THE ISOLATION AND CHARACTERIZATION OF THE FOLATE BINDING PROTEIN FROM GOAT MILK

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1. Introduction

It is now known that there are specific folate binding proteins (FABP) in various tissues such as serum, milk and intestinal mucosa [2,3,4]. Whereas the FABP have been purified from cow milk [5], human milk [6] and umbilical cord serum [7] the study of the physical characteristics and physiologic functions has been hampered by the small quantities of FABP present. Goat milk, which contains a low concentration of folic acid, was recently reported to contain high levels of unsaturated FABP [8]. Affinity chromatography has been used successfully to separate the FABP from large quantities of goat milk and the present communication describes its physical and immunological characteristics.

2. Materials and methods

Unprocessed goat milk was obtained from Pure Goat Products, Boyerstown, Pa. Cyanogen bromide activated Sepharose 4B is a product of Pharmacia, Piscataway, N J and [^{3}H]PGA, 26 Ci/mM was purchased from Amersham/Searle, Des Plaines, Ill. Radioactivity of the samples was determined as previously described [6].

Some of these studies were presented at the meetings of the Federation of the American Society of Experimental Biology, Atlantic City, New Jersey, April 15, 1975 [1].

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2.1. *Chromatographic separation of the FABP*

Folic acid was covalently linked to cyanogen bromide activated Sepharose 4B by the method of Ford et al. [5]. Goat milk, clarified by centrifugation at 45 000 X *g* for 15 min at 4°C, was passed through the column and the gel was washed until there was no detectable protein. The FABP was desorbed from the gel with 0.2 M acetic acid as previously described [6]. The fractions were dialyzed against distilled water and lyophilized to dryness. Aliquots were removed as needed for the individual experiments. Protein was determined by the method of Lowry et al. [9], FABP activity by the method of Waxman and Schreiber [10] and dihydrofolate reductase activity was measured by the method of Rothenberg [11].

2.2. *Electrophoretic analyses*

Protein solutions were subjected to polyacrylamide disc-gel electophoresis in pH 8.3 Tris–glycine buffer with the standard 7.5% gel [12]. Gels were stained for protein with Coomassie Brilliant Blue and for sugar using the periodic-acid Schiff reaction [13]. An unstained gel was fractionated in a Savant Autogel divider, Sodium dodecylsulfate electrophoresis and molecular weight calculations were carried out according to the method of Weber and Osborn [14]. Isoelectric focusing was accomplished in an LKB 110 column using 1% ampholytes with a pH-range of 3–10 on a sucrose support with an initial power output of 3.2 W.

2.3. *Gel-filtration analyses*

Molecular weights were estimated using a 2.2 X 85 cm

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Table 1
Purification of folic acid binding protein from goat milk

Stage		Volume (ml)	Total Protein (mg)	Total FABP (μg PteGlu bound)	Recovery of total FABP (%)	Specific activity (μg PteGlu bound/mg protein)	Purification
(1)	Clarified Milk[a]	1480	21 904	169.8	100	0.00775	1
(2)	Affinity chromatography: 0.2 M Acetic Acid Fraction[b]	50	17.5	42.5	25.2	2.00	260
(3)	DEAE-cellulose[c]	8	1.95	18.0	10.6	9.2	1200

[a]Raw goat milk spun at 45 000 × *g* and filtered through glass wool
[b]Binding capacity determined after fractions dialyzed
[c]Lyophilized FABP from acetic acid fraction

column of Sephadex G-200 equilibrated with 0.1 M PO_4 buffer, pH 7.2, containing 0.5 M NaCl. The columns were marked and calibrated according to the method of Laurent and Killander [15]. Concanavalin A–Sepharose chromatography and DEAE-cellulose chromatography were executed as previously described [6].

2.4. *Chemical composition determinations*

The amino acid composition was determined by acid hydrolysis of the intact protein saturated with [^{3}H]PGA using the standard two column Beckman 121 amino acid analyzer. Tryptophane was not determined. Values for mol amino acid/mol PGA was determined by assaying an aliquot of the hydrolysate for radioactivity. The amino acid sequence was determined with a Beckman sequencer. Neutral hexosanes and amino sugars were assayed by gas–liquid chromatography using the method of Allen and Mehlman [16]. Sialic acid was assayed by the thiobarbiturate method of Warren [17] after hydrolysis in 1 N HCl for 1 min at 100°C.

2.5. *Immunological studies*

Antisera to goat milk FABP was prepared by injecting New Zealand White rabbits intraperitoneally with FABP emulsified with Complete Freund's Adjuvant at seven day intervals for three weeks. The rabbits were boostered and bled thereafter at eleven day intervals. All antisera and control sera were dialyzed free of folate prior to use. Standard Ouchterlony techniques were used throughout [18].

