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Isolation and Purification of Breast Milk Folate Binding Protein: Salting-Out and Chromatography Techniques

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ABSTRACT. Folate binding protein (FBP) is a protein in breast milk that plays a role in the regulation and bioavailability of folic acid. In contrast to cow's milk FBP, information about breast milk FBP is still limited. This research aims to determine the isolation and purification methods of breast milk FBP and the molecular weight of breast milk FBP. The sample in this study was 1000 mL of breast milk. Breast milk was prepared in several stages to yield whey. Isolation and purification of FBP from whey were carried out in stages, salting-out, ion exchange chromatography, and affinity chromatography. Whey salting-out with 95% saturation of ammonium sulfate could precipitate folate-binding proteins. This precipitate showed three peaks on DEAE chromatography. Peak II DEAE 95% was thought to be a negatively charged folate-binding protein. Peak II DEAE 95% also showed the presence of two peaks on affinity chromatography. It was believed that Peak II AF 95% was a pure folate-binding protein. Peak II AF 95% showed the presence of a single band on SDS-PAGE and western blot. This indicated that the folate-binding protein was 100% pure. FBP can be isolated from breast milk by the salting-out method using 95% ammonium sulfate, DEAE chromatography, and affinity chromatography. FBP from breast milk has a molecular weight of approximately 37 kDa. The final level of FBP isolated from breast milk is approximately 0.022 mg/mL. The successful isolation of FBP from breast milk provides an opportunity to use it to understand the clinical role of FBP in increasing folic acid levels in both breast milk and infant serum, as well as to develop methods for determining folic acid levels in these fluids.

Keywords: Breast milk, folate binding protein, isolation, purification, molecular weight

INTRODUCTION

Folate binding protein (FBP) is a protein that plays an important role in the distribution of folic acid. This protein has been identified in various cells, extracellular fluids, and mammalian tissues. Folate binding protein is encoded by a gene located on the long arm of chromosome 11 at band 13 (chromosome 11q13). In 1967, this protein was first identified as a large molecule that binds folic acid in milk (Nygren-Babol & Jägerstad, 2012; Subandrate et al., 2016). FBP, also known as the folate receptor, is a family of proteins that are found in soluble or particle form in cell membranes. In humans, there are 3 families of proteins, namely folate receptor alpha (FR-α), folate receptor beta (FR-β), and folate receptor gamma (FR-γ). Breast milk FBP is included in the folate receptor alpha (Holm & Hansen, 2020; Nygren-Babol & Jägerstad, 2012).

FBP has several important roles in the regulation of the bioavailability and absorption of dietary folate. The role of FBP is to take folic acid from the blood to ensure the availability of folic acid in breast milk is sufficient for the neonate, even though the concentration of folic acid in the blood is relatively low. In addition, folate binding protein is thought to help the absorption of folate in the infant's intestines. The absorption of folate in the form of a complex with FBP is thought to be better than in the form of free folate. In the infant's blood, FBP plays a role in transporting folate to cells. The physiological role of soluble FBP is as a

transporter of folic acid in milk and breast milk. Meanwhile, FBP particulates are found in cell membranes and play a role during membrane transport. Thus, it can be said that FBP acts as a folate reservoir (Holm & Hansen, 2020; Li et al., 2021; Nygren-Babol & Jägerstad, 2012).

Utilization of folate binding protein from cow's milk, tissue, or human plasma has been extensively studied (Nygren-Babol & Jägerstad, 2012). In 2014, researchers began looking at the use of folate binding protein as a vaccine to prevent cancer recurrence (Greene et al., 2014). In 2015, research on folate binding proteins discussed the role of folate binding protein in drug delivery (Merzel et al., 2017; Merzel et al., 2015; Merzel et al., 2017). In addition, developments in cancer treatment have found evidence that FBP can be a target in ovarian cancer chemotherapy (Greene et al., 2014; Lutz, 2015). In fact, research conducted by Samadian et al. showed that FBP has antitumor capabilities both in vivo and in vitro (Samadian et al., 2022). In 2018, Budiman et al. used folate binding protein from cow's milk to measure serum folic acid levels (Budiman et al., 2018).

