

Characterization of Thiamin-Binding Protein from Wheat Germ

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ABSTRACT

Cereal Chem. 77(5):578-581

A thiamin-binding protein was isolated from wheat germ (*Triticum aestivum*). Its molecular mass was estimated as 120,000 Da consisting of two 56,000-subunits. The protein contained a large amount of glutamine or glutamic acid (15.4 mol%), arginine (12.5 mol%), and glycine (12.0 mol%). The levels of tyrosine, methionine, tryptophan, and cysteine in the protein were low. Optimum pH for its thiamin-binding activity was pH 8.0. It bound

free thiamin specifically, not thiamin phosphates. The apparent dissociation and maximum bound values for the thiamin-binding were 2.52 μ M and 9.34 nmol/mg of protein, respectively. Properties of the thiamin-binding protein from wheat germ were similar to those of the thiamin-binding protein from rice seeds, but not from buckwheat, sesame, or sunflower seeds.

Thiamin-binding proteins (TBP) are widely distributed in plants and accumulated in seeds. TBP have been isolated from rice (Nishimura et al 1984 Shimizu et al 1996), buckwheat (Mitsunaga et al 1986), sesame (Shimizu et al 1995) and sunflower seeds (Watanabe et al 1998b). Their molecular mass, subunit structures, and amino acid composition differ. Also, the optimum pH for thiamin-binding activity and the affinity for thiamin analogs differ. In addition, cross-reaction with anti-TBP from rice germ antibody indicates that TBP from buckwheat, sesame, and sunflower seeds do not have immunological homology; i.e., the TBP in plant seeds are heterogeneous. However, TBP sharing immunological homology with the TBP from rice germ have been found in gramineous seeds such as maize, foxtail millet, sorghum, and wheat through immunoblot analysis with anti-TBP from rice germ antibody (Shimizu et al 1996). This suggests that the structural and biochemical properties of TBP in closely related plant species may be similar. Therefore, we isolated TBP from wheat germ and compared the properties of wheat germ TBP (WGTBP) with TBP from other plant seeds such as rice, buckwheat, sesame, and sunflower.

MATERIALS AND METHODS

Wheat germ was donated by Nisshin Flour Milling Co. Ltd. (Tokyo, Japan). Thiamin hydrochloride was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DEAE-Sephacel, Sephacryl S-200 HR, Phenyl-Sepharose CL-4B, Q-Sepharose FF resins, and Ampholine were purchased from Amersham Pharmacia Biotech. (Uppsala, Sweden). All other chemicals were of analytical grade. Thiamin analogs such as pyrithiamin hydrobromide, oxythiamin hydrochloride, 2-northiamin, and hydroxyethylthiamin were used in the experiments, as well as thiamin derivatives such as thiamin monophosphate chloride (TMP) and thiamin pyrophosphate (TPP). Protein was assayed according to Bradford (1976) with bovine serum albumin as a standard.

Thiamin-binding activity was assayed by equilibrium dialysis. A sample solution was dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 1 μ M thiamin for 24 hr at 4°C. After equilibration, the thiamin concentrations of the inner and outer solutions were assayed by a thiochrome fluorescence method (Fujita 1955); the difference in the concentrations was taken to be the binding activity. The activity unit that bound 1 nmol of thiamin was defined as 1 unit of activity.

All operations to purify TBP from wheat germ were conducted at 4°C. Wheat germ was defatted by petroleum ether and powdered by electric mill; 200 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM dithiothreitol was added to 20 g of

powder. The mixture was stirred for 1 hr and centrifuged at 28,000 \times g for 15 min. Ammonium sulfate was added to the resulting supernatant to 90% saturation. The suspension was left for 1 hr and centrifuged at 28,000 \times g for 15 min. The precipitate was dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM dithiothreitol and centrifuged at 28,000 \times g for 15 min. The supernatant was put on a DEAE-Sephacel column (3.2 \times 50 cm) and equilibrated with the same buffer. The column was washed with 1L of the same buffer. WGTBP was eluted stepwise with 1L of 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM dithiothreitol and 100 mM potassium phosphate buffer (pH 7.0) containing 0.1M KCl. The active fractions with thiamin-binding activity were collected and dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 1M (NH₄)₂SO₄.

