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Properties of Folate Binding Protein Purified from Cow's Milk

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Folic acid played an important role in the metabolism of the body. To measure the serum folic acid levels could use the folate binding protein (FBP) from cow's milk with a technique analogous to ELISA. The aims of this study were to identify characteristics of FBP from cow's milk and binding capacity of FBP to folic acid and to purify FBP from other whey protein passed through DEAE-cellulose chromatography column. Each of DEAE-cellulose peaks was passed in affinity chromatography column. FBP was released from affinity column with sodium acetate buffer pH 3.5. The purity of obtained FBP was demonstrated by a single spot in SDS-PAGE analysis and the estimated molecular weight of FBP was around 31 kDa. Our study indicated that 1 mol FBP bound 1 mol folic acid. Alkylation with iodoacetic acid decreased the binding capacity of FBP which suggested the presence of a–SH or imidazol group in its active site. The importance of disulfide bridge was proven by decreasing of folate binding capacity of FBP after β-mercaptoethanol treatment. In contrary, the folate binding didn need Ca^{2+} ion, as indicated by EDTA test which gave the same result as control.

Key words: folate binding protein, folic acid, binding capacity

INTRODUCTION

Folate is an indispensible vitamin in our daily food. Active form of folate, tetrahydrofolate (THF), is coenzyme in various reaction involving one carbon moiety transfer (Methyl, methylene, methenyl, formyl, and formimino), whereas, CO_2 transfer needs biotin as coenzyme. Therefore, folate coenzyme is needed in nucleic acid synthesis, myelin synthesis, methionine metabolism and choline formation (Bailey 2010). Consequently, folate deficiency will disturb cell proliferation and differentiation. In blood, folate deficiency can cause megaloblastic anemia. In central nerve system, especially when it occurs during fetal life, it can lead to neural tube defect as spina bifida or event an anencephaly (Purwani & Zulaekah 2008; Bailey 2010). It is also reported that folate deficiency could affect other tissues with high cell turn over, i.e. carcinoma colorectal (Kim 2004; Babol 2007). On the other hand, excess of folate consumption is associated with the increase of the risk various malignancies and facilitates transformation from pre-malignancies into malignancies (Kim 2004; Sweeney *et al.* 2007; Sauer *et al.*2009; Bailey 2010).

It is clear from this information that the level of serum folate should be monitored especially in pregnancy, in neural disease and also in malignancy. Currently, folate is assayed by microbiological method (using *L. casei*), HPLC, radioassay, or immunoluminescence techniques. Unfortunately, all of these available folate assay are cumbersome, expensive, need special facilities or well, special trained laboratory technician. To meet the need of a simple, but specific and reliable folate assay, we can use an ELISA technique using specific antibodies toward folate itself, which is not available immediately and must be prepare in animals (Sralsjo *et al.* 2002; Nygren *et al*. 2003; Nixon *et al*. 2004; Mardiana 2005).

Several decades ago, some reports described the presence of a specific, non-antibody protein in cow's milk, which could recognize and bind specifically folic acid (Svendsen *et al.* 1979; Holm & Hansen 2001, 2003; Verwei *et al*. 2004). Some authors has used this folate binding protein (FBP) for developing a protein-ligand specific binding radioassay (Salter *et al*. 1981). Inspired by this, our laboratory has used the FBP for developing an ELISA analog assay, i.e enzyme labeled protein-ligand binding assay. The system goes well, it could detected folic acid as low as 26.4 ng/ml (0.059 μM) (Mardiana 2005). However, the detailed characters of the FBP are not all described yet. It is clear, that for using FBP as a reactant in a specific assay, some of its physicochemical properties should be well known.

The objective of our study was to identify physicochemical characters of FBP, especially molecular weight of the exact, its folate binding capacity, the need of ionic calcium for its activity, the role of disulfide bridge and also the effect of alkylating agent for its activity.

MATERIALS AND METHODS

Folate Binding Protein Isolation. Cow milk was obtained from local farm. FBP was isolated according to method described by Salter *et al.* (1981). Two liters fresh cow's milk was centrifuged at 2000 rpm for 35 minutes in a refrigerated centrifuge. The cream was removed and into 1850 ml lactoserum was added HCl 1 N until the pH value

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reach 4.6. The mixture was centrifuged at 2000 rpm for 10 minutes in a refrigerated centrifuge. The casein precipitate was removed by aspirating the supernatant (whey). Into 1520 ml whey was added 250 g ammonium sulphate/l solution (45% saturation). After 4 hour in 4 °C, the solution was filtered through Whatman paper no 42. The filtrate was collected and 190 g ammonium sulphate was added/l solution (95% saturation). Precipitate was collected and dialyzed against 0.02 M phosphate buffer pH 7.2 containing ascorbic acid in 4 °C for 7 days.

