CHEMICAL FIXATION OF FOLATE BINDING PROTEIN TO ACTIVATED SEPHAROSE

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1. Introduction

It was shown that cow's milk contains a minor protein that binds folic acid [1–3] and most of its derivatives [3–5]. This protein was recently purified by affinity chromatography, using Sepharose-bound folic acid [6]. There seems to be a growing interest in the folate binding protein, e.g. it was recently employed to determine folates in human serum [7–9]. The present communication describes the synthesis of an insoluble preparation of the folate-binding protein and discusses some of its potential uses.

2. Materials and methods

[2-^{14}C]folic acid (PGA, 55.3 μCi/μmoles) was purchased from the Radiochemical Centre (Amersham, Bucks). Sepharose-4B was a product of Pharmacia (Uppsala, Sweden). Cyanogen bromide was obtained from Eastman Organic Chemicals (Rochester, New York). Radioactivity was determined as described earlier [10].

2.1. *Preparation of folate-binding protein from goat's milk*

Goat's milk was used as it contains 2 to 4-fold more folate binder than cow's milk. The protein was purified by a procedure similar to the one described by Salter et al. [6], however, instead of folate-bound Sepharose, methotrexate bound to Sepharose was used to bind the protein. The protein was released from the column by acetic acid (0.2 M) instead of urea. Details of the purification procedure will be published elsewhere. The purified protein was concentrated by lyophilization and the acetic acid was neutralized with NaOH prior to its addition to the activated Sepharose.

2.2. *Activation of Sepharose and fixation of the folate-binding protein*

Sepharose-4B (5 ml bed volume) was washed with distilled water and activated in a 50% suspension with 1 g cyanogen bromide at pH 11 [11]. After washing with cold $NaHCO_3$ (0.1 M, 300 ml) and resuspending in 5 ml of the $NaHCO_3$ solution, the binding protein (2 ml, containing 7 mg protein with a total binding capacity of 66 000 ng of PGA) was added, and the pH adjusted to 8.5. The mixture was stirred for 4 hr in an ice bath under a stream of N_2; it was then packed into a column and washed at a rate of 4 ml/min for 48 hr with 1 M NaCl containing 0.1 M potassium phosphate buffer (pH 7.0). The Sepharose was then suspended in water to a final volume of 9 ml.

3. Results

Table 1 and 2 show the conditions for binding of radio-PGA to the Sepharose–protein complex and for releasing of the vitamin from the column. In contrast to the soluble binder, from which PGA can be released by acetic acid, a stronger acid (HCl) was required to release the vitamin from the Sepharose-bound binder. Treatment with HCl released the PGA without affecting the binding ability of the subsequently regenerated column (table 3).

To determine the capacity of the insoluble protein-binder, radioactive PGA was loaded in 100 ng aliquots

Table 1
Binding of radio-folic acid to the protein–Sepharose complex.

Fraction no	Elution conditions	Fraction volume (ml)	Radioactivity eluted %
1	Effluent	2	1.0
2	0.1 M phosphate (pH 7.0)	2	0.8
3	0.1 M phosphate (pH 7.0) containing NaCl 1 M	2	0.5
4	0.1 M phosphate (pH 7.0) containing NaCl 1 M	2	0
5	0.1 M phosphate (pH 7.0) containing NaCl 1 M	10	0
6	0.1 M acetic acid	2	7.2
7	0.1 M acetic acid	2	2.0
8	0.1 M acetic acid	2	0
9	Like no 2 but heating column content for 2 min at 100°C	3	54

The protein–Sepharose complex was packed into a Pasteur pipette (0.3 ml bed volume) and washed with 10 ml 1 M potassium phosphate buffer (pH 7.0). Radio-folic acid (20 000 cpm) in 2 ml 0.1 M potassium phosphate buffer (pH 7.0) was applied onto the column. Elution was attempted, using different conditions as given in the table.

on a column, which was prepared from 0.15 ml of the Sepharose-binder complex suspension. After each addition and appropriate washing, the amount of folate bound in the effluent was subtracted from that added, to obtain the bound PGA (fig. 1). At saturation 600 ng of folate was bound (table 4), thus the whole suspension of the protein–Sepharose complex (9 ml) was able to bind 36 000 ng PGA which corresponded to 54% of the original activity.

