

Properties of A Thiamine Binding Protein Purified from Mung Bean

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ABSTRACT

Thiamine (vitamin B1) was the first B vitamin which has been identified. It serves as a cofactor for several enzymes involved in energy metabolism. The laboratory test against thiamine deficiency can be done by measuring thiamine levels in the blood. The aim of this study was to identify the stability and the binding activity characters of TBP. The equilibrium dialysis technique was used to see the factors affecting the bond between TBP and thiamine. The MBTBP concentration of post-chromatographic affinity resulted from dilution of lyophilisate was stable for 30 days at -20°C and 3 days at 4°C. The optimal pH for binding MBTBP to thiamine was 7.5. Alkylation with iodoacetic acid decreased the binding capacity of TBP which suggested the presence of a-SH or imidazol group in its active site. The importance of disulfide bridge was proven by decreasing of Thiamine binding capacity of TBP after β-mercaptoethanol treatment. This binding activity was also affected by oxidizing agents, but it was less affected by calcium ions and heavy metals.

Key words: Thiamine; Mung bean thiamine binding protein (MBTBP); Binding capacity.

INTRODUCTION

Vitamin B1 is also known as thiamine. The chemical name for this water soluble vitamin is 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium. Thiamine consists of a pyrimidine ring and a thiazole ring, which are coupled by a methylene bridge.¹

Thiamine is an essential molecule for all life forms. It serves as a cofactor for several enzymes involved in energy metabolism. These enzymes include the pyruvate dehydrogenase, α-ketoglutarat dehydrogenase, and the transketolase. In the nervous system thiamine is needed for the stimulation of nerve cells, including g-amino butyric acid (GABA), glutamate, and aspartate.^{2,3}

The majority of thiamine in serum is bound to specific proteins. A specific binding protein called thiamine-binding protein (TBP).⁴ Gunarti⁵ showed isolation, purification, characterization of TBP derived from mung beans, which suggested the ability of TBP to measure serum thiamine levels base on principle analogous to enzyme linked immunosorbent assay (ELISA). In this study, MBTBP isolated and obtained from the fraction of affinity chromatography results were 1,446 mg/mL and its molecular weight was 103,83 kDa. The results of bioinformatics analysis and equilibrium dialysis showed every molecule of MBTBP can bind four molecules of thiamine.⁵ The results of the experiment showed that MBTBP can be used for measurement of thiamine in biological fluids using ELPLBA method. However, the detailed characters of the TBP are not all described yet.

Some of TBPs physicochemical properties should be well known before used of as a reactant in

a specific assay. The aim of this research was to identify physicochemical characters of TBP, such as the influence of temperature and storage time on MBTBP stability, effect of pH to its thiamine binding capacity, the need of ionic calcium, the role of disulfide bridge and also the effect of alkylating agent, oxidation and heavy metals on bonds between MBTBP and thiamine.

MATERIALS AND METHODS

Materials

Mung Bean Thiamine Binding Protein (MBTBP) used was the result of post-affinity chromatography with levels of 1446 µg/mL that had been identified and analyzed. KH₂PO₄, Na₂HPO₄, NaCl, NaOH, BSA, sodium EDTA, iodoacetate, hydrogen peroxide, β-mercaptoethanol, HgCl₂, AgNO₃, thiamine, buffer sulphate, dialysis tubing cellulose membrane was purchased from Sigma-Aldrich.

Methods

Protein assay

Concentration of protein was determined by the method of Warburg-Christian using bovine serum albumin as the standard.⁵

Effect of temperature and storage time on MBTBP stability

MBTBP powder was known to be concentrated and diluted to a concentration of 250 µg/mL in aliquoting and stored at -20°C, 4°C and 37°C with storage time of 3 days, 7 days, 14 days and 30 days.^{6,7} Furthermore, protein levels were measured using a standard BSA curve.

