



HUMAN SERUM FOLATE CAN BE MEASURED USING FOLATE BINDING PROTEIN LINKED TO ENZYME-LABELED PROTEIN LIGAND BINDING ASSAY (ELPLBA) AS WELL AS ELISA

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ABSTRACT

Background: Folate is an important substance used for purine and pyrimidine nucleotide synthesis. One measurement of folate that already establishes is using ELISA (Enzyme-linked immunosorbent assay) method. Folate binding protein is a protein that can bind folate, therefore it considered can be used as a tool that can replace antibody dependent ELISA method.

Objectives: The aim of this research was to create a method for folate measurement in serum called Enzyme-labeled protein ligand binding assay (ELPLBA) by replacing antibody as used in ELISA method with folate binding protein (FBP) that purified from the whey of milk.

Methods: The method is tested using 20 serum samples and compared to ELISA. Folate binding protein was purified from bovine's milk using ammonium sulfate up to 90% saturated, DEAE-cellulose anion exchange chromatography and affinity chromatography. SDS-PAGE and western blot were used to establish the protein band of FBP that has molecular weight of ~25-35 kDa. ELPLBA was arranged with stationary phase using aminohexyl-agarose, and folic acid linked on it using carbodiimide.

Results: The result show there was no significant difference of folate concentration between ELPLBA (14.804 ± 2.795) and ELISA method (13.859 ± 3.638), $p = 0.363$.

Conclusion: ELPLBA method show similarity for determination of folate in serum which was the same as standard folate measurement (ELISA).

Keywords : Serum folate, Folate binding protein, Enzyme-labeled protein ligand binding assay

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INTRODUCTION

Folate is a vitamin B9 that cannot be synthesized in the body so it is only obtained from supplements, fortified foods, and natural foods.[1] The structure of folate derivatives consists of three parts such as pteridine ring (pterin for folic acid), P-aminobenzoyl, and glutamic acid.[2] The role of folate in the body as a coenzyme is involved in nucleotide synthesis (purine and thymine) and amino acid interconversion, and as an antioxidant for decreasing endothelial damage.[2-4] Folate is required in small amounts ($\pm 400 \mu\text{g}$ for adulthood), but cannot be replaced by other substances.[5]

Some diseases caused by folate deficiency such as megaloblastic anemia, hyperhomocysteine, and preeclampsia are cured in the presence of a high intake of folate.[3,5,6] Examination of the folate level is necessary to prevent or overcome negative effects resulting from folate deficiency.[7] One of the current methods known to be accurate, sensitive, and relatively fast processing time in folate measurements is enzyme-linked immunosorbent assay (ELISA).[8,9]

Specific antibody requirement for ELISA to detect folate is important as a special protein binding folate, thus its preparation becomes a basic requirement of this method.[2,8,10] Several studies have suggested that the existence of a natural protein which is known as folate binding protein (FBP) can bind to folic acid, which is only obtained from a relatively simple separation method in order to as an alternative to ELISA antibodies.[11-13] Folate binding proteins can be found in fresh bovine's milk with levels of $211 \pm 7 \text{ nmol / L}$ ($n = 10$) and molecular weight of 30-35 kDa.[13] The homology percentage of folate binding protein from fresh bovine's milk and folate

binding protein from humans is $\pm 83\%$.[14]

Based on the potential of folate binding protein from fresh bovine's milk, it can be used to measured folate levels in the human body.[13] Folate binding protein used to measure folate levels in serum in the previous study by Mardiana.[15] However, standard solutions of folic acid are used only in the range of 25-100 ng/mL whereas the normal levels of human serum range from 6 to 20 ng/mL, therefore, Mardiana's technique cannot be used for human folate measurement.[16] Based on the description above, the steps that need to be create to refine the folate measurement technique include: purifying folate binding protein from fresh bovine's milk using ion exchange chromatography and affinity chromatography, validating enzyme labeled protein ligand binding assay (ELPLBA) technique with pure FBP instead of antibody from competitive ELISA as a standard measurement, and then comparing test, to verify between ELPLBA and competitive ELISA technique.

