Vol. 198, No. 1, 1994
January 14, 1994
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 350-358

# LACTOSE AS AFFINITY ELUENT AND A SYNTHETIC SULFATED COPOLYMER AS INHIBITOR, IN CONJUNCTION WITH SYNTHETIC AND NATURAL ACCEPTORS, DIFFERENTIATE HUMAN MILK LEWIS-TYPE AND PLASMA-TYPE α-L-FUCOSYLTRANSFERASES

E. V. Chandrasekaran, John M. Rhodes, Rakesh K. Jain, and Khushi L. Matta

Department of Gynecologic Oncology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263

Received November 30, 1993

**Summary.** Human milk Lewis-type (α1,3/4) fucosyltransferase (FT) was separated from the plasma-type by chromatography on bovine IgG glycopep-Sepharose using lactose as the selective eluent and further purified on a column of Sephacryl S-100 HR. The α1,3-FT activity towards 2'-fucosyllactose was found to be associated with α1,4-FT activity. The inherency of N-acetyl-glucosaminide α1,3-L-FT activity in the Lewis-type FT was shown by a) the emergence of both α1,3- and α1,4-FT activities from the Sephacryl S-100 HR column in the same position; b) the inhibition of the α1,3-FT activity in the Lewis-type FT by α1,4-FT specific inhibitor namely a copolymer from 3-sulfoGalß1,3GlcNAcß-0-Allyl and acrylamide; c) the inhibition of α1,4 activity in the Lewis-type FT by α1,3-FT specific acceptor. Fetuin triantennary sialoglycopeptide, the corresponding asialo glycopeptide, and bovine IgG diantennary glycopeptide served as acceptors for both FTs, the Lewis-type FT being far more active than the plasma type FT towards the triantennary sialoglycopeptide. 

Studies on the fine specificities of α-L-fucosyltransferases present in various human tissues including milk have gained much importance due to the findings that the Lewis blood group determinants are present in the tumor-associated (1,2) and differentiated antigens (3) and also have been identified as possible ligands for adhesion molecules involved in inflammatory reactions (4,5). The product of the Lewis blood group gene has been characterized as an α1,4-L-fucosyltransferase. Further, the inheritance of a Lewis blood group gene has been found to associate with the ability of α1,3-fucosylation of glucose in lactose-based oligosaccharides (6). The human milk enzyme of the plasma type which utilizes

0006-291X/94 $5.00

sialylated as well as neutral type 2 acceptors is expressed independently of the Lewis locus. However, the reports of Greenwell et al. (7), and Caillard et al. (8) are suggestive of the existence of a genetic link between the Lewis-type and plasma-type fucosyltransferases. It becomes thus important to know whether the Lewis-gene-encoded enzyme is responsible for the biosynthesis of X, Y and sialyl X structures in addition to Le a, Le b, and sialyl a structures. The present communication reports the separation of human milk Lewis-type fucosyltransferase from the plasma-type by selective elution from the affinity column with lactose followed by gel filtration on Sephacryl S-100 HR and the utilization of a copolymer from 3-sulfoGalß1,3GlcNAcß-O-Allyl and acrylamide as the inhibitor as well as various synthetic and natural acceptors, in proving the inherency of α1,3 activity in the Lewis-type enzyme.

## MATERIALS AND METHODS

Preparation of human milk (HM) samples:

HM-I (50 ml) containing exclusively α1,3-L-FT activity from one donor and HM-II (42 ml) containing both α1,3- and α1,4-L-FT activities from another donor were used in the present study. Both samples were delipidated by sedimenting the fat from centrifugation at 4°C (5000 g for 60 min) (9). The resulting supernatants were dialyzed at 4°C for 48 h against three changes of 2 liters of 25 mM Tris-HCl pH 7.0 containing 35 mM $MgCl_2$, 10 mM $NaN_3$ and 1 mM ATP.

Affinity chromatography:

The above two dialyzed milk preparations were subjected to chromatography on a bovine IgG glycopep-Sepharose column (~30 ml in bed volume) (10), which has been thoroughly washed and equilibrated with 25 mM Tris buffer containing $MgCl_2$, $NaN_3$ and ATP. After the entry of the sample, the column was washed with 100 ml of the equilibration buffer. Then sequential elution was done with 100 ml each of 0.1 M lactose and 1.0 M NaCl in the same buffer. Both eluates from each milk preparation were concentrated to ~2.0 ml by ultrafiltration and then dialyzed at 4°C against three changes of 1 liter of the Tris buffer containing $MgCl_2$, $NaN_3$ and ATP. These samples were stored at 4°C until further experimentation; under these conditions, no loss of FT activities was seen.