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Effect of pH

The effect of pH, was determined by equilibrium dialysis and pH range used was 6.0-9.0. The binding capacity of MBTBP was determined by equilibrium dialysis. At the end of this period, the thiamine concentration in dialysate was measured by spectrophotometer at the λ 280 nm. A control was used in this experiment, on to 1 mL of distilled water was added 1 ml of 50 $\mu\text{g/mL}$ thiamine. The mixtures were dialyzed against 50 ml phosphate buffer pH 7.2 at room temperature (25°C) for 2 hours. Role of pH, on to 1 ml of 250 $\mu\text{g/mL}$ TBP was added 1 ml of 50 $\mu\text{g/mL}$ thiamine and 1 ml of phosphate buffer pH 6.0. The experiment was repeated using the same amount of TBP, thiamine and phosphate buffer but with the other pH, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0. All the seven mixtures were placed in dialysis bag and dialyzed at room temperature for 2 hours against 50 ml phosphate buffer with the other pH of 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0.⁸

Effect calcium ion, alkylation, reduction, oxidation, and heavy metals agents on bonds between MBTBP and thiamine

Two controls were used in this experiment. The first was 1 mL solution of 20 $\mu\text{g/mL}$ thiamine in 1 mL of distilled water, the other was 1 ml solution of 20 $\mu\text{g/mL}$ thiamine in 1 mL of 144,6 $\mu\text{g/mL}$ MBTBP. At the end of this period, the thiamine concentration in dialysate was measured by spectrophotometer at the λ 280 nm.⁹

Role of calcium

The role of calcium ion was performed using sodium EDTA. The binding capacity of MBTBP was determined by equilibrium dialysis. On to 1 ml of 144,6 $\mu\text{g/mL}$ MBTBP was added 0.74 mg of sodium EDTA, followed by 1 ml of 20 $\mu\text{g/mL}$ thiamin. The mixtures were dialyzed against 50 ml phosphate buffer pH 7.5 at room temperature (25°C) for 2 hours.⁹

Role of-SH groups

The role of -SH groups was performed using iodoacetic acid as alkylation compound. The binding capacity of MBTBP was determined by equilibrium dialysis too. On to 1 ml of 144,6 $\mu\text{g/mL}$ MBTBP was added 0,37 mg iodoacetic acid, followed by 1 ml of 20 $\mu\text{g/mL}$ thiamin. The mixtures were dialyzed against 50 ml phosphate buffer pH 7.5 at room temperature (25°C) for 2 hours.

Role of disulfide bridge

The role of calcium ion was performed using β -merkaptoetanol. The binding capacity of MBTBP was determined by equilibrium dialysis. On to 1 ml of 144,6 $\mu\text{g/mL}$ MBTBP was added 50 μL β -merkaptoetanol, followed by 1 ml of 20 $\mu\text{g/mL}$ thiamin. The mixtures were dialyzed against 50 ml phosphate buffer pH 7.5 at room temperature (25°C) for 2 hours.⁹

Role of oxidation agent

The role of oxidation agent was performed using hydrogen peroxyde (H_2O_2). The binding capacity of MBTBP was determined by equilibrium dialysis. On to 1 ml of 144,6 $\mu\text{g/mL}$ MBTBP was added 0,067 mg H_2O_2 , followed by 1 ml of 20 $\mu\text{g/mL}$ thiamin. The mixtures were dialyzed against 50 ml phosphate buffer pH 7.5 at room temperature (25°C) for 2 hours.⁹

Role of heavy metalsagents

The role of heavy metals was performed using HgCl_2 and AgNO_3 . The binding capacity of MBTBP was determined by equilibrium dialysis. On to 1 ml of 144,6 $\mu\text{g/mL}$ MBTBP was added 0,54 mg HgCl_2 , onto another of 1 ml 144,6 $\mu\text{g/mL}$ MBTBP was added 0,34 mg AgNO_3 ,

followed by 1 ml of 20 $\mu\text{g/mL}$ thiamin. The mixtures were dialyzed against 50 ml phosphate buffer pH 7.5 at room temperature (25°C) for 2 hours.

