Purification and Some Properties of Thiamine-Binding Protein from Rice Bran

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Summary A thiamine-binding protein was purified from the extract of rice bran acetone powder by conventional procedures of acid precipitation, a series of column chromatography on DEAE-Sephadex A-50 and DEAE-cellulose, and gel filtration of Sephadex G-200. The purified thiamine-binding protein was nearly homogeneous as judged by disc gel electrophoresis and the molecular weight was estimated to be 94,000 by gel filtration on Sephadex G-200 and 50,000 by sodium dodecylsulfate (SDS) gel electrophoresis, suggesting that the protein is composed of two identical subunits. The apparent K_d and B_{max} of the binding for [¹⁴C]thiamine was $0.44 \pm 0.05 \mu M$ and 17.2 ± 0.7 nmol/mg of protein, respectively. The optimal pH for the binding is between 8.0 and 9.0. From the competition experiment using several thiamine derivatives, high binding specificity of the protein for thiamine was presumed.

Key Words thiamine-binding protein, molecular weight determination, pH optimum, rice bran

Thiamine-binding proteins are widely distributed among various organisms. In microorganisms they have been found in *Escherichia coli* (1-3), *Lactobacillus casei* (4) and *Saccharomyces cerevisiae* (5, 6). The exact function of these thiamine-binding proteins in microorganisms is still unknown, however, evidence for their involvement in thiamine transport has been discussed (7). Recently, some thiamine-binding proteins have been found in animal tissues such as chicken egg white (8) and liver (9), and it has been supposed that the binding protein in chicken egg white has a role in the deposition and transport of thiamine for the proper growth and development of chick embryo. On the other hand, a previous communication from this laboratory has indicated that there exists a thiamine-binding protein in rice bran, which was the first observation made on a plant (10). In this paper we

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describe the purification and characterization of the thiamine-binding protein from rice bran.

MATERIALS AND METHODS

Chemicals. [^{14}C]Thiamine ([thiazole-2- ^{14}C]thiamine hydrochloride, 24.3 mCi/mmol) was obtained from the Radiochemical Centre, England. Pyrithiamine hydrobromide, oxythiamine hydrochloride, thiamine monophosphate chloride and thiamine pyrophosphate chloride were the products of Sigma Chemical Co. Thiamine hydrochloride was purchased from Nakarai Chemicals Ltd. (Kyoto). Chloroethylthiamine (3-2'-methyl-4'-aminopyrimidyl-(5')-methyl-4-methyl-5-chloroethyl thiazolium chloride hydrochloride) was a gift from Sankyo Co., Ltd. (Tokyo) and *O*-benzoylthiamine disulfide from Tanabe Chemical Industries Ltd. (Osaka). Dimethialium (3-2'-methyl-4'-aminopyrimidyl-(5')-methyl-4,5-dimethyl thiazolium chloride hydrochloride), northiamine (3-2'-methyl-4'-aminopyrimidyl-4-methyl-5-hydroxyethyl thiazolium chloride hydrochloride), hydroxyethylthiamine, 2-methyl-4-amino-5-hydroxymethyl pyrimidine and 5-(2-hydroxyethyl-4-methyl)thiazole were gifts from Takeda Chemical Industries Ltd. (Osaka). [^{14}C]Thiamine monophosphate and [^{14}C]thiamine pyrophosphate were synthesized from [^{14}C]thiamine (24.3 mCi/mmol) by a method previously described (11). The radiochemical purities of [^{14}C]thiamine monophosphate and [^{14}C]thiamine pyrophosphate as found by paper chromatography in isopropanol-0.5 M sodium acetate buffer (pH 4.5)-water (65:15:20) were 96.7% and 96.1%, respectively. All other chemicals were purchased from commercial suppliers.

Assay of thiamine-binding. Unless otherwise indicated, the binding activity was assessed by an equilibrium dialysis experiment for 24 h at 4°C as previously described (12), except for the use of [^{14}C]thiamine at a final concentration of 1 μM (pH 7.5). The activity measured by this method was proportional to the amount of protein used in this experiment.