Chromatography on Sephacryl S-100 HR column:

For the sake of simplification, the FT fractions (Lactose and NaCl eluates) from HM-I were combined, concentrated to ~2.0 ml by ultrafiltration. One ml from this pool of the affinity purified fractions from HM-I and one ml each of the affinity fractions, namely lactose and NaCl eluates from HM-II were fractionated separately on a Sephacryl S-100 HR column (1.0 x 116.0 cm) which had been equilibrated with 50 mM Tris-

HCl pH 7.0 containing 0.15 M NaCl, 0.1% Triton X-100 and 0.02% $NaN_3$. Fractions of 1.0 ml were collected in each case. Aliquots of 40 µl from the alternate fractions were used in the assay of FT activities under the standard incubation conditions (11); the α1,3-L-FT activity was measured using 2'-methylLacNAc or 3'-SulfoLacNAc or 2'-FucosylLacNAcß-O-Bn as the specific acceptor and the α1,4-L-FT activity using 2-methylGalß1,3GlcNAcß-O-Bn. The quantitation of [$^{14}$C] Fuc-containing products resulting from the various acceptors was done by the Dowex-1-Cl method as described earlier (11). Protein in the fractions was measured by the BCA method (Pierce Chemical Co.).

Glycopeptides:

The diantennary glycopeptide was prepared from bovine IgG (Calbiochem) by pronase digestion, gel filtration and Con A-Sepharose chromatography as described earlier (10). A similar procedure was followed to obtain from fetuin (Sigma), the triantennary sialoglycopeptide, which did not bind to Con A-Sepharose. The asialo glycopeptide was made by heating the triantennary sialoglycopeptide at 80°C in 0.1 N HCl for 1 h and chromatography of the neutralized solution after concentration to 1.0 ml on a Biogel P2 column (1.0 x 116.0 cm) to remove sialic acid.

Synthetic sulfated copolymer:

The copolymer from 3-SulfoGalß1,3GlcNAcß-O-Allyl and acrylamide was synthesized by following the procedure of Horejsi et al. (12) This preparation contained ~1.0 µmol of the sugar unit per mg weight and was similar in molecular size to dextran of average molecular weight 39,200, as evident from column chromatography on Biogel P60.

## RESULTS

Selective elution of Lewis-type FT from affinity matrix:

The isolation of fucosyltransferase rich in α1,4 activity from HM-II was achieved by elution of the affinity column IgG glycopep-Sepharose with 0.1 M lactose prior to NaCl. The lactose eluate contained almost equal amounts of α1,3 and α1,4 activities whereas the NaCl eluate was predominantly of α1,3 activity (76%).

Fractionation on Sephacryl S-100 HR:

The exclusively α1,3-FT activity present in HM-I was separated from most of the contaminating protein and emerged from Sephacryl S-100 HR column later than ovalbumin (Fig. 1A). The lactose eluted fraction from HM-II separated into two major peaks, the first one containing both α1,4 and α1,3 activity and the second, almost exclusively α1,3 activity (Fig. 1B). The NaCl eluted fraction from HM-II separated into a small peak containing both activities and a major peak exclusively of α1,3-FT activity (Fig. 1C). As noted

