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AFFINITY CHROMATOGRAPHY OF SIALOGLYCOPROTEINS, UTILISING THE INTERACTION OF SEROTONIN WITH *N*-ACETYLNEURAMINIC ACID AND ITS DERIVATIVES

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ABSTRACT

Serotonin, immobilised on Sepharose 4B, has been used to study the affinity chromatography of neuraminic acid and its derivatives. Free *N*-acetylneuraminic acid and oligosaccharides, polysaccharides, and glycoproteins containing that sugar are specifically bound to the columns. Removal of neuraminic acid from sialoglycoconjugates, or modification of the neuraminic acid residues by periodate oxidation, abolishes their ability to bind to the ligand. The presence of the *N*-acetyl group, but not the *N*-glycolyl group, and the integrity of the side chain (C-7–C-9) of the neuraminic acid are essential for binding to serotonin.

INTRODUCTION

Serotonin (5-hydroxytryptamine) has been implicated in the binding of glycolipids and glycoproteins to synaptosomes in rat brain[1]. Neuraminic acid-containing gangliosides have been suggested to be part of the serotonin receptor on cells[2]. In the presence of lecithin, several gangliosides, particularly G_{D3} (disialosyl-lactosyl ceramide), enhance the binding of serotonin to a serotonin-binding protein[3]. A protein and a glycoprotein each having a highly specific binding capacity for serotonin have been isolated from rat platelets[4].

The lectin from haemolymph of the King crab (*Limulus polyphemus*) binds specifically to neuraminic acid residues of glycoproteins[5], but the lectin is not widely available and not in an immobilised form. Wheat-germ agglutinin interacts with neuraminic acid and glycoconjugates containing that sugar, but the lectin also interacts[6–8] with 2-acetamido-2-deoxy-D-glucosyl residues.

We now report on an affinity chromatography system which can be used specifically for the isolation of glycoconjugates containing residues of neuraminic acid. A preliminary report of this work has been published[9].

MATERIALS AND METHODS

Sepharose 6B and cyanogen bromide-activated Sepharose 4B were purchased from Pharmacia. Fetuin, *N*-glycolylneuraminic acid, heparin, colominic acid, neuraminidase, and concanavalin A–agarose were obtained from Sigma. *N*-Acetylneuraminic acid methyl β-ketoside was purchased from Boehringer. Ovomucoid was a gift from Dr. J. Beeley (University of Glasgow), methyl neuraminate was a gift from Dr. N. Baggett (University of Birmingham), and 4-*O*-methylneuraminic acid and 9-*O*-acetylneuraminic acid were gifts from Professor R. Schauer (University of Kiel).

A crude extract of carcinoembryonic antigen (CEA) was prepared from liver secondaries of primary colonic cancer by perchloric acid extraction[10], followed by ion-exchange chromatography on CM-cellulose. Prior to radiolabelling[11] with ^{125}I, CEA was purified further by chromatography on DEAE-cellulose, concanavalin A–agarose, and Sepharose 6B. CEA was assayed by a direct, double-antibody radioimmunoassay based on that of Egan *et al.*[12].

Prostatic acid phosphatase was prepared from pooled seminal fluid from normal donors by ammonium sulphate fractionation followed by chromatography on columns of CM-Biogel, concanavalin A–agarose, and L-tartrate–Sepharose[13]. The purified enzyme was radiolabelled with ^{125}I by the method of Bolton and Hunter[14].

Asialofetuin was prepared either by treatment of fetuin (10 mg) in acetate buffer (50mM, pH 4.5) with neuraminidase (2 u) in a total volume of 2 mL for 24 h, or by treatment of fetuin (10 mg) in hydrochloric acid (100mM, 5 mL) at 100° for 10 min. In each case, the liberated neuraminic acid was removed from the asialofetuin by dialysis against water.