Purification of binding protein. Unless otherwise indicated, all the operations were conducted at 4°C. One hundred grams of fresh acetone powder of commercially available rice bran were suspended in 1 liter of 0.05 M potassium phosphate buffer, pH 7.5. After stirring for 3 h, the suspension was centrifuged at $11,000 \times g$ for 15 min and the supernatant was adjusted to pH 6.0 by 5 M acetic acid. The precipitate was dissolved in 330 ml of 0.05 M potassium phosphate buffer, pH 7.0, and then added to about 15 g of DEAE-Sephadex A-50 equilibrated with the same buffer, followed by stirring for 1 h at 0°C. This mixture was set to a column (3.7×50 cm), washed with 500 ml of 0.05 M potassium phosphate buffer, pH 7.0, and eluted by an increasing linear gradient of KCl concentration to 0.3 M. The thiamine-binding protein was eluted around 0.27 M. The active fractions were combined and diluted 5 times with 0.05 M potassium phosphate buffer, pH 7.0. The diluted solution was applied to a column (3.0×22 cm) of DEAE-cellulose equilibrated with the same buffer. The active fractions eluted around 0.16 M KCl by an

increasing linear gradient of KCl concentration to 0.3 M were combined, concentrated by using an XM50 Amicon ultrafilter and then applied to a Sephadex G-200 column (20 × 90 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, containing 0.1 M KCl and eluted with the same buffer.

Estimation of molecular weight. The molecular weight of the purified binding protein was estimated by gel filtration on a Sephadex G-200 column (1.5 × 70 cm) according to Andrews (13) using 0.05 M potassium phosphate buffer, pH 7.5, containing 0.1 M KCl. Cytochrome *c* (Mr 12,500), ovalbumin (45,000), bovine serum albumin (68,000) and aldolase (158,000) served as protein markers. SDS-polyacrylamide gel electrophoresis as described by Weber and Osborn (14) was also used for the estimation of subunit molecular weight. *Escherichia coli* RNA polymerase [α -subunit (Mr 39,000), β -subunit (155,000) and β' -subunit (165,000)], bovine serum albumin (68,000) were used as the standard protein markers.

Disc gel electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out using 7% acrylamide at pH 9.5. The sample was run at 7.0 mA per tube for 1 h at room temperature. The protein was detected by staining with Coomassie brilliant blue. The binding activity of the thiamine-binding protein during polyacrylamide gel electrophoresis was detected as previously described (6).

Determination of optimum pH and K_d value. The effect of pH on the binding activity was investigated by the same procedure as described in the binding assay, except for the use of 0.05 M potassium phosphate buffer (pH 5.0–9.2), 50 mM Tris-HCl buffer (pH 7.5–9.5) and 0.05 M NaHCO₃-Na₂CO₃ buffer (pH 9.5–10.5). To determine the K_d and B_{max} values for thiamine, the binding activity was assayed by an equilibrium dialysis experiment at room temperature for 24 h in 0.05 M potassium phosphate buffer (pH 7.5) containing 0.1 to 2 μ M thiamine.

Effect of inhibitors on binding activity. Each thiamine derivative and analog was added to the dialyzing buffer simultaneously with 1 μ M [¹⁴C]thiamine at the concentration of 10 μ M, and then the thiamine-binding activity was assayed as described above.

Determination of protein. Protein was determined by the method of Lowry *et al.* (15) with albumin as a standard.

RESULTS

Purification of thiamine-binding protein from rice bran

The results of the purification of thiamine-binding protein from rice bran acetone powder are summarized in Table 1. The binding activity eluted from Sephadex G-200 coincided as a whole with a protein profile as shown in Fig. 1. The thiamine-binding protein was purified about 42-fold from the crude extract of rice bran acetone powder with a recovery of 13%. Disc gel electrophoresis at pH 9.5 of a purified thiamine-binding protein revealed a broad single band (Fig. 2a) and the location of binding activity toward thiamine coincided with the stained part of the gel in duplicate runs of the purified protein (data not shown). But this purified

Table 1. Purification of thiamine-binding protein from rice bran.

Step	Total protein (mg)	Total activity (nmol thiamine-binding)	Specific activity (nmol thiamine bound/mg of protein)	(%)
1. Crude extract	6,843	1,740	0.26	100
2. Acid precipitation	1,863	1,630	0.88	94
3. DEAE-Sephadex A-50	102	660	6.46	38
4. DEAE-cellulose	45	410	9.05	24
5. Gel filtration on Sephadex G-200	21	220	10.62	13

Fig. 1. Gel filtration on Sephadex G-200 of thiamine-binding protein from rice bran. ○, thiamine-binding activity; ●, absorbance at 280 nm. The horizontal bar represents the fractions pooled.

preparation still contained a small amount of minor protein component (Mr 33,000–35,000) detectable by SDS gel electrophoresis (Fig. 2b). This minor protein component (Mr 33,000–35,000) appeared to be produced by partial proteolysis of the major component (Mr 50,000), since it was formed from the major component by a limited proteolysis with trypsin (unpublished observation). The thiamine-binding protein did not appear to be a glycoprotein since it was unstainable using the periodic acid-Schiff base (PAS) staining method.

