EKSAKTA
Journal of Sciences and Data Analysis

E-ISSN: 2720-9326
P-ISSN: 2716-0459
DOI : 10.20885/EKSAKTA.vol5.iss2.art6

Research Article

Isolation of Thiamine-Binding Protein from Black Glutinous Rice Bran (*Oryza sativa var. Glutinosa*) Using Ammonium Sulfate Precipitation and Equilibrium Dialysis

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Received: 29 June 2024; Accepted: 17 September 2024; Published: 30 October 2024

Abstract: Thiamine is an essential cofactor in energy metabolism. Deficiency of this vitamin can cause disorders in the nervous and cardiovascular systems. Conventional methods for examining thiamine levels in the body tend to be expensive. One solution is to use a technique similar to ELISA (Enzyme-Linked Immunosorbent Assay), utilizing a specific thiamine-binding protein as a substitute for antibodies. This study aims to investigate the presence of thiamine-binding protein in black glutinous rice bran (Oryza sativa var. Glutinosa). The methods used include salting out, dialysis, and equilibrium dialysis. The results show that the thiamine-binding protein (TBP) in black glutinous rice bran precipitated with 90% ammonium sulfate. The average total protein was 5.732 mg/mL after dialysis, and equilibrium dialysis demonstrated that TBP can bind thiamine with a binding capacity ranging from 30 to 76.6 µg per 10 grams of sample.

Keywords: Black Glutinous Rice Bran, Equilibrium Dialysis, Isolation, Thiamine-Binding Protein

Introduction

Thiamine is a vitamin B group that cannot be synthesized by the human body and must be obtained from dietary sources [1]. Thiamine plays a crucial role in carbohydrate metabolism and energy production, primarily as a coenzyme in the oxidative decarboxylation of pyruvate and the citric acid cycle. Thiamine is also essential for normal nerve function as it is involved in neurotransmitter production and myelin maintenance [2]. The requirement for this vitamin varies among individuals, influenced by age, energy needs, carbohydrate intake, and body mass. Based on these considerations, the Food and Nutrition Board of the US Institute of Medicine recommends a thiamine intake of 0.5 mg/1000 kcal [3].

Thiamine deficiency impedes or severely restricts thiamine-dependent reactions, resulting in substrate accumulation for these reactions. This condition leads to decreased activity of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase enzymes and impaired adenosine triphosphate (ATP) synthesis. Reduced ATP synthesis in critical areas such as the nervous and cardiovascular systems can lead to cell death, Wernicke's encephalopathy, and impaired insulin synthesis and secretion [1]. Although thiamine deficiency is rare in various countries worldwide, due to its limited storage in the body and short half-life, a sufficient and regular supply from the diet is necessary. Under conditions of starvation or malnutrition, thiamine deficiency can trigger the onset of beriberi as the first disease to appear [4]. Therefore, it is crucial to examine thiamine levels in the human body.

Methods that accurately detect thiamine levels in biological fluids are needed to screen for thiamine deficiency. Several techniques can be used to detect thiamine levels in serum, including spectrophotometry, high-performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA) [5,6,7]. Some existing methods require special equipment and are costly to implement, hence the development of a serum thiamine assay method using a principle similar to ELISA, which utilizes thiamine-binding protein as an antibody substitute.

Several studies report that thiamine-binding protein (TBP) is found in various materials, including bacteria and other microorganisms [8,9,10,11]. TBP has also been isolated from egg white, yolk, and animal tissues [12,13]. Thiamine-binding protein can be obtained from plants that are readily available locally. Some

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sources of thiamine-binding protein that have been studied include mung beans, corn bran, and sunflower seeds [8,14,15,16]. This study aims to isolate and identify the presence of thiamine-binding protein in black glutinous rice bran (*Oryza sativa var. Glutinosa*).