Nevertheless, there is currently a lack of knowledge about how to isolate and purify folate binding proteins from breast milk. According to several studies, salting-out and affinity chromatography are adequate methods for isolating folate-binding protein from cells or tissues (O'Shannessy et al., 2011). According to the most recent study, Triton X-100, gel filtration chromatography, ion exchange chromatography, and affinity chromatography are used in the isolation and purification of FBP from breast milk (Holm & Hansen, 2020). The findings of different investigations into the molecular weight of the human folate-binding protein also differ. According to Nygren-Babol, the molecular weight of FBP derived from breast milk is around 27 kDa (Nygren-Babol & Jägerstad, 2012). In 2012, Jaiswal et al. demonstrated that the folate binding protein derived from breast milk has a molecular weight more than 30 kDa (Jaiswal et al., 2012). According to Holm and Hansen, the molecular weight of folate receptors is around 25–29 kDa and is present in breast milk, the reproductive tracts of both sexes, ascitic fluid, cyst fluid, cerebral fluid, and human saliva (Holm & Hansen, 2020). These findings differ slightly from those of a study conducted by O'Shannessy et al., which reported that the folate receptor alpha from human body tissue has a molecular weight of 38 kDa (O'Shannessy et al., 2011).

Different methods are used in the isolation and purification of folate binding protein, mostly from humans. Most of these studies additionally show the various FBP molecular weights. Furthermore, the molecular weight of proteins in gel electrophoresis is not shown in some of this research. In addition, there is currently no published information on the isolation and purification of FBP from breast milk. The presence of this protein in breast milk is significant, and information regarding the molecular weight of FBP is crucial for further research. Therefore, it is essential to conduct studies on the isolation and purification of FBP from breast milk to determine the most efficient techniques and the molecular weight of folate binding protein. This research has the potential to open up further investigations into the role of breast milk FBP in infant folate intake.

EXPERIMENTAL SECTION

Sample

The sample used in this study was 1000 mL of expressed breast milk from breastfeeding mothers in Palembang City. Breast milk was obtained from one healthy breastfeeding mother with a BMI above the normal range, whose infant was aged 2 to 6 months and not under medication. The breastfeeding mothers who participated in this study were selected randomly based on their willingness to participate after providing informed consent. Breast milk collection is carried out in stages over 7 days, with an average volume per day of 150 mL. The sample size in this study was based on previous research for the FBP characteristic assessment test (Budiman et al., 2018; Subandrate et al., 2012). This research has received an ethical certificate from the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Sriwijaya, Protocol No. 041-2023.

Tools and Materials

The tools used in this research were a micropipette (Bio-Rad), analytical balance (Shimadzu), glassware (Pyrex), pH meter (Hanna), filter paper (Whatman), cuvette (Shimadzu), UV-visible spectrophotometry (Shimadzu), centrifugation (DLAB), electrophoresis apparatus (Bio-Rad), Trans-Blot Turbo (Bio-Rad), fraction collector (Bio-Rad), chromatography column (Pyrex), and gel visualization (Shimadzu). The materials used in this research were crystals of $(NH_4)_2SO_4$ (Merck), KH_2PO_4 (Merck), K_2HPO_4 (Merck), ascorbic acid (Merck), acetic acid (Merck), sodium acetate (Merck), distilled water (Onemed), HCl (Merck), NaOH (Merck), NaCl (Merck), DEAE (diethylaminoethyl) cellulose (Solarbio), aminohexyl agarose (Sigma), carbodiimide (Sigma), folic acid (Sigma), mini protein SFX (Bio-Rad), PVDF membrane (Bio-Rad), TGS Buffer (Bio-Rad), TBST Buffer (Bio-Rad), 1% casein/TBS Buffer (Bio-Rad), Laemmli sample buffer (Bio-Rad), mercaptoethanol (Bio-Rad), Opti-4CN substrate kit (Bio-Rad), goat anti-rabbit IgG antibody HRP-conjugated (Bethyl), anti-FBP antibody (MyBioSource), and protein marker (Bio-Rad).

Isolation of FBP

Sample preparation was carried out according to the method used by Budiman et al. and Subandrate et al. (Budiman et al., 2018; Subandrate et al., 2012). A total of 1000 mL of expressed breast milk was centrifuged at 3000 rpm for 20 minutes to separate lactoserum from cream. To obtain whey, the pH of lactoserum was reduced to 4.6 by slowly adding 1 N

HCl and then centrifuging for 10 minutes at a speed of 3000 rpm.

