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Note

Analysis of reduced oligosaccharides by combined use of gel chromatography and high-performance liquid chromatography

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The oligosaccharides of mucus glycoproteins are known to be heterogeneous [1—6]. Many investigators have used high-performance liquid chromatography (HPLC) for the analysis of these oligosaccharides [7—10]. However, a single HPLC analysis does not always provide sufficient resolution for the separation of oligosaccharides, owing to the complexity of mixtures and the similar properties of the compounds investigated.

In the present work, an attempt was made to devise a method for high-resolution fractionation of an oligosaccharide mixture by combined use of gel permeation chromatography and HPLC. This method was applied to the mapping of oligosaccharides from the gastric juice of secretors with blood group A. A comparison was made with the results for gastric juice from a different blood group.

EXPERIMENTAL

Materials

Toyopearl HW 40S and Toyopearl 50S were obtained from Toyo Soda (Japan), Pronase-E from Kaken Kagaku (Japan), Bio-Gel A-1.5 m and Bio-Gel P-4 (up to 400 mesh) from Bio-Rad Labs. (U.S.A.). All other chemicals were of the highest grade available and used without further purification.

Preparation of reduced oligosaccharides from human gastric juice

Human gastric juice was collected from secretors with blood groups A and O as described previously [11]. The pooled gastric juice (1500 ml), which was donated by several individuals of each blood group (GJ-A and GJ-O), was

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dialysed extensively against distilled water under a toluene atmosphere followed by centrifugation at 1000 *g* to remove particulate material. The supernatant was concentrated to 50 ml by lyophilization. Following the addition of a 1/10 volume of 1 *M* Tris—HCl (pH 8.0) containing 10 m*M* calcium chloride, the supernatant was incubated at 50°C with 200 mg of Pronase-E. Incubation was continued for an additional three days, with 100 mg of enzyme being added each day. The reaction mixture was dialysed using Visking cellulose tubing with a molecular weight cut-off of 5000, centrifuged at 1000 *g* for 20 min to remove the insoluble material. The gastric glycopeptides were purified from the soluble digests using a Bio-Gel A-1.5 m (80 × 1.5 cm I.D.) gel permeation column and ultracentrifugation as described previously [12]. The glycopeptides (10 mg) were incubated with 2 ml of 2 *M* sodium borohydride and 0.05 *M* sodium hydroxide at 50°C for 24 h. The reaction was terminated by adjusting the pH to 5.0 with glacial acetic acid. The neutralized reaction products were applied onto connected columns of Toyopearl HW 50S (60 × 1.6 cm I.D.) and Toyopearl HW 40S (90 × 1.6 cm I.D.), and eluted with 0.1 *M* acetic acid. The effluent was monitored for hexose by the phenol—sulphuric acid method [13]. The reduced oligosaccharide fractions (molecular size somewhat less than malto-tridecaose) were pooled and concentrated.

Gel chromatography of reduced oligosaccharides

The reduced oligosaccharide mixtures from GJ-A and that from GJ-O were designated as OS-A and OS-O, respectively. A 1-ml aliquot of each (corresponding to 1 mg of hexose) was chromatographed on a Bio-Gel P-4 column (up to 400 mesh, 85 × 1.5 cm I.D.), equilibrated in and eluted with distilled water. Fractions of 0.8 ml were collected and an aliquot of the effluent was monitored for hexose by the phenol—sulphuric acid method and for amino sugar by the modified Elson—Morgan method [14] following hydrolysis.

High-performance liquid chromatographic analysis of reduced oligosaccharides

The HPLC apparatus consisted of a Tri-Rotor SR2 solvent delivery system (Japan Spectroscopic, Japan), a Uvidec-100-IV UV spectrophotometer (Japan Spectroscopic) and a Unicorder U-228 recorder (Nippon Denki Kagaku, Japan). A Chemopak Hypersil 5-μm reversed-phase column (250 × 4.6 mm I.D.) was used. The mobile phase was distilled water and the flow-rate was 0.5 ml/min. A Brownlee Lab. MPLC guard column (5 μm; 30 × 4.5 mm I.D.) was used to enhance the life and stability of the analytical column. The effluent was detected by UV absorbance at 210 nm.