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Several studies report that thiamine-binding proteins (TBP) are found in various materials, including bacteria and other microorganisms [8-11]. TBP has also been isolated from egg white, yolk, and animal tissues [12,13]. Thiamine-binding protein can be sourced from plants that are readily available in Indonesia. Some sources of TBP that have been studied include mung beans, corn bran, and sunflower seeds [8,14,15,16].

This study aims to isolate and identify the presence of thiamine-binding protein in black glutinous rice bran (*Oryza sativa var. Glutinosa*). Rice bran is a byproduct of rice milling. The bran consists of the rice husk, which is typically discarded during the rice polishing process. Although rice bran makes up only 1%–2% of the entire grain, it contains 70% of the grain's total nutrition [21]. Currently, rice bran is more commonly used as animal feed. In this study, its utilization as a source of thiamine-binding protein offers a cheaper alternative. Plant-based thiamine-binding proteins hold great potential due to their abundant availability, easy accessibility, safety, sustainability, and simpler, more cost-effective isolation process compared to animal or microbial sources.

Materials and Methods

Materials

The black glutinous rice bran used in this study was obtained from the traditional Rawamangun market and is commonly consumed by Indonesians. Other materials used include Bovine Serum Albumin (BSA) as a protein standard (Nacalai Tesque), Na_2HPO_4 (Merck), KH_2PO_4 (Merck), $BaCl_2$ (Merck), NaCl (Merck), $(NH_4)_2SO_4$ (Merck), and distilled water. The equipment used includes a balance with a 220-gram capacity (Sartorius), a blender, a 150 R sieve, a centrifuge (Hettich), a pH meter (Schott), a magnetic stirrer (Thermolyne), a UV-Vis spectrophotometer (Thermo Fisher), 1 mL and 10 µL micropipettes (Bio-Rad), 1 mL and 10 µL microtips (Extragene), 25 mL microtubes (Extragene), Falcon tubes (Extragene), 33 mm wide cellophane bags (Sigma-Aldrich), and several glassware such as test tubes and beakers (Pyrex).

Methods

Sample and Buffer Preparation

One hundred grams of black glutinous rice bran were ground using a blender until finely powdered and then sieved using a 150 R sieve. A 1-liter 0.05 M phosphate buffer with pH 7 was prepared by dissolving 0.53 grams of KH_2PO_4, 1.09 grams of Na_2HPO_4, and 4.5 grams of NaCl in distilled water while stirring with a magnetic stirrer. The pH was adjusted to 7 by adding NaOH or HCl solution.

Protein Extraction (Salting Out)

Thiamine-Binding Protein (TBP) was extracted from black glutinous rice bran samples, with three repetitions (repetition 1, 2, and 3) carried out. Each repetition weighed 10 grams of black glutinous rice bran powder, which was added to 100 mL of 0.05 M phosphate buffer solution at pH 7. The mixture was stirred using a magnetic stirrer until homogeneous for 24 hours at 4°C. To minimize protein degradation, the entire protein isolation process was conducted at 4°C. The homogenates from the three repetitions were centrifuged at 28,000 g for 15 minutes, and the supernatant (crude extract) was collected for the next stage. The protein content of each crude extract was measured using a spectrophotometer, and its concentration was calculated using a standard protein curve.

The protein in each supernatant sample was precipitated using the salting out method by adding ammonium sulfate powder until 90% saturation was reached [5]. Ammoniumsulfate is highly effective in maintaining protein stability during the purification process, thus reducing protein denaturation during the extraction stage. Ammonium sulfate powder was added gradually while stirring with a magnetic stirrer, then centrifuged at 28,000 g for 15 minutes [5]. The precipitate obtained was redissolved to the original volume by adding phosphate buffer.

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Protein Assay

Each isolation step was tested for protein using the Warburg-Christian method (λ 280 nm) with bovine serum albumin (BSA) as the standard [17].

Standard Protein Curve

The standard protein curve was made by preparing a series of BSA concentrations. The concentrations used were 0 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, and so on up to 1000 µg/mL. Each concentration was prepared in duplicate, and the average absorbance at 280 nm was measured and plotted.

