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SEPARATION OF CARBOHYDRATES AND LECTINS ON CARBOHYDRATE-IMMOBILIZED RESINS

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SUMMARY

A few carbohydrate-immobilized resins were prepared by coupling glycamines of mono- and disaccharides to an epoxy-activated methacrylate base, and the elution profiles of various carbohydrates and lectins from columns packed with the resultant resins were compared. The introduction of glucamine, maltamine or lactamine to the methacrylate base had little effect on the separation of stereoisomers of aldoses, though it enabled group separation of pentoses as well as 6-deoxyhexoses from hexoses. On the other hand, the maltamine-immobilized resin showed strong specific affinity for concanavalin A, as well as lentil and pea lectins. The lactamine-immobilized resin was specific to ricinus agglutinin, peanut agglutinin and soy been agglutinin. These carbohydrate-immobilized resins were also useful for the isolation of a lectin-like substance from rat liver.

INTRODUCTION

Recent development of specific stationary phases, bearing chiral compounds or various bioactive substances, has opened up two new areas in high-performance liquid chromatography (HPLC), chiral chromatography and affinity chromatography. Chiral stationary phases enable the separation of various enantiomers exemplified by D- and L-amino acids[1], which are difficult to separate by ordinary chromatographic methods. Affinity chromatography plays an important role in the separation of biomacromolecules, but it is generally performed by using soft bases such as agarose and polyacrylamide gels and almost exclusively used for preparative purposes. For analytical use, it needs fractionation, followed by analysis of the fractionated substances mainly by manual methods. Recently high-performance affinity chromatography was developed and applied to various biological substances[2–4], though its main use was for the determination of physical parameters of enzymes by frontal[5] and zonal[6] analyses.

Carbohydrate-bearing hard resins are attractive from the viewpoints of stereospecificity and affinity, because they will allow rapid and reproducible separation of stereoisomers and biomacromolecules. Several workers reported the separation of enantiomers of amino acids[7–9] and atropisomeric binaphthyl derivatives[10] on silica

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gels bearing 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate. On the other hand, Walters[11] described preliminary results of the separation of concanavalin A (Con A) and other proteins on a small column of glucosamine-bound silica. Abe and Ishii[12] attempted the isolation of monovalent Con A (m-Con A) derived from Con A by photochemical degradation, using a column of maltamine-bearing resin.

We have undertaken a fundamental study on the behaviour of carbohydrates and lectins on carbohydrate-immobilized hard resins. The isolation of a substance specifically bound to these resins, from animal tissue, was also attempted.

EXPERIMENTAL

Chemicals

Con A from *Canavalia ensiformis*, pea lectin from *Pisum sativum*, lentil lectin from *Lens culinaris*, peanut agglutinin (PNA) from *Arachis Hypogaea*, soy bean agglutinin (SBA) from *Glycine Max*, ricinus agglutinin (RCA60) from *Ricinus communis*, wheat germ agglutinin (WGA) from *Triticum vulgaris* and phaseolus agglutinin (PHA) from *Phaseolus vulgaris* were obtained from Hohnen Seiyu (Tokyo, Japan). Maltose, lactose and glucose were obtained from Kishida (Osaka, Japan). An epoxy-activated methacrylate resin (Shodex M-614OG, 7 μm) was supplied by Showa Denko (Tokyo, Japan). Other reagents and solvents were of the highest grade commercially available.

