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The Role and Isolation Techniques of Breast Milk Folate Binding Protein

Peran dan Teknik Isolasi Protein Ikat Folat ASI

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ABSTRACT

Breast milk folate binding protein has a different structure and molecular weight from cow's milk folate binding protein. The technique of isolation and purification of folate binding protein from various samples is different. This review examines the structure, role, and isolation techniques of breast milk folate-binding protein through a systematic literature review of studies retrieved from ScienceDirect, PubMed, the Directory of Open Access Journals (DOAJ), and Google Scholar, screened using predefined inclusion and exclusion criteria. The keywords used are "folate binding protein" or "folate receptor". The research was conducted from January to July 2025. Breast milk folate binding protein consists of 255-257 amino acids, 4 carbohydrate groups and 16 disulfide bridges. The amino acid sequence alignment between breast milk and cow's milk folate-binding protein shows approximately 74.09% similarity, although structural characteristics may vary across species and isoforms. Folate-binding protein contributes to maintaining folate availability by protecting it from enzymatic degradation, enhancing absorption, and promoting retention. Isolation and purification methods for cow's milk folate-binding protein typically involve salting out, dialysis, and one to two chromatographic techniques, whereas breast milk folate-binding protein is commonly isolated using polyethylene glycol precipitation followed by multiple chromatographic steps. Breast milk folate-binding protein differs in amino acid sequence from that of cow's milk, helps maintain folate availability, can be isolated using different techniques, and requires simpler methods for its purification.

Keywords: *Folate binding protein; Isolation techniques, Purification*.

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1. INTRODUCTION

Folic acid, also known as vitamin B9, is a vital nutrient that plays a crucial role in various bodily functions, particularly in DNA synthesis, repair, and methylation (Acharya et al., 2018; Subandrate et al., 2016). It is especially important during periods of rapid growth, such as during pregnancy and infancy,

as it supports the development of the neural tube in the fetus, significantly reducing the risk of neural tube defects (Dhalape et al., 2020; Sarella and Thammana, 2023). Folic acid can be found in a variety of foods, including leafy green vegetables, legumes, nuts, and fortified cereals, making it an accessible component of a balanced diet (Bhavya et al., 2017; Nygren-Babol and Jägerstad, 2012; Purushothaman et al., 2017). Additionally, it is recommended that women of childbearing age ensure adequate folic acid intake, either through diet or supplementation, to promote optimal health outcomes for both mother and baby (Girija et al., 2017; Purushothaman et al., 2017; Seelam et al., 2021).

Deficiencies in folic acid can lead to serious health issues, including anemia and various complications during pregnancy, underscoring its importance in overall health and wellness (Purushothaman et al., 2017; Sarella and Thammana, 2023). In milk, folate is primarily found in its bioactive form, which can be efficiently absorbed by the body (Wittiarika et al., 2022). Folate binding protein (FBP) plays a crucial role in this context, as it helps facilitate the transport and bioavailability of folate within the milk and into the bloodstream when consumed (Scaranti et al., 2020; Subandrate et al., 2016).

Folate binding protein (FBP) is one of the proteins found in milk, such as cow's milk, goat's milk, and breast milk. Folate binding protein is also present in plasma and intestinal cell membranes. In milk, FBP exists in bound form with folic acid or in free form as particulate. Folate binding protein was first discovered in cow's milk in 1967 by Ghitis. In 1969, FBP was successfully isolated from cow's milk. In addition, FBP can be isolated from various cells, such as intestinal cells and cancer cells. Folate binding protein can also be isolated from foliages. Folate binding protein levels in breast milk are around 160–250 nmol/L (Puthusseri et al., 2018b; Scaranti et al., 2020; Subandrate et al., 2016).

Folate-binding proteins comprise distinct groups with different structures and functions, including soluble folate-binding proteins (FBP), membrane-bound folate receptors (FR), and folate transporters such as the reduced folate carrier (RFC). FRs are glycosylphosphatidylinositol-anchored roteins located on cell membranes that facilitate receptor-mediated folate uptake, while RFC functions as a transmembrane transporter responsible for bidirectional folate transport across cells. In contrast, soluble FBPs, including those found in milk and plasma, act as folate carriers that help maintain folate stability and availability in biological fluids. Together, these systems contribute to the absorption, transport, and distribution of folate in the body (Bayes et al., 2019; Merzel et al., 2017; Subandrate et al., 2016). Some studies suggest that FBP is a glycoprotein. Glycosylation of FBP plays a role in preventing proteolytic digestion in the stomach. Folate binding protein has been shown to tolerate low gastric pH and resist proteolysis in newborn goats. Breast milk FBP may be just as stable in human infants. Experiments using rat intestinal cells have shown that folate absorption is higher as a complex with FBP than in free form, suggesting that FBP may facilitate folate absorption. Folate binding protein can also slow the release and absorption of folate in the small intestine to allow for the gradual release and absorption of folate, which can improve tissue utilization (Merzel et al., 2015; Nawaz and Kipreos, 2022; Subandrate et al., 2016).