Standard Thiamine Curve

The standard thiamine curve was made by dissolving 500 mg of thiamine powder in distilled water to a volume of 50 mL as stock. A series of concentrations were prepared from the stock solution by dilution, starting from 0 µg/mL, 2 µg/mL, 4 µg/mL, 6 µg/mL, and so on up to 20 µg/mL. Each concentration was prepared in duplicate, and the average absorbance at 235 nm was measured and plotted.

Dialysis

Each fraction from the salting out was dialyzed using cellophane membrane bags with a 0.05 M phosphate buffer exchange at pH 7 to separate the protein from the remaining ammonium sulfate. Dialysis was considered complete when the ammonium sulfate concentration in the dialysis buffer outside the bag was zero, using a sulfate test (1 mL solution outside the bag + 1 mL HCl + 1 mL $BaCl_2$).

Equilibrium Dialysis (TBP Binding Capacity Test)

The thiamine-binding capacity of TBP was tested using the equilibrium dialysis method. Each salting out fraction after dialysis was sampled at 198 mL to be placed in cellophane membrane bags, adding 0.02 mL of 10 mg/mL thiamine solution, and mixed until homogeneous. The bags were then placed in beakers containing 100 mL of distilled water. All bags had to be submerged. The buffer outside the bag was measured for thiamine concentration at 10-minute intervals at 235 nm, stopping the reading once equilibrium was reached, indicated by constant absorbance values.

Results

Black glutinous rice is very popular among Indonesians and is usually processed into snacks such as black glutinous rice bran porridge or black glutinous rice tape. Its relatively complete nutritional content makes it a good source of calories and vitamins. Table 1 shows the nutritional content of black glutinous rice bran (100 grams).

Table 1: Nutritional Content of Black Glutinous Rice (100 grams)

Nutrient	Nutritional Content		Nutrient	Nutritional Content	
		% Daily Value			% Daily Value
Energy	181 kkal	8,42%	Sodium	9 mg	0,60%
Protein	4 g	6,67%	Potassium	18,40 mg	0,39%
Carbohydrate	37,30 g	11,48%	Copper	100 µg	12,50%
Fat	1,2 g	1,79%	Iron (Fe)	1,70 µg	7,73%
Dietary Fiber	0,30 g	1%	Zinc	0,70 mg	5,38%
Calcium	9 mg	0,82%	Vitamin B1	0,06 mg	6%
Phosphorus	144 mg	20,57%	Vitamin B3	0,50 mg	3,33%

Compiled by author

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Protein Standard Curve

The protein standard curve was prepared by preparing a series of BSA concentrations and measuring protein levels using the Christian Welburg method at a wavelength of 280 nm. The protein standard curve will be used to measure protein concentrations. The standard curve equation is y=0.0007x+0.004; with a correlation coefficient R^2 =0.9999. The standard curve graph can be seen in Figure 1.