RESULT

Determination of MBTBP Concentration

Protein was measured at a wavelength of 280 nm with BSA as standard. The soluble MBTBP concentration was 1,115 mg/mL .

Effect of temperature and storage time on MBTBP stability

The MBTBP concentration was stable for 30 days at -20°C and 3 days at 4°C. The dissolved solution of MBTBP was unstable at 30°C (Figure 1).

Effect of pH

The binding activity between MBTBP and thiamine is influenced by pH. As shown in Figure 2, the binding activity of MBTBP at various pH value indicated that the optimum pH is 7.5.

Effect calcium ion, alkylation, reduction, oxidation, and heavy metals agents on bonds between MBTBP and thiamine

The addition of EDTA (Ca^{2+} chelating agent), were less affect the binding between TBP and thiamine (Figure 3).

However, the addition of iodoacetate (alkylating agent), β -mercaptoethanol (reduction agent) and H_2O_2 (oxydating agent), decreased the binding capacity of TBP to thiamine (Figures 4-6).

The addition of HgCl_2 and AgNO_3 were less affect the binding between TBP and thiamine (Figures 7 and 8).

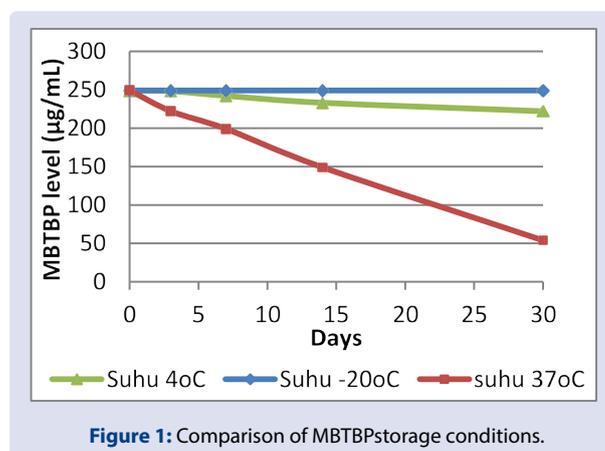


Figure 1: Comparison of MBTBP storage conditions.

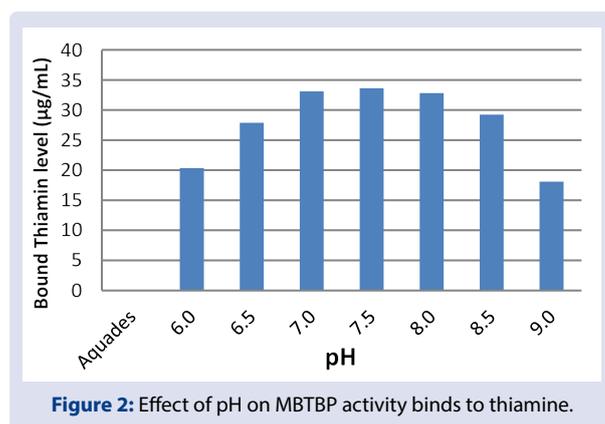


Figure 2: Effect of pH on MBTBP activity binds to thiamine.

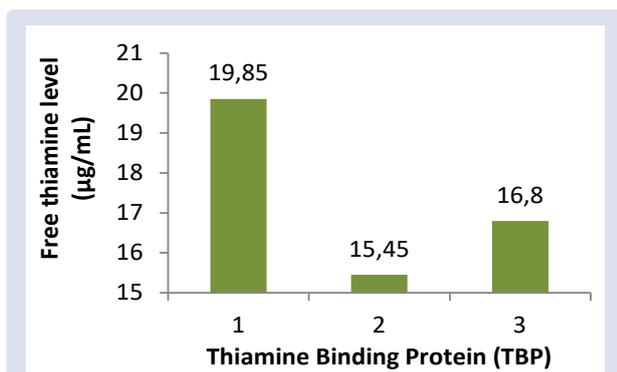


Figure 3: Free thiamine level after binding of TBP with EDTA, 1. Distilled water control, 2. TBP control, 3. TBP with EDTA.