Molecular weight of thiamine-binding protein

As shown in Fig. 3A, the molecular weight was estimated to be about 94,000 by

Fig. 2. Polyacrylamide gel (a) and SDS-polyacrylamide gel (b) electrophoresis of the purified thiamine-binding protein from rice bran. The gel was stained for protein with Coomassie brilliant blue R250.

gel filtration on Sephadex G-200 and to be approximately 50,000 by SDS gel electrophoresis (Fig. 3B). These results suggested that the thiamine-binding protein of rice bran was composed of two identical subunits.

Effect of pH on thiamine-binding protein

As shown in Fig. 4, the binding activity of the thiamine-binding protein at various pH values indicates that the pH optimum is 8.0–9.0.

Effect of ligand concentration on the binding activity

The effect of various thiamine concentrations on the binding equilibria was studied using [^{14}C]thiamine. The apparent dissociation (K_d) and maximum bound ($B_{\max}$) for the ligand were calculated from the Hughes-Klotz plot as shown in Fig. 5. The plots for the ligand showed a linear relationship and the value for apparent K_d was $0.44 \pm 0.05 \mu\text{M}$ and that of $B_{\max}$ was $17.2 \pm 0.7 \text{ nmol/mg}$ of protein.

Effect of thiamine derivatives and analogs on the binding activity

The competition of several thiamine derivatives and analogs with thiamine for binding is illustrated in Table 2. The results show that only chloroethylthiamine is an effective inhibitor to the same degree as nonlabeled thiamine for the binding of [^{14}C]thiamine, but that thiamine antagonists such as pyriethamine, dimethialium,

Fig. 3. Estimation of the molecular weight of the binding protein by gel filtration on Sephadex G-200 (A) and by SDS-polyacrylamide gel electrophoresis (B). A. The column (1.5×70 cm) was equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, containing 0.1 M KCl. 1, aldolase; 2, thiamine-binding protein from rice bran; 3, bovine serum albumin; 4, ovalbumin; 5, cytochrome *c*. B. 1; 2 and 5 are the β' -, β - and α -subunit of RNA polymerase from *Escherichia coli*, respectively; 3, bovine serum albumin; 4, thiamine-binding protein from rice bran.

Fig. 4. Effect of pH on activity of the thiamine-binding protein from rice bran. ●, 0.05 M potassium phosphate buffer; ○, 50 mM Tris-HCl buffer; △, 0.05 M NaHCO₃-Na₂CO₃ buffer. Each value is the average of duplicate determinations.

oxythiamine and northiamine were much less inhibitory on the binding of the protein to [¹⁴C]thiamine. The binding was not inhibited by *O*-benzoyl thiamine

Fig. 5. Effect of thiamine concentration on the binding activity. Each point represents the mean $\pm$ SE of triplicate determinations. Kinetic parameter K_d and B_{max} were determined using weighted fits of Hughes-Klotz plots, with the individual mean rates of triplicate experiments weighted by the factor $B^4/(SE)^2$; the standard errors for K_d and B_{max} were calculated as described by Wilkinson (18). The values of these parameters were expressed as the mean $\pm$ SE.

Table 2. Effect of thiamine derivatives and analogs on binding of thiamine to rice bran thiamine-binding protein.
Each value is the average of duplicate determinations.

Addition	Molar ratio to [14 C]thiamine	[14 C]Thiamine binding (%)	
		Expt. 1	Expt. 2
None	0	100	100
Thiamine	10	15.5	13.4
Chloroethylthiamine	10	14.1	
Dimethialium	10	60.5	
Hydroxyethylthiamine	10	98.5	
Pyrithiamine	10	78.1	
Oxythiamine	10		70.7
Northiamine	10		50.6
2-Methyl-4-amino-5-hydroxymethyl pyrimidine	10		93.5
5-(2-Hydroxyethyl-4-methyl)- thiazole	10		94.9

disulfide, hydroxyethylthiamine, 5-(2-hydroxyethyl-4-methyl)thiazol or 2-methyl-4-amino-5-hydroxymethyl pyrimidine.