Isolation was carried out using ammonium sulfate modified from the Salter method (Subandrate et al., 2012). Whey was fractionated in stages using 45%, 75%, and 95% saturation of ammonium sulfate. The concentration of ammonium sulfate is selected gradually for gradual protein separation, starting from proteins with molecular weights above 100 kDa, 50-100 kDa, and below 50 kDa. The 45% saturation of ammonium sulfate fraction was filtered to obtain Filtrate I. The 75% saturation ammonium sulfate fraction was centrifuged at a speed of 3000 rpm for 20 minutes to form Supernatant I. The 95% saturation of ammonium sulfate fraction was centrifuged at a speed of 3000 rpm for 20 minutes to form precipitate II. Precipitate II was suspended in 0.02 M phosphate buffer pH 7.2, containing ascorbic acid, and then dialyzed.

Purification of FBP

The purification of FBP was carried out using ion exchange chromatography and affinity chromatography. The ion exchange chromatography used was anion exchange chromatography (DEAE-cellulose column). Folate binding protein is negatively charged at neutral pH. A suitable matrix for column chromatography is a positively charged resin, namely DEAE cellulose. A total of 5 mL of dialysate is inserted into the column and allowed to pass to the bottom of the column. The first elution used 0.02 M phosphate buffer, pH 7.2, until the absorbance was zero at a wavelength of 280 nm. The second and subsequent elutions used 0.02 M phosphate buffer pH 7.2 containing NaCl with a concentration gradient of 5, 15, 30, 40, 60, 120, and 150 mM. The fractions resulting from the second and subsequent elutions were also collected in 2 mL, and then their absorbance was measured with a spectrophotometer at a wavelength of 280 nm. Each peak-forming fraction from the first and second elution results was then collected into one, namely peak fraction I and peak fraction II.

Affinity chromatography uses aminohexyl-agarose bound to folic acid via carbodiimide. Aminohexyl-agarose is a resin that is often used in affinity chromatography because it can be bound with specific ligands according to the type of protein being eluted. Aminohexyl-agarose is also relatively more resistant to pH and temperature fluctuations. Before passing through the affinity chromatography column, the pH of the peak of fractions I and II was adjusted to 3 by adding 1 M HCl, followed by acetate buffer pH 3.5. The peaks of fractions I and II were mixed alternately using an affinity chromatography column. The first elution used 0.02 M phosphate buffer pH 7.2 until the absorbance was zero at a wavelength of 280 nm, and the second elution used acetate buffer pH 3.5 containing 0.5 M NaCl. These fractions are called affinity fraction I and affinity fraction II.

SDS-PAGE and Western Blot

Samples such as marker, whey, peak fraction II, and affinity fraction II were run on SDS-PAGE for 100 minutes at a voltage of 100 volts. The resulting SDS-PAGE gel was then transferred to a PVDF membrane using the semi-dry trans-blot method for 6 minutes. The PVDF membrane was covered with 1% glycine in TBS. Anti-FBP antibody incubation was carried out for 14 hours at 4°C (overnight). The second antibody incubation was carried out for one hour at room temperature. Substrate was added for 30 minutes until a protein band formed. To stop the reaction, the PVDF membrane was washed with distilled water for 15 minutes according to the procedure of Opti-4CN.

RESULTS AND DISCUSSION

In this study, the amount of breast milk used was 1000 mL. Before the isolation process, the breast milk must be treated to obtain whey. At the sample preparation stage, centrifugation of breast milk at 3000 rpm for 20 minutes succeeded in separating cream and lactoserum. The cream clots at the top of the tube, while the lactoserum is at the bottom of the tube. Lactoserum collection was carried out using filter paper. Cream is a fat component found in breast milk. The centrifugation process separates fat from protein because protein has a greater specific gravity than fat (Halder et al., 2022). In general, the composition of breast milk is 87% water, 1% protein (0.9–1.2 g/dL), 4% fat (3.2–3.6 g/dL), 7% carbohydrates (6.7–8 g/dL), and 2% other components (Ballard & Morrow, 2013; Dror & Allen, 2018; Gila-Diaz et al., 2019). In this study, the amount of lactoserum obtained was 830 mL.