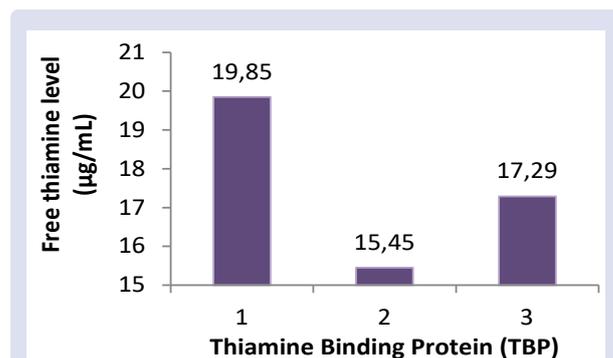


Figure 6: Free thiamine level after binding of TBP with hydrogen peroxide (H_2O_2), 1. Distilled water control, 2. TBP control, 3. TBP with hydrogen peroxide (H_2O_2).

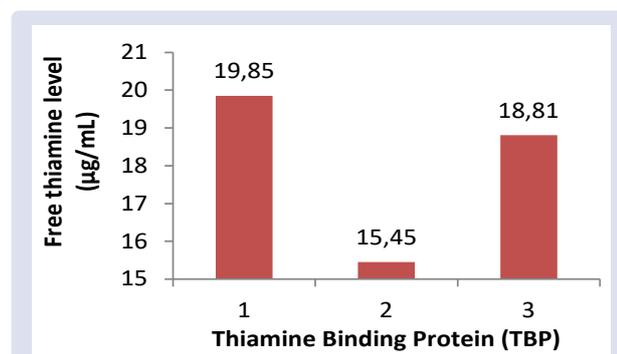


Figure 4: Free thiamine level after binding of TBP with iodoacetate, 1. Distilled water control, 2. TBP control, 3. TBP with iodoacetate.

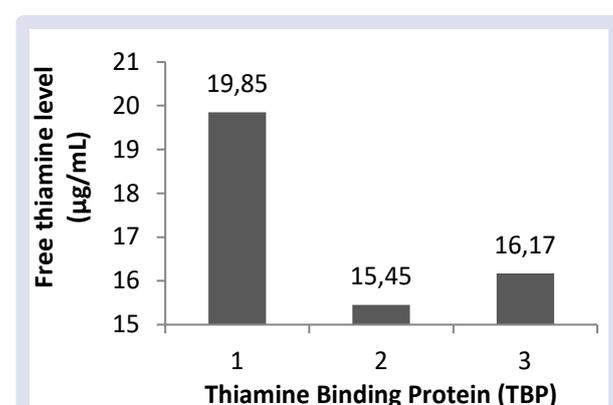


Figure 7: Free thiamine level after binding of TBP with $HgCl_2$, 1. Distilled water control, 2. TBP control, 3. TBP with $HgCl_2$.

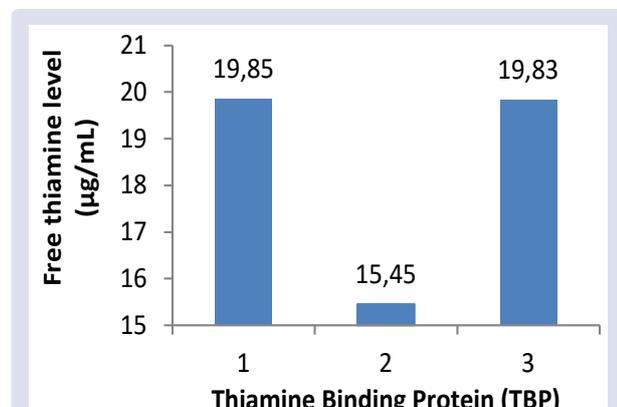


Figure 5: Free thiamine level after binding of TBP with β -mercaptoethanol, 1. Distilled water control, 2. TBP control, 3. TBP with β -mercaptoethanol.

