HAYATI Journal of Biosciences March 2013
Vol. 20 No. 1, p 1-6
EISSN: 2086-4094

Available online at:
http://journal.ipb.ac.id/index.php/hayati
DOI: 10.4308/hjb.20.1.1

Isolation and Purification of Thiamine Binding Protein from Mung Bean

DWIRINI RETNO GUNARTI, HANIFAH RAHMI*, MOHAMAD SADIKIN

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Indonesia
Jalan Salemba Raya No.6, Jakarta 10430, Indonesia

Received April 16, 2012/Accepted February 12, 2013

Thiamine has fundamental role in energy metabolism. The organs mostly sensitive to the lack of thiamine levels in the body are the nervous system and the heart. Thiamine deficiency causes symptoms of polyneuritis and cardiovascular diseases. Because of its importance in the metabolism of carbohydrates, we need to measure the levels of thiamine in the body fluids by using an easy and inexpensive way without compromising the sensitivity and selectivity. An option to it is thiamine measurement based on the principle of which is analogous to ELISA, in which a thiamine binding protein (TBP) act by replacing antibodies. The presence of TBP in several seeds have been reported by previous researchers, but the presence of TBP in mung beans has not been studied. This study was aimed to isolate and purify TBP from mung bean. The protein was isolated from mung bean through salting out by ammonium sulphate of 40, 70, and 90% (w/v). TBP has a negative charge as shown by cellulose acetate electrophoresis. The result obtained after salting out by ammonium sulphate was further purified bymeans of DEAE-cellulose chromatography and affinity chromatography. In precipitation of 90% of salting out method, one peak protein was obtained by using affinity chromatography. The protein was analyzed by SDS PAGE electrophoresis. The result of SDS PAGE electrophoresis showed that TBP has a molecular weight of 72.63 kDa.

Key words: chromatography, electrophoresis, mung bean, thiamine, thiamine binding protein (TBP)

INTRODUCTION

Thiamine is an important water-soluble vitamin which takes part in glycometabolism in the body. It has been used for the prevention and treatment of beriberi, neuralgia, Wernicke's encephalopathy, and Korsakoff's syndrome (Carpenter 2000; Eitenmiller *et al.* 2008). Thiamine deficiency is frequently seen in alcoholics because heavy drinking limits the ability of the body to absorb this vitamin from foods (Narouzi *et al.* 2010). Dietary sources considered to be primary sources to the human include fortified breakfast cereals, legumes, nuts, and meat. Recommended Dietary Allowances (RDA) as set by the Institute of Medicine in the Dietary Reference Intake (DRI) for thiamine are 1.2 mg/day for adult men and 1.1 mg/day for women (Eitenmiller *et al.* 2008). Clinically, thiamine status is indicated by measurement of urinary excretion of thiamine and erythrocyte transketolase activity. Therefore, the determination of thiamine is one of the important contents in food and clinical analysis.

Several methods have been already reported for the quantitative determination of thiamine in food and clinical analysis, including spectrophotometry (Chen & Tian 2010), spectrofluorimetry (Moore1 & Dolan 2003), chemiluminescence (Du *et al.* 2002), high-performance liquid chromatography (Anyakora *et al.* 2008), and electrochemical method (Eitenmiller *et al.* 2008). In all these methods, the most common methods used are high performance liquid chromatography, spectrofluorimetry, and spectrophotometry.

The high performance liquid chromatography method used to determine thiamine has high sensitivity, good selectivity, and the ability of simultaneous multicomponent determination. All of these methods require special equipment and high costs for the measurement of thiamine.

Therefore in this research will develop a measurement technique that utilizes thiamine binding protein (TBP) from mung bean base on principle analogous to the ELISA (Enzyme Linked Immunosorbent Assay) in which a thiamine binding protein act replace antibodies. ELISA is a popular serological test which is used as a diagnostic tool in medicine to detect the presence of a substance. ELISA technique is based on the recognation of antibody specifically against an antigen. This technique does not require expensive equipment, just by using a spectrophotometer, do not use radioactive materials, and require a relatively short time.

