Isolation and Properties of a Thiamine-Binding Protein from Buckwheat Seed

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ABSTRACT

A thiamine-binding protein was isolated from buckwheat seeds by extraction with 0.05*M* phosphate buffer (pH 7.0, containing 1% NaCl), fractionation with ammonium sulfate, ion-exchange chromatography on a diethylaminoethyl-Sephadex A-25 column, and gel filtration on a Sephacryl S-300 column. The final preparation was homogeneous by gel electrophoresis and had an apparent molecular weight of 140,000 as estimated by gel filtration. The apparent Kd and Bmax of binding for [^{14}C]thiamine were 1.1 μM and 6.94 nmol/mg of protein, respectively. The optimal pH for binding was 8.5. Competition experiments using several thiamine derivatives suggested high binding specificity of the protein for thiamine.

Proteins having thiamine-binding activity have been found in microorganisms (Nishimune and Hayashi 1971; Iwashima et al 1971, 1979; Iwashima and Nishimura 1979; Griffith and Leach 1973; Henderson et al 1977) and animal tissues (Muniyappa et al 1978, Muniyappa and Adiga 1979), and some have been isolated and characterized. In plants, thiamine-binding proteins have been found in rice bran (Nishino et al 1980), buckwheat, sesame seed, and wheat (Mitsunaga et al 1984). Among these plant sources, the thiamine-binding protein in rice bran was isolated and characterized (Nishimura et al 1984). Although the biological function of thiamine-binding proteins in plant tissues is unknown, we recently found that thiamine-binding activity decreased during germination of buckwheat seed (Mitsunaga et al 1984), suggesting that the protein may function in storage and retention of thiamine, an essential factor for germination. In this paper, we describe the isolation and partial characterization of the thiamine-binding protein from buckwheat.

MATERIALS AND METHODS

Materials

Mature common buckwheat seeds (*Fagopyrum esculentum* Moench), harvested in 1984, were stored at −20°C until used. Diethylaminoethyl (DEAE)-Sephadex A-25 and Sephacryl S-300 were from Pharmacia Fine Chemicals Co. [^{14}C]Thiamine ([thiazole-2-^{14}C]thiamine hydrochloride, 24.3 mCi/mmol) was obtained from Radiochemical Center, England. Pyrithiamine hydrobromide, oxythiamine hydrochloride, thiamine monophosphate chloride, and thiamine pyrophosphate chloride were from Sigma Chemical Co. Thiamine hydrochloride was purchased from Nakarai Chemical Ltd. (Kyoto). Chloroethylthiamine (3-2′-methyl-4′-aminopyrimidyl-(5′)-methyl-4-methyl-5-chloroethyl thiazolium chloride hydrochloride) was from Sankyo Co., Ltd. (Tokyo). Dimethialium (3-2′-methyl-4′-aminopyrimidyl-(5′)-methyl-4,5-dimethyl thiazolium chloride hydrochloride), 2-northiamine (3-4′-aminopyrimidyl-(5′)-methyl-4-methyl-5-hydroxyethyl thiazolium chloride hydrochloride), and hydroxyethylthiamine were from Takeda Chemical Industries Ltd. (Osaka). All other chemicals were purchased from commercial suppliers.

Assay of Thiamine Binding

Unless otherwise indicated, binding activity was assessed by equilibrium dialysis for 24 hr at 4°C (Iwashima et al 1971), except that thiamine concentration was 0.5 μg per ml (pH 7.5). Protein in the dialysate was measured by the procedure of Lowry et al (1951) using ovalbumin as a reference, and thiamine was determined by a thiochrome fluorescence method (Fujita 1955). The amount of thiamine bound to protein was proportional to the amount of protein under the condition used.