Preparation of glycamine-immobilized resins

Glycamines were prepared by reductive amination of parent sugars in the presence of sodium cyanoborohydride by a modification of the method of Hase *et al.*[13]. A carbohydrate sample (5 mmol), ammonium acetate (1.54 g, 20 mmol) and sodium cyanoborohydride (630 mg, 10 mmol) were added to methanol (10 ml), and the mixture was stirred for 40 h at room temperature. The mixture became a clear solution after 1 h, and a white solid mass, presumably of sodium acetate, was gradually formed. The solid was removed by filtration, and the filtrate was evaporated to dryness. The residual syrup was dissolved in a small amount of water (20 ml), and a cation-exchange resin (Amberlite CG-120, proton form, 5 ml) was added to the filtrate with constant stirring. The mixture was applied onto a column packed with the same ion-exchange resin (20 ml), and the column was washed with water (200 ml), followed by 0.6 *M* ammonia–water (200 ml). The ammonia–water eluate was evaporated to dryness to give a glycamine derivative as a syrup. Yields: glucamine (1-amino-1-deoxy-D-glucitol), 83%; maltamine (4-O-α-D-glucopyranosyl-1-amino-1-deoxy-D-glucitol), 75%; lactamine (4-O-β-D-galactopyranosyl-1-amino-1-deoxy-D-glucitol), 78%. The structures of these derivatives were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

One of the glycamines (2 mmol) obtained above was dissolved in 0.1 *M* sodium hydroxide (8 ml). An epoxy-activated resin (4 g) was added under sonification, and the suspension was swirled mechanically at 37°C for 24 h. The resin was collected on a glass filter by suction and washed with distilled water (1 l). It was suspended in distilled water and stored in a refrigerator. Just prior to chromatographic analysis, the resin was packed in a stainless-steel column (inner diameter, 4 mm) by the standard slurry method.

A reference resin was prepared in the same manner except that the glycamine was omitted.

Estimation of the contents of carbohydrates immobilized on the resins

The carbohydrate contents of maltamine and lactamine resins were estimated from the amounts of glucose and galactose, respectively, released from the resins by hydrolysis with 2 *M* trifluoroacetic acid at 100°C for 6 h. The released monosaccharides were determined as described previously[14]. Values of 75 and 180 μmol/g were obtained, respectively.

Apparatus

An Hitachi 635 high-performance liquid chromatograph was used for pumping eluent. Samples were introduced via a Rheodyne 7125 injector carrying a 20-μl loop. Carbohydrate samples were derivatized with 2-cyanoacetamide in the post-column mode using Atto SJ-2396 twin-piston pumps and the products were detected by an Atto SF-1205A UV monitor[15]. Lectin samples were directly detected by the fluorescence of the tryptophan residue at 300 nm (excitation) and 340 nm (emission), using an Hitachi 650-10LC fluoromonitor equipped with a 90-μl quartz cell.

Preparation of the homogenate of rat liver

A sample of the whole liver (7.8 g, wet weight) of a rat was rinsed in 50 m*M* Tris–HCl buffer (pH 7.5, 10 ml) and homogenized for 30 s with the same buffer. An aliquot (20 ml) of the homogenate was centrifuged at $2 \cdot 10^5$ *g* at 4°C for 3 h. Four volume of acetone were added to the supernatant, and the mixture kept at −10°C for 3 h. The resultant precipitate was collected on a filter paper, air-dried, then dissolved in a small portion of the eluent. An aliquot of the solution, equivalent to 1% of the whole rat liver, was injected on the HPLC column.

RESULTS AND DISCUSSION

Partition chromatography of carbohydrates

Capacity factors, k', of some saccharides including pentoses, hexoses and a disaccharide, eluted in partition mode from the glycamine-immobilized resins, are compared in Fig. 1. The k' value for each saccharide increased with increasing acetonitrile concentration in the eluent, but to an extent dependent on the resins. The column of the reference resin which contained no carbohydrates gave the smallest increases, that of glucamine resin showed moderate increases and the columns packed with maltamine or lactamine resin showed greater retardation of carbohydrates than the aforementioned resins at an acetonitrile concentration of 92%. Although the carbohydrate content of the glucamine resin could not be determined, the toal hydroxyl group contents in the disaccharide-immobilized resins are considered to be greater than that in the glucamine resin. The hydroxyl content of glucamine resin was naturally greater than that of the reference resin. Therefore, the order of hydrophilicity is considered to be: reference resin < glucamine resin < maltamine resin, lactamine resin. This is in accord with the order of magnitude of the increase in k'. Table I gives the elution times of some saccharides on each column.