*Corresponding author. Phone: +62-21-3910734,
Fax: +62-21-3910190, E-mail: hanifahrahmi986@yahoo.com

Thiamin-binding proteins (TBP) are widely distributed in plants and accumulated in seeds (Watanabe *et al*. 2004). TBP have been isolated from *E. coli* (Iwashima *et al.* 1971), egg yolk (Muniyappa & Adiga 1981), egg white (Muniyappa & Adiga 1979), rice (Shimizu *et al.* 1996), buckwheat (Kozik 1995), sesame (Watanabe *et al.* 2003), wheat (Adachi *et al*. 2000), maize (Adachi *et al*. 2001), and sunflower seeds (Watanabe *et al*. 2002). 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.

Mung been is widely known as one source of vitamin B1 (Escott & Stump 2008). Mung beans are popular beans in Indonesia, therefore the mung bean is easily obtained and the price is relatively inexpensive. This raises the question of whether mung bean contain proteins that capable of binding thiamine specifically (TBP). If this is true, then the mung bean can be used as an alternative source of TBP that can be developed for measuring thiamine levels with engineering *enzyme-labeled protein ligan binding specific assay* which is analogous to the ELISA assay.

MATERIALS AND METHODS

Mung bean (*Phaseolus radiatus*) were purchased from department store. We used organic mung beans from local farmers and already in sterile packaging for trafficked. Ammonium sulphate, KH_2PO_4, Na_2HPO_4, NaCl, acrylamide, N,N'-bisacryl, SDS, ammonium persulphate, TEMED, Tris-HCl, BSA, blue brilliant Coomassie, thiamin hydrochloride was purchased from Merck (IND), DEAE-Cellulose resin from Sigma (USA), NHS- Activated Separopore® 4B-CL resins from Bioworld (USA), Dialysis tubing cellulose membrane from Sigma-Aldrich (USA), Titan gel from Helena laboratories (Texas), and molecular weight protein marker from Biorad. All other chemicals were of analytical grade. Protein was assayed according to Bradford with bovine serum albumin as a standard.

Protein Extraction. Mung bean mashed with blender until smooth and then filtered with a size of 150 R (mung bean powder). To 10 g of mung bean powder was added 100 mL of 50 mM potassium phosphate buffer (pH 7, containing 1% NaCl) mixed well using stirer slowly for 24 hours at 4 °C. All operations were conducted at 4 °C. Homogenates were centrifuged at 28,000 x g for 15 minutes. Ammonium sulphate was added to the supernatant to 40, 70, and 90% saturation (Rosenberg 2005). The suspension was left for 1 h and centrifuged at 28,000 x g for 15 minutes. The resulting precipitate was dialyzed against 50 mM potassium phosphate buffer (pH 7, containing 1% NaCl). All the fractions isolated by ammonium sulphate incorporated into dialysis membrane (Rosenberg 2005). Fraction that will dialysis put into membrane and dialysis using 0.01 M potassium phosphate buffer (pH 7). Dialysis process is considered complete if the sulfate concentration in dialysis buffer is zero.

Protein Assay. Protein was assayed following the method of Warburg-Christian (λ 280 nm) and Bradford with bovine serum albumin as a standard (Rosenberg 2005).

Cellulose Acetate Electrophoresis. A set of electrophoresis tools prepared. Helena buffer was poured into positive and negative chamber until the filter paper submerged. Titan (III) cellulose gel immersed in a container that contains a buffer Helena for 20 minutes. Gel that has been soaked, then dried with filter paper. Each wells completed of 7 µL the sample solution of proteins P40, P70, P90, and filtrate. The samples applied as quickly as possible into the gel. After the last sample has been applied waited for 4 minutes to allows the sample to diffuse into the gel. The gel placed into the inner section of the chamber, cellulose side down, by gently squeezing the gel into place. Position the gel so that the edges of the cellulose are in the buffer and the application point is on the cathodic (-) side. The cover placed on the chamber and insure that the cover does not touch the gel. Electrophorese the gel at 180 volts for 15 minutes.

