PURIFICATION OF LACTOSE SYNTHETASE A PROTEIN FROM HUMAN MILK AND DEMONSTRATION OF ITS INTERACTION WITH α-LACTALBUMIN

P. ANDREWS

National Institute for Research in Dairying, Shinfield, Reading, Berkshire, RG2 9AT, England

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1. Introduction

Lactose synthetase A protein catalyses the transfer of D-galactosyl units from UDP-D-galactose to NAG *, forming *N*-acetyl-lactosamine, but in the presence of α-lactalbumin this reaction is partially inhibited and galactosyl transfer to D-glucose, forming lactose, is favoured [1–4]. The A protein occurs in a wide variety of tissues in particulate form, but is found also in milk in soluble form [5]. An easy method for its purification [3, 4, 6] has yet to be described. This paper describes its purification in good yield from human milk by a procedure involving only gel filtration and affinity chromatography [7] as the major steps.

2. Materials and methods

Human milk was collected during the period four to seven days *post partum* and the cream removed by centrifugation at 10,000 *g* for 20 min. The skim milk was filtered through glass wool to remove fat particles and stored at −20°. Bio-Gel P-200 (100–200 mesh) (Calbiochem Ltd., London) and Sepharose 6B (Pharmacia (G.B.) Ltd., London) were thoroughly washed and the fines removed before use. Sepharose–α-lactalbumin was prepared in 0.1 M $NaHCO_3$ by coupling purified bovine α-lactalbumin [8] to Sepharose 6B which had been activated with cyanogen bromide [9] and was extensively washed with 0.1 M $NaHCO_3$ containing M NaCl before use. It contained approximately 2.5 mg of α-lactalbumin/ml of gel.

Lactose synthetase activity was determined by assaying spectrophotometrically the amount of UDP formed in the reaction from UDP-galactose [10]. The standard assay contained, in a final volume of 1 ml, 50 μmoles of tris-HCl, pH 7.5, 4 μmoles of $MnCl_2$, 1 μmole of phosphoenolpyruvate, 0.1 μmole of ATP, 0.2 μmole of NADH, 0.2 μmole of UDP-galactose, 0.5 mg of crude pyruvate kinase (type 1, Sigma London Chemical Co., Ltd., London), 80 μmoles of D-glucose and 150 μg of purified human α-lactalbumin (prepared as described by Barman [8] for bovine α-lactalbumin). The standard assay for *N*-acetyl-lactosamine synthetase contained 3 μmoles of NAG instead of the glucose and α-lactalbumin. Assays were performed at 25°. One unit of enzyme is the amount of enzyme catalysing the formation of 1 μmole of UDP/min.

Protein was estimated by the spectrophotometric procedure of Warburg and Christian [11].

3. Results

3.1. *Purification procedure for A protein from human milk*

The procedure was carried out at 0–5° throughout.

A saturated solution (305 ml) of $(NH_4)_2SO_4$, previously adjusted to pH 6.8, was added slowly, with stirring, to human skim milk (250 ml). The precipitate was isolated by centrifugation at 15,000 *g* for

* *Abbreviation:*
NAG: *N*-acetyl-D-glucosamine.

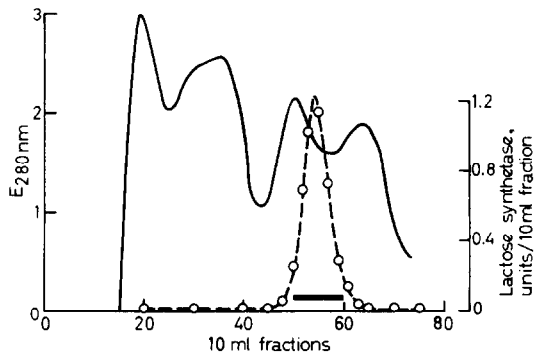

Fig. 1. Stage 3 of the purification scheme (table 1). Gel filtration of a solution (33 ml) of $(NH_4)_2SO_4$ precipitate from human skim milk on a Sepharose 6B column (5 X 50 cm), equilibrated with 0.01 M tris-HCl, pH 7.5, containing 0.2 M KCl. ——, $E_{280\,nm}$; o---o, lactose synthetase activity. Fractions were pooled as indicated by the bar.