Folate binding protein, especially those derived from cow's milk, have been extensively studied. Folate binding protein has a significant sequence similarity to chicken

riboflavin binding protein (RBP). Attempts to report the equilibrium constant of the interaction between folate and FBP have proved inconclusive over the years. The development of biosensor techniques has made it possible to study the binding kinetics of various forms of folate. These proteins have molecular weights in the range of 25,000–35,000 Da, are composed of 222–255 amino acids and have about eight disulfide bonds. This protein interacts with folic acid in the physiological pH range (Holm et al., 2015; Subandrate et al., 2016).

The discovery and role of FBP as a folate binder led to its use in serum folate analysis. Currently, the measurement of serum folic acid by microbiological methods remains the gold standard. A radioprotein binding assay was developed to replace the microbiological or folate assay in clinical samples in the mid-1970s (Bhavya et al., 2017; Dhalape et al., 2020). Another analytical application emerged during the 1980s, when FBP was introduced in affinity chromatography for the concentration and purification of folate from biological samples (Subandrate et al., 2016). Although the structure, role, and applications of folate-binding protein (FBP) from cow's milk have been extensively described, studies on breast milk FBP remain limited and fragmented. Existing literature has not comprehensively integrated structural characteristics, biological roles, and especially isolation and purification techniques specific to breast milk FBP. Therefore, this review aims to synthesize current evidence on breast milk FBP, highlight methodological differences compared to cow's milk FBP, and identify gaps in isolation approaches that warrant further development.

2. METHODS

This is a systematic literature review. The writing of this article comes from reference sources such as ScienceDirect, PubMed, Directory of Open Access Journals, and Google Scholar. Target articles are articles that have the phrases "folate binding protein" or "folate receptor". Articles from these sources were identified and then collected systematically. The articles used as writing sources are articles that are directly related to the topic searched for from 2015 to 2025. These articles are then reviewed, and general conclusions are drawn and then described in a new narrative form. The research was carried out from January to July 2025. Studies were included if they met predefined criteria (original research, 2015–2025, relevant to folate-binding protein/receptor, full-text available), while studies were excluded due to duplication, irrelevance based on title and abstract screening, or insufficient data upon full-text review. Searches for articles conducted through ScienceDirect, PubMed, Directory of Open Access Journals, and Google Scholar using the keywords "folate binding protein", or "folate receptor" result in 2,870 articles. After sorting the articles according to relevance, 124 articles were found related to the search topic. To narrow down the search topic, filtering was carried out using titles and abstracts to obtain 40 articles.

The results obtained were three research articles. The data extracted from the article are the author's name, year of publication, sample source, isolation and purification method, and SDS-PAGE results. Articles from Google Scholar, Directory of Open Access Journals, PubMed, and ScienceDirect that have been compiled for relevance to the search topic are then selected again according to the needs of writing this literature topic. The selection also saw the repetition of articles from each source. The final selection results use three reference sources from these sources (**Fig. 1**).