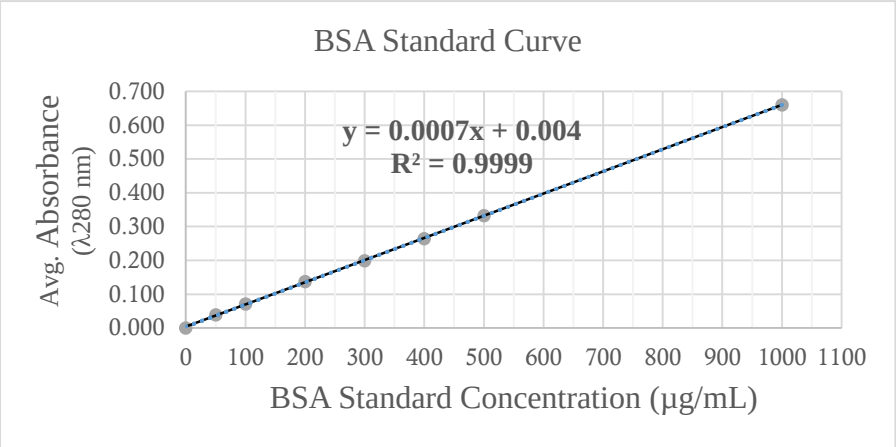

Figure 1. BSA Standard Curve

Thiamine Standard Curve

The thiamine standard curve was created by preparing a series of thiamine concentrations ranging from 0 to 10 µg/mL, read at the maximum wavelength of thiamine, 235 nm. The linear equation of the thiamine standard curve is y=0.0427x+0.0094; with a correlation coefficient R^2 =0.9969. The thiamine standard curve will be used to measure thiamine concentrations during
equilibrium dialysis. The standard curve graph is shown in Figure 2.

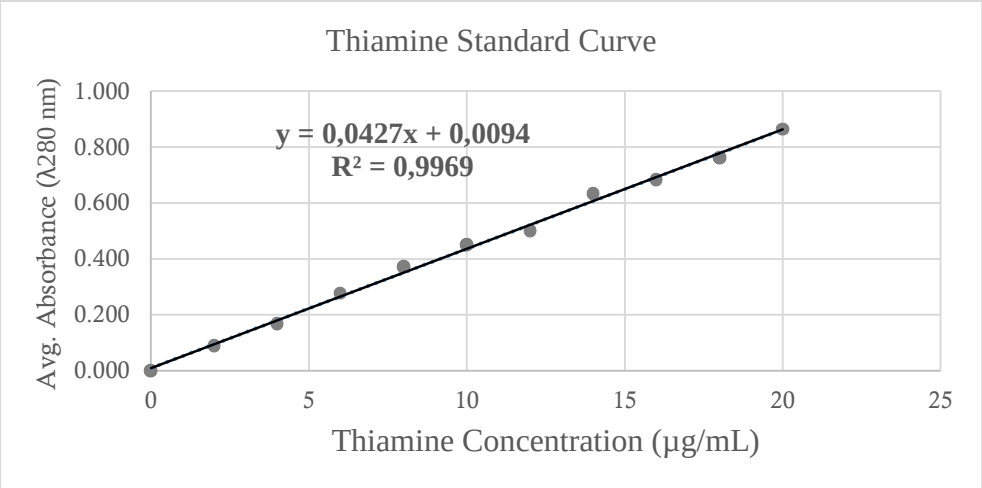

Figure 2. Thiamine Standard Curve

Protein Precipitation

The protein content of the three crude fractions from homogenized black glutinous rice samples was measured at 280 nm and calculated using the BSA standard curve. Table 2 shows the protein content of the crude fractions from each sample.

The protein content of the three crude fractions from the homogenized black glutinous rice bran sample repetitions was measured at a wavelength of 280 nm and calculated using the BSA standard curve. Table 2 shows the protein content of the crude fractions for each sample repetition. It is evident that each sample

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repetition has varying protein levels, as the protein content in each 10 grams of black glutinous rice bran powder taken also differs.

Table 2. Total Protein of Crude Fractions

Crude Sample	Absorbance (λ = 280nm)	Protein Concentration 100x Dilution (µg/mL)	Protein Concentration (µg/mL)
Repetition 1	0,056	73,57	7357,14
Repetition 2	0,076	102,86	10285,71
Repetition 3	0,045	57,86	5785,71

Salting Out and Dialysis

The protein from the crude extract was precipitated using the salting out method with ammonium sulfate at 90% saturation, then dialyzed to remove any remaining salts. Table 3 shows the protein content of each sample after salting out and dialysis.

