ISOLATION OF THE FOLATE-BINDING PROTEIN FROM COW'S MILK BY THE USE OF AFFINITY CHROMATOGRAPHY

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1. Introduction

The folate in cow's milk is strongly bound to a minor whey protein. This binding protein is present in excess and the milk has capacity to bind added folic acid [1–3]. Ford et al.[3] described the partial purification of the folate-binding protein from rennet whey by ammonium sulphate fractionation followed by chromatography on DEAE-cellulose and gel filtration in Sephadex G-150. Electrophoresis showed the product to be a mixture of several proteins, only one of which, a minor component, had folate-binding activity. This binding protein has now been further purified by affinity chromatography [4, 5], by means of folic acid covalently bound to agarose gel through 1,6-diaminohexane.

2. Materials and methods

2.1. *Analytical methods*

Folate activity was measured microbiologically with *Lactobacillus casei* as previously described [3].

Folic acid binding capacity was measured by addition of graded amounts of folic acid (Koch-Light Laboratories Ltd, Colnbrook, Bucks.) to portions of milk or protein fractions, followed by dialysis for 2 days against 6 changes of 300 ml each of 0.02 M sodium phosphate buffer pH 7.2 containing 0.15 M NaCl and 3 mM ascorbic acid (buffer A) to remove free folate. The residual bound folate was then assayed microbiologically.

Thin-layer electrophoresis of the protein fractions was carried out in starch gel containing urea at pH 2 as previously described [3].

Estimation of protein concentration: a rough measure of protein content in the various test fractions was obtained by measurement of absorbance at 280 nm.

Molecular weight was estimated by gel filtration in a column (2.4 × 50 cm) of Sephadex G-100 (Pharmacia (G.B.) Ltd, London) equilibrated with 0.01 M Tris-HCl pH 7.4 containing 0.14 M KCl and calibrated with standard proteins as described by Andrews [6].

2.2. *Preliminary fractionation of milk proteins*

Milk was taken at the morning milking from a Friesian cow of the Institute herd and rennet whey was prepared as previously described [3]. All subsequent procedures were carried out at 0–5°. To 2.29 l stirred whey were slowly added 1.87 l saturated $(NH_4)_2SO_4$ solution, pH 7. The resulting precipitate was removed by filtration through Whatman no. 42 filter paper, and to the filtrate were added 1.56 l of the saturated $(NH_4)_2SO_4$ solution. A precipitate formed, which was recovered by centrifugation at 2000 *g* for 45 min, suspended in Buffer A and dialysed against buffer of pH 7.2 containing 0.02 M sodium phosphate and 1 mM ascorbic acid until free of $(NH_4)_2SO_4$. The dialysed solution (167 ml) was applied to a column (5.4 × 27 cm) of DEAE-cellulose (Whatman, grade DE52) equilibrated with 0.02 M sodium phosphate buffer pH 7.2 containing 1 mM ascorbic acid and eluted with the same buffer.

The first protein peak, containing 76% of the folate activity and 58% of the total folate-binding capacity of the whey in 188 ml, was collected and used for affinity chromatography.

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2.3. *Preparation of folic acid derivative of ω-aminohexyl agarose*

Following the procedure of Cuatrecasas [5] for preparing ω-aminoalkyl derivatives of agarose, 25 ml (settled volume) of Sepharose 6B (Pharmacia (G.B.) Ltd, London) were activated with 5 g cyanogen bromide at pH 11, washed with 1 l cold water and added to 50 ml cold 12% (w/v) solution of 1,6-diaminohexane that had been adjusted to pH 10 with concentrated HCl. The mixture was stirred overnight in the cold, filtered, and the residue washed with 1 l water.

Folic acid was coupled to the ω-aminohexyl agarose by means of a carbodiimide condensation. The substituted agarose gel (20 ml) was suspended in an equal volume of water, folic acid (10 mg) dissolved in 0.05 M $NaHCO_3$ (10 ml) was added and the pH of the mixture adjusted to 6 with N HCl. Then, with the mixture kept in the dark at room temp and continuously stirred, 100 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Cyclo Chemicals, Los Angeles, California, USA) was added in portions of about 10 mg at 10 min intervals. The reaction mixture was kept at pH 6 by adding 0.1 N HCl as necessary. After addition of the carbodiimide stirring was continued for 1 hr. The gel, which had become pale yellow, was then filtered off and washed with 1 l 0.05 M $NaHCO_3$. The folate content of the washings, estimated from absorbance at 283 nm, indicated that about 0.3 mg folic acid/ml gel had been bound.