Depending on the concentration of oligosaccharides, 5—20 μl samples of all fractions from the Bio-Gel P-4 column were analysed by HPLC and calculations were made on the basis of peak heights. Fraction numbers of the Bio-Gel P-4 column, retention times and calculated peak heights were entered into the IBM 4341 computer system. From these results, a figure was drawn by the IBM 4341 computer system and GCONTOUR plot of the SAS/GRAPH computer graphic system.

RESULTS AND DISCUSSION

The isocratic HPLC separation of reduced oligosaccharides from the gastric

juice of blood groups A (OS-A) and O (OS-O) is illustrated in Fig. 1. However, the purity of each peak could not be confirmed definitely by a single chromatographic method. It was thus found necessary to perform a gel chromatographic fractionation prior to HPLC. The elution profiles of both OS-A and OS-O from the Bio-Gel P-4 column are shown in Fig. 2A and B. Each gel chromatographic fraction was analysed by HPLC. The fraction number was plotted as the ordinate and the retention time of HPLC as the abscissa. Absorbance was plotted as a contour line (Fig. 2C and D). The heights of the peaks eluting in less than 10 min from HPLC were not calculated, since they could not be identified as oligosaccharides. The relationship between the retention time in HPLC and fraction number in the gel chromatographic separation indicated the number of components in the oligosaccharide mixture. Some peaks fractionated by HPLC included several components, even though they appeared to be pure material according to HPLC analysis (Fig. 1). The resolution obtained by combined use of the two methods clearly exceeded that of any other conventional method, such as HPLC analysis (Fig. 1) and gel chromatography (Fig. 2A and B).

Using this method, the reduced oligosaccharide maps of the OS-A and OS-O

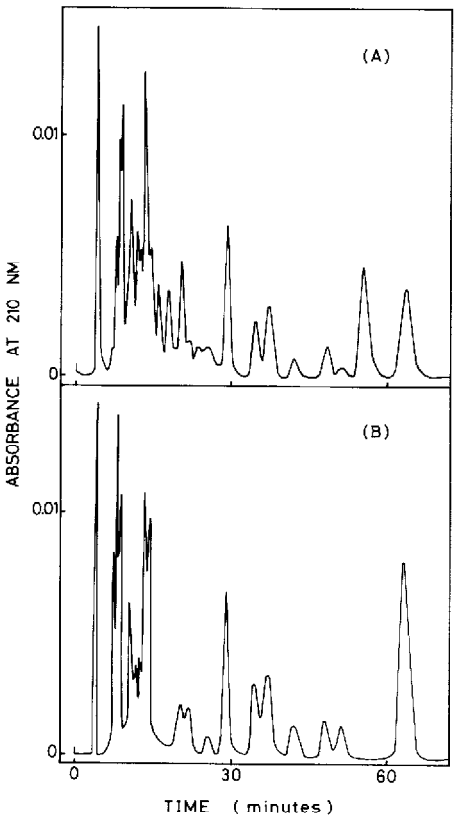

Fig. 1. HPLC analysis of reduced oligosaccharides from human gastric juice. (A) OS-A; (B) OS-O. Reduced oligosaccharides (corresponding to ca. 4 μg of hexose) were fractionated by HPLC under isocratic elution conditions as described in the text.

