Glycoconjugate Journal (1992) **9**:241–249

Purification of the Lewis blood-group gene associated α-3/4-fucosyltransferase from human milk: an enzyme transferring fucose primarily to Type 1 and lactose-based oligosaccharide chains

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Received 23 June 1992

A soluble Lewis blood-group gene associated α-3/4-L-fucosyltransferase has been purified from human milk by a series of steps involving hydrophobic chromatography on Phenyl Sepharose 4B, ion exchange chromatography on CM-Sephadex C-50, affinity chromatography on GDP-hexanolamine Sepharose 4B and gel filtration on Sephacryl S-200. The first step separated α-3-L-fucosyltransferase activity directed towards *N*-acetylglucosamine in Type 2 (Galβ1-4GlcNAc-R) acceptors from an α-3/4-fucosyltransferase fraction acting on both Type 1 (Galβ1-3GlcNAc-R) and Type 2 acceptors. Further purification of this latter fraction on CM-Sephadex and GDP-hexanolamine Sepharose gave a single peak of fucosyltransferase activity that catalysed the addition of fucose to *N*-acetylglucosamine in both Type 1 and Type 2 acceptors and to the *O*-3 position of glucose in lactose-based oligosaccharides. The enzyme preparation at this stage resembled previously described α-3/4-fucosyltransferase preparations purified from human milk. However, gel filtration of this preparation on Sephacryl S-200 or Sephadex G-150 separated further amounts of α-3-fucosyltransferase activity acting solely on Type 2 acceptors and left a residual α-3/4-fucosyltransferase that retained strong α-4 activity with the Type 1 acceptor, lacto-N-biose 1, and α-3 activity with 2′-fucosyllactose, but had relatively little α-3 activity with *N*-acetyllactosamine and virtually no capacity to transfer fucose to glycoproteins with *N*-linked oligosaccharide chains having unsubstituted terminal Type 2 structures.

Keywords: α-3/4-L-Fucosyltransferase, α-3-L-fucosyltransferase, Lewis-gene encoded enzyme, Lewis antigens, X-antigen, human milk

The biochemical characterization of the Le^a and Le^b blood-group determinants as respectively tri- [Galβ1-3(Fucα1-4)GlcNAc] [1] and tetra-saccharide [Fucα1-2Galβ1-3(Fucα1-4)GlcNac) [2] sequences containing L-fucose joined in α-anomeric linkage to the *O*-4 position of a subterminal *N*-acetylglucosamine unit in a Type 1 structure (Galβ1-3GlcNAc), led to the prediction that the product of the Lewis *Le* gene is an α-4-L-fucosyltransferase [3] that catalyses the reaction;

GDP-fucose + Galβ1-3GlcNAc → Galβ1-3(Fucα1-4)GlcNAc + GDP

Implicit in this prediction was the assumption that the fucosyltransferase utilizes only Type 1 chains as acceptor substrates. The finding of α-4-fucosyltransferase activity in human milk [4, 5] stomach tissue and submaxillary glands [6] from individuals known to be carrying the Lewis *Le* gene, and absence of this enzyme from the same tissues of those believed to be homozygous for the 'silent' allele *le*, appeared to confirm this prediction. However, experiments with saliva from individuals of known Lewis phenotype showed that the ability to add fucose to the *O*-3 position of glucose in lactose-based (Galβ1-4Glc) structures is also dependent on the inheritance of an *Le* gene [7]. Independently, Prieels *et al.* [8] showed that a highly purified fucosyltransferase preparation from human milk had the capacity to transfer fucose not only to the *O*-4 position of *N*-acetylglucosamine in Type 1 chains but also to the *O*-3 position of this sugar in Type 2 chains; these authors therefore concluded that the *Le* gene product is an

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α-3/4-fucosyltransferase. The inference from this finding is that the *Le* gene ultimately controls the synthesis of not only the Lewis Le^a and Le^b antigens but also the mono and difucosyl Type 2 structures, X (or Le^x), Galβ1-4(Fucα1-3)GlcNAc, and Y (or Le^y) Fucα-1-2Galβ1-4(Fucα1-3)GlcNAc.

The present paper describes a further purification step that removes a large proportion of the GDP-fucose: *N*-acetylglucosaminide α-3-L-fucosyltransferase activity from the *Le* gene associated α-3/4-L-fucosyltransferase isolated from human milk; the residual enzyme preparation reacts primarily with Type 1 and lactose-based substrates. This work has been briefly described elsewhere [9].

Materials and methods

Substrates

GDP-L-[U-^{14}C]fucose (254 Ci mol^{-1}) was purchased from Amersham International, UK. *N*-Acetyllactosamine (Galβ1-4GlcNAc) and lacto-*N*-biose 1 (Galβ1-3GlcNAc), synthesized by the methods of Alais and Veyrières [10] and Flowers [11], respectively, and 2′-fucosyllactose (Fucα1-2Galβ1-4Glc), lacto-*N*-fucopentaose I (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc) and lacto-*N*-fucopentaose II [Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc], recovered from human milk as described by Donald and Feeney [12] were the gifts of the late Dr A. S. R. Donald, MRC Clinical Research Centre, Harrow, UK. Lacto-*N*-biose 1 attached to a spacer arm [Galβ1-3GlcNAc-*O*-$(CH_2)_8$COOMe] was a gift from Professor R. U. Lemieux, University of Alberta, Edmonton, Canada. Lactose was purchased from BDH Biochemicals, UK, and recrystallized from ethanol before use. Transferrin and α_1-acid glycoprotein were purchased from Sigma Chemical Company Ltd., UK. Sialic acid was removed from the glycoproteins by heating a 1% solution with 0.05 M H_2SO_4 for 1 h at 80 °C. The product was thoroughly dialysed against distilled water and freeze dried.