Isolation of Binding Protein

Unless otherwise indicated, all operations were conducted at 4°C. One hundred grams of buckwheat flour was ground thoroughly with the aid of sea sand wetted with 0.05*M* potassium phosphate buffer containing 1% NaCl, pH 7.5, in a mortar. Then, 1 L of the same buffer was added to the resulting paste, and the mixture was allowed to stand for 3 hr and centrifuged at 10,000×*g* for 15 min. Solid ammonium sulfate was added to bring the solution to 50% saturation. The suspension was stirred for 2 hr before centrifuging. The precipitate was discarded, and the supernatant solution was brought to 100% saturation by the addition of ammonium sulfate, and the suspension was left for 2 hr at room temperature. The precipitate containing the activity was collected by centrifugation, suspended in 100 ml of 0.05*M* phosphate buffer (pH 7.5), and dialyzed overnight against the same buffer. A small amount of precipitate was removed by centrifugation. The clear supernatant (120 ml) was applied to a 4.5 × 100 cm column of DEAE-Sephadex A-25 equilibrated with 0.05*M* phosphate buffer, pH 7.5. Elution was carried out with 800 ml of the same phosphate buffer and then with a linear gradient consisting of 600 ml of the buffer in the mixing flask and an equal volume of buffer containing 0.25*M* NaCl in the reservoir. The thiamine-binding protein eluted around 0.15*M*. The active fraction was concentrated using a Diafilter (membrane:G-10T, fractionation range of molecular weight 10,000; Bio Engineering, Japan) and then applied to Sephacryl S-300 (1.9 × 100 cm) equilibrated with 0.05*M* potassium phosphate buffer, pH 7.5, containing 1% NaCl, and eluted with the same buffer. All the thiamine-binding activity was found in one symmetrical peak. The active fraction was stored in the buffer at −20°C.

Gel Electrophoresis

Disc gel electrophoresis was carried out as described by Reinsfeld et al (1962) on 7.0% polyacrylamide gel in β-alanine-acetic acid (pH 4.0) and Tris-glycine buffer (pH 9.4).

Molecular Weight Determination

Molecular weight was determined by molecular exclusion chromatography using a Superose 12 HR 10/30 column at a flow rate of 0.4 ml per min on a Pharmacia fast protein liquid chromatography system in a solvent system containing 0.15*M* NaCl and 0.05*M* potassium phosphate buffer, pH 7.0. Standard proteins used were catalase, aldolase, bovine serum albumin, and ovalbumin.

Amino Acid Analysis

Samples were hydrolyzed in vacuo in constantly boiling 6*M* HCl for 24 hr. Analysis was performed with a Hitachi 835 amino acid analyzer and by comparison with standard amino acid mixtures.

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Determination of Kd and Bmax Values for Thiamine Binding

To determine the Kd and Bmax values for thiamine binding, the binding activity was assayed by equilibrium dialysis in 0.05*M* potassium phosphate buffer (pH 8.5) containing 0.1 to 2 μM [^{14}C]thiamine as previously described (Iwashima et al 1971).

Effect of Inhibitors on Binding Activity

Each thiamine derivative or analog was added to dialyzing buffer at a molar ratio to thiamine of 50:1, and thiamine-binding activity was then assayed as described above.

RESULTS AND DISCUSSION

Purification of Thiamine-Binding Protein from Buckwheat Seed

The results of the purification of thiamine-binding protein from buckwheat seed are summarized in Table I.

Upon DEAE-Sephadex A-25 column chromatography (Fig. 1), the thiamine-binding protein fraction eluted in tubes 84 to 94. This fraction did not give a single band on disc electrophoresis on polyacrylamide gel (data not shown). The fraction was further purified by gel filtration on Sephacryl S-300 (Fig. 2). The active preparation was electrophoretically homogeneous (Fig. 3) and is denoted buckwheat thiamine-binding protein (BTBP). BTBP was purified fivefold from the crude extract of buckwheat seed with a recovery of 23% (Table I).

TABLE I
Purification of Thiamine-Binding Protein from Buckwheat Seed[a]

	Total Protein (mg)	Total Activity (μg thiamine bound)	Specific Activity (μg thiamine bound/mg)	Yield (%)
Extract	9,135	4,524	0.5	100
Ammonium sulfate	2,923	2,331	0.8	52
DEAE-Sephadex A-25[b]	1,635	1,842	1.2	41
Sephacryl S-300	408	1,042	2.5	23

[a] From 100 g buckwheat seed.
[b] DEAE = diethylaminoethyl.