When 92% aqueous acetonitrile was used as a mobile phase, rhamnose, xylose

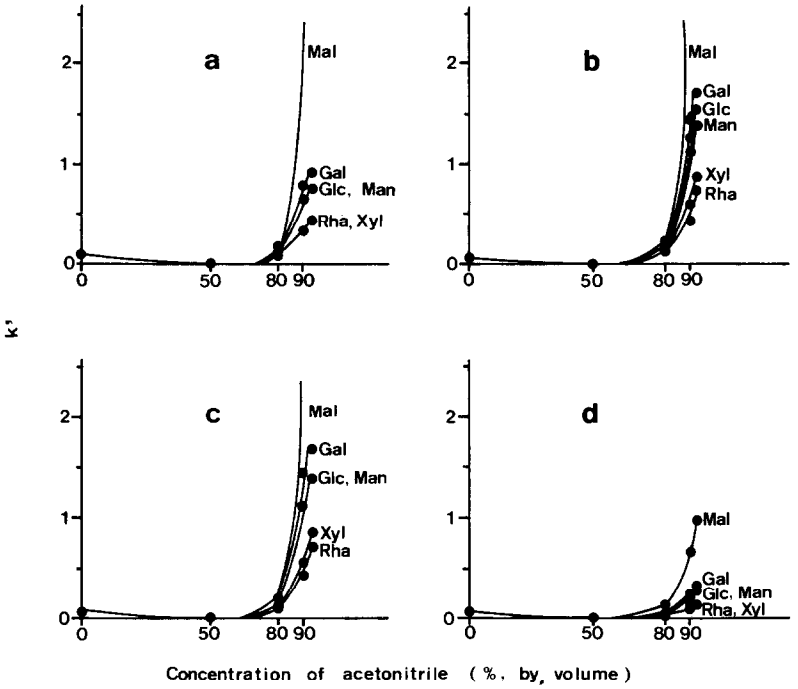

Fig. 1. Effect of the acetonitrile concentration in the eluent on the capacity factor. Columns: (a) glucamine resin; (b) maltamine resin; (c) lactamine resin; (d) reference resin (25 cm × 4 mm I.D.). Column temperature: ambient. Eluents: 0, 50, 80, 90 and 92% acetonitrile in water; flow-rate, 1.0 ml/min. Peaks were detected at 280 nm after post-column labelling with 2-cyanoacetamide[15]. Rha = Rhamnose; Xyl = xylose; Man = mannose; Glc = glucose; Gal = galactose; Mal = maltose.

and mannose were eluted almost at the same elution time on either of the columns of maltamine and lactamine resins. The elution profiles of glucose and maltose on the maltamine column were slightly different from those on lactamine. For example, glucose was retained more strongly on the column of maltamine resin. Maltose was strongly retained on both columns, but its retention time on the lactamine column was slightly longer than that on maltamine. These data suggested that there was

TABLE I

COMPARISON OF ELUTION TIMES OF VARIOUS CARBOHYDRATES FROM COLUMNS OF CARBOHYDRATE-IMMOBILIZED RESINS

Column: 250 mm × 4 mm; temperature, ambient. Eluent: acetonitrile–water (92:8). Flow-rate: 0.5 ml/min.

Resin	*Elution time (min)*					
	Rha	*Xyl*	*Man*	*Glc*	*Gal*	*Mal*
Glucamine	7.2	7.2	8.3	8.6	9.8	16.0
Maltamine	8.4	9.5	12.2	13.1	13.1, 14.6	37.0
Lactamine	8.4	9.4	12.2	12.4	14.0	39.7
Reference	6.3	6.6	6.9	6.9	7.1	9.5

stereochemical interaction between the carbohydrate samples and the carbohydrates in the resins, but the extent of this interaction was not so great as to effect separation of isomers. The galactose peak was split into a doublet presumably due to the presence of anomers. Raising the column temperature to 50°C eliminated the problem of peak splitting due to rapid equilibration of anomers.