Chromatography media

Phenyl Sepharose CL-4B, CM-Sephadex C-50, Sephadex G-150 Sephacryl S-200 and CNBr-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals Ltd, UK. GDP-Hexanolamine, synthesized by the method of Beyer *et al.* [13], was supplied by the late Dr A. S. R. Donald, MRC Clinical Research Centre, Harrow, UK, and GDP-hexanolamine Sepharose 4B was prepared by coupling GDP-hexanolamine and CNBr-activated Sepharose by the procedure of Cuatrecasas [14]. Chromatography papers No 40, and DE 81 were supplied by Whatman Ltd, UK. Blue dextran and molecular weight standards for gel filtration and SDS gel electrophoresis were purchased from Sigma Chemical Company Ltd, UK.

Gel exclusion chromatography for molecular size determination

Apparent molecular weights were determined by gel exclusion chromatography on Sephadex G-150. Samples were diluted to 1 ml with 0.15 M NaCl containing 0.01 mg ml^{-1} bovine serum albumin and run in 0.15 M NaCl on a gel column 1.5 cm × 70 cm at a rate of 20 ml h^{-1}. Blue dextran and the molecular weight markers cytochrome *c* and haemoglobin were detected by their absorbances at respectively 629 nm, 470 nm, and 430 nm. α-Lactalbumin, ovalbumin, transferrin and alcohol dehydrogenase were detected by estimation of protein.

SDS gel electrophoresis

Polyacrylamide gel electrophoresis in SDS was carried out as described by Laemmli [15] on 8% gels in 0.38 M Tris-HCl buffer, pH 8.8, containing 0.1% SDS. Gels were stained for protein as described by Dubray and Bezard [16].

Protein estimations

Protein was estimated by the method of Read and Northcote [17] with bovine serum albumin as standard.

Fucosidase treatment

α-2- and α-3/4-L-fucosidases from *Trichomonas foetus* were separated as described [18] and tested for specificity with lacto-*N*-fucopentaose I and lacto-*N*-fucopentaose II. The enzyme preparations (5 µl) were incubated at 37 °C for 1 h with 5 µl of a 1% solution of the oligosaccharides, and the products were run on Whatman No. 40 paper in propan-1-ol–ethyl acetate–pyridine–water (5:1:1:3, by vol, solvent 1). The released fucose and undigested oligosaccharides were visualized with alkaline silver nitrate [19]. For tests on the products of [^{14}C]fucose transfer 5 µl of the fucosidase preparation were incubated with 5 µl of an aqueous solution of the labelled product (approximately 10,000 counts min^{-1}). The reaction mixtures were chromatographed as above and the released [^{14}C]fucose was detected on a Packard Radiochromatogram scanner and counted in a Nuclear Chicago scintillation counter.

Fucosyltransferase assays

Standard reaction mixtures for α-3- and α-4-fucosyltransferases contained, in a total volume of 70 µl, 0.5 µmol acceptor, 0.2 nmol (70,000 counts min^{-1}) GDP[^{14}C]fucose, 1 µmol $MnCl_2$, 5 µmol sodium cacodylate-HCl buffer, pH 7.3, and 5 µg NaN_3. For assays on crude enzyme sources 1 µmol of neutral ATP was included in the reaction mixture, and for assays on highly purified enzymes 250 µg bovine serum albumin were added. *N*-Acetyllactosamine and 2′-fucosyllactose were the substrates used for routine assays of α-3-fucosyltransferase activity and lacto-*N*-biose 1 was the substrate for routine assays of α-4-fucosyltransferase activity. The reaction

mixtures were incubated at 37 °C between 30 min and 16 h according to the activity of the transferases. The reaction products were separated by chromatography on Whatman DE 81 paper in solvent 1 or, when it was necessary to establish whether 2'-fucosylated lacto-*N*-biose I or *N*-acetyllactosamine were present in addition to the 4- or 3-fucosylated products, chromatography was carried out on DE 81 paper in ethyl acetate–pyridine–water (10:4:3, by vol, solvent 2) for 40 h. Papers were scanned for radioactivity on a Radiochromatogram scanner and the product areas were cut out and counted in a scintillation counter. Mobilities (R_{Lac}) of the products were measured relative to a lactose standard.

Purification of GDP-Fucose:Galβ1-3GlcNAc-R α-3/4-fucosyltransferase from human milk

Step 1: ammonium sulphate precipitation and hydrophobic chromatography on Phenyl Sepharose CL-4B. Pooled milk from blood group Le(a − b+) donors (3450 ml) was spun for 30 min at 13 000 × **g** in a Sorvall RC-5B centrifuge in an SS34 rotor. The defatted milk was siphoned off through the accumulated fat layer and then subjected to ammonium sulfate fractionation. The fraction precipitating between 33% and 66% saturation was redissolved in 25 mM sodium cacodylate-HCl, pH 7.3, containing 0.1 M NaCl and 5% (w/v) glycerol (buffer A) and loaded on a column (10 cm × 55 cm) of Phenyl Sepharose CL-4B. The gel was washed with 1200 ml buffer A, transferred to a 2.5 cm × 100 cm column, washed with a further 1200 ml of the same buffer and then eluted with 600 ml 25 mM Tris-HCl, pH 9.1, containing 5% (w/v) glycerol (buffer B). The eluted material had fucosyltransferase activity with *N*-acetyllactosamine, but not with lacto-*N*-biose 1, and was designated PS Eluate 1. The Phenyl Sepharose CL-4B column was then washed with 200 ml 50 mM sodium cacodylate-HCl, pH 6.5, containing 5% (w/v) glycerol (buffer C), followed by 1500 ml of the same buffer containing 2% Triton X-100 (Fig. 1). The eluted material had fucosyltransferase activity with both *N*-acetyllactosamine and lacto-*N*-biose 1 and was designated PS Eluate 2.