In general, lactoserum contains whey protein and casein. The main protein in breast milk is whey (60–70%), while the main protein in cow's milk is casein (Meng et al., 2021). Casein was precipitated by decreasing the pH to get whey in lactoserum. Breast milk has a pH of around 6.4–7.7 (Basdeki et al., 2021; Golan & Assaraf, 2020). The addition of HCl solution reduces the pH of lactoserum to 4.6, resulting in casein precipitation. Casein has an isoelectric point at pH 4.6–4.9, so at this pH, casein precipitates (Blanco et al., 2022). The isoelectric point is the degree of acidity when the number of positive charges and the number of negative charges on a protein are the same, or the degree of acidity when the protein has zero charge (Lautenbach et al., 2021). To increase protein precipitation, lactoserum was centrifuged for 5 minutes at 2000 rpm. The amount of whey obtained in this study was 670 mL.

Isolation of FBP from whey uses the Salter method, namely using 45% and 75% saturation of ammonium sulfate (Subandrate et al., 2012). To achieve 45% saturation of ammonium sulfate in 670 mL of whey, 172.86 grams of ammonium sulfate crystals are needed (Duong-Ly & Gabelli, 2014). In general, 45% saturation of ammonium sulfate can precipitate

proteins with molecular weights above 60 kDa (Duong-Ly & Gabelli, 2014; Grant, 2016; Subandrate et al., 2012). Proteins with a molecular weight below 60 kDa remain suspended in lactoserum. Separation was continued by filtering with Whatman filter paper no. 42 to separate residues (proteins with a molecular weight above 60 kDa) and filtrate I (proteins with a molecular weight below 60 kDa). The amount of filtrate I obtained was 510 mL. The isolation process was continued using 75% saturation of ammonium sulfate. The number of ammonium sulfate crystals required for 510 mL of filtrate I is 96.9 grams (Duong-Ly & Gabelli, 2014). Filtrate I was centrifuged at 3000 rpm for 20 minutes to separate precipitate I and supernatant I. The amount of supernatant I obtained in this study was 490 mL. Previous research stated that FBP could be precipitated with 75% saturation of ammonium sulfate (Subandrate et al., 2012). However, the sample used was cow's milk. Meanwhile, other research states that FBP in cow's milk can be precipitated with a 95% saturation of ammonium sulfate (Budiman et al., 2018). Therefore, the isolation process was continued using 95% saturation of ammonium sulfate. The number of ammonium sulfate crystals required for 490 mL of supernatant I is 67.13 grams (Duong-Ly & Gabelli, 2014). Supernatant I was then centrifuged at 3000 rpm for 20 minutes to obtain precipitate II and supernatant II. The amount of supernatant II obtained was 475 mL. Precipitate II was suspended in 0.02 M phosphate buffer, pH 7.2, and then dialyzed to remove ammonium sulfate.

In this research, protein isolation used ammonium sulfate. Several methods are available for protein isolation, such as trichloroacetic acid (TCA), absolute alcohol, acetone, polyethylene glycol, and dextran sulfate. Holm and Hansen used triton X-100 NaCl (Holm & Hansen, 2020), while O'Shannessy et al. used NaCl (O'Shannessy et al., 2011). Some of these methods are relatively unable to be applied to some proteins because they cause the protein to denature and make it difficult to redissolve. Apart from that, the cost factor is the reason why these materials cannot be used widely (Duong-Ly & Gabelli, 2014; Grant, 2016).

Salting-out is one method of protein precipitation. This method is used because it is relatively easy, cheap, and does not damage the protein structure. The principle of this method is protein precipitation because the gram draws water from the protein. Protein is a hydrophilic colloid, so it is surrounded by a water coat. The larger the size of the protein molecule, the less water it has and the easier it can be deposited. Likewise, the smaller the size of the protein, the more water the coat surrounds it, so it requires a high concentration of salt to precipitate it. Salting-out can use various types of salt, such as sodium chloride, potassium chloride, magnesium sulfate, and ammonium sulfate. Salting-out generally uses ammonium sulfate crystals because ammonium sulfate has the ability to precipitate more quickly compared to other salts such as $MgSO_4$, KCl, and NaCl. To be able to precipitate protein, ammonium sulfate is needed with a concentration of up to 50–100%, depending on the weight of the isolated protein (Grant, 2016; Lee, 2017).

