Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 224-229

ISOLATION OF GALACTOSYLTRANSFERASE FROM HUMAN MILK AND THE DETERMINATION OF ITS N-TERMINAL AMINO ACID SEQUENCE

Hubert E. Appert[1]*, Thomas J. Rutherford[2], George E. Tarr[3], Neil R. Thomford[1] and D. James McCorquodale[2]

[1]Medical College of Ohio at Toledo, Department of Surgery
[2]Medical College of Ohio at Toledo, Department of Biochemistry
C.S. 10008, Toledo, Ohio 43699
[3]The University of Michigan, Department of Biochemistry,
Ann Arbor, Michigan 48109

Received May 5, 1986

Galactosyltransferase (EC 2.4.1.22), purified to homogeneity from human milk by affinity chromatography, had an apparent molecular weight of 53,000 as determined by denaturing polyacrylamide gel electrophoresis. Subtraction of the estimated contribution of the oligosaccharide portion of the molecule leaves a M_r of 47,000. An N-terminal amino acid sequence analysis of the isolated protein revealed a sequence similar to that found near the 5' end of a cDNA clone isolated by Shaper et al (11), which encodes a 35,500 molecular weight protein. Either the molecular weight of galactosyltransferase, has been overestimated, or a discrepancy exists between the actual molecular weight of galactosyltransferase and that predicted by the bovine cDNA clone isolated by Shaper et al (11). © 1986 Academic Press, Inc.

Lactose synthetase catalyzes the terminal step in the synthesis of lactose according to the reaction:

$$\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP}$$

Enzymatic activity resulting in the synthesis of the galactose $\beta1\rightarrow4$ glucose linkage in lactose was first demonstrated by Watkins and Hassid (13) in particulate fractions obtained from bovine and guinea pig lactating mammary glands. Lactose synthetase activity was later demonstrated in bovine milk by a number of subsequent investigators (1, 2, 3, 6, 10). Galactosyltransferase isolated from bovine milk whey by affinity chromatography demonstrates the presence of three species of enzyme having approximate molecular weights of 54,000, 49,000 and 43,000 daltons (1). Gel filtration studies show that all

*To whom correspondence should be addressed. Supported by an ACS grant and Biomedical Research Grants (NIH) #2 S07 RR05700-15 and -16.

Abbreviations: SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate; IEF, isoelectric focusing; CNBr, cyanogen bromide.

0006-291X/86 $1.50
Copyright © 1986 by Academic Press, Inc.
All rights of reproduction in any form reserved.

three components are enzymatically active (10). Magee et al. (8) reported that the higher molecular weight form of the enzyme found in bovine milk could be converted to a lower molecular weight form by proteolysis. Gerber, Kozdrowski, Wyss and Berger (5) reported that galactosyltransferase, isolated from human milk, was homogeneous when analyzed by SDS-PAGE, but could be resolved into 13 different forms using isoelectric focusing. Since galactosyltransferase is a glycosylated protein, the heterogeneity that Berger and associates observed could be a result of differences in glycosylation resulting from post-translation processing of the enzyme. The low-degree of heterogeneity of the enzyme using SDS-PAGE suggested that the non-glycosylated portion of the enzyme occuring in human milk could be homogeneous. The isolation of galactosyltransferase from human milk was therefore attempted in order to determine whether enough homogeneous enzyme could be obtained for amino acid sequencing.

MATERIALS AND METHODS

Galactosyltransferase Assay. Galactosyltransferase was measured by determining the rate of transfer of $[^3H]$-galactose from UDP-$[^3H]$-galactose to the sugar acceptor, N-acetylglucosamine. The unreacted UDP-$[^3H]$-galactose was separated from the radioactive product of the reaction, $[^3H]$-galactosyl-N-acetylglucosamine, by ion exchange chromatography as described by Barker et al. (1).

Preparation of Affinity Columns. The hexanolamine derivative of N-acetylglucosamine was prepared as described by Barker et al (1) by condensing N-trifluoroacetyl-6-amino-1-hexanol with 3, 4, 6 tri-O-acetyl-2-acetamido-2-deoxy-β-D-glucopyranosyl chloride. The resultant compound, 6-amino-1-hexyl-2-acetamido-2-deoxy-β-D-glucopyranoside, was coupled to Sepharose 4B using CNBr. The ligand concentration was 1.2 mg of N-acetylglucosamine derivative per ml of wet Sepharose. Bovine α-lactalbumin was similarly coupled to Sepharose at a concentration of 3.5 mg per ml of Sepharose.