3. Results

FABP was clearly separated from the goat milk by affinity chromatography and purified 260-fold. Further purification on DEAE-cellulose yielded FABP with a specific activity of 9.2 μg [^{3}H]PGA bound/mg protein and represents a 1200-fold purification with a recovery of 10.6% of the total FABP present in clarified milk (table 1). The FABP recovered was unable to convert [^{3}H]PGA to [^{3}H]tetrahydrofolate and is clearly not dihydrofolate reductase. Polyacrylamide electrophoresis reveals a distinct protein band in the upper one-third of the gel which bound [^{3}H]PGA. A similar gel, following exhaustive washing with 10%

Fig.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of 30 μg of the isolated FABP.

trichloroacetic acid stained distinctly pink with PAS in the same region. Passage through Sephadex G-200 revealed a molecular weight of 37 000. SDS-electrophoresis of 30 μg FABP revealed one single band with a molecular weight estimation of 38 000 (fig.1). 47% of the purified FABP was retained by the concanavalin A–Sepharose and was rapidly eluted with α-methyl mannoside. Isoelectric focusing revealed three major peaks of FABP with a p*I* of 6.6, 7.3 and 8.4. The peak at 4.7 is charcoal adsorbable and presumable represents free PGA (fig.2). Isoelectric focusing of the FABP eluted with α-methyl mannoside yielded two peaks of FABP with a p*I* of 6.6 and 7.3. Only a remnant of the peak at 8.4 remained. The results of the amino acid and carbohydrate analysis are shown in table 2. Using the molecular weights of the inidividual amino acids and carbohydrates we determined that the FABP contains 22% carbohydrate and contains approx. 39 200 X *g* of amino acid and carbohydrate/mol bound PGA. Sequencing revealed

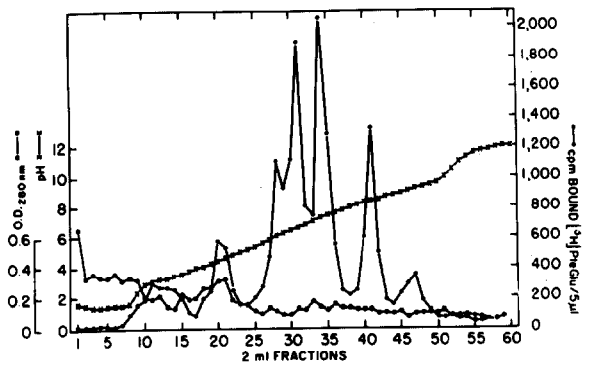

Fig.2. Isoelectric focusing of the isolated FABP labeled with [^{3}H]PGA in a pH-range of 3–10. The peak of radioactivity at pH 4.7 is charcoal adsorbable and represents free [^{3}H]PGA.

Table 2
Amino acid and carbohydrate composition of goat milk FABP

Amino acid[a]	Residues/mol bound PteGlu
Lysine	17
Histidine	9
Arginine	12
Aspartic acid	26
Threonine	12
Serine	21
Glutamic acid	24
Proline	22
Glycine	29
Alanine	22
Half-Cystine	4
Valine	16
Methionine	3
Isoleucine	10
Leucine	15
Tyrosine	7
Phenylalanine	7
Total	256
Carbohydrate	
Fucose	4
Galactose	3
Mannose	11
Galactosamine	6
Glucosamine	9
Sialic acid	3
Total	36

[a]Tryptophan not determined

Fig.3. Precipitin reaction of rabbit anti-goat milk FABP (center well) to the FABP purified from various sources. Each outer well was filled with 20 µl of purified FABP. (1) Human milk low molecular weight FABP. (2) Human serum FABP. (3) Human placenta FABP. (4) Goat milk FABP. (5) Goat milk FABP saturated with PGA. (6) Bovine milk FABP.

a single N-terminal residue of alanine while position three revealed a single residue of valine. Antibody to FABP gave a single precipitin line when placed against FABP alone or FABP saturated with PGA, Methotrexate or *N*-5-methyl tetrahydrofolate (fig.3). The antibody to goat FABP will block PGA binding to FABP and will also precipitate the PGA–FABP complex. The antibody will block the binding of PGA to human and bovine FABP but does not precipitate the PGA–FABP complex or form precipitin lines with the FABP of these other species.

4. Discussion

Goat milk has proven to be a rich source of FABP and affinity chromatography an excellent technique for its isolation. For the first time a specific folate binding protein has been isolated to homogeneity and its composition characterized. Goat milk FABP is a glycoprotein with 22% carbohydrate. It is probable that the several isoelectric peaks characteristic of the isolated goat milk FABP (and purified human milk FABP) represent isoproteins. Its physical characteristics are similar to those found in human milk [6] and

human serum [7] as well as the milks from other species [5]. The molecular weight, glycoprotein nature and physical characteristics clearly show that FABP is distinct from dihydrofolate reductase. The FABP isolated binds 9.2 μg PGA/mg protein and based on a molecular weight comparison appears to be on a 1:1 ratio. The data suggests that the FABP binding site is shared by many species. The preparation of large amounts of homogeneous FABP which can now be iodinated will facilitate the study of the disposition of tissue FABP and the effect of FABP on folate absorption, plasma clearance and cellular metabolism.

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