15 min and dissolved in 0.01 M tris-HCl, pH 7.5, giving 66 ml of turbid solution. This solution, in two equal portions, was submitted to gel filtration on a column of Sepharose 6B (fig. 1). Protein in the pooled fractions (269 ml) was concentrated by addition of $(NH_4)_2SO_4$ (105 g) and the precipitate isolated by centrifugation and dissolved in buffer (20 ml) as before. The solution was dialysed against 0.01 M tris-HCl, pH 7.5, for 4 hr to remove $(NH_4)_2SO_4$, otherwise the salt caused contraction of the gel

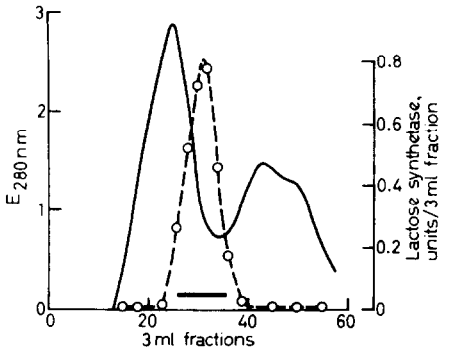

Fig. 2. Stage 5 of the purification scheme (table 1). Gel filtration of a solution (16 ml) of partially purified lactose synthetase A protein on a Bio-Gel P-200 column (2.5 X 43 cm) equilibrated with 0.01 M tris-HCl, pH 7.5, containing 0.04 M KCl. ——, $E_{280\,nm}$; o----o, lactose synthetase activity. Fractions were pooled as indicated by the bar.

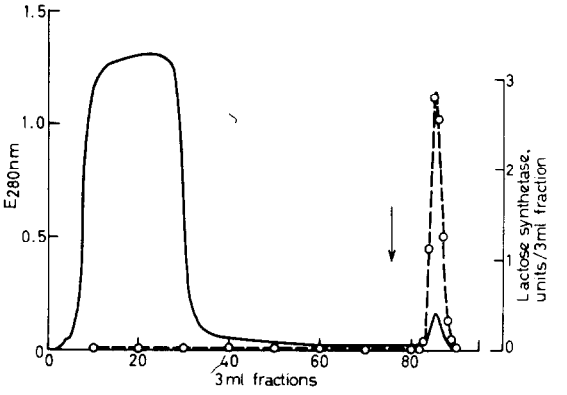

Fig. 3. Stage 6 of the purification scheme (table 1). Affinity chromatography of a solution (72 ml) of partially purified lactose synthetase A protein on a column (1.1 X 23 cm) of Sepharose–α-lactalbumin equilibrated with 0.01 M tris-HCl, pH 7.5, containing 0.04 M KCl and 3 mM NAG. Elution was continued with the same buffer after application of the sample until 76 fractions had been collected (arrow), then elution was with the tris buffer containing 0.04 M KCl only. ——, $E_{280\,nm}$; o-----o, lactose synthetase activity.

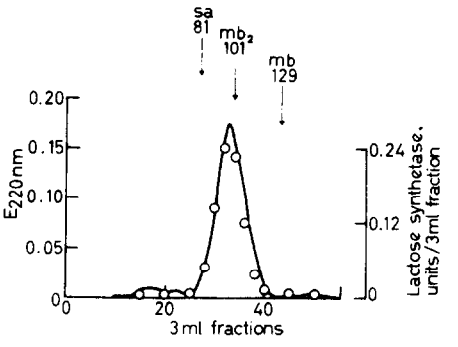

Fig. 4. Gel filtration of lactose synthetase A protein (0.43 mg) on a Bio-Gel P-200 column (2.5 X 43 cm) equilibrated with 0.01 M tris-HCl, pH 7.5, containing 0.04 M KCl. ——, $E_{220\,nm}$; o o, lactose synthetase activity. For estimation of its molecular weight, the A protein (50 μg) was added to myoglobin (mb; 0.5 mg), myoglobin dimer (mb_2; 0.5 mg) and bovine serum albumin (sa; 5 mg) and the mixture run as above. The position of the A protein was determined by the lactose synthetase assay and the positions of the standard proteins by extinction measurements. The elution volume of the A protein was 98 ml and those of the standard proteins (in ml) were as indicated by the arrows and figures. To study interaction between the A protein and α-lactalbumin, the mixture of A protein and standard proteins was run in the same buffer, with additions as described in the text. The elution volume of A protein was shifted to 84 ml in the presence of α-lactalbumin and NAG, whereas those of the standard proteins were unchanged.

Table 1
Purification of lactose synthetase A protein from human milk.

Stage	Volume (ml)	Protein (mg)	Lactose synthetase activity (units)	Recovery (%)	Specific activity (units/mg of protein)	Purification
(1) Skim milk	250	4500	21.7	100	0.005	1
(2) $(NH_4)_2SO_4$ precipitate (0–55% saturation)	66	2490	19.5	90	0.008	1.6
(3) Gel filtration on Sepharose 6B	269	603	19.7	91	0.033	6.6
(4) $(NH_4)_2SO_4$ precipitate (0–60% saturation) and dialysis	32	374	15.4	71	0.041	8.2
(5) Gel filtration on Bio-Gel P-200	72	84	10.8	50	0.129	26
(6) Affinity chromatography on Sepharose–α-lactalbumin	12	1.36	7.8	36	5.74	1150

column and seriously reduced the flow-rate in the next gel filtration step.