Fig. 2 shows the elution profile of a mixture of seven monosaccharides on a column of glucamine resin. Pentoses (xylose and arabinose) and 6-deoxyhexoses (rhamnose and fucose) were eluted at approximately the same elution time. Hexoses (mannose, glucose and galactose) were eluted more slowly than pentoses and 6-deoxyhexoses. However, no isomers were separated under these conditions. The addition of calcium chloride to the mobile phase in an attempt to effect separation in the ligand-exchange mode was also unsuccessful. Columns of maltamine and lactamine resins gave similar results to those obtained on glucamine resin.

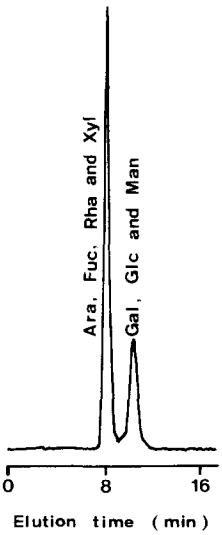

Fig. 2. Separation of aldoses on a column of glucamine resin. Eluent: 92% (v/v) acetonitrile in water. Other analytical conditions and abbreviations, except for Ara and Fuc, as in Fig. 1. Ara = Arabinose; Fuc = fucose.

High-performance affinity chromatography

Fig. 3 shows the behaviour of some lectins on the maltamine resin. Each of the lectins, PNA, SBA, RCA60, WGA nad PHA, showed no affinity for this resin, giving a single peak at the void volume. On the other hand, Con A as well as pea and lentil lectins, all of which are known to be specific to α-glucoside and α-mannoside, showed strong affinity for this column. For example, Con A (3 μg) was injected on the column as indicated by the ↑, and the injector was washed with the eluent at the point ◭. The excess of the lectin was eluted at the void volume. Subsequently 0.1 *M* lactose (20 μl) was injected, and again after 4 min, as indicated by ▲. No peak appeared, suggesting that the retained material specifically recognized the maltamine moiety of the resin. Finally 0.1 *M* maltose (20 μl) was repeatedly injected at the points △. As expected, intense peaks of Con A were observed. The