DEAE-Cellulose Chromatography. To 18 g of resin was swelled in 65 mL distilled water for 45 minutes in room temperature. At the end of this periode, the supernatant was decanted and the resin was treated extensively according to procedure described by Sigma. Briefly, the resin was washed repeatedly with great volume various solution containing salt, NaOH and HCl. Before using, the difference of pH between the resin and the solution should not exceed 0.15. The resin was poured carefully into a 30 x 1.5 cm column. Then, 50 mM potassium phosphate buffer (pH 7) was added onto the gel until the flow was stable ($A_{280} = 0$). Two mL filtrate from the dialysis bag were placed onto the gel. The bound protein were eluted firstly with phosphate buffer (pH 7). Fractions of 2 mL were collected and A_{280} was read (Ahmed 2005).

If the $A_{280} = 0$, the 2nd elution was made by adding potassium phosphate buffer (pH 7) containing 10 mM NaCl. The fraction collection continued until A_{280} gave a constant value. The elution continued

progressively with potassium phosphate buffer (pH 7) containing 15, 20, 30, and 40 mM NaCl. Fractions forming peak were pooled for the further analysis.

Affinity Chromatography. Thiamine is immobilized on to NHS- Activated Separopore® 4B-CL. For 20 mL NHS-Activated Separopore medium, 10% (w/v) thiamin hydrochloride was added drop by drop on to stirred medium suspension. Then the free sites of medium were blocked by addition ethanolamine. After free washed with large of 0.1 M Tris buffer (pH 8), then 2 mL of peak fraction in DEAE-cellulose column was put on NHS- Activated Separopore® 4B-CL column and eluted with 0.1 M Tris buffer (pH 8). The active fraction of 1 mL were collected and A_{280} of each fraction was measured when the absorbance was 0, the column washed again with the same buffer. After the washing, buffer containing 1 M NaCl was added onto column and the fraction of 1 mL were collected (Dennison 2002). A_{280} was measured and each fraction was pooled.

Crude protein, precipitate of 90% ammonium sulphate, second peak of DEAE-cellulose column at 90% saturation, and second peak of NHS- Activated Separopore® 4B-CL column at 90% saturation was runned in SDS-PAGE 10% together with molecular weight marker protein and BSA. The gel was coloured by Coomasie brilliant blue solution. The molecular weight of TBP was determined by interception of each Rf with a curve of made from molecular weight marker protein.

RESULTS

Three saturation of 40, 70, dan 90% obtained from salting out by ammonium sulphate. Each saturation of 40, 70, dan 90% dialyzed to remove ammonium sulphate. Protein was measured at at a wavelength of 280 nm with bovine serum albumin as a standard (Table 1).

Cellulose Acetate Electrophoresis. Saturation of 40, 70, 90%, and filtrat was analyzed to determine the protein charge of each sample. Protein band in 90% saturation was thicker than other protein band and has negative charge (Figure 1).

Precipitate with 40, 70, and 90% saturation of ammonium sulphate, followed by DEAE-cellulose

Table 1. Total protein concentration after dialysis

Sample protein	Protein concentration (mg/mL)
40% saturation	9.60
70% saturation	5.10
90% saturation	2.50

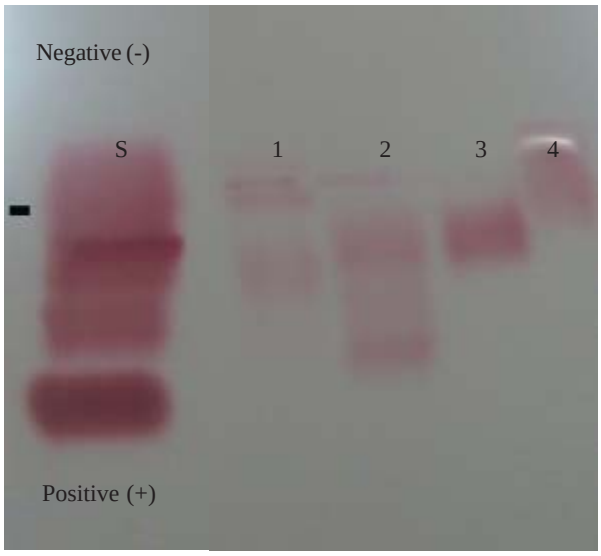

Figure 1. Cellulose acetate electrophoresis of protein from mung bean. S: Serum, 1: Presipitat 40%, 2: Presipitat 70%, 3: Presipitat 90%, and 4: Filtrat.