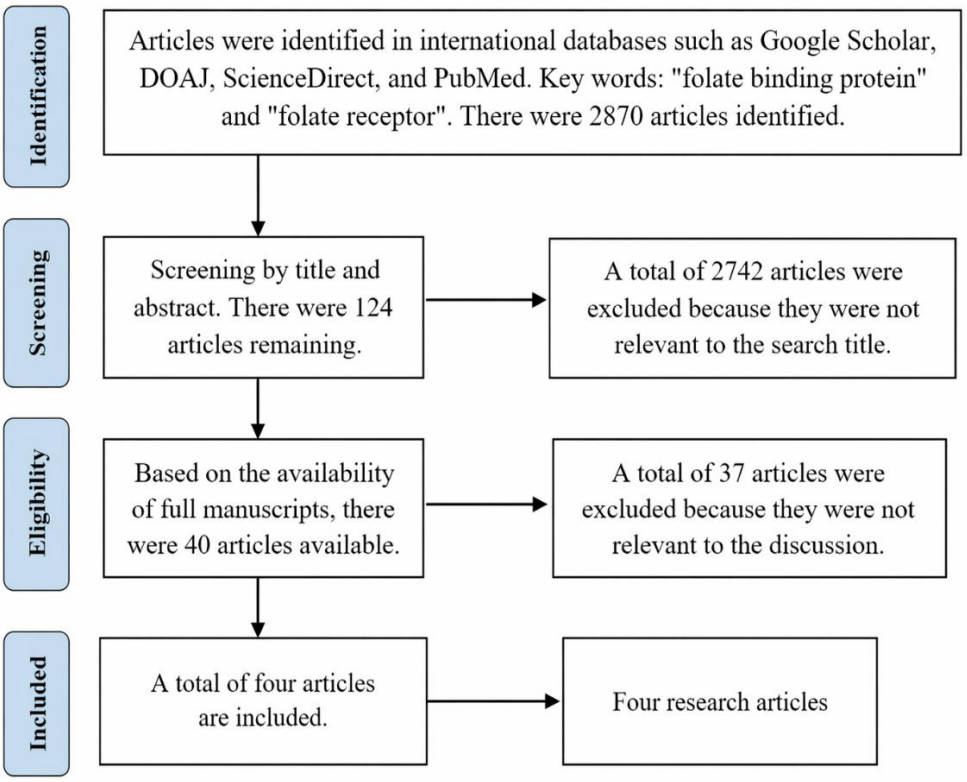

Figure 1. The flow of database searches of publications

3 RESULTS AND DISCUSSION

Folate-binding protein (FBP) plays a central role in folate transport and distribution and has been identified in various mammalian cells, extracellular fluids, and tissues. Although FBP is often used interchangeably with folate receptor (FR), these entities differ in form and function, with FRs (FR-α, FR-β, and FR-γ) representing membrane-bound or soluble isoforms with distinct biological roles.

Breast milk FBP is generally classified as FR-α; however, structural and functional variability has been reported across studies, suggesting possible differences in isoform expression and post-translational modifications (Holm and Hansen, 2020; Subandrate et al., 2016; Wang et al., 2018). Quantitative studies indicate that FBP levels in cow's milk (~250 nmol/L) are comparable to those in breast milk (180–250 nmol/L), although variations may arise due to differences in analytical methods, sample handling, and lactation stages (He et al., 2016; Subandrate et al., 2016).

Structurally, breast milk FBP differs from cow's milk FBP in molecular weight and amino acid composition, which may influence binding affinity and stability, although mechanistic explanations for these differences remain limited (**Fig. 2, Fig. 3, Table 1**).

Methodologically, isolation techniques vary substantially between studies. Cow's milk FBP is commonly isolated using salting-out and dialysis followed by chromatographic purification, whereas breast milk FBP often requires polyethylene glycol precipitation and more complex chromatographic steps. These differences may reflect variations in protein composition and matrix complexity, but also highlight a lack of standardized protocols.

SDS-PAGE is widely used to confirm purification; however, inconsistencies in reported band patterns suggest variability in purity and possible protein degradation (**Table 2**). Importantly, there is a lack of comparative studies directly evaluating isolation efficiency, structural integrity, and functional activity between methods. Future research should focus on developing standardized, simplified isolation techniques and clarifying the relationship between structural variation and biological function of breast milk FBP.

