AFFINITY CHROMATOGRAPHY OF CARBOHYDRATE-SPECIFIC IMMUNOGLOBULINS: COUPLING OF OLIGOSACCHARIDES TO SEPHAROSE *

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A simple method has been developed for the coupling of oligosaccharides to Sepharose. The sugars are reacted with β-(p-aminophenyl)-ethylamine to form N-alkylglycosides which are then reduced with sodium borohydride to stable secondary amines. The derivatives are then coupled to cyanogen bromide-activated Sepharose through their arylamino groups. Yields are essentially quantitative based on starting oligosaccharides. An affinity column containing lacto-*N*-difucohexaose I coupled to Sepharose by this method was used for the purification of an antibody directed against this oligosaccharide. The antibody is absorbed by the gel and is specifically eluted by the free sugar.

Reducing oligosaccharides dissolve in alkylamines to form N-alkylglycosides (2). These compounds are easily hydrolyzed but can be reduced to stable secondary amines. The oligosaccharides do not react with arylamines in the absence of catalyst. Therefore, when oligosaccharides are dissolved in β-(p-aminophenyl)-ethylamine and reduced, only the secondary alkylamine derivatives are produced. As the remaining primary arylamine reacts readily with cyanogen bromide-activated Sepharose under the usual conditions (3), these reactions provide a convenient method for making affinity columns with carbohydrate ligands.

The method described in the present paper is relatively rapid, proceeds under mild conditions, and gives essentially quantitative yields based on the starting sugar. Therefore, it offers certain advantages over existing

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* Part of this work was presented at the 9th FEBS Meeting in Budapest, Hungary, August 1974 (1).

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methods for coupling carbohydrates to large carriers (4-9), especially with sugars that contain labile linkages or whose supply is limited.

MATERIALS AND METHODS

β-(p-Aminophenyl)-ethylamine was purchased from the Aldrich Chemical Company, cyanogen bromide-activated Sepharose 4B from Pharmacia Fine Chemicals, and rabbit anti-goat serum from Miles Laboratories. 2'-Fucosyllactose, lacto-*N*-tetraose, lacto-*N*-fucopentaose I, and lacto-*N*-difucohexaose I were isolated from human milk (10). Oligosaccharides were labeled with tritium by reduction with sodium borotritide to alditol derivatives (11). Goat antiserum directed against lacto-*N*-difucohexaose I was prepared by repeated immunization with a conjugate of the sugar covalently linked to polylysine (12) and complexed with succinylated hemocyanin (details of the immunization procedure and characterization of the antibody will be presented in a future publication). Binding of tritium-labeled oligosaccharides by the goat antiserum was studied using a nitrocellulose filter assay (13). Immunoelectrophoresis was performed by standard methods (14).

RESULTS AND DISCUSSION

Formation of secondary amines. In a typical experiment, 85 mg of lacto-*N*-fucopentaose I (0.1 mmoles) is added to 0.5 ml of β-(p-aminophenyl)-ethylamine (3.5 mmoles) and stirred in a sealed flask for 15 hours, during which time the sugar slowly dissolves and the viscosity of the solution increases. To this mixture is added 0.5 ml of absolute ethanol, followed immediately by 12 mg of sodium borohydride in 1 ml of absolute ethanol. The mixture is stirred for 5 hours in a vented flask, diluted with 4 ml of water, chilled in ice, and adjusted to pH 5.6 by dropwise addition of glacial acetic acid. After removal of ethanol under reduced pressure the mixture is taken up in 5 ml H_2O, applied to a column of Sephadex G-10 (2.5 cm x 100 cm), and eluted with 1 M acetic acid adjusted to pH 5.0 with pyridine. Adequate separation of product from the free amine is demonstrated by spotting 5 μl aliquots of the column fractions on filter paper and developing with Ekman's reagent (15) which gives a positive reaction with both the coupled sugar derivative and unreacted amine. Additional 5 μl aliquots are spotted on silica gel plates, sprayed with concentrated sulphuric acid, and heated. Fractions giving a charring reaction (indicating the presence of sugar) are pooled and lyophilized. The yield of product based on starting lacto-*N*-fucopentaose I is essentially quantitative as measured by weight, fucose determination (16), or by UV absorption (assuming ε_{285} = 1020 in 0.1 M sodium bicarbonate buffer, pH 8.0). Derivatives of 2'-fucosyllactose, lacto-*N*-tetraose, and lacto-*N*-difucohexaose I prepared in the above manner were also obtained in high yield.