DEAE-Cellulose Ion-Exchange Chromatography. Twelve grams of resin was swelled in 100 ml distilled water for 1 hour at room temperature (25 °C). At the end of this periode, the supernatant was decanted and the resin was treated extensively according to procedure described by Sigma. Briefly, the resin was washed repeatedly with great volume various solution containing salt, NaOH and HCl. Before using, the difference of pH between the resin and the solution should not exceed 0.15. The resin was poured carefully into a 30 x 1.5 cm column. Then, phosphate buffer 7.2 was added onto the gel until the flow was stable (A_{280} = 0). Two ml filtrate from the dialysis bag were placed onto the gel. The bound protein were eluted firstly with phosphate buffer pH 7.2. Fractions of 2 ml were collected and A_{280} was read. If the A_{280} = 0, the 2nd elution was made by adding phosphate buffer pH 7.2 containing 5 mM NaCl. The fraction collection continued until A_{280} gave a constant value. The elution continued progressively with phosphate buffer pH 7.2 containing 10, 20, 30, and 40 mM NaCl. Fractions of the peak were pooled for the further analysis.

Affinity Chromatography. Folic acid was immobilized on to pre-activated agarose. For each 1 ml pre-activated agarose, 0.47 µmol folic acid was added drop by drop on to stirred agarose suspension. Then the free sites of agarose were blocked by addition ethanolamine. After free washed with large of phosphate buffer pH 7.2, peak of pool fraction was added and mixed well by stirring the mixture at room temperature (25 °C) for 3 hours. Then, all the mixtures were poured into a column and fractions of 1.5 ml were collected and A_{280} of each fraction was measured when the absorban was 0, the column washed again with the same buffer. After the washing, 0.1 M acetate buffer pH 3.5 was added onto column and the fraction of 1.5 ml were collected. A_{280} was measured and each fraction was pooled.

SDS-PAGE 10%. Filtrate of 45% ammonium sulphate, filtrate of dialysis 95% precipitate and peak two of affinity chromatography was runned in SDS-PAGE 10% together with molecular weight marker protein and BSA. The gel was colored by Coomasie brilliant blue solution. The molecular weight (MW) of FBP was determined by interception of each Rf with a curve of made from molecular weight marker protein.

Folate Binding Capacity of FBP. The binding capacity of FBP was assessed by an equilibrium dialysis. Onto 1 ml of 0.23 µmol folic acid was added 2 ml of 20.7 nmol FBP. The experiment was repeated using the same amount of folic acid but with the other amount of FBP i.e. 2 ml with concentrate of 10.4 and 5.1 nmol. All the tree mixtures were placed in dialysis bag and dialyzed against 49 ml phosphate buffer pH 7.2 at room temperature for 3 hours (equilibrium state). At the end of this period, the folic acid concentration in the dialysate was measured by spectrophotometer at the λ280 with a mixture 1 ml of 0.23 µmol folic acid and 2 ml distilled water as a control. Using FBP concentration (20.7, 10.4, and 5.1 nmol) based on concentration of FBP that isolated and purified in this research.

Role of Calcium. The role of calcium was determined by equilibrium dialysis too. Onto 1 ml of 20.7 nmol FBP was added 0.77 mg of sodium EDTA, followed by 1 ml of 0.46 µmol folic acid. The mixtures were dialyzed against 50 ml phosphate buffer pH 7.2 at room temperature (25 °C) for 3 hours. At the end of this period, the folic acid concentration in the dialysate was measured by spectrophotometer at the λ280. Two controls were used in this experiment. The first was 1 ml solution of 0.46 µmol folic acid in distilled water, the other was 1 ml solution of 0.46 µmol folic acid in 20.7 nmol FBP.