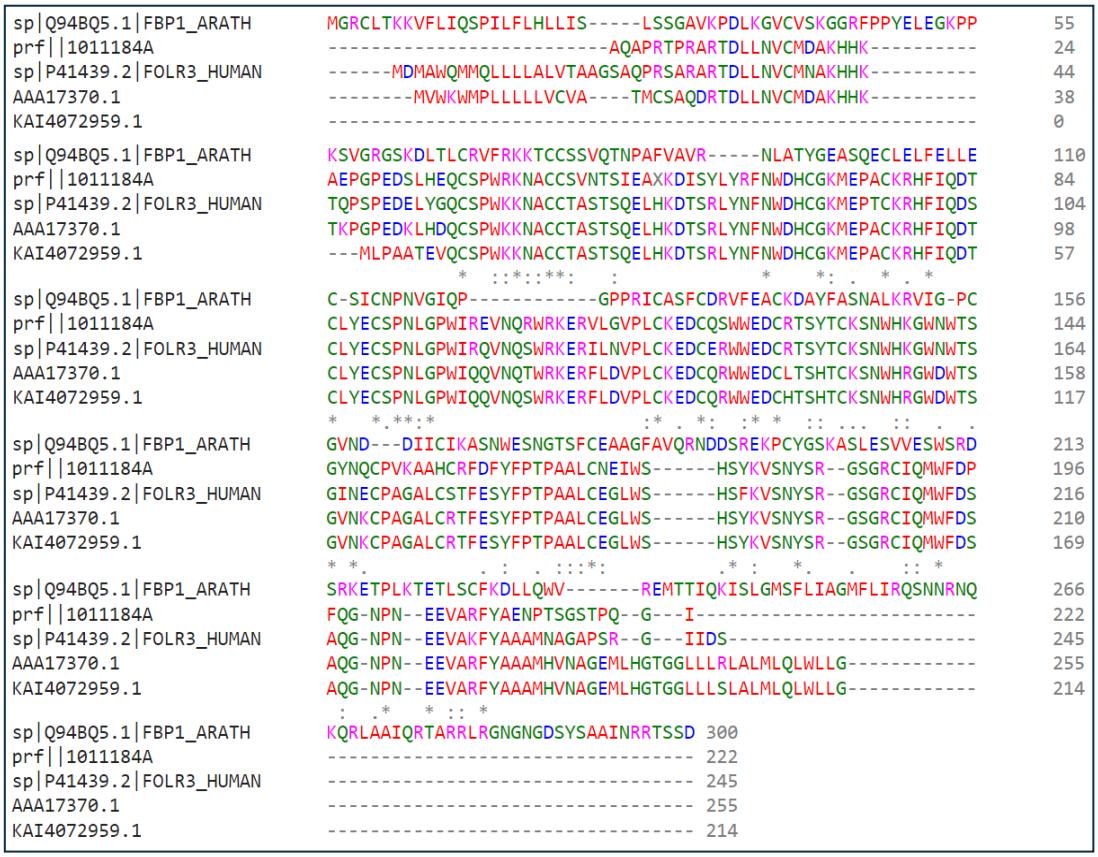

Figure. 2. Amino Acid Chain Alignment Test for Folate Binding Proteins. sp|Q94BQ5.1: FBP from plant leaves, prf||1011184A: FBP from Cow's Milk, sp|P41439.2: FR-γ, AAA17370.1: FBP, KAI4072959.1: FR-β (EBI, 2022).

3.1 Structure of folate binding protein

Folate-binding protein in cow's milk consists of approximately 220–237 amino acids with a molecular weight of about 25–34 kDa. Its concentration in cow's milk has been reported in the range of 10–60 mg/kg (approximately 10 mg/L). Structurally, FBP contains multiple disulfide bonds—typically around 8 disulfide bridges (formed by conserved cysteine residues)—and is glycosylated at specific asparagine residues (e.g., Asn49 and Asn141). The carbohydrate moiety, which accounts for approximately 10% of the total molecular mass, mainly consists of N-acetylglucosamine, N-acetylgalactosamine, fucose, mannose, galactose, and sialic acid. (Holm et al., 2015; Subandrate et al., 2016).

Folate binding protein from cow's milk has an amino acid sequence like the amino acid sequence of chicken riboflavin binding protein (RBP). All tryptophan residues and sixteen cysteine residues in FBP are also present in RBP. This indicates that there are structural and functional similarities between FBP and RBP. In RBP, all cysteine residues and some tryptophan residues are in the vicinity of the ligand-binding domain. Therefore, the binding site between FBP and folic acid may also be at the amino acid position. This is due to the structural similarity of folic acid and riboflavin, both of which have a hydrophobic aromatic ring (Chen et al., 2016; Holm and Hansen, 2020; Merzel et al., 2017; Subandrate et al., 2016). A previous study conducted by Subandrate et al. indicates that the active group of FBP contains an imidazole or a sulfhydryl group. In addition, the disulfide bridge in FBP is important for maintaining the three-dimensional structure of folic acid binding to FBP. The binding of FBP to folic acid does not require calcium ions as a cofactor. Folate binding protein is negatively charged at neutral pH, soluble in milk and other body fluids, and has resistance to digestive enzymes. Folate binding protein, isolated from cows, has a constant affinity for folic acid. The degree of bond dissociation between FBP and folic acid is 10-9-10-10 M, and the stoichiometric ratio between FBP and folic acid is 1:1 mol (Holm et al., 2015; Subandrate et al., 2016).

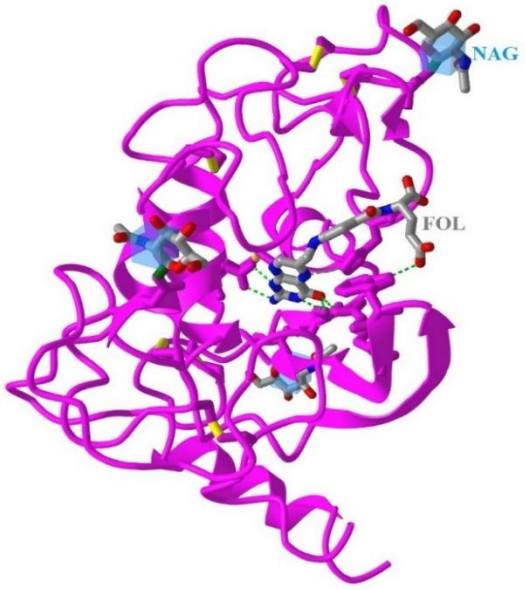

Figure 3. Crystallographic structure of FBP (4LRH). Folic acid is gray (FOL), N-Acetyl-D-Glucosamines are blue (NAG), disulphide bonds are yellow, folate binding sites are orange, and the chemical bond between folic acid and FBP is the dotted green line (Ke et al., 2016; NCBI, 2022).