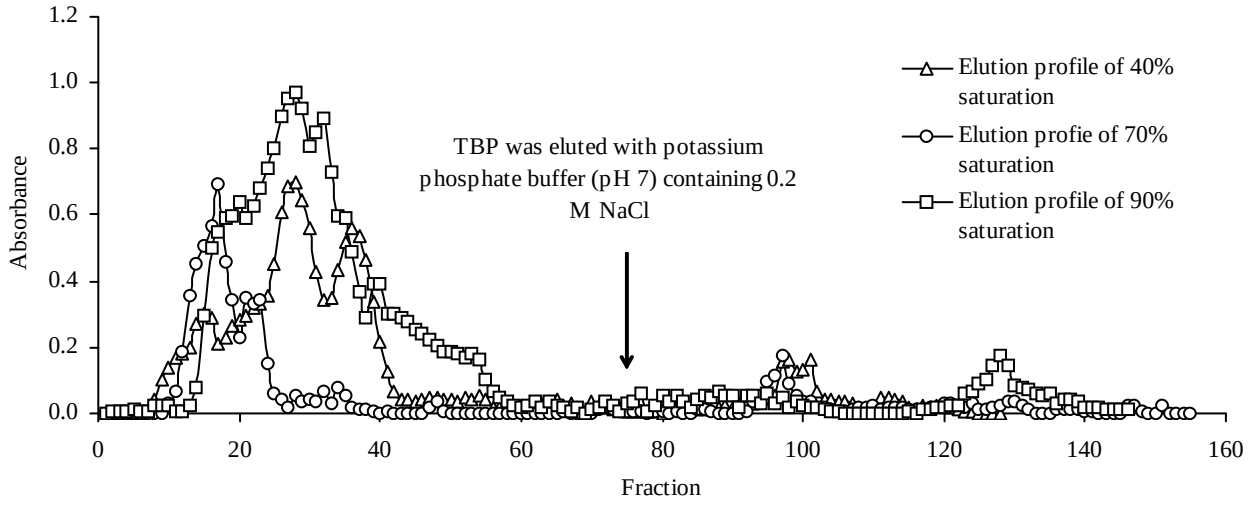

Figure 2. Elution profile from a DEAE-cellulose column.