The trisaccharide α-Neu*p*NAc-(2→3)-β-D-Gal*p*-(1→3)-D-GalNAcol was prepared by alkaline borohydride treatment of fetuin[15]. Fetuin (50 mg) in sodium hydroxide (100mM, 10 mL) containing sodium borohydride (10 mg) was kept at 4° for 48 h. Residual sodium borohydride was decomposed with acetic acid (100mM), and the mixture was concentrated by rotary evaporation to 2 mL, and separated into a fraction of high molecular weight and an oligosaccharide fraction by chromatography on a column of Sephadex G-50 (1 × 50 cm) with water as eluant. The oligosaccharide fraction was separated from residual inorganic material by re-chromatography on a column (1 × 50 cm) of Biogel P2 with water as eluant.

Colominic acid (10 mg) was oxidised to NeuNAc-7-colominic acid by treatment with sodium periodate (20mM) for 3 h at room temperature. Oxidation was terminated by the addition of glycerol (0.2 mL). The aldehyde groups generated were then reduced with sodium borohydride (10 mg) for 1 h, followed by dialysis against distilled water.

Neuraminic acid (free or bound) was determined by the 3-methyl-2-benzothiazolone hydrazone[16] or orcinol[17] methods. Hexose was determined by the orcinol–sulphuric acid method[18], and protein by a modified Lowry procedure[19].

The affinity column was prepared by immobilising serotonin (10 mg) on

Sepharose 4B-CNBr (2 g) according to the manufacturer's instructions. Unreacted, activated groups on the Sepharose were inactivated by treatment with an excess of aqueous ethanolamine. The immobilised serotonin was packed into small columns (bed volume, 4 mL). The columns were washed with water and then loaded with sialoglycoproteins (~3 mg). Elution was continued with water until the u.v. absorption at 280 nm returned to zero, when the eluant used was phosphate or Tris