Table 3. Total Protein of Salting Out Fractions After Dialysis

Salting Out Sample	Absorbance (λ = 280nm)	Protein Concentration 100x Dilution (µg/mL)	Protein Concentration (µg/mL)	Protein Concentration Percentage (%)/10gram
Repetition 1	0,037	47,14	4714,29	0,047
Repetition 2	0,040	51,43	5142,86	0,051
Repetition 3	0,044	57,14	5714,29	0,057

Equilibrium Dialysis

Equilibrium dialysis was conducted on the three samples to determine the presence of thiamine-binding protein and the concentration of thiamine it could bind. Thiamine absorbance was read in the solution outside the cellophane bag at 235 nm during equilibrium dialysis. Repetition 1 reached equilibrium at 120 minutes with an absorbance reading of 0.029 (Table 4). Repetition 2 reached equilibrium at 140 minutes with an absorbance reading of 0.069 (Table 5). Repetition 3 reached equilibrium at 140 minutes with an absorbance reading of 0.062 (Table 6). The three samples, after reaching equilibrium, are shown as a flat line in Figures 3, 4, and 5.

Table 4. Equilibrium Dialysis Repetition 1

Time (minutes)	Absorbance (λ=280 nm)	Time (minutes)	Absorbance (λ=280 nm)
0	0		
10	0,014	90	0,027
20	0,019	100	0,03
30	0,024	110	0,028
40	0,022	**120**	**0,029**

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Time (minutes)	Absorbance (λ=280 nm)	Time (minutes)	Absorbance (λ=280 nm)
50	0,023	130	0,029
60	0,024	140	0,029
70	0,025	150	0,029
80	0,03	160	0,029

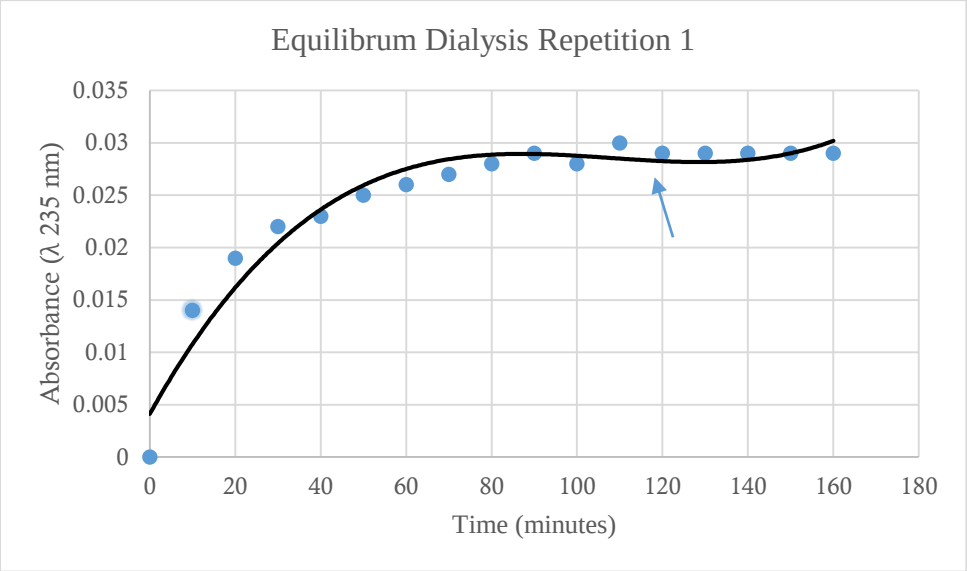

Figure 3. Equilibrium Dialysis Graph for Repetition 1. Equilibrium reached at the 120th minute with an absorbance reading of 0.029 that is marked with blue (Table 4)

Table 5. Equilibrium Dialysis Repetition 2

Time (minutes)	Absorbance (λ=280 nm)	Time (minutes)	Absorbance (λ=280 nm)
0	0		
10	0,019	90	0,056
20	0,027	100	0,058
30	0,033	110	0,061
40	0,037	120	0,066
50	0,044	130	0,067
60	0,045	**140**	**0,069**
70	0,049	150	0,069
80	0,052	160	0,069