Step 2: cation exchange chromatography of PS Eluate 2 on CM-Sephadex C-50. The pooled PS Eluate 2 fractions were loaded on a column (1.5 cm × 25 cm) of CM Sephadex C-50 which had been equilibrated with buffer C. The gel was washed with 180 ml buffer C and then a linear gradient (0–0.4 M) of NaCl was applied. A sharp peak of fucosyltransferase activity (CM Eluate) reacting with both *N*-acetyllactosamine and lacto-*N*-biose 1 emerged at a concentration of approximately 0.18 M NaCl (Fig. 2).

Step 3: affinity chromatography of CM Eluate on GDP-Hexanolamine Sepharose 4B. The pH of the pooled CM eluate fractions was adjusted to 7.3 with Tris-HCL buffer, pH 9.1, and the solution was then pumped at a rate of 10 ml h^{-1} on a column (1 cm × 5 cm) of

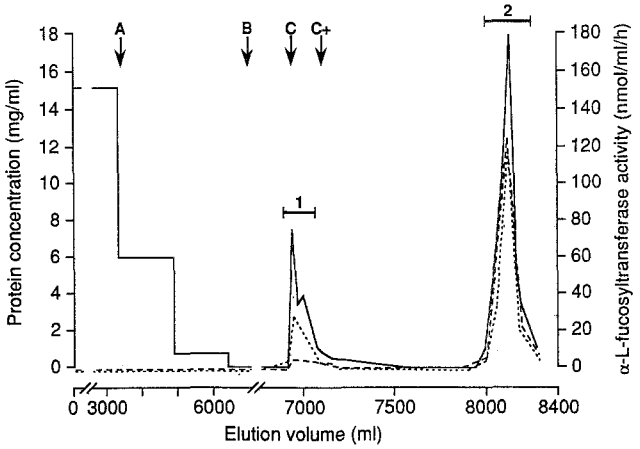

Figure 1. Elution profile of α-3/4-fucosyltransferase from a Phenyl Sepharose CL 4B column. α-3- and α-4-Fucosyltransferase activities were assayed under standard conditions: ....., α-3-fucosyltransferase activity measured with Galβ1-4GlcNAc as substrate; ----, α-4-fucosyltransferase activity measured with Galβ1-3GlcNAc as substrate; ——, protein concentration. The arrows indicate the points of application of the buffers all of which contained 5% (w/v) glycerol; A, 25 mM Na cacodylate-HCl, pH 7.3, containing 0.1 M NaCl; B, 25 mM Tris-HCl, pH 9.1; C, 50 mM Na cacodylate-HCl, pH 6.5: C+, buffer C containing 2% Triton X-100. The bars show the fractions pooled to give PS Eluate 1 and PS Eluate 2.

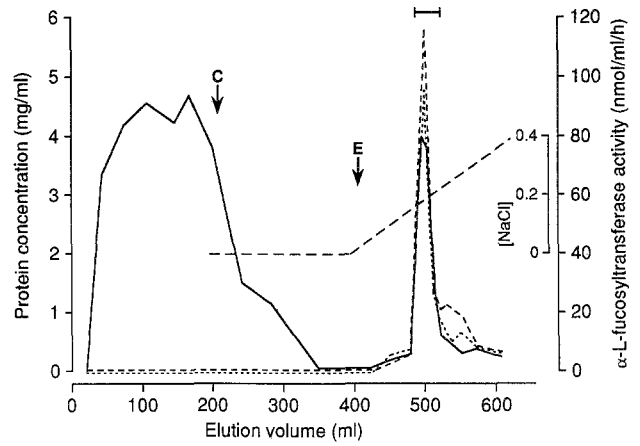

Figure 2. Elution profile obtained by chromatography of PS-Eluate 2 on a CM-Sephadex C-50 column. Fucosyltransferase assays were carried out under standard conditions. ----, NaCl gradient; ....., α-3-fucosyltransferase activity measured with Galβ1-4GlcNAc as substrate; ----, α-4-fucosyltransferase activity measured with Galβ1-3GlcNAc as substrate; ——, protein concentration. The arrows indicate the point of application of 50 mM Na cacodylate-HCl buffer, pH 6.5, containing 5% glycerol (C) and the start of the NaCl (0–0.4 M) gradient (E). The bar shows the fractions pooled to give CM-Eluate.