In an attempt to reduce the basicity, and hence the ion-exchange capacity, of any unsubstituted ω-amino groups remaining on the gel, the agarose-folic acid preparation was resuspended in 20 ml water and 50 mg sodium acetate added. The pH was then adjusted to 6 and a further 100 mg of the carbodiimide added in 10 mg portions as before. The gel was filtered off, washed with water, and packed into a column (1.2 × 20 cm), which was further washed for several days with 0.02 M sodium phosphate buffer pH 7.2 containing 0.1 M NaCl (5 l) until the folic acid concentration in the effluent was < 0.5 ng/ml.

2.4. *Isolation of the folate-binding protein*

Agarose-folic acid (19 g wet wt) was added to the protein fraction containing the folate-binding protein from whey (188 ml, see above) and the mixture stirred. The pH was lowered to 3.6 with N HCl to dissociate the native 5-methyl-5, 6, 7, 8-tetrahydrofolate from the binding protein [3], then raised to 7.0 with NaOH to allow the binding protein to become attached to the agarose-bound folic acid. About 12% of the folate-binding protein remained in the solution. The gel was then packed into a column (1.2 × 19 cm) and washed with 0.05 M sodium phosphate buffer pH 7.2. It was then eluted successively with 0.05 M sodium phosphate pH 7.2 containing 1 M NaCl, 0.05 M sodium phosphate pH 6.0, 0.1 M sodium acetate pH 5.0, 0.1 M sodium acetate pH 5.0 containing 8 M urea and finally with 1 N HCl containing 8 M urea. All solutions contained 3 mM 2-mercaptoethanol to stabilise folate in the reduced form. The first buffer removed unbound protein (peak A, fig.1) which contained very little folate and had no capacity to bind added folic acid. Elution with 1 M NaCl at pH 7.2 yielded 2 protein fractions (peaks B and C), both of which contained small amounts of bound folate but had no additional folate-binding capacity. No protein was eluted with dilute buffers at pH 6 or pH 5. Elution with 8 M urea at pH 5 gave protein (peak D) containing only a trace of folate but with a high capacity to bind added folic acid. Further elution yielded only small amounts of material (peaks E and F) with no capacity to bind folic acid.

The purification scheme leading to the isolation of the folate-binding protein in peak D is summarised in table 1.

2.5. *Characteristics of the folate-binding protein*

Analysis of the material in peak D by thin-layer starch gel electrophoresis in the presence of urea at pH 2 (fig.2) indicated that it contained one main component with traces of impurities. The major component coincided with the band identified as the folate-binder in earlier preparations [3]. Electrophoresis also showed the presence of traces of this component in peak C, accounting for the folate content of this peak.

To estimate the molecular weight of the folate-binding protein by gel-filtration, excess folic acid was added to a portion of the material from peak D, free folic acid was removed by dialysis, and a sample containing 0.017 absorbance units of protein and 46 ng of bound folic acid was run on the Sephadex G-100 column with a mixture of bovine γ-globulins (1.5 mg), bovine serum albumin (2.5 mg), ovalbumin (2.5 mg),