In humans, FBP consists of three protein families, namely alpha folate receptors, beta folate receptors, and gamma folate receptors. Alpha folate receptors consist of 255-257 amino acids, beta folate receptors consist of 214 amino acids, and gamma folate receptors consist of 245 amino acids (Chen et al., 2016; EBI, 2022; Ke et al., 2016; NCBI, 2022). An amino acid sequence alignment between FBP (folate receptor alpha) and folate receptor beta, folate receptor gamma, cow's milk FBP, and plant leaf FBP showed sequence similarities of

94.86%, 81.17%, 74.09%, and 19.56%, respectively (**Fig. 2**). The alignment was performed using sequences retrieved from the NCBI and EBI databases and analyzed using a pairwise alignment tool (e.g., BLASTp/Clustal Omega) with default parameters (**Fig. 2**) (EBI, 2022; NCBI, 2022).

Folate binding protein does not require ions to bind folic acid, while FR-γ requires chloride and potassium ions. About 20% of FBP is glycosylated. Folate binding protein has four carbohydrate groups (N-acetylglucosamine) and sixteen disulfide bridges (Fig. 3) (Chen et al., 2016; EBI, 2022; Jiang et al., 2020; NCBI, 2022). At high concentrations and low ionic strength, pure FBP precipitates rapidly. Folate binding protein binds folate tightly in the pH range of 5.5 to 8.0 (1 mole of folate per mole of protein at pH 7.2) (Chen et al., 2020; Holm and Hansen, 2020; Merzel et al., 2017).

Folate binding protein releases folate at an acidic pH but retains its binding activity and can re-form the folate complex after neutralization (Chen et al., 2020; Wang et al., 2018; Wynn and Sheehy, 2022). The observed differences between cow's milk and breast milk folate-binding proteins are likely attributable to a combination of species-specific variation, the presence of different folate receptor isoforms, and post-translational modifications such as glycosylation and disulfide bond formation, which may influence molecular weight, amino acid length, and carbohydrate composition. Comparison of the structure of FBP from cow's milk and breast milk (**Table 1**).

Table 1. Comparison of the structure of cow's milk and breast milk FBP

Folate binding protein	Cow's milk (Holm et al., 2015; Subandrate et al., 2016)	Breast milk (Holm and Hansen, 2020; Subandrate et al., 2025)
Levels	250 nmol/L	160-250 nmol/L
Amino acids	220-237	255-257
Molecular weight	25-34 kDa	25-29 kDa/37 kDa
Disulfide bonds	6-8	16
Carbohydrates	10%	20%
The main sugar	N-acetylglucosamine, N-acetylgalactosamine, fucose, mannose, galactose, sialic acid	N-acetylglucosamine

3.2 Role of folate binding protein

Folate binding protein has several important roles in the regulation of the bioavailability and absorption of dietary folate (Puthusseri et al., 2018b; Subandrate et al., 2016). The primary function of FBP is to take folate from the blood and secure an adequate folate supply for the neonate, despite the relatively low concentration of folate in breast milk(Lwin et al., 2022). Folate transport in serum or milk is thought to be a physiological function of soluble FBP. Particulate FBP is found in cell membranes and plays a role during membrane transport (Froese et al., 2019; Holm and Hansen, 2020; Subandrate et al., 2016). The role of FBP as a folate binder in intestinal has been well documented in several studies. Experiments using rat intestinal cells have shown that folate absorption is higher in the FBP complex in comparison to the free form. This confirms the evidence that FBP can facilitate folate

absorption (Alpers, 2016; Maruvada et al., 2020). Similarly, evidence from goat studies suggests that FBP-bound folate plays a physiological role through its absorption during lactation, potentially contributing to neonatal folate supply. In humans, breast milk folate bound to protein is also suggested to be absorbed in relatively intact form through the infant's permeable intestine, with reported concentrations ranging from 109 to 204 nmol/L, which are higher than maternal plasma levels.