GDP-hexanolamine Sepharose 4B which had been equilibrated with buffer A. When all the solution had been loaded, the column was first washed with 52 ml 25 mM sodium cacodylate-HCl, pH 7.3, buffer containing NaCl (0.1 M) and glycerol (25% w/v) (buffer D) and then eluted

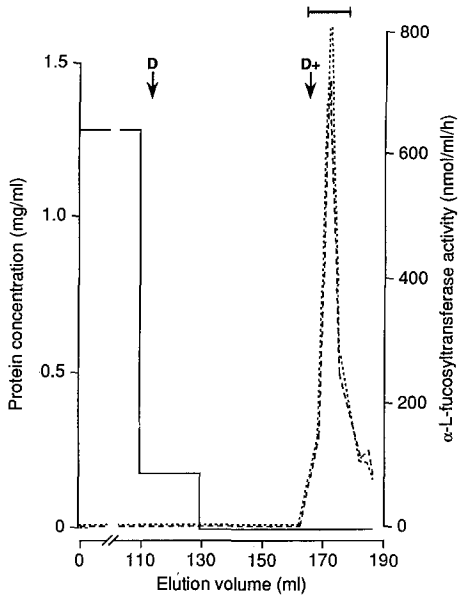

Figure 3. Elution profile obtained by chromatography of CM-Eluate on GDP-hexanolamine Sepharose 4B column. Fucosyltransferase assays were carried out under standard conditions. ....., α-3-Fucosyltransferase activity measured with Galβ1-4GlcNAc as substrate; – – – –, α-4-fucosyltransferase activity measured with Galβ1-3GlcNAc as substrate; ——, protein concentration. The arrows indicate the point of application of the elution buffers; D, 25 mM Na cacodylate, pH 7.3, containing 0.1 M NaCl and 25% glycerol; D+, buffer D to which had been added 33 μM GDP and 1 mM $MnCl_2$. The bar shows the fractions that were pooled to give GDP-HS Eluate.

with 30 ml buffer D to which had been added GDP (33 μM) and $MnCl_2$ (1 mM). The eluted fucosyltransferase (GDP-HS Eluate) reacted with both *N*-acetyllactosamine and lacto-*N*-biose 1 (Fig. 3).

Step 4: further separation of GDP-Fucose:Galβ1-4GlcNAc-R α-3-L-fucosyltransferase from the GDP-Fucose:Galβ1-3-N-GlcNAc-R α-3/4-L-fucosyltransferase

Method 1: Chromatography of GDP-HS Eluate on Sephacryl S-200 in buffer containing 25% glycerol. GDP-HS Eluate (50 μl) was diluted to 1 ml by the addition of 0.1 ml 0.5 M sodium cacodylate-HCl, pH 5.5, and 0.85 ml 25 mM sodium cacodylate-HCl, pH 5.5, containing 0.2 M NaCl, 25% (w/v) glycerol and 0.01% bovine serum albumin and loaded on a column (1.5 cm × 64 cm) of Sephacryl S-200. The column was eluted with the same buffer at a rate of 20 ml h^{-1} and the resulting 1 ml fractions were brought to neutral pH by the addition of 50 μl 50 mM Tris-HCl buffer, pH 9.1, and tested for fucosyltransferase activity with *N*-acetyllactosamine, lacto-*N*-biose 1 and 2′-fucosyllactose; in contrast to the activity profile obtained with the eluate from the GDP-hexanolamine Sepharose 4B column the eluted fractions gave a profile consisting of two overlapping peaks (Fig. 4a). The first peak to emerge (S-200 Eluate 1, fractions 51–56) had higher activity with lacto-*N*-biose 1 and 2′-fucosyllactose than with *N*-acetyllactosamine and was partially resolved from a second larger peak (S-200 Eluate 2, fractions 89–110) which had much stronger activity with *N*-acetyllactosamine. The fractions constituting each of these peaks were pooled and concentrated by loading them on mini-columns containing 0.5 ml GDP-hexanolamine Sepharose 4B and eluting the enzyme with 0.9 ml 50 mM sodium cacodylate-HCl buffer, pH 6.5, containing 33 μM GDP, 1 mM $MnCl_2$, 25% glycerol and 0.1% bovine serum albumin. The volume of the eluted fractions was adjusted to 1 ml with 0.5 M cacodylate-HCl buffer, pH 5.5, and each was re-chromatographed on the same Sephacryl S-200 column in the same buffer system. Rechromatography of

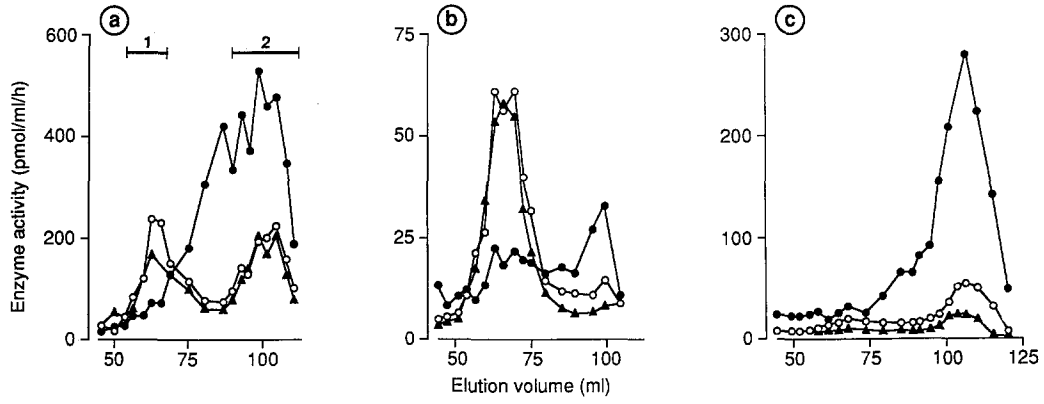

Figure 4. Chromatography of GDP-HS Eluate on Sephacryl S-200; elution with buffer, pH 5.5, containing 25% glycerol. (a) First elution from column; the fractions indicated by the bars were pooled to give S-200 Eluates 1 and 2. (b) Rechromatography of S-200 Eluate 1. (c) Rechromatography of S-200 Eluate 2. Fucosyltransferase assays were carried out under standard conditions: ●, α-3-Fucosyltransferase activity with Galβ1-4GlcNAc as acceptor substrate; ▲, α-3-fucosyltransferase activity measured with Fucα1-2Galβ1-4Glc as acceptor substrate; ○, α-4-fucosyltransferase activity measured with Galβ1-3-GlcNAc as acceptor substrate.