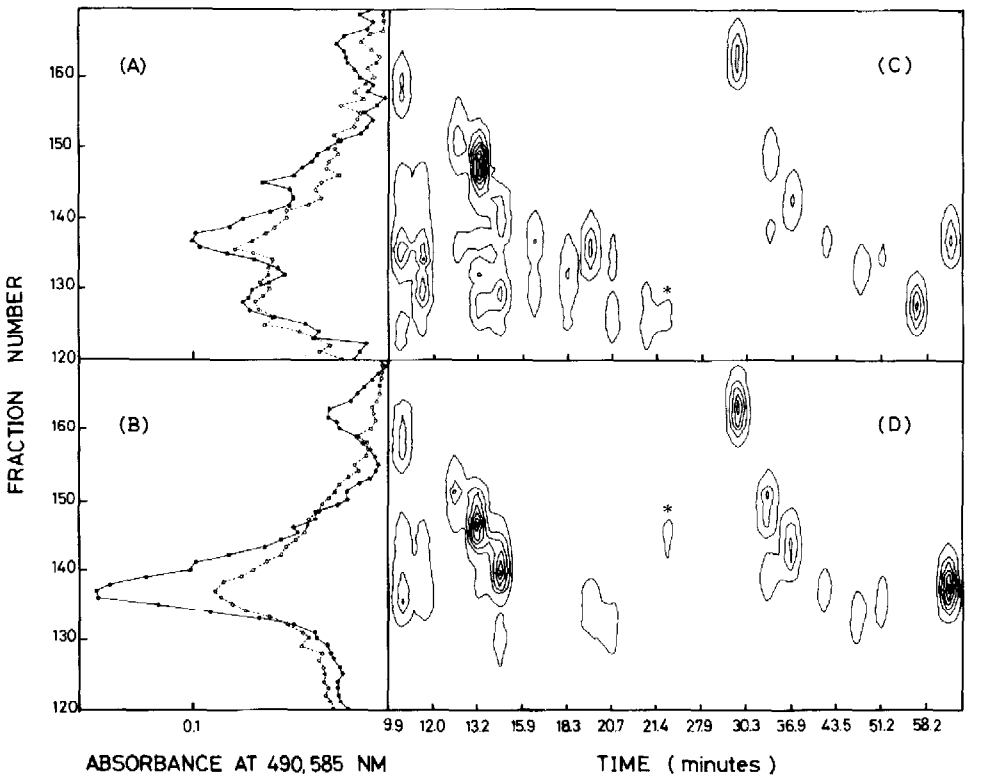

Fig. 2. Gel chromatography and two-dimensional representation of the separation of reduced oligosaccharides. Bio-Gel P-4 chromatography of OS-A (A) and OS-O (B). Reduced oligosaccharide (1 mg as hexose) was applied onto a column (85 × 1.5 cm I.D.) of Bio-Gel P-4 (up to 400 mesh) and developed under the conditions described in the text. Solid line, phenol—sulphuric acid reaction (490 nm); broken line, modified Elson—Morgan method (585 nm). Experimental details of the two-dimensional representation of the OS-A (C) and OS-O (D) are described in the text.

groups were compared (Fig. 2C and D). It was consequently possible to visualize the differences between the oligosaccharide side-chain compositions of GJ-A and GJ-O mucus glycoproteins. Several spots in Fig. 2C are characteristic of OS-A and thus the mucus glycoproteins from GJ-A have oligosaccharide side-chains not present in OS-O. It became evident that a peak in OS-A, indicated by the asterisk, was not the same component as that of OS-O, even though both eluted with the same retention time in HPLC (Fig. 2C and D). This clearly demonstrates the applicability of this method to oligosaccharide mapping.

Oligosaccharides apparently can be divided into two groups of similar molecular weights, but different hydrophobicities. Spot distribution in each group on the two-dimensional chromatogram indicates that both groups may possibly contain structurally similar oligosaccharides. Since smaller oligosaccharides eluted faster in HPLC and the peaks in each group appeared to form a line, it is likely that each group consisted of a series of degradation products and/or biosynthetic intermediates derived from parent oligosaccharide. By higher peak resolution, the mapping method should also be found use-

ful for preparative separation of oligosaccharides and for further structural analysis.

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