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Note

Improved method for the isolation of 2'-fucosyllactose from human milk

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The oligosaccharides obtained from human milk are of intrinsic interest because some of them carry the H, Le^a and Le^b blood group determinants. They have also been used extensively as well-characterised structures for determination of the specificity of antibodies[1] and lectins[2], as substrates for establishing the specificity of glycosidases[3] and as acceptor substrates in studies on the glycosyltransferases of the ABO and Lewis blood group systems[4]. In particular 2'-fucosyllactose (Fuc-α(1 → 2)Gal-β(1 → 4)Glc) is used as acceptor substrate in assays of the blood group A and B gene specified glycosyltransferases, and for elution of these enzymes from biospecific absorbents[5,6]. Established methods for the isolation of this disaccharide use either charcoal columns[7] or gel filtration on Sephadex[8] or Bio-Gel[9] columns, and produce a mixture of 2'-fucosyllactose and 3-fucosyllactose (Gal-β(1 → 4)-[Fuc-α(1 → 3)]Glc). The subsequent fractionation of this mixture by paper chromatography is very time-consuming and impractical when gram-scale amounts are involved. This paper describes a relatively rapid method for the isolation of 2'-fucosyllactose by a combination of recycling chromatography on a column of K^+ form cation-exchange resin[10] and fractionation on a charcoal–Celite column. The recycling procedure also has applications for separation of other milk sugars in the di- to pentasaccharide range.

EXPERIMENTAL

Uridine diphosphate [^{14}C]galactose (UDP-[^{14}C]galactose, specific activity 307 Ci/mole) was purchased from the Radiochemical Centre (Amersham, Great Britain). The DE-52 diethylaminoethyl cellulose and DE-81 diethylaminoethyl paper were products of Whatman Biochemicals (Maidstone, Great Britain), and the charcoal was from BDH (Poole, Great Britain). The Celite 535 was obtained from Koch-Light Labs. (Colnbrook, Great Britain). The Zerolit 225-X4, minus 200 mesh resin was a product of Diamond Shamrock (Isleworth, Great Britain). The resin was fractionated by repeatedly collecting the fraction which sedimented in 0–40 min. This was washed in turn with 1 *M* KOH, water, 1 *M* HCl, water, 1 *M* KOH and finally with water until the washings were neutral. The columns were obtained from Pharmacia (London, Great Britain) and the peristaltic pump from LKB (Croydon, Great Britain). The differential refractometer was a Model 1103 from LDC (Stone, Great

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Britain). The three-way slide valves were products of Locarte (London, Great Britain).

The following reference milk oligosaccharides were either prepared in this laboratory or were supplied by Dr. A. Gauhe, Heidleberg, G.F.R.: 2′-fucosyllactose; 3-fucosyllactose; lactodifucotetraose, Fuc-α(1 → 2)Gal-β(1 → 4) [Fuc-α(1 → 3)]Glc; lacto-N-tetraose, Gal-β(1 → 3)GlcNAc-β(1 → 3)Gal-β(1 → 4)Glc; lacto-N-neotetraose, Gal-β(1 → 4)GlcNAc-β(1 → 3)Gal-β(1 → 4)Glc; lacto-N-fucopentaose I, Fuc-α(1 → 2)Gal-β(1 → 3)GlcNAc-β(1 → 3)Gal-β(1 → 4)Glc; lacto-N-fucopentaose II, Gal-β(1 → 3)-[Fuc-α(1 → 4)]GlcNAc-β(1 → 3)Gal-β-(1 → 4)Glc.

Fractions which were shown by paper chromatography to contain either 2′- or 3-fucosyllactose were distinguished using an assay in which the 2′-, but not the 3-, isomer is converted into a radioactive tetrasaccharide by a blood group B gene specified α-3-galactosyltransferase. Solutions to be assayed were incubated with UDP-[^{14}C]galactose and the serum of a blood group B person using the conditions described by Sabo *et al.*[4]. The incubation mixture was separated by descending chromatography on DE-81 paper in *n*-propanol–ethyl acetate–pyridine–water (5:1:1:3) for 18 h. The radioactive tetrasaccharide product (R_{lactose} 0.68) was located with a Packard 7201 radiochromatogram scanner and counted in a Nuclear Chicago scintillation counter Mark II.

Descending paper chromatography, except as described above, was carried out on Whatman No. 40 paper with ethyl acetate–pyridine–water (12:5:4, solvent a; 10:4:3, solvent b). Sugars were located with $AgNO_3$ reagent[11].