The dialyze was put on a Phenyl-Sepharose CL-4B column (1.8 \times 30 cm) and equilibrated with the same buffer. The column was washed with 400 mL of the same buffer. WGTBP was eluted stepwise with 250 mL of the same buffer containing 0.8M (NH₄)₂SO₄ and 150 mL of the same buffer without ammonium sulfate. The active fractions with thiamin-binding activity were collected and dialyzed against 50 mM Tris-HCl buffer (pH 9.0) containing 0.1M NaCl. The dialyze was put on a Q-Sepharose FF column (1.5 \times 20 cm) and equilibrated with the same buffer. The column was washed with 80 mL of the same buffer. WGTBP was eluted stepwise with 80 mL of the same buffer containing 0.2M NaCl and 20 mL of the same buffer containing 1M NaCl. The active fractions with thiamin-binding

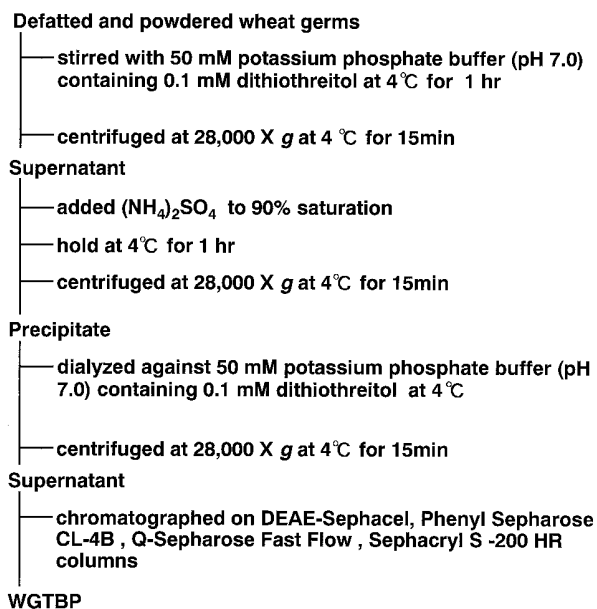


Fig. 1. Purification procedure for thiamin-binding proteins from wheat germ (WGTBP).

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activity were collected and dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 1% (w/v) NaCl. The dialyzate was put on a Sephacryl S-200 HR column (2.2 × 80 cm) and equilibrated with the same buffer. WGTBP was eluted with 350 mL of the same buffer (Fig. 1).

Native-PAGE was conducted in 12.5% (w/v) slab gel as described by Laemmli (1970). For two-dimensional-PAGE (2D-PAGE), 5% (w/v) gel containing 2% (v/v) Ampholine (pH 6–8), and 20% (w/v) glycerol was used for isoelectric focusing. After the focusing, the gels were equilibrated with 10 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol, and 0.001% (w/v) bromophenol blue. For the second dimension, 12.5% (w/v) polyacrylamide slab gel (4.5% [w/v] stacking gel) was used. WGTBP (20 µg) was loaded for the first dimension. The gel was stained with Coomassie brilliant blue R-250.

The molecular mass of WGTBP was estimated by gel filtration on TSK-GEL G3000SW (0.75 × 60 cm) using a Shimadzu LC-6A liquid chromatograph (Watanabe et al 1998a). The HPLC mobile phase was 50 mM potassium phosphate buffer (pH 7.0) containing 0.2M KCl with a flow rate of 1.0 mL/min. Detection was set at 280 nm on a SPD-10A UV spectrophotometric detector (Shimadzu). Proteins standards were obtained from Oriental Yeast (Tokyo, Japan).

For amino acid analysis, WGTBP was hydrolyzed under reduced pressure in 6N HCl at 105°C for 24 and 48 hr and analyzed using a Hitachi 8500 amino acid analyzer. Tryptophan was measured spectrophotometrically in 0.1M NaOH (Shimizu et al 1995).

To determine optimum pH for thiamin-binding activities, different pH values were measured following the thiamin-binding assay described above, except that WGTBP was dialyzed using a Britton and Robinson (1931) buffer (pH 6.0–10.0).