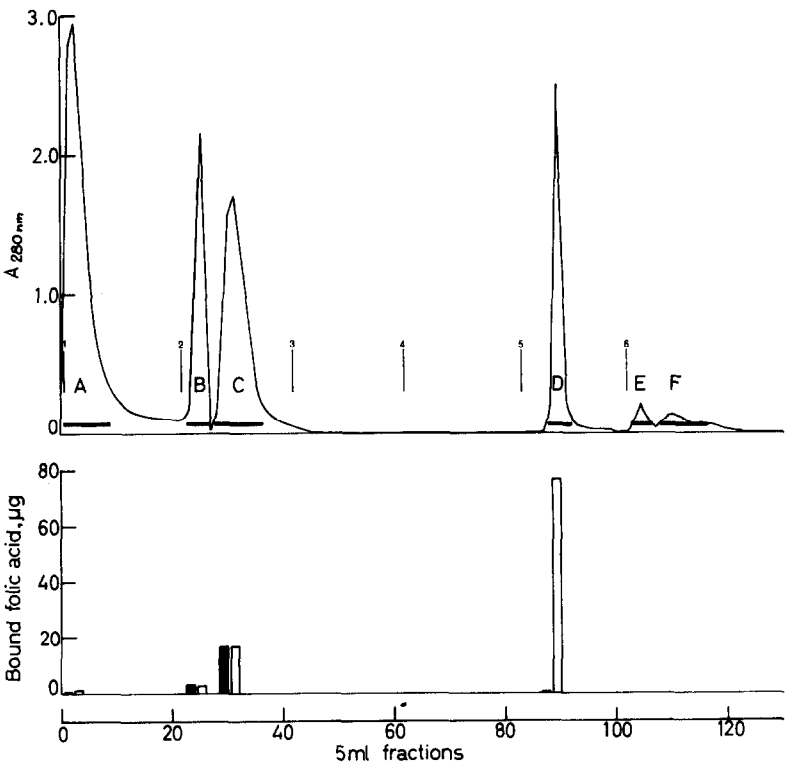

Fig.1. Affinity chromatography of partially purified folate-binding protein from whey on a column (1.2 × 19 cm) of agarose-folic acid. Proteins were first adsorbed to the gel as described in the text. The column was eluted with the following buffers, all containing 3 mM 2-mercaptoethanol, the point of application of each buffer being as indicated: 1) 0.05 M sodium phosphate pH 7.2; 2) 0.05 M sodium phosphate pH 7.2 containing M NaCl; 3) 0.05 M sodium phosphate pH 6.0; 4) 0.1 M sodium acetate pH 5.0; 5) 0.1 M sodium acetate pH 5.0 containing 8 M urea; 6) N HCl containing 8 M urea. (———): A_{280}. Fractions were pooled as indicated by the bars, dialysed against buffer A (see text) and the bound folate content of the resulting solutions measured before (■) and after (□) the addition of an excess of folic acid.

Table 1
Purification of folate-binding protein from whey.

Stage	Volume (ml)	Protein (Absorbance units: Vol. × A_{280})	Folate content (µg)	Total folate-binding capacity * (µg folic acid)	Recovery of total folate-binding capacity (%)	Specific folate-binding capacity (µg folic acid/absorbance unit of protein)	Purification
(1) Whey	2286	34,800	107	214	100	0.006	1
(2) DEAE-cellulose fraction	188	633	81	124	58	0.195	32
(3) Peak D from affinity chromatography (fig.1)	15	21	0.35	77	36	3.64	601

* Including native bound folate.