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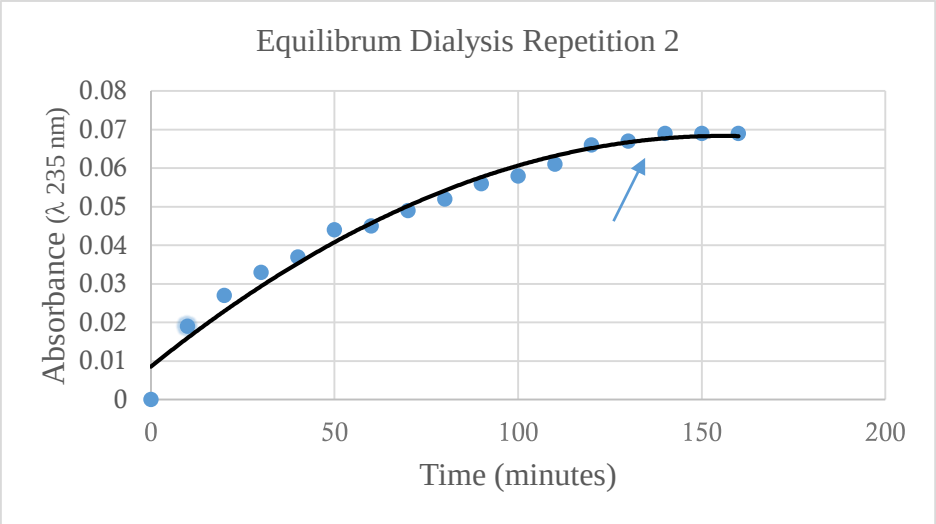

Figure 4. Equilibrium Dialysis Graph for Repetition 2. Equilibrium reached at the 140th minute with an absorbance reading of 0.069 that is marked with blue (Table 5).

Table 6. Equilibrium Dialysis Repetition 3

Time (minutes)	Absorbance (λ=280 nm)	Time (minutes)	Absorbance (λ=280 nm)
0	0		
10	0,02	90	0,047
20	0,021	100	0,051
30	0,028	110	0,054
40	0,039	120	0,057
50	0,036	130	0,057
60	0,043	**140**	**0,062**
70	0,042	150	0,062
80	0,048	160	0,062

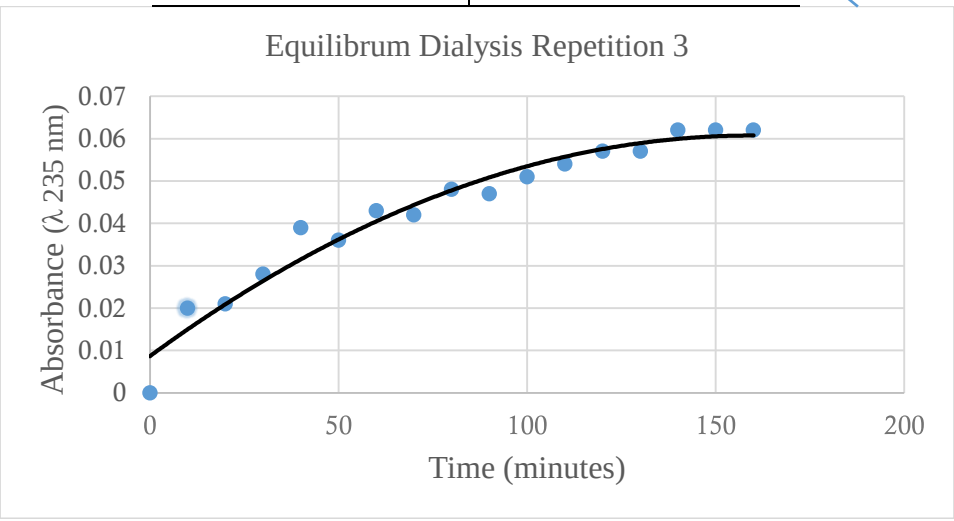

Figure 5. Equilibrium Dialysis Graph for Repetition 3. Equilibrium reached at the 140th minute with an absorbance reading of 0.062 that is marked with blue (Table 6).