Previous studies (2) as well as nmr spectra and mass spectra derived from the present work are consistent with the reactions shown in Fig. 1 in

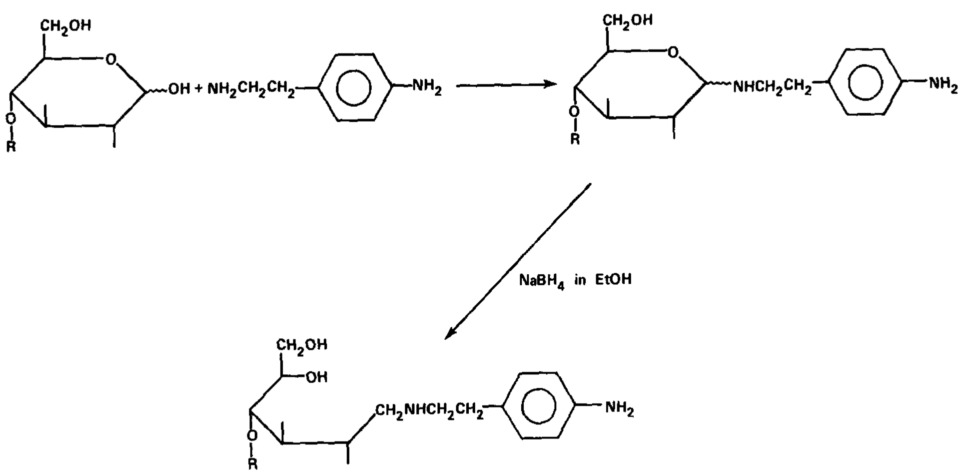

FIG. 1. *Formation of N-alkyl-1-amino-1-deoxyalditols from oligosaccharides and β-(p-aminophenyl)-ethylamine.*

which the N-glycosylamine formed by dissolving the oligosaccharide in β-(p-aminophenyl)-ethylamine is reduced by sodium borohydride to an N-alkyl-1-amino-1-deoxyalditol.

Coupling to Sepharose. The deoxyalditol derivatives are coupled to Sepharose as follows: In a typical preparation 20 μmoles of lacto-*N*-fucopentaose I derivative in 5 ml of 0.1 M $NaHCO_3$ buffer, pH 8.0, is added to