Protein isolation causes the target protein to mix with or bind with the salt used. In protein isolation and purification, salt needs to be removed because the presence of salt interferes with the affinity or interaction of the protein with its ligand. To remove salt from the isolated protein, dialysis is carried out. Dialysis can be considered complete if the sulfate test in the buffer solution is negative or no white precipitate forms (Grant, 2016; Subandrate et al., 2012). In this study, dialysis was carried out for 3 days, with the buffer solution changed twice a day. The results of this dialysis are called dialysate, which is used for the next stage.

FBP fractionation was then carried out using anion exchange chromatography (DEAE chromatography) (Budiman et al., 2018; Subandrate et al., 2012). Protein fractionation with DEAE Chromatography using 0.02 M phosphate buffer pH 7.2 containing NaCl with a concentration gradient of 5, 15, 30, 40, 60, 120, and 150 mM. Fractionation of precipitate I with DEAE chromatography showed that only one peak was formed (Peak I DEAE 75%) (**Figure 1**). Elusion with increasing salt concentration does not show any more protein. Fractionation of precipitate II with DEAE chromatography showed that three peaks were formed (**Figure 2**). Peak I (DEAE 95%) is a protein that has the same charge as the DEAE matrix. Peak II (Peak II DEAE 95%) and Peak III (Peak III DEAE 95%) are proteins that are negatively charged or have a charge that is not the same as the DEAE matrix. Peak II was eluted with a NaCl concentration of 15 mM, and peak III was eluted with a NaCl concentration of 150 mM.

Folate binding protein is a negatively charged protein at neutral pH. To separate folate binding proteins, anion exchange chromatography can be used with diethylaminoethyl (DEAE) and quaternary aminoethyl (QAE) matrix. Purification of folate binding protein from cow's milk often uses DEAE-cellulose chromatography (Budiman et al., 2018; Subandrate et al., 2012), but there are also researchers who use QAE-cellulose chromatography (Holm & Hansen, 2020). The use of salt as an eluent in chromatography causes a high salt concentration in the protein solution. High salt affects the structure, solubility, and function of proteins so that they can be released from the DEAE matrix. The dialysis process is used again to remove salts from protein solutions (Grant, 2016; Lee, 2017; Subandrate et al., 2012).

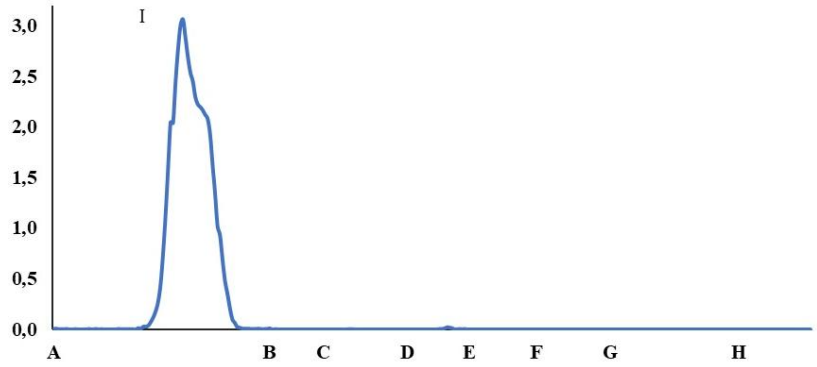

Figure 1. DEAE chromatography of precipitate I (75% saturation of ammonium sulfate). Eluent: A. Phosphate buffer pH 7.2; B. Phosphate buffer pH 7.2 containing NaCl with a concentration gradient of 5 mM; C.15 mM; D. 30 mM; E. 40 mM; F. 60 mM; G. 120 mM; H. 150 mM.

Fractionation of precipitate I by DEAE chromatography showed that only one peak was formed. This strengthens the notion that FBP cannot be precipitated with 75% saturation of ammonium sulfate. The formation of two or more peaks in DEAE chromatography indicates that precipitate II contains a protein thought to be FBP. Peaks II or III are negatively charged proteins that are enabled by FBP. This strengthens the notion that FBP can be precipitated with 95% saturation of ammonium sulfate. There is no data showing the use of ammonium sulfate for isolating protein from breast milk. Some studies do not show the saturation of the salt used (Holm & Hansen, 2020; O'Shannessy et al., 2011). However, research from Budiman et al. and Subandrate et al. shows that FBP from cow's milk can be precipitated with 95% and 75% saturations of ammonium sulfate (Budiman et al., 2018; Subandrate et al., 2012). Thus, the results of this research can be a new reference in isolating folate binding protein from breast milk, namely using a 95% or higher saturation of ammonium sulfate.