To determine the effect of thiamin concentration on thiamin-binding activity, WGTBP was dialyzed against 50 mM potassium phosphate buffer (pH 8.0) containing 0.4–1.0 µM thiamin, and thiamin-binding activity was measured following the thiamin-binding assay described above. WGTBP was dialyzed against 1 µM thiamin derivative or analog for 24 hr, and binding activity to WGTBP was assayed in the same manner as thiamin-binding assay. TMP and TPP in the inner and outer solutions were hydrolyzed to free thiamin with 0.05% (w/v) Takadiastase B for 12 hr at 37°C before the assay.

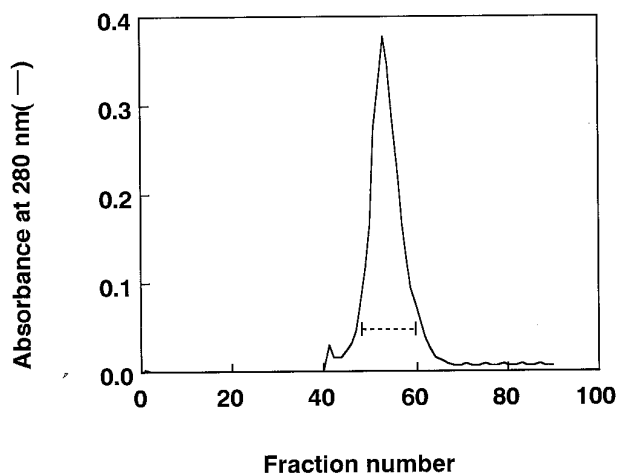


Fig. 2. Gel filtration on HPLC of thiamin-binding proteins from wheat germ (WGTBP). Thiamin-binding activity indicated by dotted line.



Fig. 3. Native PAGE of thiamin-binding proteins from wheat germ (WGTBP).

Thiamin derivative or analog was added to a dialysis buffer at a concentration of 10 µM simultaneously with 1 µM thiamin, and the thiamin-binding activity of WGTBP was measured as described above.

RESULTS

One protein peak with thiamin-binding activity was obtained using Sephacryl S-200 column chromatography (Fig. 2). This peak produced a single protein band on native PAGE (Fig. 3). The findings of the purification of WGTBP are summarized in Table I. WGTBP was purified 33-fold from crude TBP, with a recovery of 27.5%.

The molecular mass of WGTBP was estimated as 120,000 Da by gel filtration on HPLC (Fig. 4) and 56,000 Da using 2D-PAGE (Fig. 5). These findings suggest that WGTBP consisted of two subunits of the same size. The molecular mass of WGTBP was close to that of rice germ TBP (107,000 Da) (Shimizu et al 1996); lower than the TBP of buckwheat (140,000 or 320,000 Da) (Mitsunaga et al 1986, Watanabe et al 1998a) and sunflower (230,000 Da) seeds (Watanabe et al 1998b); and higher than the TBP of sesame seed (17,000 and 19,000 Da) (Shimizu et al 1995).

Table II shows that the amino acid composition of WGTBP is rich in glutamine (or glutamic acid), glycine, and arginine, but poor in tyrosine, methionine, tryptophan, and cysteine. The amino acid composition of WGTBP was similar to that of rice germ TBP (Shimizu et al 1996).

Thiamin-binding activity of WGTBP at various pH values indicated that the optimum pH for the activity was pH 8.0 (Fig. 6). The optimum pH of WGTBP was similar to that of rice germ TBP (pH 8.5) (Shimizu et al 1996), buckwheat seed (pH 8.5) (Mitsunaga et al 1986), and sunflower seed (pH 8.0) (Watanabe et al 1998b).

The effect of thiamin derivatives and analogs on the binding of thiamin to WGTBP is shown in Table III. The binding activity of WGTBP to thiamin was not inhibited by thiamin derivatives and analogs. The observations were similar to those for rice germ (Shimizu et al 1996), buckwheat seed (Mitsunaga et al 1986), and sunflower seeds (Watanabe et al 1998b).

Binding activity of thiamin derivatives and analogs to WGTBP is shown in Table IV. WGTBP bound slightly to 2-northiamin but not to thiamin derivatives and hydroxyethylthiamin. These observations were similar to those for rice germ (Shimizu et al 1996).