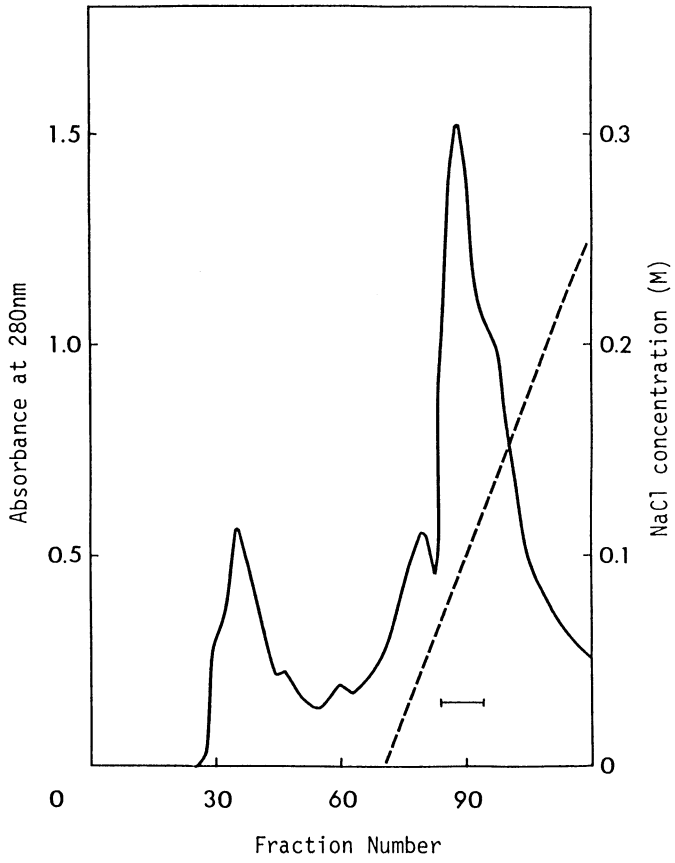

Fig. 1. Elution profile of crude thiamine-binding protein from buckwheat seeds from diethylaminoethyl-Sephadex A-25. Bound material was eluted with a linear NaCl gradient. Active fractions used for further studies are indicated by a bar (⊢).

Molecular Weight of BTBP

The molecular weight of BTBP was estimated to be about 140,000 by gel filtration on a Superose 12 HR 10/30 column. This value is the same as for thiamine-binding protein from *Saccharomyces cerevisiae* (Iwashima et al 1979), but thiamine-binding proteins from other sources have different molecular weights—39,000 in *Escherichia coli* (Matsuura et al 1973), 38,000 in egg yolk (Muniyappa and Adiga 1981), and 94,000 in rice bran (Nishimura et al 1984).

Amino Acid Analysis

The results of the amino acid analysis show the high content of acidic amino acids and the low content of sulfur-containing amino acids in BTBP (Table II).

Effect of pH on BTBP

As shown in Figure 4, the binding activity of BTBP at various pH values indicates that the optimum pH is 8.5. This value is similar to the pH optimum of thiamine-binding protein isolated from rice bran (Nishimura et al 1984).