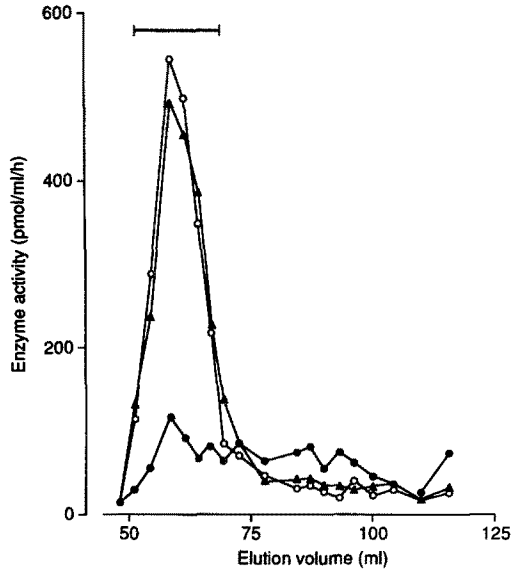

Figure 5. Chromatography of GDP-HS Eluate on Sephacryl S-200; elution with buffer, pH 5.5, containing 5% glycerol. Fucosyl transferase assays were carried out under standard conditions. ●, α-3-Fucosyltransferase activity with Galβ1-4GlcNAc as acceptor substrate; ▲, α-3-fucosyltransferase activity with Fucα1-2Galβ1-4Glc as acceptor substrate; ○, α-4-fucosyltransferase activity with Galβ1-3GlcNAc as acceptor substrate. The bar indicates the fractions pooled to give S-200 Eluate 3.

S-200 Eluate 1 gave a major peak of activity which reacted strongly with lacto-*N*-biose I and 2′-fucosyllactose and only very weakly with *N*-acetyllactosamine (Fig. 4b) and a second minor peak that had activity primarily with *N*-acetyllactosamine. Rechromatography of S-200 Eluate 2 yielded a major peak of fucosyltransferase reacting primarily with *N*-acetyllactosamine (Fig. 4c).

Method 2: Chromatography of GDP-HS Eluate on Sephacryl S-200 in buffer containing 5% glycerol. Chromatography on Sephacryl S-200 was repeated exactly as described above except that the buffer used for elution contained 5% glycerol in place of 25% glycerol. The fractions were tested for fucosyltransferase activity with the same three substrates as in method 1; only one major peak of activity, S-200 Eluate 3, was observed (Fig. 5). The enzyme preparation reacted strongly with lacto-*N*-biose I and 2′-fucosyllactose and only very weakly with *N*-acetyllactosamine.

Method 3: Chromatography of GDP-HS Eluate on Sephadex G-150. GDP-HS Eluate (50 µl) was diluted to a volume of 1 ml with 0.15 M NaCl containing 0.01% bovine serum albumin, loaded on a column of Sephadex G-150 (1.5 cm × 70 cm) and eluted wih 0.15 M NaCl at a flow rate of 20 ml h^{-1}. The emerging 1 ml fractions tested with the same three substrates as in methods 1 and 2 once again showed partial separation of the enzyme activities (Fig. 6a). The peak fractions were pooled and concentrated by loading them on 0.5 ml columns of GDP-hexanolamine Sepharose 4B followed by elution with 1 ml 0.25 mM sodium cacodylate-HCl buffer, pH 7.3, containing 0.1 M NaCl and 25% glycerol. Re-chromatography on the same Sephadex G-150 column of the first peak to emerge (peak 1) resulted in a fucosyltransferase preparation (Fig. 6b) with similar acceptor specificity to the enzyme preparation obtained by rechromatography of S-200 Eluate 1 (Fig. 4b). Rechromatography of the second peak of fucosyltransferase activity (peak 2) gave an enzyme that reacted preferentially with *N*-acetyllactosamine (Fig. 6c).

Results and discussion

Milk samples from Le(a − b+) donors were stored at −40 °C until sufficient had been collected to be pooled and used for purification of the fucosyltransferases. Preliminary assays on the pooled milk showed that it contained enzymes that catalysed the transfer of fucose from GDP-L-fucose to lacto-*N*-biose 1 and *N*-acetyllactosamine and the