Role of –SH Groups. The role of SH groups was also determined by equilibrium dialysis. Onto 1 ml of 20.7 nmol FBP was added 0.38 mg of iodoacetate, followed by 1 ml of 0.46 µmol folic acid. The mixtures were dialyzed against 50 ml phosphate buffer pH 7.2 at room temperature (25 °C) for 3 hours. At the end of this period, the folic acid concentration in the dialysate was measured by spectrophotometer at the λ280. Two controls were used in this experiment. The first was 1 ml solution of 0.46 µmol folic acid in distilled water, the other was 1 ml solution of 0.46 µmol folic acid in 20.7 nmol FBP.

Role of Disulfide Bridge. The role of disulfide bridge was also determined by equilibrium dialysis. Onto 1 ml of 20.7 nmol FBP was added 50 µl of 2-mercaptoethanol, followed by 1 ml of 0.46 µmol folic acid. The mixtures were dialyzed against 50 ml phosphate buffer pH 7.2 at room temperature (25 °C) for 3 hours. At the end of this period, the folic acid concentration in the dialysate was measured by spectrophotometer at the λ280. Two controls were used in this experiment. The first was 1 ml solution of 0.46 µmol folic acid in distilled water, the other was 1 ml solution of 0.46 µmol folic acid in 20.7 nmol FBP.

RESULTS

FBP could be isolated and purified from cow's milk using salting out method followed by ion exchange DEAE-cellulose chromatography and affinity chromatography. In the DEAE-cellulose, there were two early peaks eluted from column (Figure 1). Affinity chromatography of DEAE-cellulose peak one gave no any clear peak after the addition of acetate buffer pH 3.5 (Figure 2). On the other hand, the same treatment gave clear peak post of the addition of acetate buffer pH 3.5 in DEAE-cellulose peak two (Figure 3). Obtained FBP had molecular weight about 31,000 Dalton showed by SDS-PAGE analysis (Figure 4).

FBP from cow's milk could bind folic acid with stoichiometry ratio is 1:1 mol (Figure 5 & Table 1). The

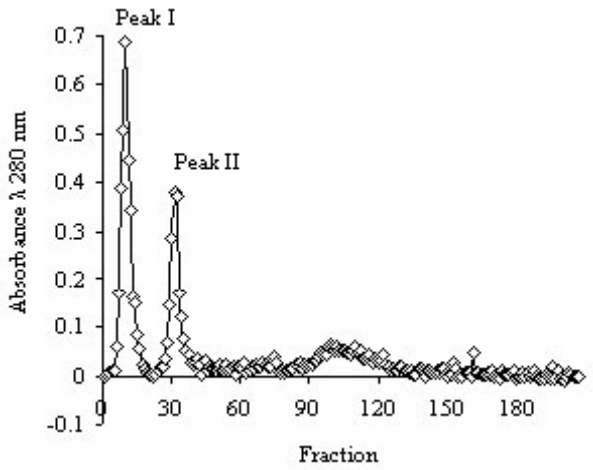

Figure 1. Fractionation with DEAE-cellulose chromatography.

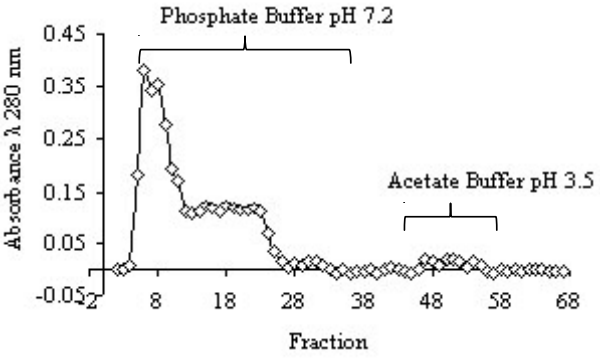

Figure 2. Fractionation of DEAE-cellulose peak one with affinity chromatography.

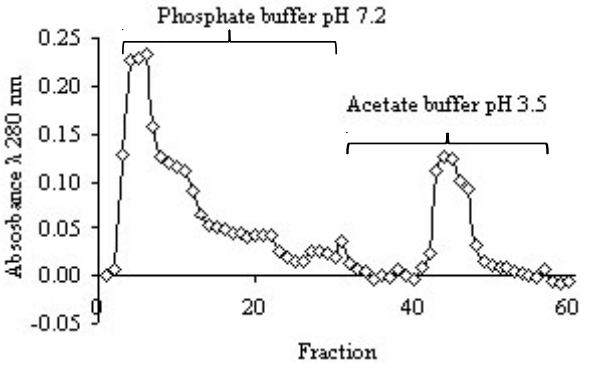

Figure 3. Fractionation DEAE-cellulose peak two with affinity chromatography.

addition of EDTA, Ca^{2+} chelating agent, did not affect the binding between FBP and folic acid (Figure 6). However, the addition of iodoacetic acid (alkylating agent) and β-mercaptoethanol (reductor agent) decreased the binding capacity of FBP to folic acid (Figure 7 & 8).