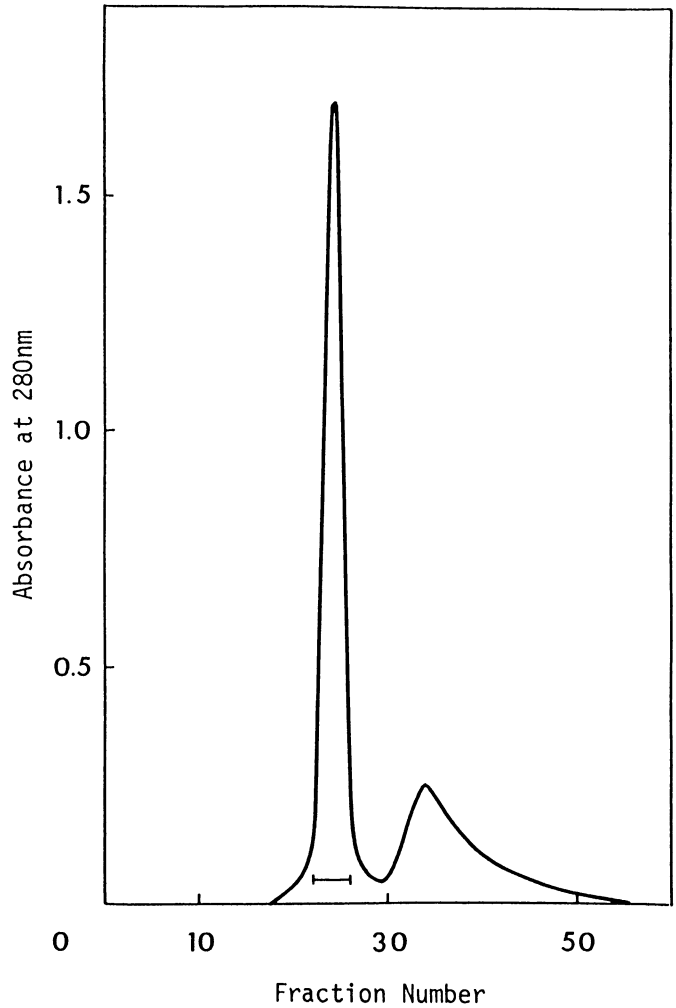

Fig. 2. Elution profile of a partially purified buckwheat thiamine-binding protein from diethylaminoethyl-Sephadex A-25 chromatography on Sephacryl S-300.

Effect of Ligand Concentration on Binding Activity

The effect of various thiamine concentrations on the binding equilibria was studied using [^{14}C]thiamine. The apparent dissociation (Kd) and maximum binding (Bmax) for the ligand were calculated from a Lineweaver-Burk plot, as shown in Figure 5. The plots for the ligand were linear, the apparent Kd was 1.1 μM and Bmax was 6.94 nmol/mg of protein. The Kd of this protein was markedly higher than that of microbial thiamine-binding proteins (*E. coli*, 9.2–50 nM [Griffith and Leach 1973, Matsuura et al 1973, Nishimune and Hayashi 1971]; *S. cerevisiae*, 29 nM [Iwashima et al 1979]; and *L. casei*, 10 nM [Henderson et al 1977]), whereas its value was near to the Kd value of rice bran thiamine-binding protein (Nishimura et al 1984). Calculation based on an apparent molecular weight of about 140,000 and a Bmax of 6.94 nmol/mg of protein suggest binding of 1 mole of thiamine per mole of BTBP.

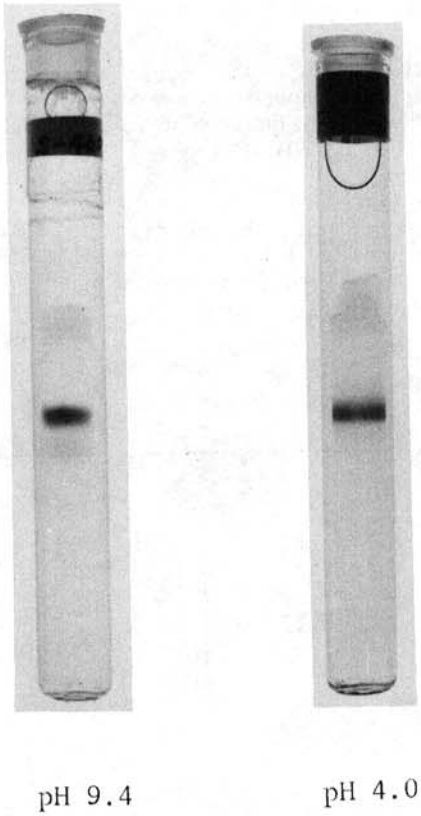

Fig. 3. Polyacrylamide gel electrophoresis of purified buckwheat thiamine-binding protein. Experimental procedures are described in the text.

Effect of Thiamine Derivatives and Analogs on the Binding Activity

Competition of several thiamine derivatives and analogs with thiamine for binding is illustrated in Table III. Binding activity of BTBP to thiamine was not inhibited significantly by thiamine phosphates and thiamine analogs. Binding activity of thiamine-binding protein from microorganisms was generally inhibited by thiamine phosphates and other thiamine analogs, however. These results indicate that BTBP is highly specific for thiamine.

TABLE II
Amino Acid Composition of Buckwheat Thiamine-Binding Protein

Amino Acid	Molar %
Aspartic acid	11.8
Threonine	3.4
Serine	7.0
Glutamic acid	17.8
Glycine	8.6
Alanine	5.5
Half-cystine	0.5
Valine	7.4
Methionine	0.6
Isoleucine	4.2
Leucine	6.9
Tyrosine	1.9
Phenylalanine	4.8
Lysine	3.4
Histidine	1.9
Arginine	9.8
Proline	4.5
Total	100.0

Table III
Effect of Thiamine Derivatives and Analogs on Binding of Thiamine to Buckwheat Thiamine-Binding Protein[a]

Addition	Molar Ratio to Thiamine	Thiamine-Binding (%)
None	0	100.0
TMP	50	89.5
TPP	50	98.2
Oxythiamine	50	95.6
Pyrithiamine	50	99.3

[a]Each value is the average of duplicate determinations.