However, much of the mechanistic evidence is derived from animal models, and caution is needed when extrapolating these findings to human infants due to species differences in intestinal permeability, folate metabolism, and receptor expression (Holm and Hansen, 2020; Maruvada et al., 2020). There is a positive relationship between breast milk folate and folate binding protein concentrations. The folate binding capacity of breast milk is reported to range between 180 and 270 nmol/L. Excess folate binding capacity may concentrate breast milk folate for secretion against a concentration gradient. Plasma folate concentrations in breastfed infants are generally high (45–68 nmol/L) in the first 6 months of life and decline to 23–45 nmol/L at 12 months when foods other than breast milk are consumed (Holm and Hansen, 2020; Subandrate et al., 2016). This shift demonstrates the important role of FBP in increasing folate bioavailability from breast milk.

Studies in rats using the folate complex of cow's milk FBP show that although FBP releases folate under gastric acid conditions, the complex is regenerated in the jejunum. However, FBP-bound folate is poorly absorbed in the jejunum, whereas free folate is rapidly absorbed. Both free and bound forms of folate are absorbed at the same rate in the ileum. The rapid rate of absorption of free folate leads to high blood folate levels, which, in turn, leads to rapid urinary and biliary folate excretion (Alpers, 2016). The lower controlled blood folate levels achieved using FBP-bound folate increase folate retention time, enabling it to reach its target tissues. Thus, FBP can be said to increase folate retention and bioavailability (Hansen et al., 2015; Sensharma et al., 2018; Subandrate et al., 2016).

Goat's milk FBP inhibits enzymatic digestion in the gastrointestinal system and mediates folate absorption through interactions with particular receptors at the intestinal brush border, according to research on the impact of goat's milk FBP on folate absorption in neonatal goats. The mucosal surface microenvironment's acidic circumstances make it easier for FBP receptors to bind and enable the release of bound folate.

Folate binding protein also increases folate bioavailability by protecting bound folate from folate-requiring gut bacteria as well as sequestering some folate synthesized by other microorganisms in the gut microflora from the colon. Thus, milk FBP may contribute in several ways to maintaining the folate status of the neonate (Bayes et al., 2019; Maruvada et al., 2020; Sensharma et al., 2018; Subandrate et al., 2016).

The role of folate binding protein is not limited to its role in the body as a carrier or receptor for folate absorption. Folate binding protein has been known to increase in uterine and ovarian cancer, so it is used as a marker in determining the diagnosis of uterine and ovarian cancer (Saito et al., 2023).

In addition to its nutritional role, it is important to distinguish milk-derived folate-binding protein (FBP) from cellular folate receptors (FRs), which are widely studied in disease contexts. While milk FBP primarily functions as a soluble carrier that maintains

folate stability and availability, membrane-bound FRs—particularly FR-α and FR-β—have been explored as therapeutic targets in cancer and inflammatory diseases. For example, FR-α is overexpressed in certain solid tumors, including ovarian cancer, making it a target for antifolate-based chemotherapy (Lutz, 2015; Varaganti et al., 2023; Young et al., 2023) and drug delivery systems (Thai et al., 2022). Similarly, FR-β expressed on activated macrophages has been investigated as a delivery route for antifolate therapy in rheumatoid arthritis (Jiang et al., 2023; Merzel et al., 2017, 2015). However, these applications relate to membrane-bound folate receptors rather than milk-derived FBP and should be interpreted within that distinct biological context.

Knowledge of the structure and role of FBP as a folate binding protein has prompted efforts to develop FBP for serum folate analysis. Currently, the gold standard method for serum folate measurement is the microbiological assay. In the mid-1970s, radio-protein binding assays were developed to replace folic acid assays in microbiological clinical samples (Bhavya et al., 2017; Dhalape et al., 2020; Savitha and Ravichandran, 2020; Seelam et al., 2021). In the 1980s, FBP was introduced through affinity chromatography for the purification of folic acid from biological materials, resulting in the emergence of its applications for testing folate levels in clinical samples (Subandrate et al., 2016). Previous study succeeded in using folate binding proteins from cow's milk to measure serum folic acid levels. In this study, standard serum folic acid levels ranged from 25–100 ng/mL, whereas normal serum folic acid levels were between 6–20 ng/mL. In addition, this study used folate binding proteins from impure cow's milk (Budiman et al., 2018; Subandrate et al., 2016). In 2018, Budiman et al. used folate binding proteins from purified cow's milk. The results showed that folate binding proteins from cow's milk can be used to measure serum folic acid levels.

These results also indicate that the measurement with folate binding protein produces relatively similar outcomes to the ELISA method. Budiman et al. used the enzyme-labeled protein ligand binding assay (ELPLBA) method (Budiman et al., 2018). In 2020, Chen et al. measurd serum folic acid levels using ruthenium-labeled folate binding protein (Chen et al., 2020).