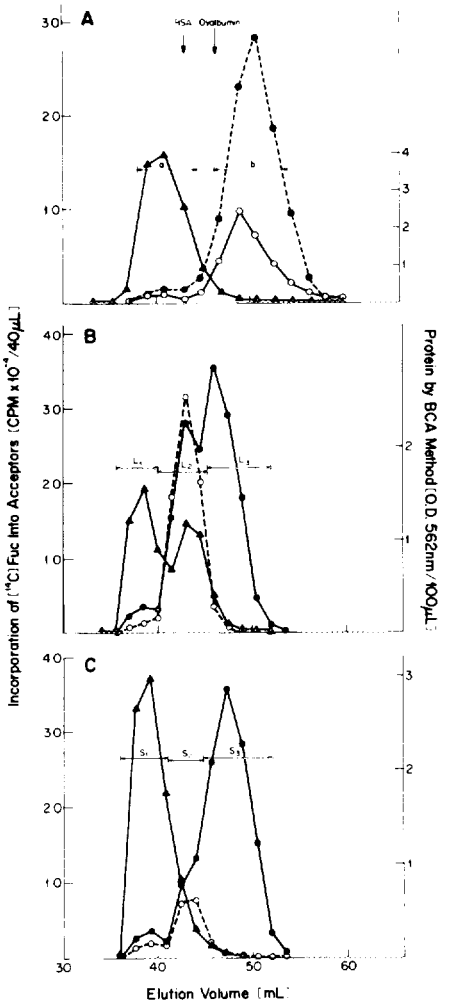


Fig. 1. Chromatography on Sephacryl S-100 HR column

A. Fractionation of the affinity-purified pooled preparation from HM-I.

○—○—○ Incorporation of [$^{14}$C] Fuc into 2'-methylLacNAc
●--●--● Incorporation of [$^{14}$C] Fuc into 3'-sulfoLacNAc
▲—▲—▲ Protein

B and C. Fractionations of the lactose and the NaCl eluted materials isolated from HM-II respectively.

●—●—● Incorporation of [$^{14}$C] Fuc into 2'-FucosylLacNAcß-0-Bn
○--○--○ Incorporation of [$^{14}$C] Fuc into 2-methylGalß1,3GlcNAcß-0-Bn
▲—▲—▲ Protein

for HM-I α1,3 activity, the exclusively α1,3 activity present in the lactose and NaCl eluted fractions from HM-II emerged from Sephacryl S-100 HR column later than ovalbumin whereas the peaks containing both α1,3 and α1,4-FT activities (Fig. 1B and 1C) emerged from the column in a position similar to bovine serum albumin.

Characteristics of the enzyme fractions:

From HM-II, 26.6%, 27.2% and 32.4% of α1,3-FT activity were recovered in $L_2$, $L_3$ and $S_3$ whereas 74.9% and 14.8% of α1,4-FT activity were found in $L_2$ and $S_2$. The ratio of α1,3-FT activity/α1,4-FT activity in $L_2$, $L_3$ and $S_3$ were 1.5, 76.6 and 53.2, respectively. A similar analysis of a and b from HM-I indicated very little α1,4-FT activity, the ratios being 250 in both cases.

$L_2$, $L_3$ and $S_3$ from HM-II and b from HM-I were tested with synthetic carbohydrates for FT activity (Table I); $L_2$ showed activity with α1,3- specific acceptors (2'-Fucosyl LacNAcß-0-Bn and 3'-SulfoLacNAc) and with α1,4-FT-specific acceptor (2-methylGalß1,3GlcNAcß-0-Bn). It did not react with the synthetic compound Galß1,3(4-0-Me)GlcNAcß-0-Bn, which had a methyl group substituted on C-4 hydroxyl and as well as with the disubstituted analog Galß1,3(4,6-di-OMe)GlcNAcß-0-Bn. $L_2$ showed activity with Galß1,3(6-0-Me)GlcNAcß-0-Bn indicating that C-6 substitution did not prevent the transfer of Fuc to C-4. $L_3$, $S_3$ and HM-I-b reacted only with α1,3-FT-specific acceptors. $L_2$ was the only one which showed considerable activity with 2'-fucosyllactose.

Inhibition of fucosyltransferases by the sulfated copolymer as well as competitive inhibition (Table II):

No inhibition of α1,3 activity was seen in $L_3$, $S_3$ and HM-Ib. It is explainable since the copolymer is a specific

TABLE I

Reactivity of Sephacryl S-100 HR major fractions isolated from human milk with synthetic acceptors

| | Enzyme Activity Sephacryl S-100HR Fractions | | | |
|---|---|---|---|---|
| | $L_2$ | $L_3$ | $S_3$ | HM-Ib |
| 2'-FucosylLacNAcß-0-Bn | 100 | 100 | 100 | 100 |
| 2'-FucosylLac | 25.9 | 0.2 | 1.1 | 0 |
| 3'-SulfoLacNAc | 110.5 | 86.7 | 97.8 | 86.4 |
| 2-methylGalß1,3GlcNAcß-0-Bn | 67.6 | 1.3 | 1.9 | 0.4 |
| Galß1,3(4-0-Me)GlcNAcß-0-Bn | 4.9 | | | |
| Galß1,3(6-0-Me)GlcNAcß-0-Bn | 63.4 | | | |
| Galß1,3(4,6-di-0-Me)GlcNAcß-0-Bn | 1.4 | | | |