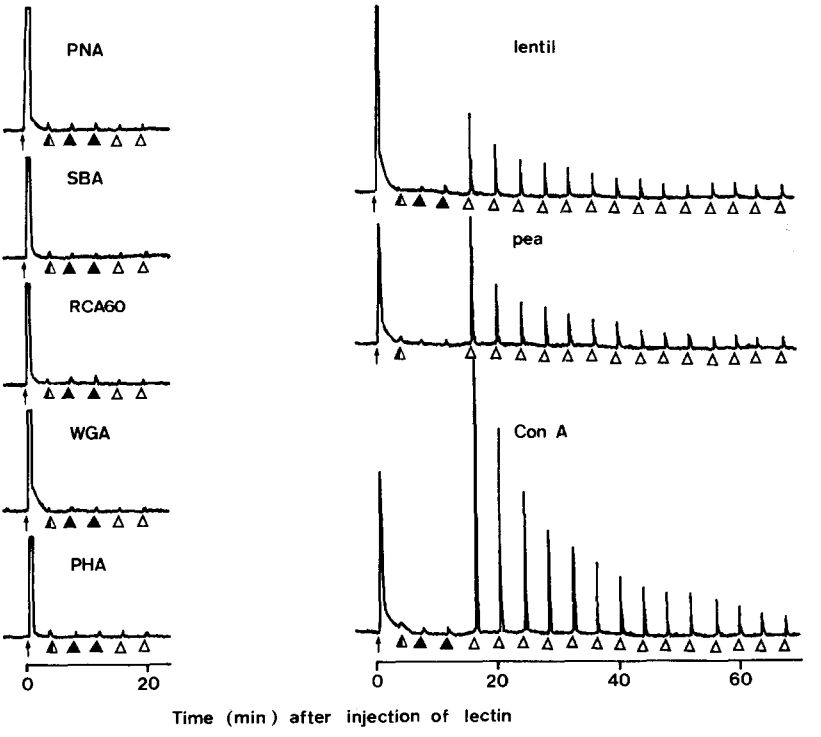

Fig. 3. Behaviour of various lectins on maltamine resin. Column: 50 mm × 4 mm I.D.; temperature, ambient. Eluent; 50 m*M* Tris–HCl buffer (pH 8.0) containing 200 m*M* sodium chloride, 1 m*M* calcium chloride and 1 m*M* magnesium chloride. Wavelenths for detection; 300 nm (excitation) 340 nm (emission). Sample: each 2–3 μg. The symbols ↑, ◭, ▲ and △ indicate the injection of 20 μl each of a lectin solution, the eluent, 0.1 *M* lactose and 0.1 *M* maltose, respectively.

peak height decreased with the number of injections, and all the Con A retained on the column could be recovered after twenty injections. Similar results were obtained in the cases of lentil and pea lectins, though the amounts retained, calculated by comparing the peak area at the void volume with that observed on a column of reference resin, were less than the amount of Con A.

On the other hand, PNA, SBA and RCA60 known to be specific to β-galactoside were retained on a column of lactamine resin (Fig. 4), whereas lentil lectin, pea lectin, Con A, WGA and PHA appeared at the void volume. Retained lectins were not eluted with a maltose solution, but were eluted with a lactose solution.

The results obtained with these disaccharide-immobilized resins were not contradictory to previous knowledge of lectins, and ensure analytical use of these resins. The separation of lectins on these resins was very rapid and reproducible. Non-specific retention and elution could be neglected and the durability of the resins was excellent. Abe and Ishii[12] reported a different preparation of a maltose resin, involving coupling of maltose to an α-aminoalcohol-bearing resin produced by ring opening of an epoxy resin. The amount of maltose introduced (35–40 μmol/g) was only half that in our maltose resin, and the binding capacity was naturally lower.

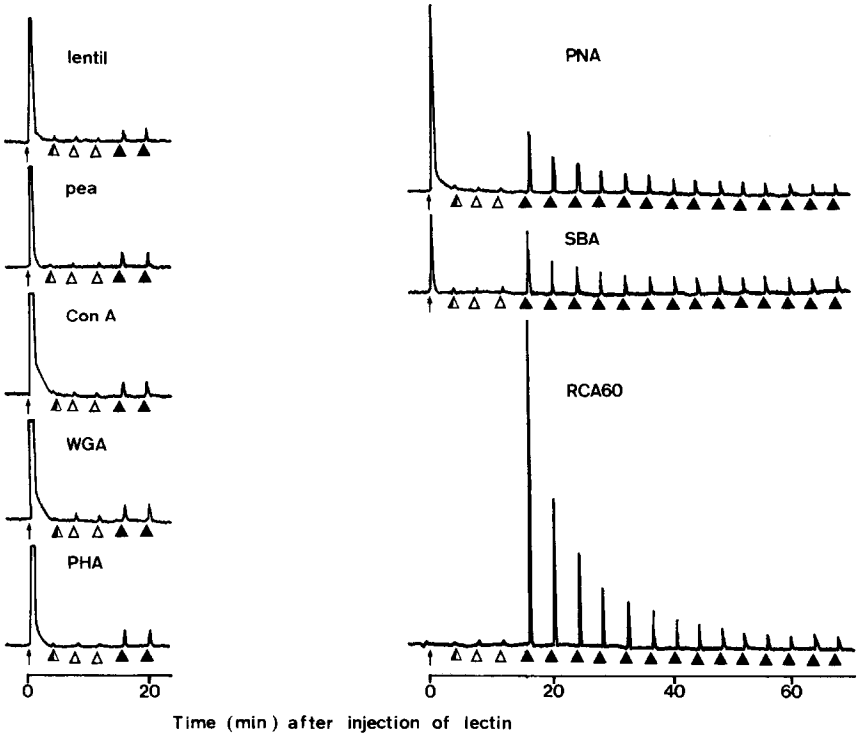

Fig. 4. Behaviour of various lectins on lactamine resin. The analytical conditions and symbols are as in Fig. 3.