RESULTS AND DISCUSSION

Pooled human milk (1 litre) was dialysed against water (6 × 4 litre changes over 3 days). The diffusate was concentrated to about 300 ml and sialic acid containing oligosaccharides were removed by passing the solution through a column (20 × 3 cm I.D.) of DE-52 previously equilibrated with water. The eluate and washings were concentrated to approximately 75 ml and the mixture was left at 3°C overnight. The lactose which crystallised out was removed by filtration. The filtrate and washings were then adjusted to a volume of 100 ml and the solution was fractionated in two batches by recycling chromatography on a cation-exchange resin (K^+ form) with water as eluent. The principle involved in the separation appears to be primarily one of molecular sieve chromatography[10] although it is possible that ligand-exchange effects[12] exert a modifying influence on the elution positions of the various sugars. The apparatus used for the recycling consisted of the following connected in series: (1) a peristaltic pump; (2) two columns (80 × 5 cm I.D.) filled with the Zerolit (K^+) resin equilibrated with water: (3) a differential refractometer; (4) two 3-way slide valves. The valves were arranged so that the column eluate could be either returned to the pump for recycling or collected and the pump fed instead with water from a reservoir. The pump was set at a flow-rate of approximately 100 ml/h. During the first cycle the eluate in region A of the chromatogram shown in Fig. 1 was collected, and paper chromatography indicated that it contained hexasaccharides and larger oligosaccharides. The rest of the eluate was recycled. When material began to emerge from the column for the second time the eluate was collected in fractions (50 ml). Paper chromatography established that the region marked B in Fig. 1 was composed

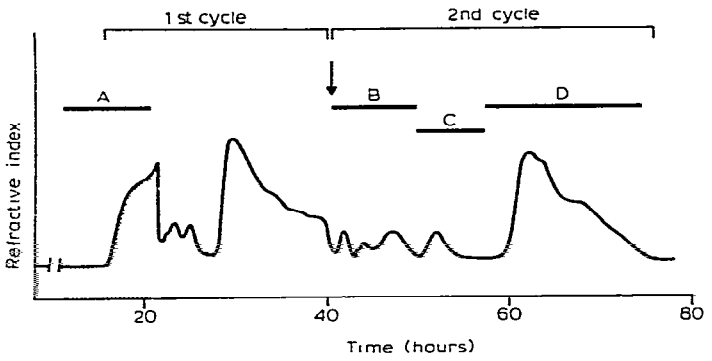

Fig. 1. Fractionation of crude milk oligosaccharides. The diffusate from human milk, freed from excess lactose as described in the text, was applied to two columns (80 × 5 cm I.D.) of Zerolit 225 (K^+), connected in series. The columns were eluted with water at 100 ml/h and the eluate passed through a differential refractometer. The eluate indicated by the hatching on the chromatogram was removed, and the rest was recycled. During the second cycle fractions were collected beginning at the point indicated by the arrow. The position at which the components emerged was established as described in the text.

of tri- to pentasaccharides and regions C and D contained mainly 2′-fucosyllactose and lactose, respectively. The elution position of 2′-fucosyllactose was confirmed by an assay in which this trisaccharide serves as an acceptor for [^{14}C]galactose transferred from UDP-[^{14}C]galactose by the blood group B gene specified α-3-galactosyltransferase present only in the serum of blood group B individuals.

Fractions were pooled as indicated by the bars on Fig. 1, and the pools from the two runs were combined. Fraction C which contained the 2′-fucosyllactose was then further fractionated on a charcoal–Celite (1:1) column (30 × 2 cm I.D.). The column was eluted first with water (1 litre), and then with portions (1 litre) of 1%, 2.5%, 5%, 7.5% and 10% (v/v) ethanol in water, at a flow-rate of 60 ml/h maintained by a pump. The elution position of 2′-fucosyllactose was established as described above. One troublesome contaminant of 2′-fucosyllactose is an unidentified compound which co-chromatographs with it on paper and accepts [^{14}C]galactose in β-linkage from UDP-[^{14}C]galactose by means of a β-galactosyltransferase present in the serum of most individuals irrespective of their blood group[13]. Each of the fractions from the charcoal–Celite column was therefore tested for this contaminant using the assay conditions described for 2′-fucosyllactose, but using the serum of a blood group O person instead of that of a blood group B. Fractions containing 2′-fucosyllactose but free of the β-galactose-accepting contaminant were combined, concentrated, and then subjected to further recycling chromatography. For this a single column (90 × 2.5 cm I.D.) of the Zerolit 225 (K^+) resin was used at a flow-rate of 25 ml/h. The 2′-fucosyllactose was eluted during the third cycle as indicated on Fig. 2. Small amounts of other sugars, mainly 3-fucosyllactose were removed by this procedure, and the 2′-fucosyllactose so obtained (yield approx. 1.5 g/litre of milk) migrated as a single spot when examined by paper chromatography in solvents a and b. The identity of this compound was confirmed by gas–liquid chromatographic analysis of the constituent sugars[14] and by methylation[15].