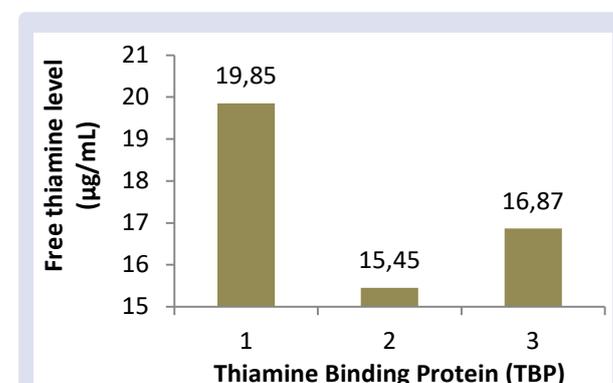


Figure 8: Free thiamine level after binding of TBP with $AgNO_3$, 1. Distilled water control, 2. TBP control, 3. TBP with $AgNO_3$.

DISCUSSION

Purified proteins often need to be stored for an extended period of time while retaining their original structural integrity and/or activity.¹⁰ From the previous study, protein levels after purification were 1,446 mg/mL. The extent of storage 'shelf life' can vary from a few days to more than a year and is dependent on the nature of the protein and the storage conditions used.^{7,10}

The protein concentration from dilution of lyophilisate was 1,015 mg/mL. Protein was assayed following the method of Warburg-Christian (λ 280 nm) with bovine serum albumin as a standard.⁵ The lyophilization allows for long-term storage of protein with very little threat of

degradation, but the protein must be reconstituted before use and may be damaged by the lyophilization process.⁶

Optimal conditions for storage are distinctive to each protein.¹⁰ The TBP of lyophilisate dilution when stored at 4°C was stable up to day three and thereafter a slower concentration decrease occurred than when stored at 30°C. Generally, proteins are best stored at $\leq 4^\circ C$. such proteins may not be stable for more than a few days or weeks. Storage at room temperature often leads to protein degradation and/or inactivity, commonly as a result of microbial growth, for short term storage (1 day to a few weeks).^{7,10} The TBP of lyophilisate dilution results was more stable when stored at -20°C. Frozen at -20°C or -80°C is the more common form of cold protein storage. Because freeze-thaw cycles

decrease protein stability, samples for frozen storage are best dispensed and prepared in single-use aliquots so that, once thawed, the protein solution will not have to be refrozen.

Like the TBPs of sesame seeds¹¹, the mung bean TBP exhibits the maximal thiamine-binding activity at pH 7,5. In contrast, the thiamine-binding by TBPs from buck wheat⁸, sun flower¹¹, wheat germ¹², Maize seeds¹³ and rice¹⁴ are optimal at pH 8.0-9.0.

Measurement of binding capacity MBTBP against thiamine through equilibrium dialysis showed a value of 1: 4.⁵ To bind thiamine, the TBP apparently need Ca²⁺ ion. This conclusion was obtained from the experiment with EDTA. With EDTA, TBP bound practically the less amount of thiamine, which means the presence of Ca²⁺ ion is essential for the binding.

The experiment with iodoacetic acid showed that binding activity of TBP decrease significantly. Iodoacetic acid is well-known as an alkylating agent. One of the binding sites of MBTBP to thiamine is histidine.⁷ The addition of iodoacetate compound causes alkylation in MBTBP histidine residues. This means that histidine residues play a role in the thiamin binding process by MBTBP. Part of amino acid histidine could be alkylated by iodoacetic acid.^{15,16}

β-Mercaptoethanol is known for reducing disulfide bridge in any protein. This research found that addition of this reducing agent decreased significantly the binding activity of TBP (almost 100% as compared to distilled water control). It has been reported that TBP has several disulfide bridges. The experiment showed clearly that the maintaining of the disulfide bridge for the binding capacity of TBP is very important. The Subandrate research (2011) found that addition of this reducing agent decreased significantly the binding activity of TBP.