TABLE II

Inhibition of the fucosyltransferase activities present in Sephacryl S-100HR fractions from human milk by synthetic compounds

| Synthetic compound in the reaction mixture | Inhibition of fucosyltransferase activity Sephacryl S-100HR fraction | | | |
|---|---|---|---|---|
| | $L_2$ | $L_3$ | $S_3$ | HM-Ib |
| α1,3-fucosyltransferase activity: 2'-FucosylLacNAcß-0-Bn as the acceptor in presence of SGGA & Acrylamide copolymers | | | | |
| a) 10 μg | 14.5% | 0 | 0 | 0 |
| b) 100 μg | 24.3% | 0 | 0 | 0 |
| α1,4-fucosyltransferase activity: 1) 2-methylGalß1,3GlcNAcß-0-Bn as the acceptor in presence of SGGA & Acrylamide copolymers | | | | |
| a) 10 μg | 16.2% | | | |
| b) 100 μg | 82.2% | | | |
| 2) Galß1,3(6-0-Me)GlcNAcß-0-Bn as the acceptor in presence of | | | | |
| a) 3'-SulfoLacNAc (3.0 mM) | 29.1% | | | |
| b) 3-SulfoGalß1,3GlcNAcß-0-Bn (3.0 mM) | 92.0% | | | |

inhibitor of α1,4-FT activity. On the contrary, $L_2$ which contained both α1,3 and α1,4 activity gave a different picture; both α1,3 and 1,4 activities were inhibited by 14.5% and 16.2% respectively at 10 μg level of the copolymer and 24.3 and 82.2% at 100 μg level. The competitive inhibitors namely 3'-sulfo LacNAc and 3-sulfoGalß1,3GlcNAcß-0-Bn for α1,3 and α1,4-FT activity respectively, when tested for the inhibition of α1,4 activity present in $L_2$, both inhibited the α1,4-FT activity by 29.1% and 92.0% respectively.

Activity towards glycopeptide acceptors (Table III):

At 20 μg levels, fetuin triantennary sialoglycopeptide was about 50% more active and bovine IgG diantennary glycopeptide was 40-60% less active than fetuin triantennary asialoglycopeptide when tested for the acceptor activity towards $L_2$, $L_3$ and $S_3$; at 200 μg level, fetuin triantennary asialo glycopeptide and bovine IgG glycopeptide were 3-8 fold more active. Fetuin triantennary sialo glycopeptide showed lower activity at 200 μg level as compared to 20 μg level, the least activity being seen with $L_3$, $S_3$ and HM-Ib. On a molar basis, fetuin triantennary sialo glycopeptide (20

TABLE III

Reactivity of Sephacryl S-100HR major fractions from human milk with glycopeptides

| Glycopeptide | α1,3-L-fucosyltransferase activity* Sephacryl S-100HR fractions | | | |
|---|---|---|---|---|
| | $L_2$ | $L_3$ | $S_3$ | HM-Ib |
| Fetuin sialoglycopeptide (Triantennary with 3'-sialylLacNAc terminals): | | | | |
| 20 μg | 30.4 | 15.5 | 18.4 | 9.2 |
| 200 μg (0.40 mM)[a] | 18.6 | 2.3 | 2.9 | 1.0 |
| Fetuin asialoglycopeptide: | | | | |
| 20 μg | 20.3 | 10.5 | 12.8 | 8.8 |
| 200 μg (0.48 mM)[b] | 125.5 | 76.9 | 102.5 | 31.5 |
| Bovine IgG glycopeptide: (Diantennary) | | | | |
| 20 μg | 7.8 | 6.9 | 5.8 | 5.1 |
| 200 μg (0.56 mM)[c] | 56.5 | 51.1 | 58.7 | 17.3 |

* Expressed as percent of the activity towards 2'-fucosylLacNAcβ-O-Bn (3.0 mM).

[a,b and c] denote the concentration of glycopeptides in the reaction mixture based on approximate molecular weights of 5000, 4100 and 3600 daltons, respectively.

μg = ~0.04 mM) appeared to be an efficient acceptor for these enzymes.

## DISCUSSION

Prieels et al. (13) were first to propose the existence of α1,3- and α1,4-FT activities in a single molecular species of human milk. Subsequently, a partial separation of α1,3/4-FT and α1,3-FT from human milk was reported by Johnson and Watkins (14) and Eppenberger-Castoti et al. (9). Recently, Johnson et al. (15) reexamined the activities of human milk α1,3/4-FT at two stages of purification and then raised a doubt whether the remaining α1,3-FT activity in Sephacryl S-200 eluate was really inherent to the α1,4-FT species.