Table 2
Effect of acids on the release of radio-folic acid from the Sepharose–protein complex.

Fraction no	Elution conditions	Fraction volume (ml)	Radioactivity eluted %
1	Effluent	2	7.5
2	0.1 M phosphate (pH 7.0)	2	2.5
3	0.2 M acetic acid	2	11.5
4	0.2 M acetic acid	2	2.5
5	0.1 M HCl	2	73
6	0.1 M HCl	2	3

Protein–Sepharose complex (0.1 ml bed volume) was packed into a column with non-activated Sepharose-4B* (0.5 ml bed volume) and washed with 0.1 M potassium phosphate buffer pH 7.0 (10 ml). Radio-folic acid (20 000 cpm) was added to the column. After washing with phosphate buffer to remove unbound folate, the column was washed with acids as described in the table.

*Non-activated Sepharose-4B was employed to increase the bed volume.

4. Discussion

According to Goldstein [12], proteins bound to water-insoluble polymers are suitable for repeated and continuous use since they are usually stable and they do not contaminate the reaction products; they can also be employed for the adsorption and purification of inhibitors of the activities of these proteins. The insoluble folate-binding protein described above also shows these properties. The rapidity by which PGA is absorbed to, and desorbed from, the column and the regeneration of the column renders the preparation suitable for purification of folates. The method can also be applied to remove endogenous folates

Table 3
Binding of radio-folic acid to a regenerated Sepharose–protein complex.

Fraction no	Elution conditions	Fraction volume (ml)	Radioactivity eluted %
1	Effluent	2	8.6
2	0.1 M phosphate (pH 7.0)	2	1.8
3	0.1 M HCl	2	69
4	0.1 M HCl	2	8

The column employed in table 2 was washed with 0.1 M phosphate buffer pH 7.0 till the pH of the effluent was neutral. Radioactive folic acid (20 000 cpm) was applied to the column and then eluted as described in the table.

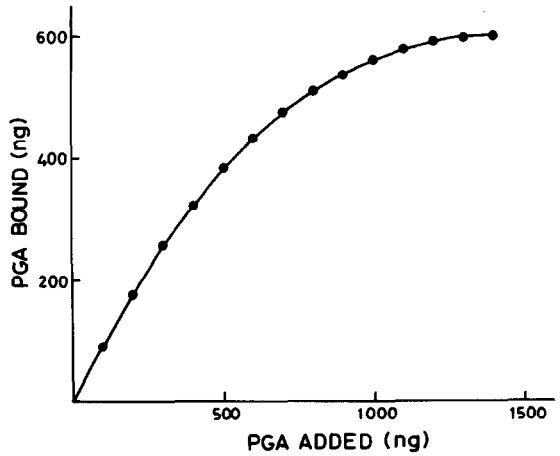

Fig. 1. Determination of folate-binding capacity of the protein–Sepharose complex. Protein–Sepharose complex, (0.15 ml suspension, 0.075 ml bed volume) was packed with non-activated Sepharose-4B (0.8 ml bed volume) into a Pasteur pipette column. The column was then washed sequentially with 5 ml 0.1 M HCl, 5 ml 1 M potassium phosphate buffer (pH 7.0) and finally with 5 ml 0.1 M phosphate buffer (pH 7.0). Aliquots (0.25 ml) of radio-folic acid (400 ng/ml) were applied repeatedly to the column followed by washing each time with 2.75 ml 0.1 M potassium phosphate buffer (pH 7.0). The effluent was assayed for radioactivity. The difference in values between added and eluted radioactivity represents the measure of bound folate. Addition of radio-folate was discontinued when the above difference approached zero.

from enzymatic reactions in which folate is involved as a coenzyme. The column can also be used to measure the transfer of one carbon unit to or from tetrahydrofolates. We are currently investigating the feasibility of using this column for folate radioassay.

Table 4
Comparison between calculated and eluted bound radio-folate at saturation*.

	cpm
Calculated	132 580
Found after elution with 0.1 M HCl	133 000

* Details of this experiment are given in fig. 1.

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