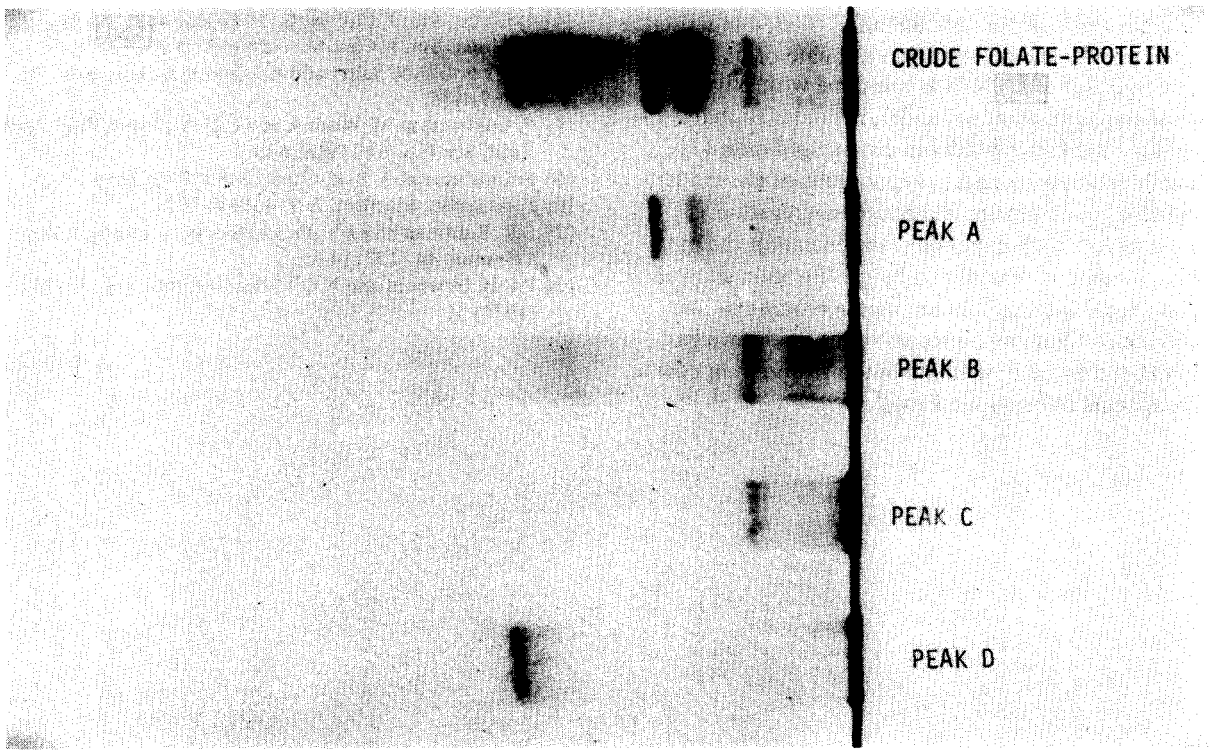

Fig.2. Thin-layer starch gel electrophoresis of protein fractions obtained from the affinity chromatography column (see fig.1). Electrophoresis was carried out in formic–acetic acid buffer of pH 2 in the presence of 5 M urea as described previously [3].

chymotrypsinogen (1.5 mg) and cytochrome c (1.5 mg), the proteins being dissolved in 5 ml of the eluting buffer. Folic acid was eluted in a symmetrical peak (V_e: 160 ml) between ovalbumin (V_e: 152 ml) and chymotrypsinogen (V_e: 174 ml), indicating a molecular weight of about 35,000 for the binding protein.

3. Discussion

The use of affinity chromatography in the preparation of the folate-binding protein in milk has resulted in a marked improvement in the purity of the product compared with previous methods [3]. An analogous procedure has been described for the preparation of dihydrofolate reductase by affinity chromatography with aminohexylagarose-methotrexate [7, 8]. We chose to link folic acid to agarose because it binds to the protein more strongly than does the natural milk folate [3], and this strong attachment of the binding protein to the affinity column proved an advantage in separating it from other whey proteins which also became adsorbed to the column, possibly by attraction to basic groups on the gel or to the remaining free carboxyl group on the bound folic acid itself. These proteins have not been identified, although the electrophoretic mobilities of the main components of peaks B and C are similar to those of bovine α-globulin and γ-globulin, respectively, when run under the same conditions. The recovery of folate-binding capacity in peak D from stage 2 of the purification procedure (table 1) indicates that the treatment with 8 M urea caused, at most, a loss of 38% of the binding capacity of the protein. As in the purification of dihydrofolate reductase [7], a preliminary fractionation of crude material was necessary before affinity chromatography could be used satisfactorily.

The yield of folate-binding capacity associated

with the protein eluted in peak D suggests that this protein is the major, if not the only, folate-binder in cow's milk. The gel-filtration estimate of its molecular weight (35,000), which was done with physiological concentrations of binder, is similar to that found for the folate-binder in centrifuged milk [3]. As with previous less pure preparations of the binder, reversible concentration-dependent aggregation [3] was observed; with increasing concentration the molecular weight as determined by gel-filtration showed a progressive increase. Assuming the binding of one molecule of folate by a protein of molecular weight 35,000, the amount of folate-binding protein in cow's milk appears to be about 8 mg/l.

References

[1] J. Ghitis, Am. J. Clin. Nutr. 18 (1966) 452.
[2] J. Ghitis, Am. J. Clin. Nutr. 20 (1967) 1.
[3] J.E. Ford, D.N. Salter and K.J. Scott, J. Dairy Res. 36 (1969) 435.
[4] P. Cuatrecasas, M. Wilchek and C.B. Anfinsen, Proc. Natl. Acad. Sci. U.S. 61 (1968) 636.
[5] P. Cuatrecasas, J. Biol. Chem. 245 (1970) 3059.
[6] P. Andrews, Biochem. J. 91 (1964) 222.
[7] B.T. Kaufman and J.V. Pierce, Biochem. Biophys. Res. Commun. 44 (1971) 608.
[8] P.C.H. Newbold and N.G.L. Harding, Biochem. J. 124 (1971) 1.