The present study used two milk samples; one contained strictly α1,3-FT activity and the other, both α1,3- and α1,4-FT activities; this strategy enabled us to ascertain the specificity of the real non-Lewis type α1,3-FT present in one milk and compare this with that of the non-Lewis type obtainable from the other milk sample. A specific elution of the affinity column (bovine IgG glycopep-Sepharose) with lactose resulted in the separation of α1,4-FT activity-rich

fraction from the bulk of α1,3-FT activity. The chromatographic fractionation using Sephacryl S-100 HR instead of Sephacryl S-200 column resulted in a clear separation of α1,4-FT containing the inherent α1,3-FT activity (Lewis-type) from the strictly α1,3 activity (plasma-type). The inherent α1,3-FT activity of the Lewis-type enzyme was demonstrated by us from comparing the inhibition of α1,3- and α1,4-FT activities in $L_2$ and α1,3-FT activity in $L_3$, $S_3$ and HMI-b by an α1,4-FT specific inhibitor namely the copolymer from SGGA and acrylamide. While α1,3 FT activity in addition to the expected α1,4-FT activity present in $L_2$ was inhibited by this copolymer, the α1,3-FT activities of $L_3$, $S_3$ and HMI-b were not inhibited at all. Further, both 3'-sulfoLacNAc and 3-sulfoGalß1,3GlcNAcß-O-Bn which are the specific acceptors respectively for 1,3- and α1,4-FT activities, inhibited the α1,4-FT activity present in $L_2$. We also showed (Table III) that even though both Lewis-type and plasma-type FT of human milk can utilize fetuin triantennary sialoglycopeptide, the corresponding asialo glycopeptide and bovine IgG diantennary glycopeptide as acceptors, the triantennary sialo glycopeptide served as an efficient substrate for the Lewis-type enzyme only.

Acknowledgments: The authors are thankful to Dr. Tariq S. Malik, M.D. for providing the human milk specimens. This investigation was supported by Grant CA35329, National Cancer Institute, U.S.A.

## REFERENCES

1. Fukushi, Y., Nudelman, E., Levery, S., and Hakomori, S. (1984) J. Biol. Chem. 256, 10456-10463.
2. Hakomori, S. (1985) Cancer Res. 45, 2405-2414.
3. Feizi, T. (1988) J. Biol. Chem. 263, 10186-10191.
4. Brandley, B.K., Swiedler, S.J., and Robbins, P.W. (1990) Cell 63, 861-863.
5. Springer, T.A., and Laskey, L.A. (1990) Nature 349, 196-197.
6. Johnson, P.H., Yates, A.D., and Watkins, W.M. (1981) Biochem. Biophys. Res. Commun. 100, 1611-1618.
7. Greenwell, P., Johnson, P.H., Edwards, J.M., Reed, R.M., Moore, P.P., Bird, A., Graham, H.A., and Watkins, W.M. (1986) Blood Transfusion Immunohemat. 29, 223-249.
8. Caillard, T., LePendu, J., Ventura, M., Mada, M., Rault, G., Mannoni, P., and Oriol, R. (1988) Exp. Cli. Immunol. 5, 15-23.

9. Eppenberger-Castori, S., Lotscher, H., and Finne, J. (1989) Glycoconjugate J. 6, 101-114.
10. Yazawa, S., Madiyalakan, R., Chawda, R.P., and Matta, K.L. (1986) Biochem. Biophys. Res. Commun. 136, 563-569.
11. Chandrasekaran, E.V., Jain, R.K., and Matta, K.L. (1992) J. Biol. Chem. 267, 23806-23814.
12. Horejsi, V., Smolek, P., and Kocourek, J. (1978) Biochem. Biophys. Acta 538, 293-298.
13. Prieels, J.P., Monnom, D., Dolmans, M., Beyer, T.A., and Hill, R.L. (1981) J. Biol. Chem. 256, 10456-10463.
14. Johnson, P.H., and Watkins, W.M. (1982) Biochem. Soc. Trans. 10, 445-446.
15. Johnson, P.H., Donald, A.S.R., Feeney, J., and Watkins, W.M. (1992) Glycoconjugate J. 9, 251-264.