On the basis of these results, a tentative survey for carbohydrate-binding substances in animal tissues was performed. Fig. 5 illustrates the result obtained from a rat liver homogenate. When a 1% aliquot of the whole liver homogenate from a rat was applied onto a column of lactamine resin, and the column was eluted with a lactose solution, only small peaks were detected, as shown in Fig. 5b. However, when the same quantity of sample was applied onto a column of maltamine resin and the column was repeatedly eluted with a maltose solution, small but clear peaks were observed as in Fig. 5a. The substance giving these peaks were collected and concentrated by ultrafiltration, and the concentrate was applied again onto the same column and eluted with a maltose solution. Intense peaks of a maltamine-specific lectin-like substance were observed on the chromatogram. Several workers, *e.g.*, refs. 16–19, reported the presence of lectin-like substances in animal tissues, based on the characterization of isolated substances. However, the isolation procedures were tedious and time-consuming. The use of carbohydrate-immobilized resins described in this paper is highly promising for small-scale isolation of plant as well as animal lectins, and will stimulate biochemical study of such materials.

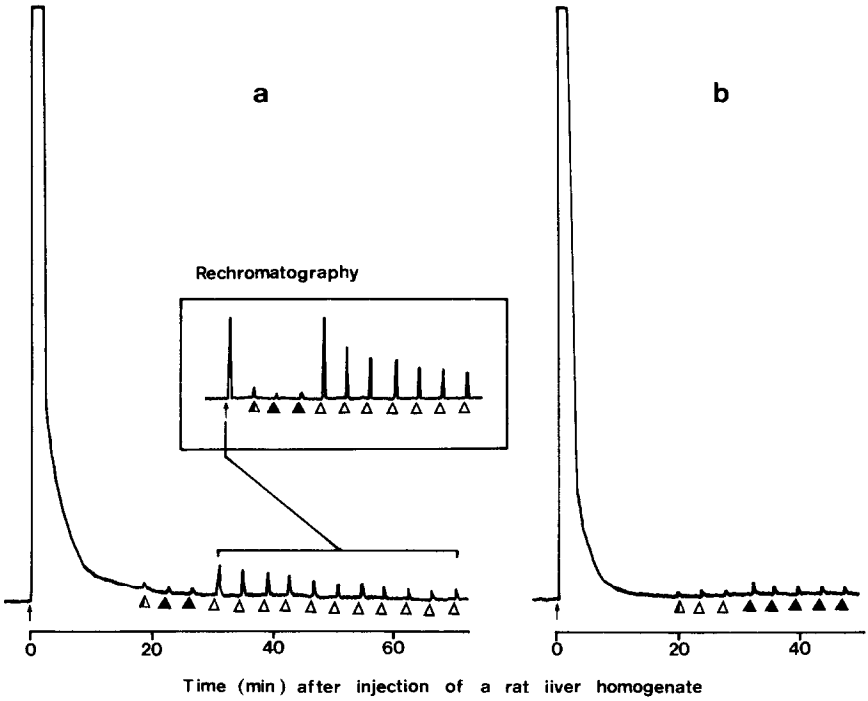

Fig. 5. Attempted isolation of a lectin-like substance from a rat liver homogenate using columns of maltamine (a) and lactamine (b) resins. Sample: equivalent to 1% of the whole liver homogenate of a rat in both cases. Analytical conditions and symbols as in Fig. 3.

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