DISCUSSION

In general we used isolation technique for FBP as described by Salter *et al.* (1981). Briefly, the whey is precipitate with 95% saturation of ammonium sulphate, followed by DEAE-cellulose chromatography. To obtain

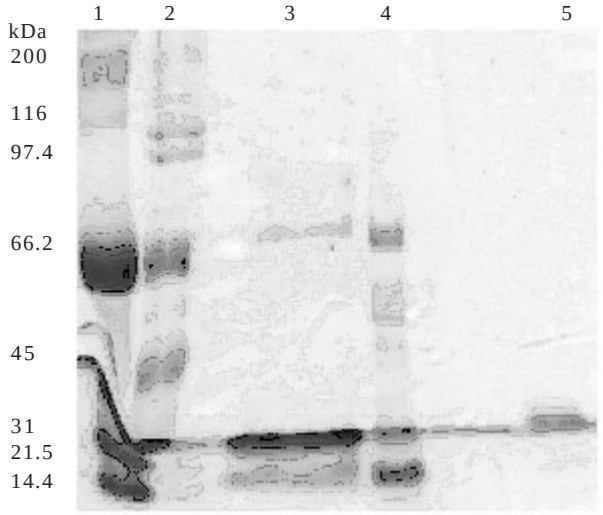

Figure 4. Electrophoresis SDS-PAGE. 1: BSA, 2: Protein marker, 3: Filtrate of 45% ammonium sulphate, 4: Filtrate of dialysis 95% precipitate, 5: Peak two of affinity chromatography (FBP).

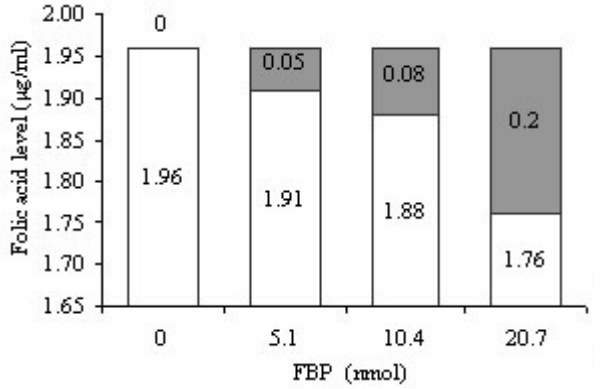

Figure 5. Folic acid level after binding of FBP. ■: Bound folic acid, □: Free folic acid.

Table 1. Ratio binding of FBP to folic acid

FBP	5.1 nmol	10.4 nmol	20.7 nmol
Total folic acid	230	230	230
Free folic acid	224.71	220.7	207.7
Folic acid bound to FBP	5.29	9.3	22.3
Ratio FBP:Folic acid	0.93:1	1.11:1	0.97:1

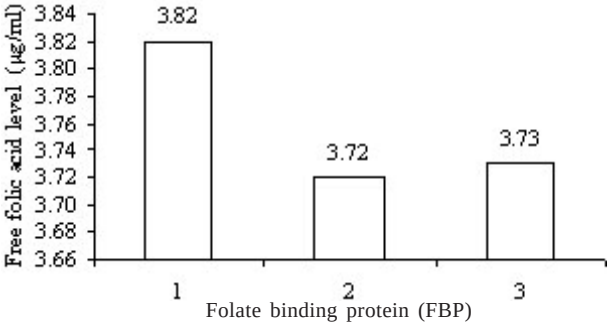

Figure 6. Free folic acid after binding of FBP with EDTA. 1. Distilled water control, 2. FBP control, 3. FBP with EDTA.