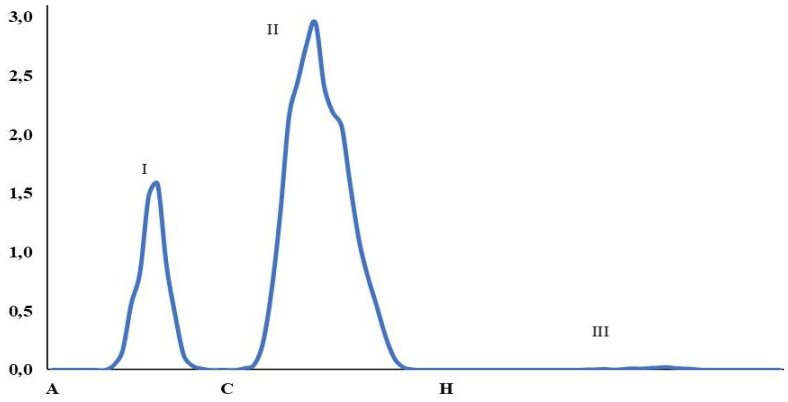

Figure 2. DEAE chromatography of precipitate II (95% saturation of ammonium sulfate). Eluent: A. Phosphate buffer pH 7.2; B. Phosphate buffer pH 7.2 containing NaCl with a concentration gradient of C.15 mM; H. 150 mM.

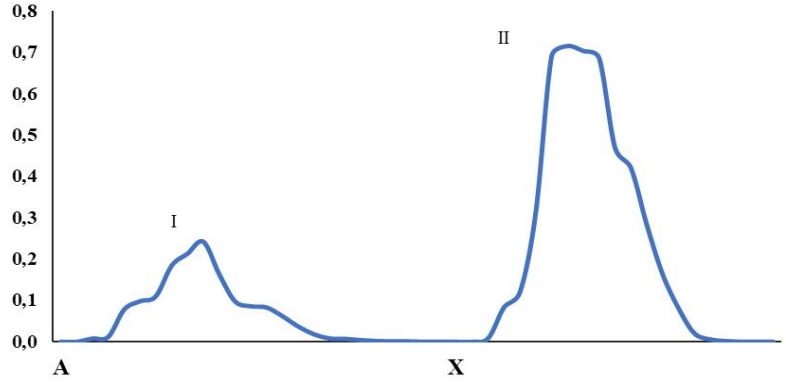

Figure 3. Affinity chromatography of peak II DEAE 95%. Eluent: A. Phosphate buffer pH 7.2; X. Acetate buffer pH 3.5.

To prove that peak II DEAE 95% is a folate binding protein, fractionation was carried out using affinity chromatography. The chromatogram shows the presence of two peaks from affinity chromatography (**Figure 3**). The first peak (Peak I AF 95%) is a protein that does not bind to the affinity chromatography matrix, while the second peak (Peak II AF 95%) is a protein that binds to the affinity chromatography matrix or folate binding protein. Folate binding protein binds to folic acid at a normal pH. In this study, elution used acetate buffers at pH 3.5. At low pH (pH3.5), there is a conformational change in the folate binding protein so that it is released from the folic acid and chromatography matrix (Grant, 2016; Merzel, Frey, et al., 2017; Subandrate et al., 2016; Yi et al., 2013).