Gel filtration of the dialysed solution in two equal portions on a column of Bio-Gel P-200 (fig. 2) yielded a solution from the pooled fractions which was used for affinity chromatography on a column of Sepharose–α-lactalbumin (fig. 3) after addition of NAG to 3 mM concentration. Lactose synthetase A protein was retained on the column while other proteins were eluted with buffer containing 3 mM NAG, but was eluted sharply when the NAG was omitted from the buffer. The effluent fractions containing most of the A protein were free from NAG.

Further details of the purification procedure are given in table 1.

3.2. *Properties of the A protein preparation*

About 95% of the A protein preparation was eluted from a column of Bio-Gel P-200 as a single peak, with constant lactose synthetase specific activity over the peak (fig. 4). Comparison of the gel filtration behaviour of the A protein with that of "standard" proteins of known molecular weight [12], as shown in fig. 4, indicated that its molecular weight is 40,000–42,000. Its behaviour was unchanged when run in buffer containing bovine α-lactalbumin (100 μg/ml) but in the presence of bovine α-lactalbumin (100 or 200 μg/ml) plus NAG (3 mM or 6 mM) its elution volume corresponded to a molecular weight of about 60,000.

Sedimentation velocity measurements in the Spinco Model E ultracentrifuge at 20°, using absorption optics and a solution of the A protein (0.15 mg/ml) in 0.01 M tris-HCl containing 0.1 M KCl, gave sedimentation coefficients of 3.37 S and 3.52 S at 42,040 and 59,780 rpm, respectively.

The A protein showed characteristic [2] activities with D-glucose and NAG as galactosyl acceptor substrates at different α-lactalbumin concentrations (fig. 5). With 80 mM glucose as acceptor, activity in the lactose synthetase assay without added α-lactalbumin was 3.4% of that in the presence of 150 μg/ml of added α-lactalbumin.

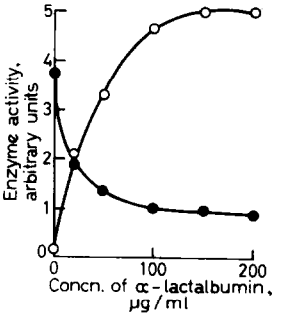

Fig. 5. Lactose synthetase (o——o) and *N*-acetyl-lactosamine synthetase (•——•) activities of purified lactose synthetase A protein at different human α-lactalbumin concentrations. Other substances in the assays were at the concentrations listed for standard assays in the Materials and methods section.

4. Discussion

The purification procedure described above provides a fairly simple means of isolating lactose synthetase A protein from human milk in good yield. The conditions used for affinity chromatography are preferred to those mentioned by Trayer et al. [6] because the passage of A protein through the Sepharose–α-lactalbumin column is retarded much more in the presence of NAG than in the presence of glucose, when either sugar is used at optimum concentration (P. Andrews, unpublished work). The product still showed activity in the lactose synthetase assay without added α-lactalbumin, as does the A protein from cow's milk [2–4]. The specific activity of the product (table 1) indicates that human milk generally contains 10–20 mg of A protein/l.

The sedimentation coefficient of about 3.5 S for human milk A protein is consistent with the molecular weight of about 40,000 estimated by gel filtration. A similar molecular weight recently reported for cow's milk A protein was obtained in denaturing solvents [6]. The difference between these values and estimates of about 70,000 for both proteins [4, 13] may be due to dissociation phenomena [6].

Preliminary kinetic studies with partially purified A protein from human milk, in which it was found that α-lactalbumin acted as an uncompetitive inhibitor of the *N*-acetyl-lactosamine synthetase reaction [13], implied that α-lactalbumin combined with an A protein-substrate complex rather than with the A protein alone. Affinity chromatography and the gel filtration experiments with purified A protein in buffers containing either α-lactalbumin alone or α-lactalbumin plus NAG support this view. In addition, the gel filtration experiments indicate that one molecule each of A protein (MW about 40,000) and α-lactalbumin (MW 14,437) participate with NAG in forming a complex, since its molecular weight appears to be about 60,000.

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Summary

A fairly simple procedure for isolating lactose synthetase A protein from human milk in 36% yield is described. The A protein has a molecular weight of about 40,000. Its interaction with α-lactalbumin in the presence of *N*-acetylglucosamine has been demonstrated by affinity chromatography and gel filtration.

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