Figure 7 shows a Hughes-Klotz plot for thiamin (Nishimura et al 1984) that is linear. The apparent dissociation (K_d) and maximum bound (B_{max}) values were calculated as 2.52 µM and 9.34 nmol/mg

TABLE I
Purification of Wheat Germ Thiamin-Binding Protein (TBP)

Purification Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)
Crude TBP	1,488	40.8	0.027	100
DEAE-Sephacel	164.5	21.7	0.132	53.2
Phenyl-Sepharose CL-4B	105.4	14.9	0.141	36.5
Q-Sepharose Fast Flow	21.0	11.5	0.550	28.4
Sephacryl S 200 HR	12.6	11.2	0.889	27.5

TABLE II
Amino Acid Composition of Wheat Germ Thiamin-Binding Protein^a

Amino Acid	mol%	Amino Acid	mol%
Asx	6.9 ± 0	Tyr	0.6 ± 0
Thr	3.9 ± 0	Phe	4.3 ± 0
Ser	7.0 ± 0.1	Lys	3.4 ± 0.1
Glx	15.4 ± 0.1	His	4.4 ± 0.1
Gly	12.0 ± 0.3	Arg	12.5 ± 0.4
Ala	6.8 ± 0	Pro	4.4 ± 0.2
Val	7.0 ± 0	Met	0.9 ± 0
Ile	3.6 ± 0.1	Cys	1.2 ± 0
Leu	5.5 ± 0.1	Trp	0.2 ± 0

^a Values ± standard deviation (n = 3).

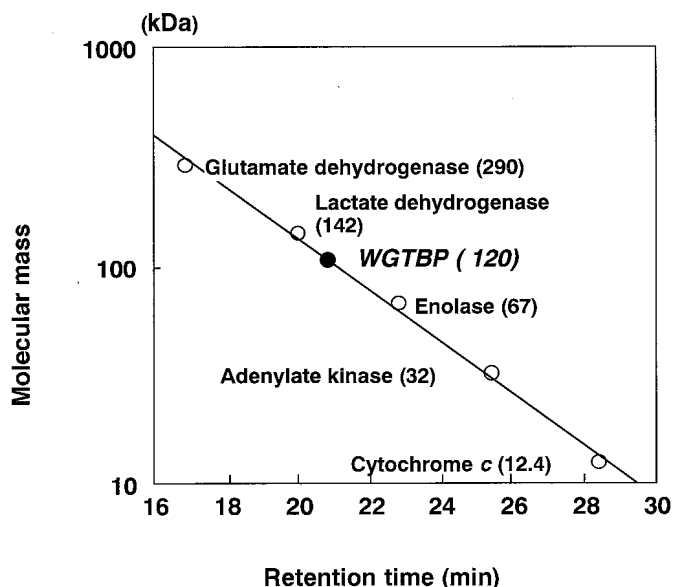


Fig. 4. Gel filtration on HPLC to estimate molecular mass of thiamin-binding proteins from wheat germ (WGTBP).

TABLE III
Inhibitory Effect of Thiamin Derivatives and Analogs on Wheat Germ Thiamin-Binding Protein^a

Addition	Molar Ratio to Thiamin	Residual Thiamin-Binding Activity (%)
None (control)	0	100
Thiamin monophosphate chloride	10	115. ± 6.2
Thiamin pyrophosphate	10	105.3 ± 3.1
Oxythiamin	10	105.3 ± 12.4
Pyriothiamin	10	94.7 ± 13.6

^a Values ± standard deviation ($n = 3$).

TABLE IV
Binding Activity of Thiamin Derivatives and Analogs to Wheat Germ Thiamin-Binding Protein^a

Substrates Tested	Binding Activity (μg/mg)
Thiamin	0.19 ± 0.01
Thiamin monophosphate chloride	0
Thiamin pyrophosphate	0
2-Northiamin	0.05 ± 0.01
Hydroxyethylthiamin	0

^a Values ± standard deviation ($n = 3$).