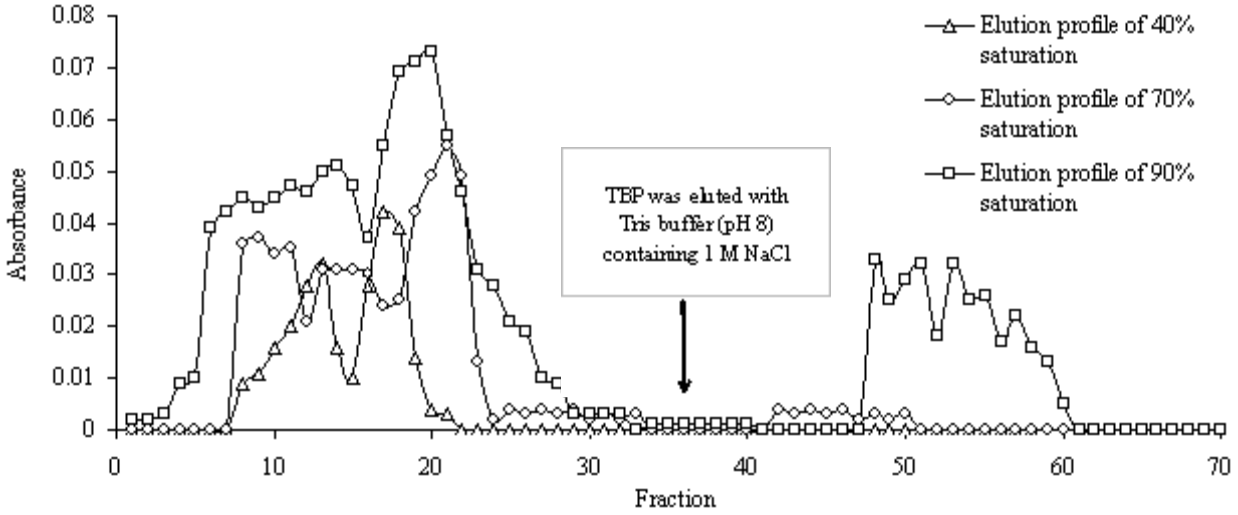

Figure 3. Elution profile results of affinity chromatography.

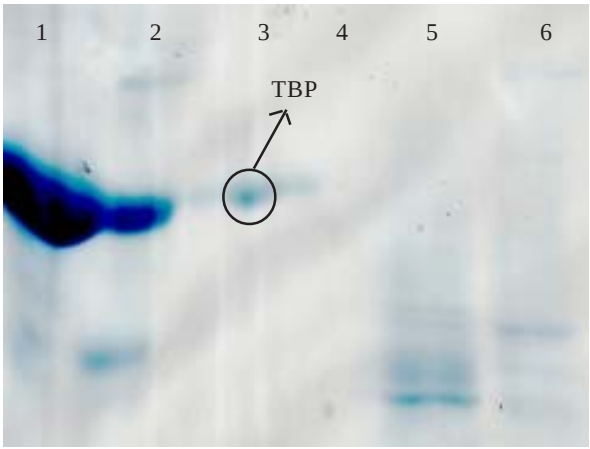

Figure 4. SDS-PAGE of TBP from mung bean. Well 1: Bovine serum albimine, 2: marker protein, 3: peak protein of 90% saturation by affinity column, 4: peak protein of 90% saturation by DEAE-Cellulose column, 5: 90% saturation by ammonium sulphate, 6: crude protein of mung bean.

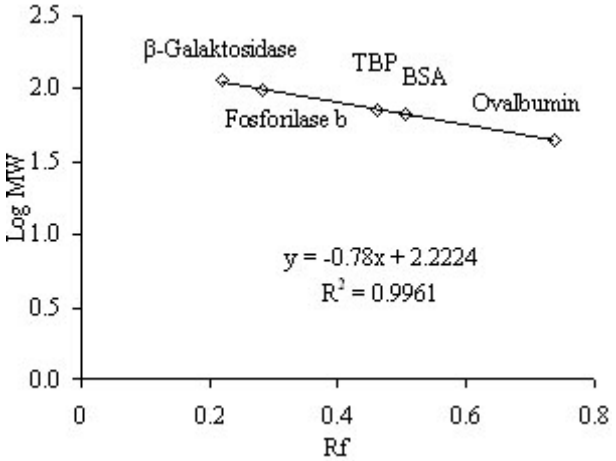

Figure 5. Molecular weight (MW) estimation of TBP from mung bean by SDS PAGE.

chromatography. To obtain the TBP in pure state, we proceeded an affinity chromatography method.

DEAE-Cellulose Chromatography. Thiamin is positively charge in neutral pH, therefore the TBP should have a relatively negative charge. Two peak protein was obtained using DEAE-cellulose column chromatography (Figure 2). In the DEAE-cellulose the first peak eluted from the column usually have positive charge and TBP with negative charge will eluted by potassium phosphate buffer containing 0.2 M NaCl. Because that, we purified second peak fatherly by passing them in affinity chromatography column. Each second peak of DEAE-cellulose was passed in the affinity column in separate experiment.