Purification Procedure. One liter of pooled human milk was defatted by centrifugation at 17,000 x g for 20 minutes at 4°C. The defatted milk was brought to pH 4.6 by the addition of 4 M acetic acid, and dialyzed for 48 hours against 2 changes of 20 liters of distilled water containing 10 ml of chloroform to prevent bacterial growth. After completion of the dialysis, the casein precipitate was removed by centrifugation at 17,000 x g for 20 minutes at 4°C. The clear supernatant was brought to 25 mM in sodium cacodylate (pH 7.4), 25 mM in $MnCl_2$ and 1 mM in 2-mercaptoethanol. The preparation was applied to a 500 ml (5 x 25 cm) N-acetylglucosamine-Sepharose column previously equilibrated with 25 mM sodium cacodylate, 25 mM $MnCl_2$, 1 mM 2-mercaptoethanol and 0.8 mM UMP. The column was washed with 6 bed volumes of the equilibration buffer. Elution was effected with 25 mM sodium cacodylate (pH 7.4), 8 mM N-acetylglucosamine, 1 mM 2-mercaptoethanol and 25 mM EDTA. In order to restore the Mn^{++} concentration, the eluted enzyme was concentrated to 3 ml using an Amicon PM 30 membrane, and then returned to a volume of 15 ml using a buffer solution containing 25 mM sodium cacodylate, (pH 7.4), 25 mM $MnCl_2$, 1 mM 2-mercaptethanol and 8 mM N-acetylglucosamine. The

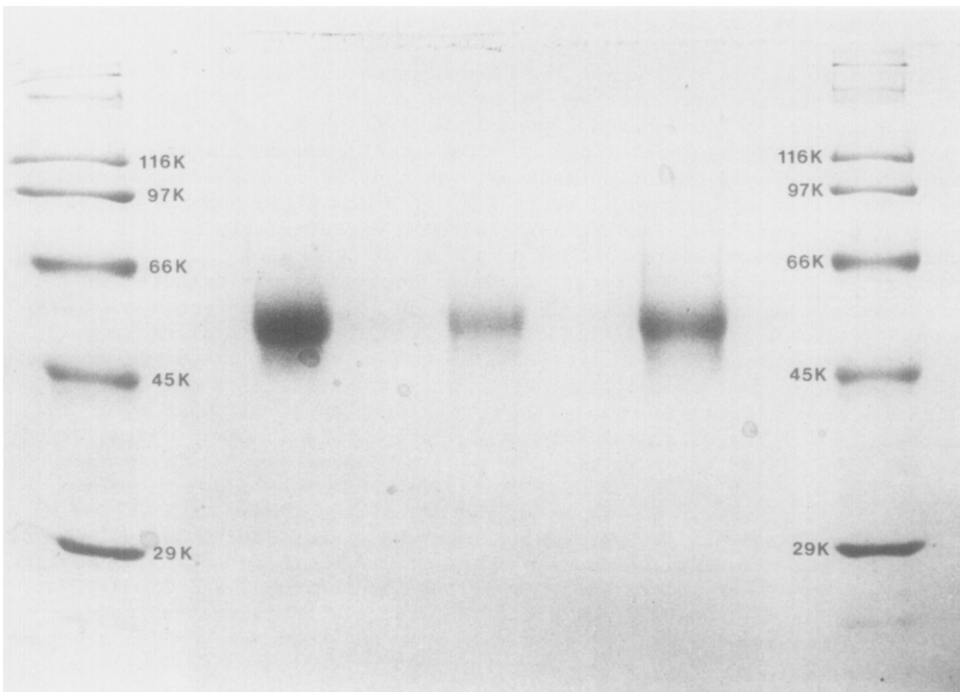
reconstituted enzyme solution was applied to a 1 x 19 cm α-lactalbumin-Sepharose column previously equilibrated with the same buffer as that used to reconstitute the enzyme preparation. The column was washed with 30 bed volumes of equilibration buffer, and the enzyme was eluted with equilibration buffer in which the N-acetylglucosamine was omitted. The eluted enzyme was collected in tubes containing a concentrated solution of N-acetylglucosamine which restored the concentration of that sugar to 8 mM. The elute was concentrated to 5 ml, applied to a second α-lactalbumin column, and affinity purified as just described. The enzyme that eluted from the second α-lactalbumin affinity column was concentrated to 1 ml and retained 90% of its activity for one month when stored at 4°C.