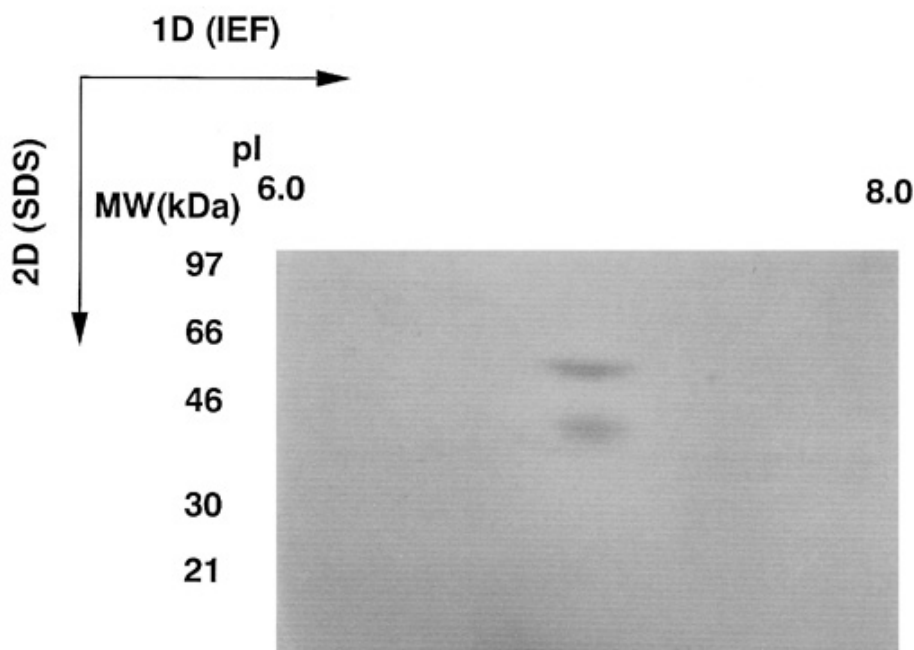


Fig. 5. Two-dimensional PAGE of thiamin-binding proteins from wheat germ (WGTBP).

of protein, respectively. Calculation using the molecular mass of 120,000 Da and the B_{\max} indicated that 1.12 moles of thiamin bound to 1 mole of WGTBP.

DISCUSSION

Thiamin is necessary for human and animal nutrition. TBP may enhance the nutritive value in plant seeds. TBP have additional functions such as retention of thiamin in dormant seeds and provision of a nitrogen source at germination. The TBP from rice germ (Shimizu et al 1996a), sesame seeds (Shimizu et al 1995), buckwheat seeds (Mitsunaga et al 1986, Watanabe et al 1998a), and sunflower seeds (Watanabe et al 1998b) differ in their structural, biochemical, and immunological properties, although they all bind only free thiamin, not thiamin phosphates. This specificity for thiamin is characteristic

of TBP from plant seeds. The molecular mass of WGTBP was estimated to be 120,000 Da using gel filtration (Fig. 4). WGTBP consists of two identical 56,000 Da subunits (Fig. 5). WGTBP contains a large amount of glutamine (or glutamic acid), glycine, and arginine (Table II). Amino acid composition of WGTBP is also similar to TBP from rice germ (Shimizu et al 1996). The 40,000 Da polypeptide that appeared on the SDS-PAGE (Fig. 5) is assumed to be a partial digestion product of the 56,000 Da subunit because rice bran TBP are also a partial digestion products of subunits (Nishimura et al 1984). In rice bran TBP, a partial digestion was formed from the subunit by a limited proteolysis with trypsin. However, the molecular mass of TBP from buckwheat seeds is 320,000 or 140,000 Da. It is composed of six subunits consisting of polypeptides linked by disulfide bonds (Watanabe et al 1998a) and contains a large amount of glutamine (or glutamic acid), asparagine (or aspartic