Hydrogen peroxide (H₂O₂) is a strong oxidizing compound. This oxidizing compound can cause the oxidation of some amino acid residues and protein polymerization.¹⁷ The test results showed unbound thiamine MBTBP of 41% when compared to control. Amino acid residues that are susceptible to oxidation reactions are histidine and tyrosine.¹⁷ The result of Gunarti's research (2016) is the binding site of MBTBP1 with thiamine i.e., threonine, tyrosine, arginine, histidine and MBTBP2 with thiamine i.e., tyrosine, arginine and glutamine.

Heavy metals like Hg²⁺, Ag⁺, may also disrupt disulfide bonds because of their high affinity and attraction for sulfur and will also lead to the denaturation of proteins. Since salts are ionic, they disrupt salt bridges in proteins. The reaction of a heavy metal salt with a protein usually leads to an insoluble metal protein salt.^{17,27}

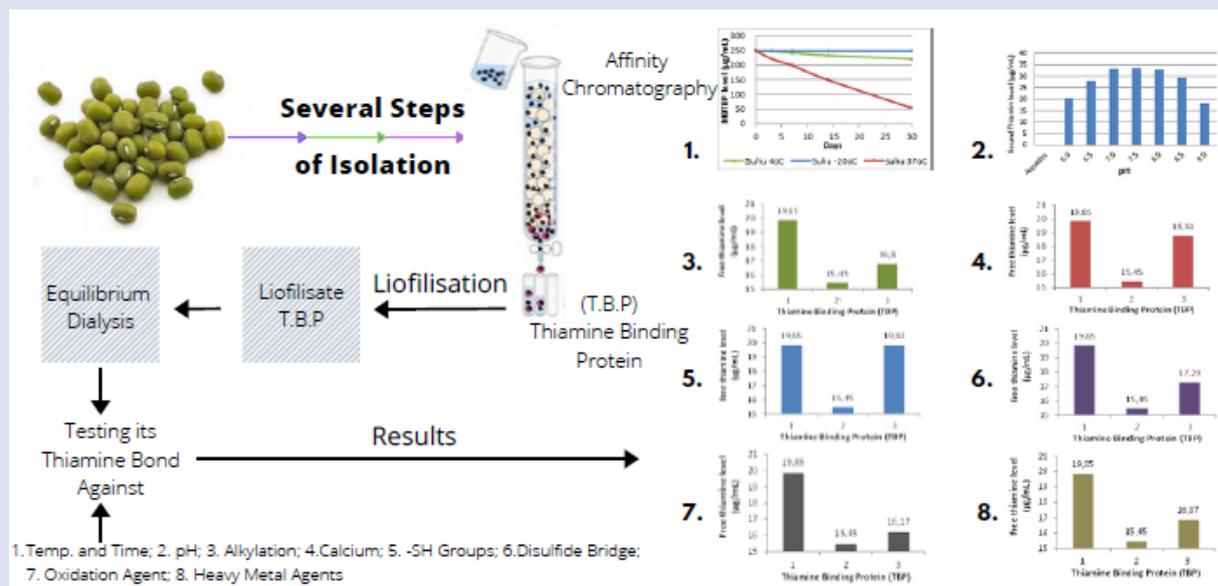
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GRAPHICAL ABSTRACT



SUMMARY

T.B.P which has been isolated from mung beans can be used as a laboratory test for thiamine deficiency. The aim of this research was to identify the stability and the binding activity characters of TBP. The equilibrium dialysis technique was used to see the factors affecting the bond between TBP and thiamine. In this research, temperature, time, alkylation, calcium, -SH groups, disulfide bridge, oxidation, and heavy metal shown to be decreasing the stability of M.B.T.B.P bonds with thiamine.

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