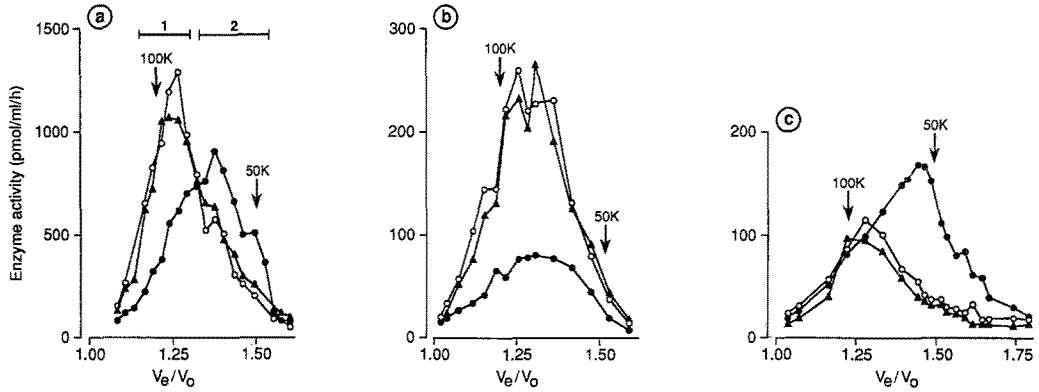

Figure 6. Chromatography of GDP-HS Eluate on Sephadex G-150. (a) First elution with 0.15 M NaCl. The bars indicate the fractions pooled to give peaks 1 and 2. (b) Rechromatography of peak 1. (c) Rechromatography of peak 2. Fucosyltransferase activities were assayed under standard conditions: ●, α-3-Fucosyltransferase activity with Galβ1-4GlcNAc as substrate; ▲, α-3-fucosyltransferase activity with Fucα1-2Galβ1-4Glc as substrate; ○, α-4-fucosyltransferase activity with Galβ-1-3GlcNAc as substrate. The arrows indicate molecular sizes deduced from the elution volumes of molecular weight markers.

Table 1. Purification of α-3/4-fucosyltransferase activity from human milk of Le(a − b+) donors.

Purification step	*Volume (ml)*	*Protein (mg)*	*Acceptor*							
			N-acetyllactosamine				*Lacto-N-biose 1*			
			Total activity (μmol min^{-1})	*Specific activity (μmol min^{-1} mg^{-1})*	*Purification (fold)*	*Recovery (%)*	*Total activity (μmol min^{-1})*	*Specific activity (μmol min^{-1} mg^{-1})*	*Purification (fold)*	*Recovery (%)*
Whole milk	3450	60 500	0.34	5.6×10^{-6}	1	100	0.19	3.0×10^{-6}	1	100
Phenyl Sepharose CL-4B	170	813	0.08	1.1×10^{-6}	19	28	0.11	1.4×10^{-4}	44	60
CM-Sephadex C-50	100	150	0.07	4.9×10^{-4}	88	21	0.08	5.1×10^{-4}	165	41
GDP-hexanolamine-Sepharose 4B	6	0.030	0.05	1.7	310 000	16	0.05	1.6	520 000	27

chromatographic mobilities ($R_{Lac} = 0.7$) of the products on DE 81 paper in solvent 2, together with the release of fucose by the *T. foetus* α-3/4-L-fucosidase and not by α-2-L-fucosidase, indicated that the products were Galβ1-4(Fucα1-3)GlcNAc and Galβ1-3(Fucα1-4)GlcNAc, respectively. The Type 1 and Type 2 disaccharides are both substrates for the blood-group *H/Se* gene associated α-2-fucosyltransferases [20], but no products susceptible to α-2-fucosidase, or migrating as expected of α-2-linked products ($R_{Lac} = 1.0$ on Whatman DE 81 paper in Solvent 2), were detected. The pooled milk preparation therefore contained α-3- and α-4-fucosyltransferase activity and no measurable quantity of α-2-fucosyltransferase activity. Although α-2-fucosyltransferase would be expected to be present in milk from Le(a − b+) donors [21], in our experience this enzyme activity is considerably lower in fresh milk than the α-3 and α-4 activities, and is less stable to storage.

Purification of GDP-Fucose:Galβ1-3GlcNac-Rα-3/4-L-fucosyltransferase from the milk of a blood group Le(a − b+) donor

Earlier investigations on fucosyltransferases in human saliva and milk demonstrated the co-presence of an α-3-fucosyltransferase catalysing the transfer of fucose only to *N*-acetylglycosamine in Type 2 chains and an α-3/4-fucosyltransferase which has a much wider acceptor specificity [7, 22]. The α-3-fucosyltransferase was found in all saliva and milk samples examined, whereas the broader specificity α-3/4 enzyme was present in these secretions only when the donor carried a Lewis *Le* gene. Partial separation of these two enzyme species in milk was previously achieved by chromatography on CM-Sephadex C50 at pH 6.5; the unbound fraction had a greatly increased ratio of α-3- to α-4-*N*-acetylglucosaminide:α-fucosyltransferase activity [22]. In the present investigation hydrophobic chromatography on Phenyl Sepharose CL-4B was chosen as the first purification step of the α-3/4-fucosyltransferase because this absorbent provided a more efficient means than chromatography on CM-Sephadex for concentrating the enzymes from large volumes of milk. Both the α-3- and α-4-fucosyltransferase activities bound to the Phenyl Sepharose CL 4B but preliminary experiments showed that elution at high pH, in the absence of sodium chloride, released an enzyme fraction that transferred fucose preferentially to Type 2 acceptors. Elution of the Phenyl Sepharose 4B column with Tris-HCl buffer, pH 9.1, yielded a fucosyltransferase preparation that had strong activity with *N*-acetyllactosamine but only marginal activity with lacto-*N*-biose 1 or 2′-fucosyllactose (Fig. 1). The chromatographic mobility ($R_{Lac} = 0.70$) of the *N*-acetyllactosamine product on Whatman DE 81 paper in solvent 2, and its susceptibility to α-3/4-fucosidase, indicated that the fucose had been transferred to the *O*-3 position of the *N*-acetylglucosamine residue in the disaccharide. The first enzyme eluted from the Phenyl Sepharose 4B column was thus a GDP-fucose:Galβ1-4GlcNAc-R α-3-L-fucosyltransferase. Subsequent elution of the Phenyl Sepharose 4B with buffer, pH 6.5, containing Triton X-100 released a second, sharp peak of fucosyltransferase activity that utilized *N*-acetyllactosamine, 2′-fucosyllactose and lacto-*N*-biose 1, and therefore had the properties of an α-3/4-fucosyltransferase (Fig. 1).