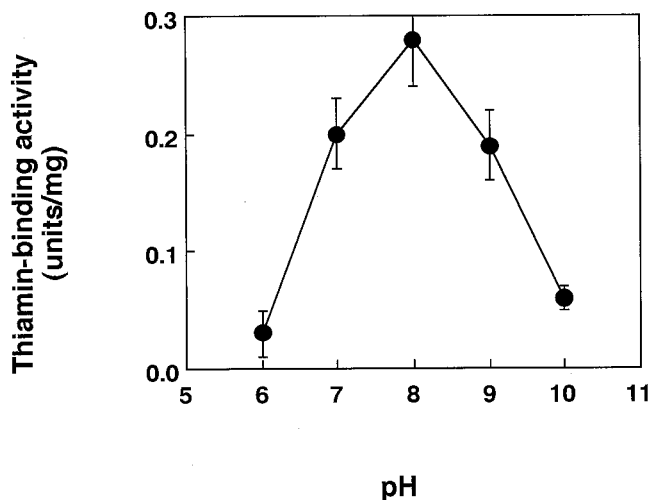


Fig. 6. Effect of pH on thiamin-binding activity of thiamin-binding proteins from wheat germ (WGTBP). Mean \pm standard deviation ($n = 3$).

acid), and arginine (Mitsunaga et al 1986). The molecular mass of TBP from sesame seeds is 17,000 or 19,000 Da. It is composed of two subunits consisting of polypeptides linked by disulfide bonds and have a large amount of glutamine (or glutamic acid), arginine, and serine (Shimizu et al 1995). The molecular mass of TBP from sunflower seeds is 230,000 Da. It is composed of six subunits consisting of polypeptides linked by disulfide bonds and contains a large amount of glutamine (or glutamic acid), asparagine (or aspartic acid), and glycine (Watanabe et al 1998b).

The optimum pH for thiamin-binding of WGTBP was pH 8.0 (Fig. 6), where the TBP from buckwheat, rice, and sunflower seeds also exhibited maximum thiamin-binding activity. In contrast, the maximum thiamin-binding activity of TBP from sesame seeds is pH 6–8. The thiamin-binding activity of WGTBP was not inhibited by thiamin derivatives and analogs (Table III) as was the case for TBP from rice, buckwheat, and sunflower seeds. WGTBP bound slightly to 2-northiamin (Table IV), which is similar to rice seed TBP (Nishimura et al 1984).

The structural and biochemical properties of WGTBP are similar to those of rice seed TBP (Nishimura et al 1984, Shimizu et al 1996), except for the ratio of binding TBP and thiamin. It has been reported that WGTBP has immunological homology with rice germ TBP (Shimizu et al 1996). Also, sharing the immunological homology are TBP from gramineous seeds such as maize, foxtail millet, and sorghum (Shimizu et al 1996). These findings indicate that the properties of WGTBP are similar to that of rice seed TBP, suggesting that the TBP in closely related plant species may have the same evolutionary origin.

CONCLUSIONS

The molecular mass, subunit structure, and amino acid composition of WGTBP were similar to those of rice seed TBP. Also, the

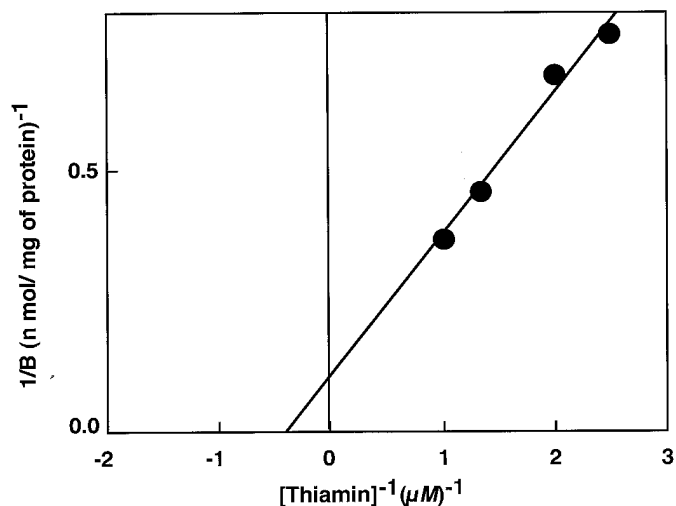


Fig. 7. Effect of thiamin concentration on thiamin-binding activity of thiamin-binding proteins from wheat germ (WGTBP) using values calculated from the Hughes-Klotz plot ($n = 3$).

optimum pH for thiamin-binding and the affinity for thiamin and thiamin-related compounds of WGTBP and rice seed TBP were similar. These findings suggest that the properties of TBP in individual plant species differed, but the properties of the TBP in closely related plant species were similar.

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[Received August 30, 1999. Accepted March 15, 2000.]