Affinity chromatography in this study used a matrix of aminohexyl-agarose and folic acid linked to carbodiimide (Budiman et al., 2018; Puthusseri et al., 2018; Subandrate et al., 2012). Slightly different from the method used by Holm and Hansen which uses CNBr-activated methotrexate-sepharose 4B as an affinity chromatography matrix (Holm & Hansen, 2020; O'Shannessy et al., 2011). O'Shannessy et al. also used a different matrix for affinity chromatography namely anti-folate receptor alpha antibody (O'Shannessy et al., 2011). Folic acid (molecular weight 441.4) and methotrexate (molecular weight 454.4) have a similar structure; namely, they both consist of a pteridine ring, p-aminobenzoic acid, and glutamic acid. The different structures are the groups attached to the pteridine ring and p-aminobenzoic acid. The hydroxyl group in the pteridine ring of folic acid is substituted for the amine group in methotrexate. The 10th nitrogen of p-aminobenzoic acid in methotrexate has an additional methyl group, whereas in folic acid there is no additional group (Lima et al., 2014; Wong & Choi, 2015). The main advantage of affinity chromatography is that the folate binding protein binds very specifically to folic acid, thus providing very clean chromatogram results (Budiman et al., 2018; Grant, 2016; Subandrate et al., 2012).

SDS-PAGE was carried out, followed by a western blot to prove the purity of the isolated FBP. Western blot results showed that peak II AF 95% formed a band that was almost parallel to the 37 kDa marker band. The western blot results also showed the consistency of one band, which was almost parallel to the 37 kDa marker band (Peak II AF95%, Peak II DEAE95%, Precipitate II, and Whey). This proves that the isolated protein was a folate binding protein (**Figure 4**).

The isolated FBP in this study showed a molecular weight of around 37 kDa. This molecular weight is in accordance with the isolation method used, namely 95% saturation of ammonium sulfate (Budiman et al., 2018). This result is somewhat different from research conducted by Nygren-Babol and Jägerstad who found that breast milk FBP has a molecular weight of 27 kDa (Nygren-Babol & Jägerstad, 2012). Meanwhile, Holm and Hansen stated that the molecular weight of FBP is 25 kDA (Holm & Hansen, 2020). However, this research is in line with research by Jaiswal et al., in 2012, which showed that the molecular weight of folate binding protein in breast milk is above 30 kDa kDa (Jaiswal et al., 2012). O'Shannessy et al. also stated that the alpha folate receptor from human body tissue has a molecular weight of 38 kDa (O'Shannessy et al., 2011). These studies stated the molecular weight of FBP only based on the cut-off size of the filtration gel used, not based on SDS-PAGE or western blot results. In this study, the isolation and purification results were confirmed by SDS-PAGE and western blot, thus providing more provable results.

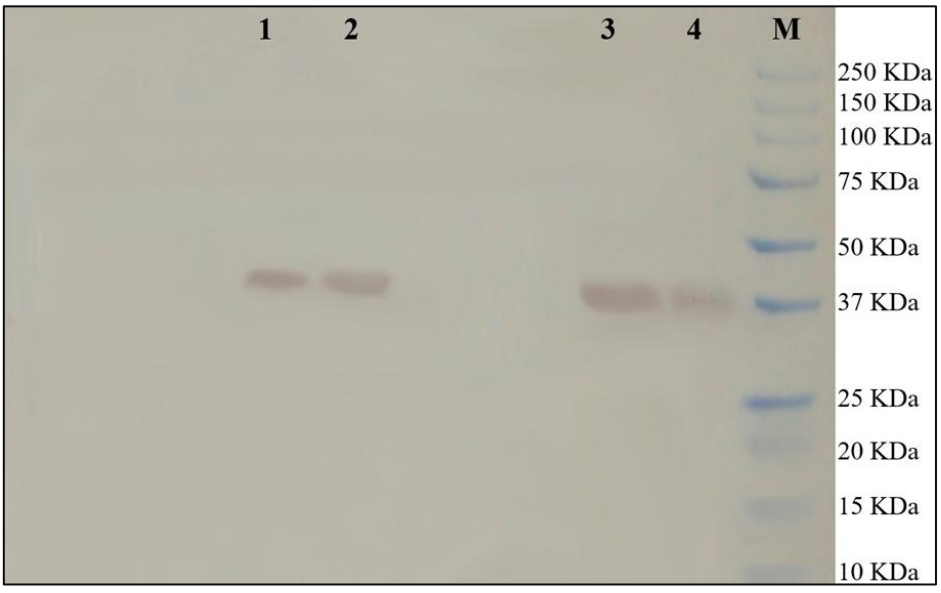

Figure 4. Result of western blot. 1. Peak II AF95%; 2. Peak II DEAE95%; 3. Precipitate II; 4. Whey; M. Marker.