MATERIAL AND METHODS

Before comparison of ELPLBA with ELISA method, the first step was preparation of ELPLBA. It needed to purify first the FBP from whey of bovine milk. The FBP used as capture molecule to bind folate in ELPLBA as same as a specific antibody in ELISA method.

Purification protocol of FBP started by isolation of whey from bovine milk using ammonium sulfate, following by ion exchange chromatography, and affinity chromatography. To ensure the purification result SDS-PAGE and Western-blot were used.

Purified FBP as capture molecule placed in the surface of microplate well. The FBP will bind the serum folate and its competitor avidin-linked folate. The last step was adding the conjugated enzyme biotin peroxidase. The next step was same as in ELISA method measure the absorbance of the product, oxidized chromogen.

Isolation and purification of FBP

Bovine's milk of 2.5L has centrifuged at 2000 rpm then decreasing its pH to form whey by adding $(\text{NH}_4)_2\text{SO}_4$. Ammonium sulfate was added to whey up to 90% saturated and then centrifuged 2000 rpm to separate supernatant and precipitate. The precipitate was put inside a cellophane bag for dialysis.[7,11]

Purification of ion exchange chromatography

The DEAE-cellulose phase stationary preparation was prepared according to manufacture instruction (Santa Cruz). The prepared gel was then suspended in 0.02 M phosphate buffer saline pH 7.2 and packed into a chromatographic column with a diameter of 1.5 cm and a length of 15 cm. After the column was ready to use, 1 mL of $(\text{NH}_4)_2\text{SO}_4$ -free dialysate was added to the column, then eluted with phosphate buffer saline pH 7.2 with concentrations of 5 mM; 20 mM; 40 mM; 60 mM; 80 mM; and 100 mM. Elution fraction each of 2 mL was collected and measured at λ 280 nm. Elution was discontinued and replaced with subsequent phosphate buffer saline concentration when the absorbance was near 0.[7,11,15]

Purification of affinity chromatography

Preparation of stationary phase affinity chromatography using aminohexyl-agarose and folic acid linked

using carbodiimide. The yellow gel was decanted and washed with 0.05M NaHCO_3 . The medium that did not stick to folic acid was blocked by the addition of ethanolamine 1 M pH 7.2 for two hours at room temperature and washed 2-3 times with 0.02 phosphate buffer saline pH 7.2 containing 0.1 M NaCl.[17,18]

DEAE-cellulose ion exchange chromatography fractions pH was changed to be 3 by adding H_3PO_4 1M solution and followed by acetate buffer pH 3.5 to separate folate from FBP. It was run the following the protocol. The DEAE-cellulose dialysate (second peak) was mixed with folic acid-bound agar resins in a beaker and added with 1 M NaOH solution to adjust its pH around 7.2, stirred for 3 hours at room temperature and replaced into chromatography column with a capacity of 10 mL. After that, the column was eluted with 0.02 M of phosphate buffer saline pH 7.2 and the absorbance of fractions was measured until the fraction that shows absorbance near to 0 at λ 280. Then, column was re-eluted using acetate buffer with pH 3.5 and 0.5 M NaCl. Folate Binding Protein fractions were collected at the second highest peak, and then inserted into a cellophane pouch and immersed in 0.02 M of phosphate buffer saline pH 7.2 in order to neutralize the acidic pH.[7,11,17]

SDS-PAGE and Western blot

The FBP purification was confirmed using SDS-PAGE based on molecular weight and western blot based on antigen-antibody interaction of FBP. [19,20]

Application of FBP in ELPLBA method on folate measurement.