3.3. Folate binding protein isolation and purification techniques

Isolation of folate-binding protein (FBP) presents specific challenges due to its low abundance, glycosylation, and strong binding affinity to folate, which can affect recovery and stability. In cow’s milk, FBP is relatively easier to isolate using salting-out and dialysis followed by chromatographic purification, with generally higher yield and purity. In contrast, breast milk FBP requires more complex approaches, such as polyethylene glycol precipitation combined with multiple chromatographic steps, likely due to differences in protein composition and matrix complexity. Variations in reported yields and purity across studies reflect differences in isolation protocols, highlighting the need for more efficient and standardized methods, particularly for breast milk FBP. (Dharmatti et al., 2018; Grant, 2016; Kim et al., 2019).

Table 2. Comparison of folate binding protein isolation and purification techniques

Author	Sample	Isolation	Purification	SDS-PAGE
Holm dan Hansen (2020).	Breast Milk/Serum	Polyethylene glycol (Triton X-100)	Gel filtration chromatography, cation-anion exchange chromatography, and ethotrexate affinity chromatography	25 kDa/ 29 kDa
Budiman et al (2018)	Cow's Milk	Salting out by (NH4)2SO4	DEAE-cellulose chromatography and affinity chromatography using aminohexyl-agarose and folic acid linked using carbodiimide	25-35 kDa
Puthusseri et al (2018)	Arabidopsis plant leaves	Salting out by CaCl2 and polyethylene glycol (Triton X-100)	Methotrexate-sepharose 4B CNBr-activated affinity chromatography	43 kDa
Subandrate et al (2025)	Breast Milk	Salting out by $(NH_4)_2SO_4$	DEAE-cellulose chromatography and affinity chromatography using aminohexyl-agarose and folic acid linked using carbodiimide	37 kDa

Protein isolation is not a simple and short process. Apart from the source factor, there are several reasons that may complicate protein isolation. First, there are no general properties that can be applied in protein purification, unlike the nucleic acid isolation method, which can use a positive charge adsorption technique owing to nucleic acids' negative charge. In protein isolation, the isolation principle is limited by the specific hydrophobicity or electrostatic properties that can be found in various molecules, including lipids, nucleic acids, and carbohydrates. Second, the isolated protein content tends to be low.

In some cases, the target protein may be present in picomole or femtomole concentrations. Spectroscopic or colorimetric methods usually require protein samples with concentrations ranging from 0.01 to 1 mM (or up to tens of mg/mL). Third, there is no way to increase protein quantity. If a protein is not produced in excess, it can be lost continuously during the isolation process, to the point of becoming undetectable at the final analysis stage. Fourth, the problem of contamination is inevitable. Certain contaminant proteins are a very large part of the proteins present in cells. Proteins classified as contaminants are often present in significantly greater amounts than the target protein, which often makes data analysis difficult. Fifth, proteins are unstable. Proteins can be degraded by enzymes both in vivo and in vitro. It is not easy to inhibit all proteolytic activity by adding a protease inhibitor. If factors such as temperature, pH, or salt concentration are not appropriate, the protein will most likely be denatured. If the experimental conditions of the buffer are not optimal, the protein may aggregate before or after analysis (Holm and Hansen, 2020; Lee, 2017; Subandrate et al., 2016).

In general, the stages of protein isolation and purification consist of salting out, dialysis, chromatography, and electrophoresis. Isolation and purification of folate binding proteins also follow these steps. Previous study stated that in general, researchers use salting out when isolating folate binding proteins from milk, either breast milk, cow's milk, or goat's milk. The most commonly used chromatography in the isolation of FBP is affinity chromatography, which can be optimized by ion exchange chromatography or

column chromatography (**Table 2**) (Holm and Hansen, 2020; Lee, 2017; Subandrate et al., 2016).

Table 2 shows that different isolation and purification strategies yield varying molecular weight profiles and likely reflect differences in purity and protein integrity. Affinity chromatography, particularly using folate or methotrexate ligands, appears to provide higher specificity for FBP compared to conventional methods such as salting-out and ion-exchange chromatography, although it requires more complex preparation and higher cost.

Polyethylene glycol precipitation is frequently used in breast milk samples to improve protein recovery from complex matrices, but may result in lower purity without subsequent chromatographic steps. In contrast, ammonium sulfate fractionation combined with ion-exchange chromatography is relatively simple and cost-effective but may yield lower specificity. However, direct comparisons of yield and purification efficiency across studies remain limited due to differences in protocols and reporting, highlighting the need for standardized evaluation of FBP isolation methods.