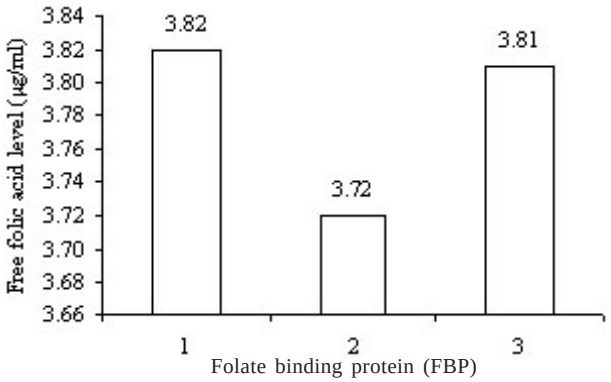

Figure 7. Free folic acid after binding of FBP with iodoacetic acid. 1. Distilled water control, 2. FBP control, 3. FBP with iodoacetic acid.

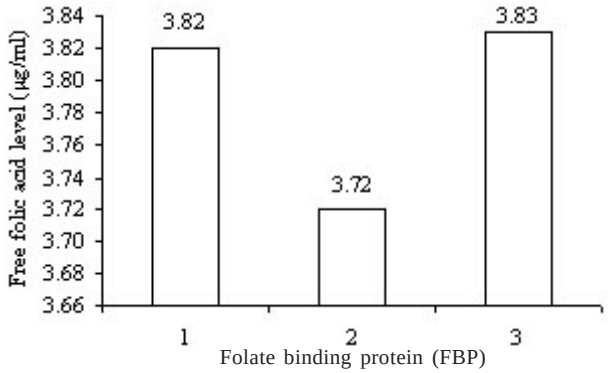

Figure 8. Free folic acid after binding of FBP with β-mercaptoethanol. 1. Distilled water control, 2. FBP control, 3. FBP with β-mercaptoethanol.

contamination, an affinity chromatography method was applied. Folic acid is negatively charge in neutral pH, therefore the FBP should have a relatively positive charge. In the DEAE-cellulose the first peak eluted from the column usually have positive charge. Two early peaks eluted from column were purified by passing them in affinity chromatography column. For this aims, a certain amount of folic acid was fixed on activated agarose followed by saturating the free sites of agarose with ethanolamine. Each DEAE-cellulose peak was passed in the affinity column in separate experiment. Affinity chromatography of DEAE-cellulose peak gave clear peak post of the addition of acetate buffer pH 3.5 in DEAE-cellulose peak two. Hence, it could be said that the post acetate buffer peak contain only the pure FBP fraction. The purity of obtained FBP was showed by a single spot in SDS-PAGE analysis. From this technique, it could be estimated the MW of FBP which migrated about the same distance as carbonic anhydrous. This result practically is identical with Salter (35 kDa) and Nygren (31 kDa) (Salter *et al.* 1981; Babol 2007). This research used our pure FBP further analysis.

Equilibrium dialysis of folic acid and FBP showed that there was practically 1:1 mol ratio binding between FBP and folic acid. Salter *et al.* (1981) found also the same binding as observed in this research.

To bind folic acid, the FBP apparently didn't need Ca^{2+} ion. This conclusion was obtained from the experiment with EDTA. With or without EDTA, FBP bound practically the same amount of folic acid, which means the presence of Ca^{2+} ion is not essential for the binding.

Iodoacetic acid is well-known as an alkylating agent. Usually, iodoacetic acid reacted with an -SH group as found in cystein moiety. If the cystein moiety is found in an active site of any protein then the alkylating of the –SH groups (iodoacetic acid addition), it would disturb the biological activity of protein. The experiment with iodoacetic acid showed that binding activity of FBP decrease significantly (almost 100% as compared to distilled water control). It had been reported that FBP contain no free –SH group. Though the presence of –SH group in FBP should still be considered, there is another possible interpretation. Imidazol, part of amino acid histidine could be alkylated by iodoacetic acid (Fruchter & Crestfield 1967; Chase & Tubbs 1970; Maziarz *et al.* 1999). If histidine is found in active site in any protein i.e. enzyme, the alkylating process will inhibit the enzyme activity. In this experiment, it is not impossible that active site of FBP contain histidine moiety. Histidine has a very important role in supporting biological activity of any protein. Many enzyme especially proteases and other hydrolases need the presence of histidine in their catalytic site. Alkylation of this enzyme will block their activities and it is one of the principles of using alkylating agent as insecticide. Further research is needed to determine whether cystein or histidine is found as active site of FBP.

β-Mercaptoethanol is known for reducing disulfide bridge in any protein. This research found that addition of this reducing agent decreased significantly the binding activity of FBP. It has been reported that FBP has several disulfide bridge. Some author reported that it has 6 disulfide bridges while other reported the presence of 8 disulfide bridges. This observation had not count the number of disulfide bridges in FBP, but the experiment showed clearly that the maintaining of the disulfide bridge for the binding capacity of FBP is very important.