Recycling chromatography on X4 cross-linked K^+ form resin is also useful for the fractionation of other milk oligosaccharides. Fig. 3 shows the chromatogram

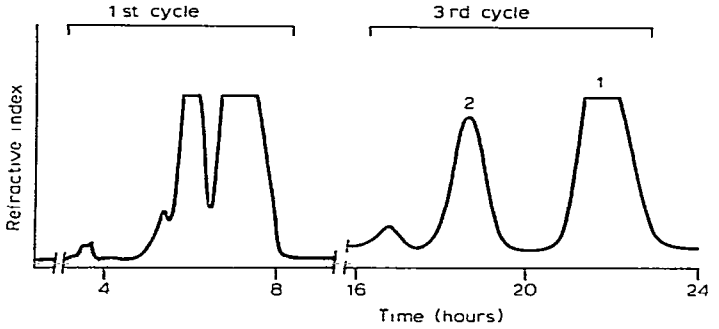

Fig. 2. Final purification of 2′-fucosyllactose by recycling chromatography. The 2′-fucosyllactose fraction from the charcoal–Celite column was loaded onto a column (90 × 2.5 cm I.D.) of Zerolit 225 (K^+) resin, and the column was eluted with water at a flow-rate of 25 ml/h. The portion of the eluate that is shown hatched on the chromatogram was removed during the first cycle. The remainder was allowed to remain on the column for two cycles, and was then eluted during the third. 1 = 2′-Fucosyllactose; 2 = 3-fucosyllactose.

obtained by recycling an artificial mixture of 2′-fucosyllactose, 3-fucosyllactose, lactodifucotetraose, lacto-N-tetraose, lacto-N-neotetraose, and lacto-N-fucopentaoses I and II on a column (90 × 2.5 cm I.D.) of the resin. As can be seen from Fig. 3, lacto-N-tetraose and lacto-N-neotetraose, which are separated only with difficulty by paper chromatography, are well resolved after 4 cycles. Of the pairs of compounds which do run together on recycling chromatography, lacto-N-tetraose and 3-fucosyllactose can be separated easily by preparative paper chromatography or on a charcoal–Celite column as described above. The lactodifucotetraose–lacto-N-neotetraose mixture may be similarly resolved. Lacto-N-fucopentaoses I and II are best separated by paper chromatography, and the lacto-N-fucopentaose II so obtained is probably contaminated[8] with lacto-N-fucopentaose III (Gal-β(1 → 4)-[Fuc-α(1 → 3)]GlcNAc-β(1 →3)Gal-β(1 → 4)Glc, but may be freed from it by O-acetylation and separation of the acetylated product by thin-layer chromatography[16].

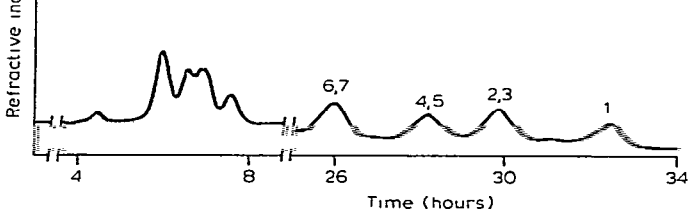

Fig. 3. Separation of some milk oligosaccharides by recycling chromatography. An artificial mixture of oligosaccharides was fractionated by recycling chromatography using the conditions described in Fig. 2. The eluate indicated by the hatching was removed during the first cycle, and the oligosaccharides were eluted on the fourth cycle. The elution position of each of the components was established by repeating the run first with 2′-fucosyllactose, and then with mixtures of 2′-fucosyllactose and each of the other components in turn. 1 = 2′-Fucosyllactose; 2 = 3-fucosyllactose; 3 = lacto-N-tetraose; 4 = lacto-N-neotetraose; 5 = lactodifucotetraose; 6 = lacto-N-fucopentaose I; 7 = lacto-N-fucopentaose II.

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