Budiman et al. succeeded in isolating folate binding protein from cow's milk using ammonium sulfate at a concentration of 75% (salting out) (Budiman et al., 2018). Puthusseri et al. succeeded in isolating the folate binding protein from Arabidopsis leaves by salting them out using $CaCl_2$ and polyethylene glycol (Triton X-100)(Puthusseri et al., 2018a). Meanwhile, Holm and Hansen isolated folate binding proteins from breast milk or serum in 2020 using polyethylene glycol (Triton X-100) (Holm and Hansen, 2020). In contrast, Subandrate et al. (2015) successfully isolated folate-binding protein from expressed human milk using 95% ammonium sulfate precipitation (Subandrate et al., 2025).

Salting out is one method of protein isolation. This method is used because it is relatively easy, inexpensive, and does not damage the protein structure. Salting out generally uses ammonium sulfate crystals. The principle of this method is protein deposition due to the attraction of water from the protein. To be able to precipitate protein, ammonium sulfate is required at a concentration of up to 50–75%, depending on the weight of the isolated protein. The removal of salt from isolating proteins is known as dialysis. Dialysis is carried out using a cellophane bag so that salt (small molecular weight) can diffuse out of the protein solution (Holm et al., 2015; Lee, 2017; Subandrate et al., 2016).

The purification of folate binding proteins can be carried out using various chromatographic techniques. Budiman et al. and Subandrate et al. purified folate-binding protein from cow's milk and breast milk, respectively, using two chromatographic techniques: anion-exchange chromatography and affinity chromatography (Budiman et al., 2018; Subandrate et al., 2025). Meanwhile, Puthusseri et al. succeeded in purifying folate binding proteins from plant leaves just by affinity chromatography (Puthusseri et al., 2018a).

In contrast to Budiman et al. and Puthusseri et al., Holm & Hansen and Puthusseri et al. used three types of chromatography to purify folate binding proteins from breast milk or serum, namely gel filtration chromatography, cation-anion exchange chromatography, and affinity chromatography (Budiman et al., 2018; Holm and Hansen, 2020; Puthusseri et al., 2018a).

The affinity chromatography used by Budiman et al. was aminohexyl-agarose and folic acid linked using carbodiimide, while Puthusseri et al. and Holm & Hansen used methotrexate-sepharose 4B, which was activated with CNBr, as the affinity chromatography matrix (Budiman et al., 2018; Holm and Hansen, 2020; Puthusseri et al., 2018a). Folic acid and methotrexate have a similar structure in that 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 (Grant, 2016).

Chromatography is a molecular separation technique based on differences in the pattern of movement between the mobile phase and the stationary phase. In protein isolation and purification, the purification process not only measures molecular levels but also collects isolated proteins. Therefore, the chromatography used should not be one that discards the results of the separation or only measures concentration. In this case, chromatography methods that are generally suitable for protein purification include gel filtration chromatography, ion exchange chromatography, and affinity chromatography (Dharmatti et al., 2018; Holm et al., 2015; Lee, 2017).

The final step in protein isolation and purification is to assess protein purity, commonly performed using SDS-PAGE, which separates proteins based on their apparent molecular weight. FBP from cow's milk typically appears as a single band at approximately 25–35 kDa (Budiman et al., 2018; Holm et al., 2015), while FBP from plant leaves is reported around 43 kDa (Puthusseri et al., 2018a), and FBP from breast milk or human serum ranges from approximately 25 to 37 kDa. These variations in apparent molecular weight may reflect not only differences in amino acid sequence but also post-translational modifications, particularly glycosylation, which can significantly alter protein mobility in SDS-PAGE. Western blot analysis can be used to confirm protein identity through specific antigen–antibody interactions (Lee, 2017; Subandrate et al., 2025). Therefore, accurate determination of FBP molecular characteristics requires complementary approaches, including protein sequencing and characterization of post-translational modifications.

4. CONCLUSIONS

Breast milk folate-binding protein (FBP) demonstrates structural differences compared to cow's milk FBP, which are attributable not only to variations in amino acid sequence but also to differences in post-translational modifications such as glycosylation. Across studies, FBP consistently functions to maintain folate stability and availability in biological fluids, although its precise role in intestinal folate absorption in human infants remains incompletely understood, with current evidence largely derived from animal models.

Isolation and purification of FBP from both cow's milk and breast milk have been successfully achieved using combinations of precipitation and chromatographic techniques; however, breast milk FBP generally requires more complex procedures, reflecting differences in sample composition and protein characteristics. Despite these advances, direct comparisons of yield, purity, and functional integrity across methods are limited due to variability in protocols and reporting. Key knowledge gaps include the lack of standardized isolation techniques for breast milk FBP, insufficient characterization of its structure–function relationship, and limited human-based evidence on its role in folate absorption. Future research should focus on

developing simplified and reproducible purification methods, conducting comparative methodological studies with quantitative yield and purity metrics, and elucidating the physiological role of FBP in human infants.

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