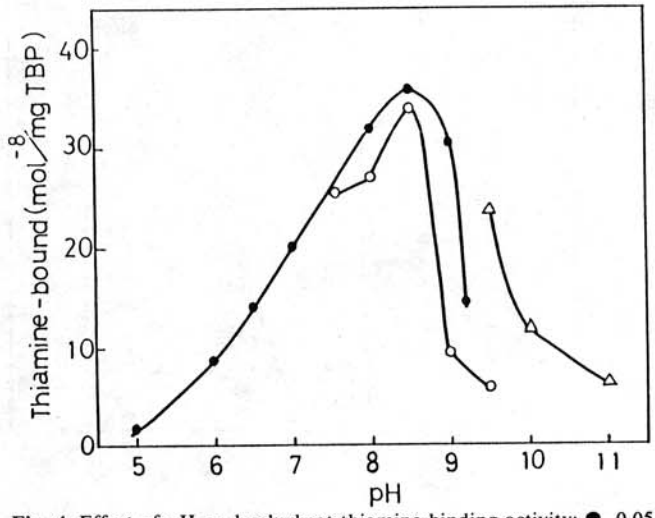

Fig. 4. Effect of pH on buckwheat thiamine-binding activity: ●, 0.05M potassium phosphate buffer; O, 0.05M Tris-HCl buffer; △, 0.05M $NaHCO_3$-Na_2CO_3 buffer. Each value is the average of duplicate determinations.

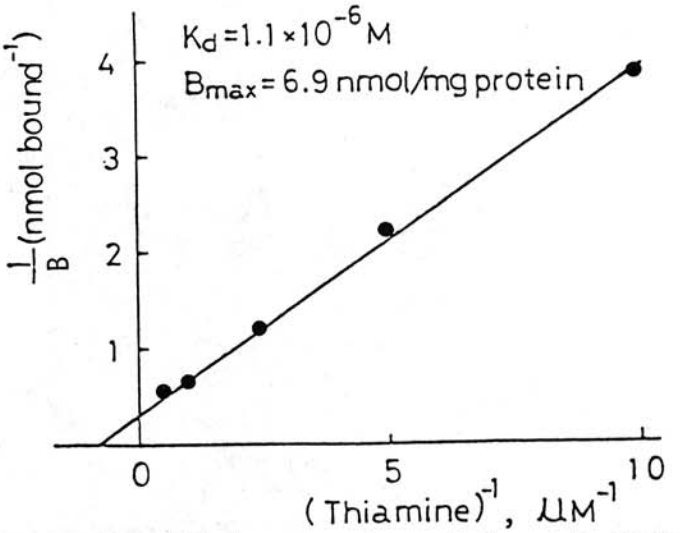

Fig. 5. Effect of thiamine concentration on binding activity. Kinetic parameter Kd and Bmax were determined using Lineweaver-Burk plots of the binding as a function of [^{14}C]thiamine concentration.

Binding of Thiamine Derivatives and Analogs to BTBP

Only 2-northiamine bound to BTBP to the same degree as thiamine. Chloroethylthiamine inhibited the binding of thiamine to the thiamine-binding protein from rice bran (Nishimura et al 1984) but did not bind to BTBP. Thiamine monophosphate chloride, thiamine pyrophosphate chloride, and dimethialium had no affinity to BTBP. These results suggest that a quaternary nitrogen atom, a β-hydroxyethyl group of the thiazole moiety, and an amino group of pyrimidine moiety of thiamine influence binding of BTBP to thiamine. The specificity of the protein to thiamine is particularly interesting, because most thiamine is present in the nonesterified form in buckwheat, whereas it is found as its pyrophosphate ester in microorganisms and animal tissues. Although it is unknown whether all buckwheat thiamine occurs bound to the binding protein, the amount of binding protein is estimated to be sufficient to bind most of free thiamine.

Although the biological function of BTBP in buckwheat is unknown, roles both for retaining thiamine, which is essential for germination and growth, and for supplying it to the germ when required should be considered.

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