Affinity Chromatography. Two peak protein obtained by affinity chromatography of 90% saturation (Figure 3). The first peak from affinity column has protein which unbound thiamin. Whereas the second peak from affinity column has only the pure TBP. On the other hand, only one peak protein unbound thamin by affinity chromatography of 40 and 70% saturation (Figure 3). The purity of obtaining TBP was showed by single spot in SDS-PAGE analysis (Figure 4). From this technique, we could estimate the molecular of TBP. Molecular weight TBP can be calculated by entering the Rf value of 0.46 into the standard curve equation (Figure 5). The results of the calculation of molecular weight proteins bind thiamine obtained for 72.63 kDa. TBP obtained by concentration of 34 µg/mL (Table 2).

DISCUSSION

Thiamin is necessary for human and animal nutrition. TBP may enhance the nutritive value in plant seeds. TBP have additional functions such as

Table 2. Purification of thiamine binding protein from mung bean at 90% saturation

Purification step	Total protein (µg/mL)
Extract	70103
Ammonium sulfate	6845
Dialysis	2500
DEAE-cellulose	67
NHS-Activated Separopore® 4B-CL	34

retention of thiamin in dormant seeds and provision of a nitrogen source at germination (Watanabe *et al*. 2004). In this research to isolate TBP from mung bean, respectively, using ammonium sulfate salts with a saturation of 40, 70, and 90%, followed by purification by DEAE-cellulose ion exchange chromatography and affinity chromatography.

The early stages of purification was centrifugation. Pellet was dissolved with 0.05 M potassium phosphate pH 7. Protein deposition is done by addition of ammonium sulfate at different concentrations. Concentration of protein obtained at this stage can be seen in Table 1. Dialysis is carried out on the next step to eliminate the salt ammonium sulphate from each presipitate. The TBP has negative charge, this is evidenced by doing an analysis of cellulose acetate electrophoresis. These results are supported by Adachi *et al*. (2001) research that TBP from maize seed had negative charge.

Purification continued by ion exchange chromatography method using resin DEAE celullose. In this process there will be competition between proteins with Cl^- that have negative charge equally bound to matrix DEAE celullose that have positive charge (Bonner 2007). Every precipitate from this stage showed 2 peak protein, the first peak is positively charged protein and the second peak is composed of negatively charged proteins (Figure 2). Protein levels for 90% saturation at each purification steps are shown in Table 2.

To obtained pure TBP, each peak of negative protein from DEAE cellulose chromatography was separated by affinity chromatography techniques. NHS-Activated Separopore@4B-CL used as resin affinity chromatography. Thiamin hydrochloride used as ligand which binding TBP on affinity column, although TBP from plant seed bind only free thiamin not thiamin phosphates (Watanabe *et al*. 2003). Thiamin which has positive charge would bound with TBP of mung bean. TBP was eluted with 1 M NaCl solution. At this step, TBP obtained by concentration of 34 µg/mL (Tabel 2).

The level of purification is analyzed by SDS-PAGE electrophoresis technique. The molecular mass of TBP from mung bean was estimated to be 72.63 kDa using SDS PAGE (Figure 4, 5). The TBP from rice germ, sesame seeds, buckwheat seeds, maize seed, wheat, and sunflower seeds differ in their structural, biochemical, and immunological properties. The TBP from rice germ has a molecular mass of 50 kDa on the SDS-PAGE (Shimizu *et al*. 1996). The TBP from wheat germ has a molecular mass of 56 kDa (Watanabe *et al*. 2003). The TBP from maize seed has a molecular mass of 96 kDa (Adachi *et al*. 2000). The TBP from sunflower seed has a molecular mass of 46 kDa (Watanabe *et al*. 2002) and TBP from sesame seed has has a molecular mass of 17 and 19 kDa (Watanabe *et al*. 2003).

ACKNOWLEDGEMENT

This research is part of the project "Utilization thiamine binding protein (TBP) from bekatul for measuring vitamin B_1 (thiamine)", supported from DRPM UI which has funded this research through Riset Awal Grant 2010.

REFERENCES

Adachi T, Watanabe K, Mitsunaga T. 2000. Characterization of thiamin-binding protein from wheat germ. *Cereal Chem* 77:578-581. http://dx.doi.org/10.1094/CCHEM.2000.77.5.578

Adachi T, Watanabe K, Mitsunaga T. 2001. Characterization of thiamin-binding protein from maize seeds. *Plant Physiol Biochem* 39:99-105. http://dx.doi.org/10.1016/S0981-9428(00)01220-1

Ahmed H. 2005. *Principles and Reaction of Protein Extraction, Purification, and Characterization*. London: CRC Pr.