Folate binding protein of 100 μL was coated to the microplate and incubated it for 24 hours at 4°C. After incubation and

washing step, 150 μ L of 1% BSA was added into each well (blocking), then incubated 2 hours at room temperature. Furthermore, 50 μ L of serum was incorporated into each well followed by adding 50 μ L of 1 μ g/L folic acid-avidin. Subsequently, the microplate was incubated for 1 hour at room temperature and 37°C (for optimization temperature). Next, conjugated enzyme biotin peroxidase was diluted with PBS pH 6.0 (final concentration of 20 μ g/mL of), then plated at approximately 50 μ l /well in a microplate. Next, the microplate was incubated for 1 hour at room temperature. [2,15]

After incubation and washing, OPD was added (measured maximum absorbance first) 100 μ L/well and incubated for 15 minutes at room temperature in the dark. The last, 100 μ L of 2 N Sulphuric acid was added to stop enzymatic reaction and light absorption was read using microplate reader at 490 nm.[15]

Sample preparation

This study was conducted using 20 serum blood of normal patients. All patients (both men and women) were about 24-50 years old. The blood obtained from the patient was then collected in an SST tube and incubated for 20-30 minutes to allow clotting process, after that the serum was separated from blood by centrifugation 2000 rpm for 10-15 minutes. Serum was stored in microtube at -20°C. This study was reviewed and ethically approved by Faculty of Medicine Universitas Indonesia Research Committee (No. 790/ UN2.F1/ETIK/2016).

Statistical analysis

Statistical analysis comparative test was performed with independent sample T-test or Mann-Whitney test. The test

results in each group will be considered statistically significant at 95% confidence level.[21]

The accuracy test was considered to be accurate with the percentage of recovery was around 80-120%.

$$\% \text{Recovery} = \frac{\text{Measured concentration}_{\text{spiked}} - \text{Measured concentration}_{\text{test sample}}}{\text{Theoretical concentration}_{\text{spiked}}} \times 100$$

The data's repeatability test was measured from the % CV value which should not exceed 10%, and the regression analysis of the error data was generally measured using independent t-test with 95% confidence level.[22] The linearity of serum sample was considered normal if the percentage of recovery values was still approximately 80%-120%.[23]

RESULTS

The result of FBP isolation from bovine's milk after dialysis that was purified in ion exchange chromatography produced two peaks (figure 1). Then, its purified using affinity chromatography, and show result two peaks. The second peak that eluted with acetate buffer was suspected as FBP (figure 2). The FBP purification result was confirmed using SDS-PAGE and western blot generated 3 bands in the range of 25-35 kDa (figure 3 and 4).