Binding of [^{14}C]thiamine phosphates to rice bran thiamine-binding protein

Since thiamine phosphates such as thiamine monophosphate and thiamine pyrophosphate did not significantly inhibit the binding of [^{14}C]thiamine to crude thiamine-binding protein from rice bran (10), the binding of ^{14}C -labeled thiamine phosphates synthesized from [^{14}C]thiamine to the purified thiamine-binding protein was examined. As shown in Table 3, the affinity of [^{14}C]thiamine monophosphate and [^{14}C]thiamine pyrophosphate to rice bran thiamine-binding protein was negligibly small.

DISCUSSION

Although the occurrence of thiamine-binding proteins in animal and microbial sources has been known for recent years, there has been no information forthcoming on purification of thiamine-binding protein from plants. In this study, a thiamine-binding protein was purified from rice bran in an almost homogeneous state. Thiamine-binding proteins thus far isolated from various sources have been found to have different molecular weights, *i.e.*, 39,000 from *Escherichia coli* (16, 17), 140,000 from *Saccharomyces cerevisiae* (5) and 38,000 from egg yolk (8), but all of them are reported to be a single polypeptide. It is of interest to note that the molecular weight of the rice bran thiamine-binding protein was estimated to be 94,000 by gel filtration on Sephadex G-200 and to be 50,000 by SDS gel electrophoresis, indicating that the binding protein is composed of two identical subunits.

The K_d and $B_{\max}$ values of the thiamine-binding protein from rice bran were calculated to be $0.44 \pm 0.05 \mu\text{M}$ and $17.2 \pm 0.7 \text{ nmol/mg}$ of protein, respectively. The K_d of this protein was markedly higher than that of microbial thiamine-binding protein (*E. coli*: 9.2–50 nM, *S. cerevisiae*: 29 nM and *L. casei*: 10 nM), whereas its value was similar to the K_d value ($0.3 \mu\text{M}$) of thiamine-binding protein isolated from egg white (8). Calculation from the molecular weight of 94,000 obtained by gel filtration on Sephadex G-200 and the $B_{\max}$ of 17.2 nmol/mg of protein, revealed the binding of 1.62 mol of thiamine per mol of the thiamine-binding protein. Furthermore, it was presumed that each subunit of rice bran thiamine-binding protein composed of two identical subunits (Mr 50,000) binds one molecule of thiamine when the $B_{\max}$ obtained above was corrected to 19.1 nmol/mg of protein at optimal pH.

The binding activity of thiamine-binding proteins from microorganisms was generally inhibited by thiamine phosphates and many other thiamine analogs. On the other hand, the binding activity of the thiamine-binding protein from rice bran to [^{14}C]thiamine was not markedly inhibited by thiamine phosphates and thiamine analogs except for chloroethylthiamine. In fact, the affinity of the thiamine-binding

Table 3. Binding of [^{14}C]thiamine, [^{14}C]thiamine monophosphate and [^{14}C]thiamine pyrophosphate to rice bran thiamine-binding protein. Each value is the average of duplicate determinations.

Substrate tested	Binding (%)
[^{14}C]Thiamine	100
[^{14}C]Thiamine monophosphate	4.9
[^{14}C]Thiamine pyrophosphate	0

protein to thiamine phosphates was much less than that of thiamine. These results indicate that the thiamine-binding protein from rice bran is highly specific to the thiamine molecule. Furthermore, the strong inhibitory effect of chloroethylthiamine on thiamine binding is attributable to the similarity of the electronegativity between the oxygen atom and the chlorine atom in the thiamine structure. The specificity to thiamine of the protein may be particularly interesting since thiamine present in the free form in rice bran differed from the coenzyme form found in microorganisms and animal tissues. Although it is unknown whether all of the thiamine in rice bran is in the bound-form with the binding protein, the amount of the binding protein in rice bran is roughly estimated to be sufficient to bind most of the free thiamine present in rice bran.

Although the biological function of thiamine-binding protein in rice bran is unknown at present, roles both for retaining thiamine which is essential for germination and growth of rice and for supplying it to the endosperm when necessary should be considered.

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