Table 1. Protein isolation and purification

Sample	Volume (mL)	Protein Levels (mg/mL)	Stage
Breast Milk	1000	-	Preparation
Lactoserum	830	10.2	Preparation
Whey	670	7.5	Preparation
Filtrate I (45%)	510	5.8	Isolation
Supernatan I (75%)	490	2.9	Isolation
Sepernatan II (95%)	475	1.4	Isolation
Precipitate II (95%)	22	0.73	Isolation
Peak I DEAE 95%	-	0.10	DEAE-Chromatography
Peak II DEAE 95%	-	0.21	DEAE-Chromatography
Peak III DEAE 95%	-	0.10	DEAE-Chromatography
Peak I AF95%	-	0.18	Affinity Chromatography
Peak II AF95%	-	0.022	Affinity Chromatography

To see protein levels at each stage of isolation and purification, protein levels were measured. During the isolation and purification process, changes in protein levels or activity occur. Protein levels will decrease, activity will decrease, but specific activity will increase (Grant, 2016). Protein levels were measured using the direct or Warburg-Christian method at a wavelength of 280 nm. The Warburg-Christian method is more suitable for measuring protein levels in protein isolation and purification (Grant, 2016; Purwanto, 2014). In the isolation and purification of folate binding protein, all research uses direct methods for measuring protein levels (Budiman et al., 2018; Nygren-Babol & Jägerstad, 2012; Puthusseri et al., 2018; Subandrate et al., 2012). As a standard curve, bovine serum albumin was 1.0 mg/mL, 0.8 mg/mL, 0.6 mg/mL, 0.4 mg/mL, and 0.2 mg/mL ($y = 1.8859x + 0.0369$ with $R^2 = 0.9986$). Protein levels can be seen in **Table 1**. **Table 1** shows that protein levels always decrease according to the isolation and purification stages carried out. The initial protein level in lactoserum was 10.2 mg/mL, then decreased to 7.5 mg/mL in whey, until finally it became 0.022 mg/mL in peak II AF 95%. Merzel et al. stated that there is around 100 nM FBP in breast milk (Merzel, Frey, et al., 2017), while Nygren-Babol and Jägerstad stated that the FBP level in breast milk is around 180–250 nmol/L (Nygren-Babol & Jägerstad, 2012).

This study provided an overview of the amount of FBP in breast milk. The presence of this protein in breast milk may depend on maternal diet and folate levels. Increased folate intake during pregnancy and lactation may contribute to higher levels of FBP in breast milk, which is important for supporting infant growth and development. Additionally, breastfeeding behavior and infant factors may also influence FBP levels in breast milk. Thus, this study not only provides basic information on FBP but also opens up possibilities for further exploration of the relationship between maternal nutrition, breast milk protein levels, and child health (Holm & Hansen, 2020; Li et al., 2021).

In this study, the known characteristics of FBP include a molecular weight of 37 kDa with one monomer. Additional characteristics of FBP can be studied further, such as its binding capacity, amino acid sequence, and the factors that affect its ability to bind folic acid. FBP is a natural folate-binding protein. With the successful isolation of this protein, further development of this discovery includes its potential use in measuring serum folic acid levels. This approach is expected to provide a simple, accurate, and inexpensive method for measuring folic acid (Budiman et al., 2018).

This study focused solely on the technique for obtaining pure folate-binding protein. The salting-out stages utilized in this study are tiered and rather lengthy. There is a need to optimize these simple procedures to achieve faster and more efficient results. Additionally, this study constitutes basic research in the field of biochemistry. Clinical research related to folate levels, breastfeeding behavior, infant aspects, maternal aspects, and nutrition presents significant potential for further exploration.

CONCLUSIONS

Folate binding protein can be isolated from breast milk. Efficient methods that can be used for the isolation and purification of FBP are salting-out with 95% saturation of ammonium sulfate, DEAE chromatography, and affinity chromatography. Breast milk FBP has a molecular weight of approximately 37 kDa. The final FBP level that can be isolated from breast milk is 0.022 mg/mL, for a total of 7 mL. Further research on the characteristics of FBP, its relationship to maternal and infant folate levels and nutrition, as well as its application in developing folate measurement methods, is needed to gain a broader understanding of the role of this protein.

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