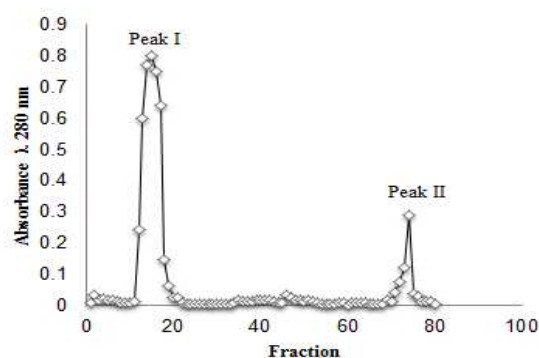


Figure 1. Fractionation with ion exchange chromatography

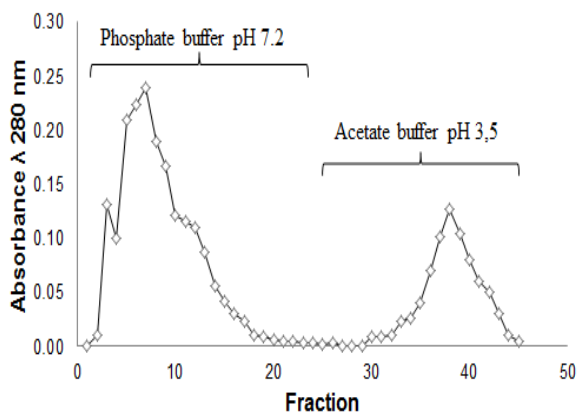


Figure 2. Fractionation with affinity chromatography

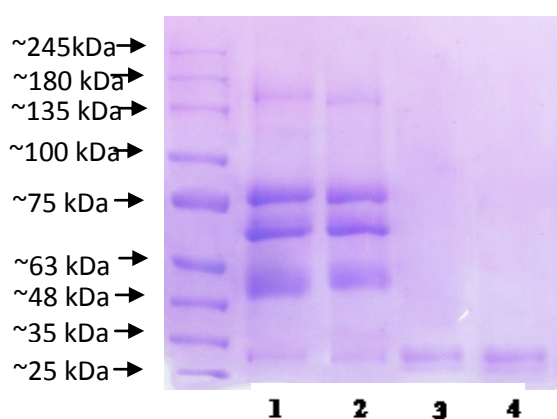


Figure 3. Electrophoresis SDS-PAGE
1: marker, 2,3: dialysate 4,5: second peak eluate

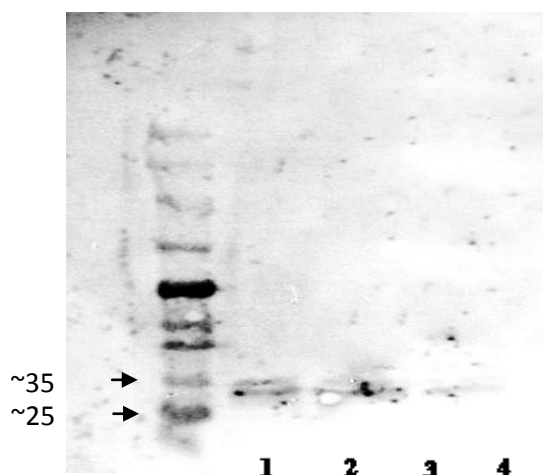


Figure 3. Western blot
1: marker protein, 2,3,4 : second peak of affinity chromatography (FBP) bands.

The temperature optimization techniques of ELPLBA were performed, using purified FBP from the affinity chromatography fraction (data not shown) that also used in competitive ELISA. The optimum temperature that generated based on the highest relative coefficient value was FBP at 25°C with dilution 1/10 that show coefficient of determination (R2) = 0.917.

The repeatability test of ELPLBA on serum folate measurements was performed right after purification in the same day. The result of repeatability analysis (table 1) was 9.8%.

Table 1. Repeatability test ELPLBA

Sample (N=20)	Folate level (ng/mL)		CV (%)	CVstd
	means	SD		
Serum	0,913	0,084	9,8	<10%

The reproducibility test of ELPLBA on folate measurements serum after 5 days was not good, the mean value had significantly different by independent T-test which can be seen in table 2.

Table 2. Reproducibility test ELPLBA

Sampel	N	date	Mean (ng/mL)	SD	p value
Serum	20	5 Mei 2017	14.804	2.79	<0.05
	20	9 Mei 2017	10.307	3.82	

The accuracy and linearity of the ELPLBA on serum folate measurements was performed by the addition of 10 ng/mL of known folic acid concentration (recovery test) can be seen in table 3.

Table 3. The accuracy and linearity of the ELPLBA

Sample	Folate level (ng/mL)	Folate level after spiked	%recovery
Serum a	10.57	19.85a	92.8
1:2		10.67b	107
1:4		5.97 b	120
1:8		2.98 b	119
Serum b	11.30	19.45a	81.5
1:2		11.61 b	119
1:4		5.534 b	113
1:8		2.4 b	123.58

Comparative test between ELPLBA and ELISA technique showed that both techniques were not significantly different with $p=0.363$ at 95% confidence level which can be seen in table 4.