Amino Acid Sequence Analysis. The purified protein was dialyzed against 5 mM ammonium bicarbonate and subsequently dried under vacuum. The preparation was then reduced under nitrogen in 0.2 M N-ethylmorpholine/acetate buffer, pH 8.3, containing 6 M guanidine and 1 mM DTT and then alkylated with 4-vinyl-pyridine. Amino acid composition analysis and manual amino acid sequencing was then conducted as described by Tarr (12).

Electrophoretic Analysis. Denatured proteins were separated by 10% SDS-PAGE (7). After electrophoresis, proteins were stained with Coomassie brilliant blue and destained in 7.5% acetic acid. The two dimensional isoelectric focusing separation was performed as described by Kristjansson et al. (6).

RESULTS AND DISCUSSION

The yield of enzyme prepared by affinity chromatography ranged from 0.9 to 1.3 mg per liter of milk in 4 consecutive runs. The preparation was homogeneous on SDS-PAGE (Fig. 1), and had an apparent M_r of about 53,000. When



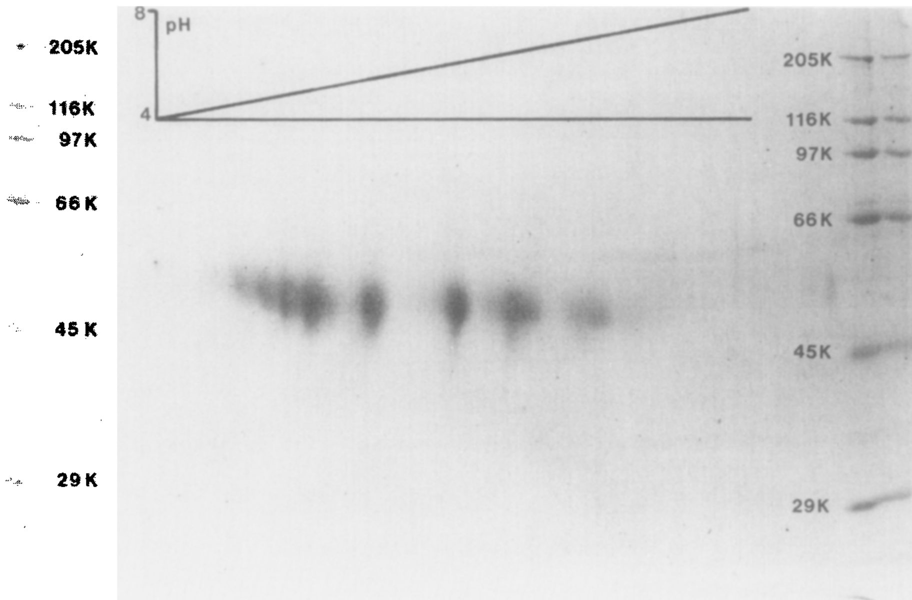


Fig 2. Protein staining pattern of purified galactosyltransferase transferase after two-dimensional IEF separation of proteins. A mixture of ampholines (2.5% wt/vol; pH range 3.5-10.0) and sucrose (5% wt/vol final concentration) was added to the dialyzed, lyophiled samples. The samples were applied to a 5% polyacrylamide gel (0.15% bisacrylamide) containing ampholines (pH range 3.5-10.0; final concentration 2.5% wt/vol). A sample was applied to an IEF slab gel for separation in the first dimension. After running for 16 hours, a whole track was cut out and mounted on top of a SDS-PAGE slab gel for separation in the second dimension by size. After electrophoresis, the proteins were stained with fast green. At the top, the pH in the IEF gel is indicated as measured in a co-migrating track. The position of molecular weight marker proteins are indicated.

the purified galactosyltransferase was subjected to isoelectric focusing with a pH gradient of 3.5-10, it could be resolved into eight fractions (Fig. 2) which is a finding similar to that reported by Berger and associates (5). The estimated amino acid composition is shown in Table I. The total number of residues was estimated allowing for the carbohydrate composition of the glycoprotein which was assumed to be 10% by weight using a previous literature report (4). The results of the N-terminal amino acid analysis are shown in Table II. The N-terminus of this preparation of galactosyltransferase was

Fig 1. SDS-PAGE electrophoresis of purified galactosyltransferase. Protein samples were dialyzed, lyophilyzed and redissolved with heating (100°C for 5 minutes) in sample preparation buffer containing 2% 2-mercaptoethanol. Electrophoresis on 10% polyacrylamide gels was performed at 25 milliamps for 5 hours at room temperature. Proteins were stained with Coomassie brillant blue R. Three different concentrations of purified protein were applied to the gel.