Cation exchange chromatography of PS Eluate 2 on CM-Sephadex C-50 resulted in a 3–4-fold increase in purification and yielded a preparation (CM-Eluate) that still reacted well with both the Type 1 and Type 2 disaccharides (Fig. 2). Major purification of this fraction was achieved by affinity chromatography of the CM Eluate on GDP-hexanolamine Sepharose 4B. Elution with buffer, pH 7.3, to which had been added GDP and $MnCl_2$, gave a preparation of fucosyltransferase purified 500 000-fold (GDP-HS Eluate) with respect to activity with lacto-*N*-biose 1 (Fig. 3). The enzyme recoveries and extent of purification achieved up to this stage are summarized in Table 1. Whereas the degrees of purification and recoveries

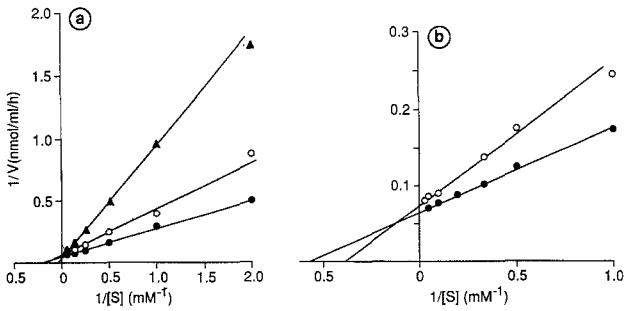

Figure 7. Competition experiments with GDP-HS Eluate. (a) Fucα1-2Galβ1-4Glc substrate: ●, no inhibitor; ▲, plus 5 mM Galβ1-3GlcNAc; ○, plus 5 mM Galβ1-4GlcNac, (b) Galβ1-4GlcNAc substrate: ●, no inhibitor; ○, plus 0.9 mM Galβ1-3GlcNAc-*O*-$(CH_2)_8$COOMe. Fucosyltransferase assays were carried out under standard conditions except that the substrate concentration was varied as shown, the GDP-fucose concentration was adjusted to 0.8 nmol (140 000 counts min^{-1}) and the inhibitors were added to each tube at the concentrations given above.

of enzyme activity measured with *N*-acetyllactosamine and lacto-*N*-biose 1 were dissimilar at this stage of the purification (Table 1), the recovery of activity with 2′-fucosyllactose as substrate (500 000-fold purification and 39% yield) closely paralleled that obtained with the Type 1 acceptor, lacto-*N*-biose 1.

Evidence of lack of homogeneity of α-3/4-fucosyltransferase

SDS polyacrylamide gel electrophoresis of the most active fraction eluted from the GDP-hexanolamine Sepharose 4B column showed three distinct protein bands with molecular weights of approximately 75 000, 67 000 and 61 000 stained with the sensitive silver stain reagent [16], indicating that the enzyme had not been purified to homogeneity (data not shown). The fucosyltransferase preparation did not enter the polyacrylamide gel in the absence of SDS, and it was therefore not possible to attempt to correlate enzyme activity with a migrating protein band in a nondenaturing gel.

Competition experiments with the trisaccharide 2′-fucosyllactose as acceptor substrate and *N*-acetyllactosamine or lacto-*N*-biose 1 as competitive substrates indicated that both these disaccharides were competitive inhibitors of the α-3-fucosyltransferase activity in the GDP-HS Eluate (Fig. 7a). Similar experiments with *N*-acetyllactosamine as acceptor and lacto-*N*-biose 1 as inhibitor were rendered difficult because the chromatographic mobilities of the two products of [^{14}C]fucose incorporation are almost identical and, hence, they are difficult to separate. However, with *N*-acetyllactosamine as substrate and lacto-*N*-biose attached to a spacer arm [Galβ1-3GlcNAc-*O*-$(CH_2)_8$COOMe] as a competitor only weak inhibition was observed, and a double reciprocal plot of substrate concentration versus initial reaction velocity suggested that the inhibition was mixed or noncompetitive (Fig. 7b). This result therefore supported the idea that the preparation might still contain more than one enzyme.

Further purification of the eluate from GDP-hexanolamine Sepharose

The fucosyltransferase preparation obtained at the affinity chromatography stage resembled in its acceptor specificity the enzyme described by Prieels *et al.* [8]; these authors found that the three activities, that is the transfer of fucose to the *O*-3 positions of *N*-acetylglucosamine and glucose, and to the *O*-4 position of *N*-acetylglucosamine, co-purified over 500 000-fold on affinity chromatography on GDP-hexanolamine agarose, and proposed that the three activities are contained in a single enzyme. However, in the present experiments, chromatography of the GDP HS Eluate on Sephacryl S-200 in buffer pH 5.5 containing 25% glycerol (method 1), partially split these activities and gave two overlapping peaks (S-200 Eluates 1 and 2) with differing ratios of α-3- and α-4-fucosyltransferase activities (Fig. 4a). Rechromatography of S-200 Eluate 1 on the same column of Sephacryl S-200 further resolved the two peaks and gave a major peak of enzyme activity reacting primarily with lacto-*N*-biose 1 and 2′-fucosyllactose (Fig. 4b). Rechromatography of S-200 Eluate 2 on Sephacryl S-200 gave a sharp peak of fucosyltransferase activity that reacted primarily with *N*-acetyllactosamine and had only marginal residual activity with lacto-*N*-biose 1 and 2′-fucosyllactose (Fig. 4c). This gel filtration step had therefore separated a substantial further amount of GDP-fucose:Galβ1-4GlcNAc α-3-fucosyltransferase from the α-3/4-fucosyltransferase preparation.