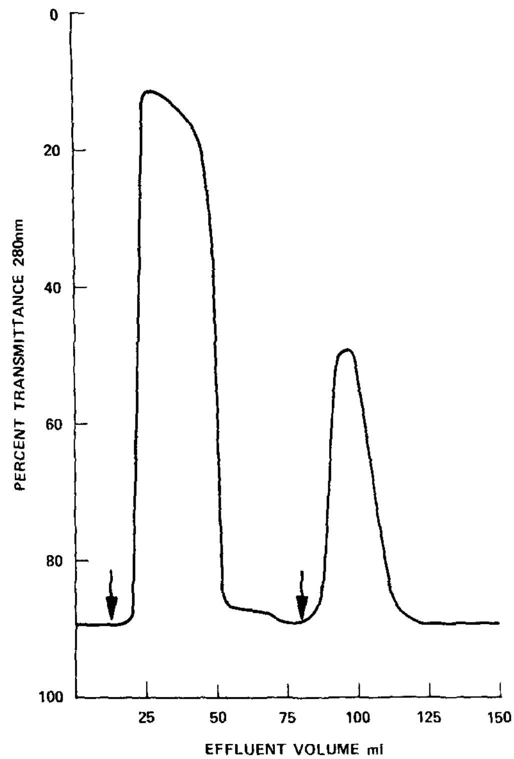

FIG. 2. *Purification of goat anti-lacto-N-difucohexaose I antibody by affinity chromatography.* A column of Sepharose 4B (0.8 cm x 3.0 cm) containing 10 μmoles of covalently bound lacto-*N*-difucohexaose I derivative was loaded (left arrow) with 2.0 ml of a 4-fold concentrate of goat antiserum in PBS at 25°. The column was eluted at 10 drops/minute and washed with PBS until the UV transmittance of the elute returned to baseline. Hapten elution (right arrow) was carried out with free lacto-*N*-difucohexaose I (2 mg/ml in PBS).

1 g of cyanogen bromide-activated Sepharose 4B previously washed with 100 ml of 0.001 N HCl, followed by 10 ml of H_2O. As the suspension is slowly rotated for 2 hours, the optical density of the aqueous phase at 285 nm drops from about 4.0 to less than 0.10. Ethanolamine, 0.5 ml of a 10 M solution adjusted to pH 8.0 with acetic acid, is added, and the suspension slowly rotated for an additional 2 hours. The derivatized gel is then washed sequentially with 25 ml aliquots of 0.1 M $NaHCO_3$, 0.1 M sodium acetate buffer, pH 5.0, and phosphate-buffered saline (PBS) containing 0.85% NaCl in 0.02 M sodium phosphate buffer, pH 7.4.

Purification by affinity chromatography of antibodies directed against lacto-N-difucohexaose I. The immunoglobulins of a goat serum containing

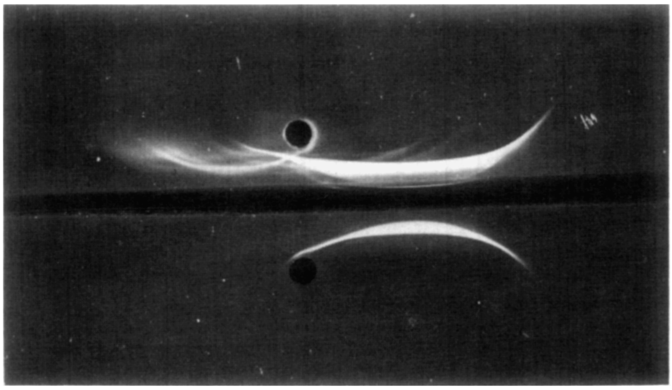

FIG. 3. *Immunoelectrophoresis of goat anti-lacto-N-difucohexaose I serum fractions.* The top well contains the protein applied to the affinity column in Fig. 2. The lower well contains the protein eluted from the column by free hapten. The center trough contains rabbit anti-goat serum.

1.1 mg/ml of antibodies precipitable by polylysine-lacto-*N*-difucohexaose I conjugate were concentrated 4-fold by precipitation with 40% $(NH_4)_2SO_4$ followed by dialysis of the precipitated protein against PBS. Two ml of the immunoglobulin concentrate was passed through a column of lacto-*N*-difucohexaose I coupled to Sepharose 4B as shown in Fig. 2. The protein eluted from the column by free lacto-*N*-difucohexaose I was concentrated by vacuum dialysis against PBS to 3 ml and passed through a column of Biogel P-6 (2.5 cm x 35 cm) using PBS as eluent. The protein obtained in this way accounts for over 90% of the precipitable antibody in the original serum and over 95% of its binding activity towards ^{3}H-labeled lacto-*N*-difucohexaitol. Immunoelectrophoresis of the eluted protein (Fig. 3) reveals a single arc migrating in the position of goat IgG. In control experiments, no protein is eluted from loaded, washed columns by lactose in PBS (5 mg/ml) and columns derivatized with lacto-*N*-tetraose or lacto-*N*-fucopentaose I do not bind the goat antibody.

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