Table 4. Comparison test between ELPLBA and ELISA

Sample	N	Technique	mean (ng/mL)	SD	p value
Serum human	20	ELPLBA	14.804	2.795	>0.05
	20	ELISA	13.859	3.637	

DISCUSSION

The isolation of FBP from bovine's milk is aimed to separate the fat and casein through centrifugation and precipitation in order to produce whey. Whey contains many proteins such as immunoglobulin, β -lactoglobulin, α -lactoalbumin, folate binding protein, lactoferrin.[24]

The addition of ammonium sulfate salt to whey is performed in two stages to precipitate the whole casein and whey protein with greater solubility, while the second step to precipitate whey protein with a smaller solubility. The second

precipitate resulted in is then suspended and dialyzed using a cellophane pouch with 12 kDa MWCO aiming to restore the protein condition in its initial state.[7,11,25]

The result of dialysis process is further purified by ion exchange chromatography. Folate binding proteins are negatively charged at a neutral pH that can be bound by a positively charged cellulose DEAE. The result of the first peak protein elution is a positive charge protein that does not bind to DEAE cellulose such as lactoferrin, which can come out directly from the column using the phosphate buffer saline eluent pH 7.2. Then, the result of second peak protein elution using phosphate buffer saline pH 7.2 and 0.1 M NaCl was suspected as a negative charge protein containing a folate binding protein. [7,26]

Full purification of folate binding protein from fresh bovine's milk will be done by affinity chromatography based on procedure by Salter et al. The stationary phase used is a crosslinked agarose with folic acid. Crosslinker in the binding of folic acid-agar is carbodiimide.[17,27] The elution result on affinity chromatography shows two peaks, namely the first peak is protein that is not bound by the stationary phase while the second peak is a binding protein with a stationary phase. The second peak is believed to be pure FBP.[7] The result of FBP purification confirmation test using affinity chromatography are similar to Nygren *et al* and Iwai *et al* studies because of glycosylation differences leading to the molecular weight of FBP from $\pm 25,700$ Da to 30,000-35,000 Da.[19,20]

Validity technique used in this research is a precision test (repeatability, reproducibility), accuracy, and linearity. The repeatability test of ELPLBA

technique on serum folate measurements was performed within the same day. Results of repeatability analysis of 9.8%. The % C.V (coefficient of variance) value of repeatability is considered good if the value of coefficient of variance (C.V) is less than 10%.[21]

On the other hand, the reproducibility test of ELPLBA on serum folate measurement obtained using independent T-test indicates poor error in the measurements of serum folate stored within five days. The measurement of serum folate is generally unstable over the long period of time because it is easily degraded to p-aminobenzoylglutamic acid (PABG) affecting the measurement of the technique itself. Eugène H.J.M. Jansen *et al.* explained that folate storage for 4 days, showing a decrease of stability up to 60% and 83% in the next day.[28]

The accuracy test is measured from the recovery value in the presence of the addition of a certain concentration of folic acid to the sample. The recovery test by addition of folic acid (spiked) for the serum a shows a recovery percentage of 92.9% and the second serum reaches recovery percentage of 81.7%. Based on the recovery test indicates that both serum can be accepted based on acceptable recovery limit (80%-120%).[23]

The linearity of the serum folate sample was performed by the addition of 10 ng/mL of the folate standard (spike) then diluted with a ratio of 1: 2, 1: 4, and 1: 8. On the basis of linearity, the serum response after adding standard folate (10 ng/mL) and diluting on serum (a) still produces a proportional response in the range or the range of the standard curve with a recovery rate range is about 80%-120%. However, the recovery value of serum (b) at 1: 8 dilution does not include the limit of the recovery range leading not

to produce an appropriate dilution response in the standard curve range. This may be due to a matrix component (serum protein) interfering serum folate detection at 1: 8 dilution.[23]

The comparative test results between the two techniques showed no significant differences. This indicates that the results obtained from ELPLBA do not conflict with the results obtained by competitive ELISA technique in the measurement of serum folate at a 95% confidence level.[8,21]

CONCLUSION

The method ELPLBA is eligible enough and relatively simple for determination of folate in serum which was the same as standard folate measurement such as ELISA. FBP from bovine's milk can serve as candidate alternative to antibody in ELISA. However, we consider this study only used ELISA method as standard measurement folate and only applied for small amount subject studies so that can't be used as a base for standardization efforts yet and needed next research plan with modifying the sample preparation.

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