structure of the 56-kDa subunit are needed.

Previously, it was reported that the molecular weight of BTBP had been estimated to be about 140-kDa (5). However, the molecular weight of the purified BTBP was estimated to be about 320-kDa by molecular-sieve HPLC, indicating the possibility that the protein with a molecular mass of 140-kDa might be associated to the 320-kDa protein during the purification process (8, 9). Further experiments are needed to determine the exact molecular weight of BTBP.

**Immunological homology between BTBP and other TBP s from plant seeds**

Figure 3 shows the result of double-immunodiffusion using a specific antiserum against BTBP. The TBPs from rice seeds and sesame seeds did not give any precipitin line, indicating that BTBP was not immunologically homologous with the TBPs from rice seeds and sesame seeds. This result was confirmed by Western blotting analyses of the purified BTBP.

**Binding of thiamin to BTBP**

The modification of carboxyl groups in BTBP by EDAC resulted in a considerable loss of its thiamin-binding activity (Fig. 4). BTBP treated with EDAC for 30 min did not bind thiamin. On the other hand, the thiamin-binding activity of the BTBP which had bound to thiamin was not inhibited by treatment with
Fig. 3. Double-immunodiffusion analysis of TBPs. The center well contains the antiserum to BTBP. Wells 1 and 4, BTBP; wells 2 and 5, TBP from rice seeds; wells 3 and 6, TBP from sesame seeds.

Fig. 4. Inactivation of BTBP by EDAC. ○, control; ●, treated with 0.5 mM EDAC; and △, treated with 1 μM thiamin and then with 0.5 mM EDAC. Each value is the mean of duplicate determinations.

EDAC. It thus seems likely that carboxyl groups susceptible to EDAC occur in the thiamin-binding site of BTBP as well.

BTBP was composed of 50 and 56-kDa subunits, each of which consisted of polypeptides linked by disulfide bond(s). In contrast, the TBP from rice seeds was composed of two 50-kDa subunits which had no disulfide bonds (1, 2) and the TBPs from sesame seeds were composed of two 17 or 19-kDa subunits, each of which consisted of a large polypeptide and a small polypeptide linked by disulfide bond(s) (4). BTBP did not have immunological homology with the TBPs from rice seeds or sesame seeds. However, the binding of thiamin to BTBP was inhibited by
chemical modification of the carboxyl groups, similarly to the TBP from rice seeds (3). These results indicate that the subunit structure of BTBP differs from those of the TBPs from rice seeds and sesame seeds. Such a difference was also reflected by no cross-reactivity of the antiserum against BTBP with rice or sesame TBPs, while the mechanism of binding thiamin had resemblance between BTBP and other TBPs. Further studies are required to appreciate the differences in the properties of the TBPs present in each plant.

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