TABLE I: Estimated number of residues per peptide from acid hydrolysis of galactosyltransferase

Ala	23.2	Leu	34.1
Cya	.0	Met	8.5
Asp	56.3	Pro	41.0
Glu	34.0	Arg	31.1
Phe	16.4	Ser	29.5
Gly	40.1	Thr	16.0
His	11.4	PeC	4.8
Ile	18.4	Val	30.1
Lys	9.2	Tyr	17.6

TABLE II: N-terminal amino acid sequence of human galactosyltransferase

THR	GLY	GLY	ALA	ARG	PRO	PRO	PRO
PRO	LEU	GLY	ALA	SER	SER	GLN	PRO

not blocked since the amino acid sequence of that portion of the enzyme could be determined by sequential Edman degradations. The galactosyltransferase amino acid composition found in the current investigation showed fairly good correspondence to that of serum galactosyltransferase reported by Fujita-Yamaguchi and Yoshida (4). On the other hand, the amino acid composition of human milk galactosyltransferase had little resemblance to that reported by Podolsky and Weiser (9) for either of the enzymes that they designated as galactosyltransferase I and II.

Shaper et al. (11) recently reported partial amino acid sequences for bovine galactosyltransferase, from which they constructed oligonucleotide probes for isolation of a cDNA clone specific for the bovine enzyme from a λgt11 bovine expression library. Our N-terminal sequence is partially homologous to a predicted sequence near the 5'-end of their cDNA clone (i.e. near the N-terminus of the predicted amino acid sequence). Nine of the 16 amino acids in our N-terminal sequence agree in type and position with the predicted sequence near their N-terminus. However, the molecular weight that the nucleotide sequence of their cDNA clone predicts from this partially homologous sequence to their translational stop codon is only about 35,500, whereas the apparent molecular weight of our purified enzyme containing this partially homologous N-terminal amino acid sequence is 53,000. When we subtract the estimated portion of the molecular weight contributed by the oligosaccharide component (~6,000) from 53,000, we are left with a molecular weight, due to the amino acids alone, of 47,000 rather than 35,500. We are currently attempting to resolve this discrepancy.

REFERENCES

1. Barker, R., Olsen, K.W., Shaper, J.H. and Hill, R.L. (1972) J. Biol. Chem. 247, 7135-7147.
2. Brew, K., Vanaman, T.C., Hill, R.L. (1968) Proc. Nat. Acad. Sci. 59, 491-497.
3. Brodbeck, U. and Ebner, K.E. (1966) J. Biol. Chem, 241, 762-764.
4. Fujita-Yamaguchi, Y. and Yoshida, A. (1981) J. Biol. Chem. 256, 2701-2706.
5. Gerber, AC, Kozdrowski, I, Wyss, S.R., Berger, E.G. (1979) Eur. J. Biochem. 93, 453-460.
6. Kristjansson, G.I., Zwiers, H., Oestreicher, A.B., and Gispen, W.H. (1982) J. Neurochem. 39, 371-378.
7. Laemmli, U.K., (1970) Nature 227, 680-685.
8. Magee, S.C., Mawal, R. Ebner, K.E., (1973) J. Biol. Chem. 248, 7565-7569.
9. Podolsky, D.K. and Weiser, M.M. (1979) J. Biol. Chem. 254, 3983-3990.
10. Powell, J.T. and Brew, K. (1974) Eur. J. Biochem. 48, 217-228.
11. Shaper, N.L., Shaper, J.H., Neuth, J.L., Fox, J.L., Chang, H., Kirsch, I.R., Hollis, G.F. (1986) Proc. Natl. Acad. Sci. 83, 1573-77.
12. Tarr, G.E. (1986) Microcharacterization of Polypeptides, pp. 155-194, Humana Press Inc., Clifton, NJ.
13. Watkins, W.M. and Hassid, W.Z. (1962) J. Biol. Chem. 237, 1432-1440.