Anyakora C, Afolami I, Ehianet T, Onwumere F. 2008. HPLC analysis of nicotinamide, pyridoxine, riboflavin and thiamin in some selected food products in Nigeria. *Afr J Pharm Pharmacol* 2:029-036.

Bonner PLR. 2007. *Protein Purification*. New York: Taylor & Francis Group.

Carpenter KJ. 2000. *Beriberi, white rice, and vitamin B: a disease, a cause, and a cure*. London: University of California Pr.

Chen Y, Tian F. 2010. Enzymatic catalytic spectrophotometric determination of thiamine in food. *Food Anal Methods* 3:7-11. http://dx.doi.org/10.1007/s12161-008-9070-z

Dennison C. 2002. *A Guide to Protein Isolation*. New York: Kluwer Acad.

Du J, Li Y, Lu J. 2002. Flow injection chemiluminescence determination of thiamine based on its enhancing effect on the luminol-hydrogen peroxide system. *Talanta* 57:661-665. http://dx.doi.org/10.1016/S0039-9140(02)00079-6

Eitenmiller RR, Ye L, Landen WO. 2008. *Vitamin analysis for the health and food sciences*. 2nd ed. New York: CRC Pr.

Escott S, Stump. 2008. *Nutrition and diagnosis-related care*. 6th ed. USA: Lippincott Williams and Wilkins.

Iwashima A, Matsuura A, Nose Y. 1971. Thiamine-binding protein of *Escherichia coli*. *J Bacteriol* 108:1419-1421.

Kozik A. 1995. Thiamin-binding protein from buckwheat seeds: some molecular properties, ligand-protein interaction, bioanalytical applications. *Buckwheat Research* 118:823-831.

Moore1 JC, Dolan KD. 2003. Optimization of oxidation steps used in fluorometric determination of thiamin in soft wheat flour. *Cereal Chem* 80:238-240. http://dx.doi.org/10.1094/CCHEM.2003.80.2.238

Muniyappa K, Adiga PR. 1979. Isolation and characterization of thiamin-binding protein from chicken egg white. *Biochem J* 177:887-894.

Muniyappa K, Adiga PR. 1981. Nature of the thiamin-binding protein from chicken egg yolk. *Biochem J* 193:679-685.

Narouzi P, Garakani TM, Rashedi H, Zamani HA, Ganjali MR1. 2010. Ultrasensitive flow-injection electrochemical method using fast fourier transform square-wave voltammetry for detection of vitamin B1. *Int J Electrochem Sci* 5:639-652.

Rosenberg IM. 2005. *Protein Analysis and Purification*. Boston: BirkhÓuser.

Shimizu M, Yoshida T, Toda T, Iwashima A, Mitsunaga T. 1996. Isolation of a thiamin-binding protein from rice germ and distribution of similar proteins. *Biosci Biotech Biochem* 60:453-457. http://dx.doi.org/10.1271/bbb.60.453

Watanabe K, Konishi A, Mitsunaga T. 2002. Molecular characteristics of thiamin-binding protein from sunflower seeds. *Plant Physiol Biochem* 40:417-421. http://dx.doi.org/10.1016/S0981-9428(02)01387-6

Watanabe K, Nishida N, Adachi T, Ueda M, Mitsunaga T, Kawamura Y. 2004. Accumulation and degradation of thiamin-binding protein and level of thiamin in wheat seeds during seed maturation and germination. *Bisci Biotech Biochem* 68:1243-1248. http://dx.doi.org/10.1271/bbb.68.1243

Watanabe K, Takahashi H, Ampo M, Mitsunaga T. 2003. Change of thiamin-binding protein and thiamin levels during seed maturation and germination in sesame. *Plant Physiol Biochem* 41:973-976. http://dx.doi.org/10.1016/j.plaphy.2003.07.005