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Absorbance readings at the end of equilibrium dialysis for the three samples were recorded and converted to thiamine concentrations using the thiamine standard curve. The thiamine concentration was then calculated using the formula (1) to determine the thiamine concentration bound to the thiamine-binding protein in the cellophane bag. The calculation results can be seen in Table 7

$$(1)\quad [\text{Bound Ligand}] = \frac{\text{Total ligand Mol} - \text{Free ligand Mol}}{\text{The volume of the compartment in the cellophane bag}}$$

Total ligand moles are the amount of thiamine moles introduced into the cellophane bag. Free ligand moles are obtained from the thiamine concentration in the outer compartment multiplied by the total volume of both compartments. The total ligand moles used in this study are:

$$\frac{0,2}{337300} = 5,93 \text{ x } 10^{-7}.$$

Table 7. Free and Bound Thiamine Concentrations

Sample	Free Thiamine Concentration (mg/mL)	Bound Thiamine Concentration (mg/mL)	Bound Thiamine Percentage (%)
Repetition 1	0,46 x 10^{-3}	7,66 x 10^{-2}	1,62 %
Repetition 2	1,37 x 10^{-3}	3,72 x 10^{-2}	0,73 %
Repetition 3	1,23 x 10^{-3}	3,00 x 10^{-2}	0,53 %

Discussion

Several studies have isolated thiamine-binding proteins from other sources. One such study by Gunarti et al. isolated thiamine-binding protein from mung beans, achieving a total protein concentration of 2.5 mg/mL after dialysis [5]. When compared to mung beans, black glutinous rice bran, with an average total protein concentration of 5.732 mg/mL after dialysis, has twice the concentration, suggesting a higher potential for isolating thiamine-binding protein from black glutinous rice bran.

Another source of thiamine-binding protein studied is corn. The total protein obtained from corn in the crude sample was 792 mg [15]. In comparison, the average total protein from black glutinous rice bran in the crude sample was 750 mg, which is slightly less. However, another study by Mitsunaga et al. found that the thiamine-binding capacity of corn protein was not as good as black glutinous rice bran, with 20.5 µg per 10 grams of sample compared to black glutinous rice, which ranged from 30-76.6 µg per 10 grams of sample [19].

Thiamine-binding protein has also been isolated from buckwheat (*Fagopyrum esculentum*), with a total protein of 292 mg after salting out and dialysis [20]. In comparison, black glutinous rice provided twice as much total protein at the salting out stage, with 593 mg.

There are many other sources of thiamine-binding protein. Mitsunaga et al. [19] studied 18 plant species containing thiamine-binding protein. Plants with thiamine-binding capacities comparable to black glutinous rice bran (30-76.6 µg per 10 grams of sample) include soybeans (34.8 µg), brown rice (44.5 µg), *Vigna sinensis L.* (46.4 µg), peas (52.5 µg), *Phaseolus vulgaris L. var. cultiva Kintoki* (57.9 µg), *Phaseolus vulgaris L. var. cultiva Aschers* (61.9 µg), and *Phaseolus coccineus L. var. albus Bailey* (64.5 µg) [19]. Plants exceeding the binding capacity of black glutinous rice bran (>76.6 µg per 10 grams of sample) include wheat germ (87.6 µg), *Fagopyrum esculentum* Moench. (104.1 µg), bran (147.7 µg), sunflower seeds (288.5 µg), and sesame seeds (675.0 µg) [19].

The thiamine-binding protein isolated from black glutinous rice bran in this study is still impure. Further purification steps are needed to obtain pure TBP. Pure TBP can be used as a substitute for antibodies in a thiamine measurement tool similar to ELISA. This tool can be used to measure thiamine levels in food and biological fluids.

Conclusion

Thiamine-binding protein can be found in extracts of black glutinous rice bran and has been shown to bind thiamine effectively.

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Acknowledgment

The authors would like to express their appreciation to the analysts and fellow laboratory staff at the Department of Biochemistry and Molecular Biology, Universitas Indonesia, for their technical assistance during the experiments.

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