When a similar aliquot of the GDP HS Eluate was chromatographed on Sephacryl S-200 in the same buffer but in the presence of only 5% glycerol in place of 25% (method 2) a single major peak (S-200 Eluate 3) of fucosyltransferase activity was eluted; this preparation had almost equal activity with lacto-*N*-biose 1 and 2′-fucosyllactose but only marginal activity with *N*-acetyllactosamine (Fig. 5). This result was reproducible and it is not known whether the loss of the GDP-fucose:Galβ1-4GlcNAcα-3-fucosyltransferase activity was due to the enzyme binding irreversibly to the column or whether it had been denatured. Chromatography of an aliquot of the GDP HS Eluate on Sephadex G-150 (method 3) again gave partial separation of the fucosyltransferases (Fig. 6a) and rechromatography of the two major peaks also yielded two preparations, one which had activity primarily with lacto-*N*-biose 1 and 2′-fucosyllactose and a second which had activity primarily with *N*-acetyllactosamine (Fig. 6b, c).

Apparent M_r of α-3- and α-3/4-fucosyltransferases

Estimates of the apparent molecular sizes of the two major peaks separated on the Sephadex G-150 column gave a value of about M_r 54 000 for the α-3-fucosyltransferase

Table 2. Acceptor specificities of the α-3/4-fucosyltransferase preparations obtained by chromatography on GDP-hexanolamine Sepharose (GDP-HS Eluate) and Sephacryl S-200 (S-200 Eluate 3).

Substrate	*α-3/4-Fucosyltransferase preparation (Relative activity*[a]*)*	
	GDP-HS Eluate	S-*200 Eluate 3*
Galβ1-4GlcNAc	100	11
Galβ1-3GlcNAc	105	100
Fucα1-2Galβ1-4Glc	69	91
Asialo-α_1-acid glycoprotein	30	0.3
Asialo-transferrin	16	0.2

[a] Incorporation into 0.5 μmol of oligosaccharide acceptor, or 100 μg of glycoprotein acceptor, relative to the incorporation into 0.5 μmol Galβ1-3GlcNAc under the same conditions.

and about M_r 90000 for the α-3/4-fucosyltransferase (Fig. 6a, b). Eppenberger-Castori *et al.* [23] isolated α-3/4-fucosyltransferase from human milk and their final purification step involving HPLC on a Superose 12 gel filtration column separated two fractions; one with α-3/4-fucosyltransferase activity with an apparent M_r 98000 and a second fraction with an apparent M_r 47000 which had only *N*-acetylglucosaminide α-3-fucosyltransferase activity. This gel filtration step therefore appears to have brought about a separation similar to that described in this paper but, although these authors reported only limited specificity studies [23], the α-3/4-fucosyltransferase fraction they isolated appears to retain considerably more activity with Type 2 acceptors than was found for the preparations obtained by gel filtration of GDP-HS Eluate.

Acceptor specificities of GDP-HS Eluate and S-200 Eluate 3

The relative activities of the GDP-HS Eluate and the S-200 Eluate 3 with the three low molecular weight substrates, *N*-acetyllactosamine, lacto-*N*-biose 1 and 2′-fucosyllactose and the asialo-glycoproteins transferrin and α-1 acid glycoprotein, which both have terminal Type 2 structures on *N*-linked oligosaccharide chains [24, 25], are summarized in Table 2. The activities with GP-HS Eluate were similar to those recorded by Prieels *et al.* [8] in that the Type 1 and Type 2 disaccharides, and the glycoprotein, asialo-transferrin, were all good acceptors for the enzyme. In contrast, the α-3/4-fucosyltransferase preparation obtained after Sephacryl S-200 chromatography showed only weak activity with the free Type 2 disaccharide and the activity with the asialo-glycoproteins transferrin and α-$_1$ acid glycoprotein had virtually disappeared (Table 2).

Insufficient material was available to examine the homogeneity of the α-3/4-fucosyltransferase preparations obtained at the final gel filtration stage. However, the current wide acceptance that the Lewis-gene associated fucosyltransferase is responsible for the synthesis on cell surfaces of not only the Lewis blood-group determinants Le^a and Le^b but also of Type 2 X (Le^x), Y(Le^y) and sialyl-X determinants, makes the more limited acceptor substrate specificity of the enzyme preparations described in this paper unexpected. This is especially the case since a cloned human cDNA that encodes an α-3/4-fucosyltransferase [26], believed to be the product of the Lewis blood-group gene, is reported to determine the expression in COS-1 cells of both Type 1 and Type 2 fucosylated structures. In order to characterize more precisely the α-3/4-fucosyltransferase preparations obtained at the stage of elution from the GDP-hexanolamine Sepharose 4B (GDP-HS Eluate) column and after Sephacryl S-200 chromatography (S-200 Eluate 3), the general properties, acceptor specificities, and structures of the products formed, have been examined in greater detail and are reported in the following paper [27].

Acknowledgements

We are indebted to Professor R. U. Lemieux for the gift of a synthetic substrate and wish to acknowledge the advice and oligosaccharides received from our late colleague, Dr A. S. R. Donald.

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