As conclusion, we could suggest that FBP purified from cow's milk has a MW around 31 kDa, binds folic acid in 1:1 mol ratio, needs the intact –SH or imidazol groups in its active site, disulfide bridges for maintaining the three dimension structure and therefore the biological activity, but does not need the presence of Ca^{2+} ion.

REFERENCES

Babol NL. 2007. Folate binding protein in bovine milk (Doctoral thesis). Uppsala: Swedish Univ.

Bailey LB. 2010. Folate in Health and Disease. 2nd ed. Boca Roton: CRC Pr.

Chase JFA, Tubbs PK. 1970. Specific alkylation of a histidine residue in carnitine acetyltransferase by bromoacetyl-L-carnitine. *Biochem J* 116:713-720.

Fruchter RG, Crestfield AM. 1967. The specific alkylation by iodoacetamide of histidine-12 in the active site of ribonuclease. *J Bio Chem* 242:5807-5812.

Holm J, Hansen SI. 2001. Binding of radiolabeled folate and 5-methyltetrahydrofolate to cow's milk folate binding protein at pH 7.4 and 5.0. *Biosc Rep* 21:733-743. http://dx.doi.org/10.1023/A:1015576522416

Holm J, Hansen SI. 2003. Ligand binding and polymerization characteristics of human milk folate binding protein depend on concentration of purified protein and presence of amphiphatic substances. *Biosc Rep* 23:77-85. http://dx.doi.org/10.1023/A:1025572207004

Kim YI. 2004. Will mandatory folic acid fortification prevent or promote cancer? *Am J Clin Nutr* 80:1123-1128.

Mardiana. 2005. Penggunaan protein ikat folat (PIF) dari susu sapi untuk mengukur kadar folat serum [Thesis]. Jakarta: University of Indonesia.

Maziarz KM, Monaco HL, Shen F, Ratnam M. 1999. Complete mapping of divergent amino acids responsible for differential ligand binding of folate receptors α and β. *J Bio Chem* 274:11086-11091. http://dx.doi.org/10.1074/jbc.274.16.11086

Nixon P, Jones M, Winzor DJ. 2004. Quantitative description of the interaction between folate and the folate-binding protein from cow's milk. *Biochem J* 382:215-221. http://dx.doi.org/10.1042/BJ20040411

Nygren L, Sternesjo A, Bjorck L. 2003. Determination of folate-binding proteins from milk by optical biosensor analysis. *Intern Dairy J* 1:283-290. http://dx.doi.org/10.1016/S0958-6946(02)00193-0

Purwani E, Zulaekah S. 2008. Risiko lahirnya bayi cacat pembuluh syaraf pada ibu hamil yang kekurangan asam folat. *J Kesehatan* 1:19-28.

Salter DN, Scott KJ, Slade H, Andrews P. 1981. The preparation and properties of folate-binding protein from cow's milk. *Biochem J* 193:469-476.

Sauer J, Mason JB, Choi SW. 2009. Too much folate – a risk factor for cancer and cardiovascular disease? *Curr Opin Clin Nutr Metab Care* 12:30-36. http://dx.doi.org/10.1097/MCO.0b013e32831cec62

Sralsjo L, Arkbage K, Witthoft C, Jagerstad M. 2002. Evaluation of a radioprotein binding assay for folate analysis in berries and milk. *Food Chem* 709:525-534. http://dx.doi.org/10.1016/S0308-8146(02)00392-8

Svendsen IB, Martin B, Pedersen TG, Hansen SI, Holm J, Lyngbye J. 1979. Isolation and characterization of the folate-binding protein from cow's milk. *Carlsberg Res Commun* 44:89-99. http://dx.doi.org/10.1007/BF02906524

Sweeney MR, McPartlin J, Scott J. 2007. Folic acid fortification and public health: report on threshold doses above which unmetabolised folic acid appear in serum. *BMC Public Health* 7:1-7. http://dx.doi.org/10.1186/1471-2458-7-1

Verwei M, Arkbåge K, Mocking H, Havenaar R, Gr J. 2004. The binding of folic acid and 5-methyltetrahydrofolate to folate-binding proteins during gastric passage differs in a dynamic in vitro gastrointestinal model. *J Nutr* 134:31-37.