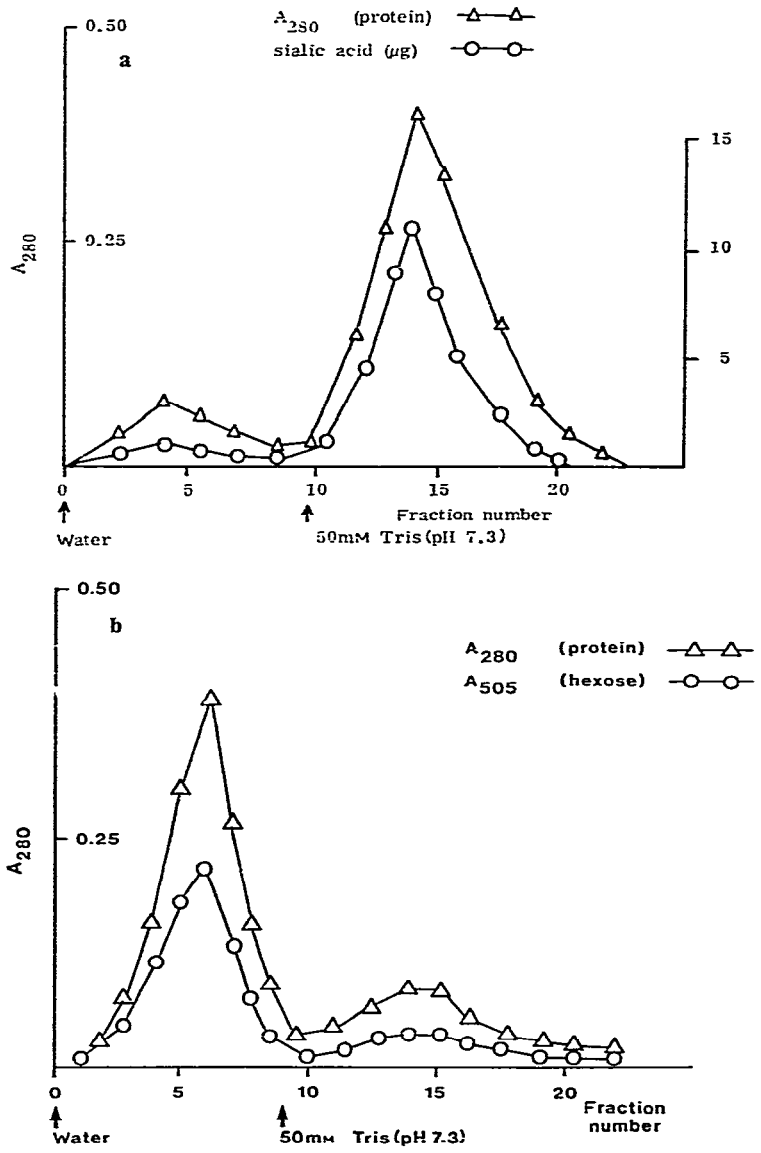

Fig. 1. Chromatography of (*a*) fetuin and (*b*) asialofetuin on immobilised serotonin. Glycoproteins (3 mg) were loaded on the columns, which were eluted successively with water and buffer (fraction vol., 2.0 mL).

buffer (100mM, pH 7.0), followed by the same buffer containing sodium chloride (100mM). Columns were regenerated by washing with acetate buffer (100mM, pH 3.0) followed by water.

RESULTS AND DISCUSSION

Chromatography of fetuin (3-mg samples) on immobilised serotonin is shown in Fig. 1a. Elution of the bound material with 50mM phosphate or Tris-HCl buffer (pH 7.3) gave an almost quantitative recovery of fetuin. Asialofetuin has no affinity for the immobilised serotonin (Fig. 1b). Similar results were obtained for asialo-ovomucoid. The small amount of glycoprotein bound to the column and released by the buffer at pH 7.3 probably arises from the presence on fetuin of a small number of neuraminic acid residues resistant to acid hydrolysis or neuraminidase treatment. Results similar to those shown in Fig. 1a were obtained on chromatography of *N*-acetylneuraminic acid methyl β-ketoside, 4-*O*-methylneuraminic acid, 9-*O*-acetyl-neuraminic acid, ovomucoid, carcinoembryonic antigen (CEA), prostatic acid phosphatase, colominic acid, and the *O*-glycosidically linked trisaccharide-chains of fetuin [α-Neu*p*NAc-(2→3)-β-D-Gal*p*-(1→3)-D-GalNAcol].

Neuraminic acid residues on fetuin and on colominic acid were selectively modified to the seven-carbon analogue (5-acetamido-3,5-dideoxy-L-*arabino*-heptulosonic acid, Neu*p*NAc-7) by mild oxidation with periodate followed by borohydride reduction[20]. Although colominic acid bound to the affinity column, the NeuNAc-7 analogue of colominic acid did not. Since colominic acid is a linear polymer of (2→8)-α-linked *N*-acetylneuraminic acid residues, only the residue at the non-reducing terminus is modified by the treatment with sodium periodate. Thus, the integrity of the C-7–C-9 chain of the neuraminic acid residue appears to be essential for binding to serotonin. Ion-exchange binding of neuraminic acid residues to serotonin must be negligible, since each residue of the Neu*p*NAc-7-colominic acid has a charged carboxyl group. N.m.r. spectroscopic studies are now being undertaken to elucidate the mechanism of interaction between serotonin and neuraminic acid.

Further evidence of the lack of involvement of charge in the chromatographic system is shown by the binding of the methyl ester of neuraminic acid (methyl *N*-acetylneuraminate) to immobilised ligand. This is in contrast to the results of Ochoa and Bangham, who concluded that the interaction between serotonin and neuraminic acid is mainly electrostatic in nature[21]. Since the interaction between wheat-germ agglutinin and glycoconjugates containing *N*-acetylneuraminic acid has been reported to be dependent, in part, on a charge effect due to the high isoelectric point of the lectin[8], the possibility of the affinity chromatography involving ion-exchange phenomena cannot be overlooked. *N*-Glycolylneuraminic acid, which is found occasionally in sialoglycoconjugates, does not bind to the affinity support, suggesting that the *N*-acetyl group and not the *N*-glycolyl group of the neuraminic acid is essential for binding. Similar conclusions concerning the binding of neuraminic acid and wheat-germ agglutinin have been reported[6].

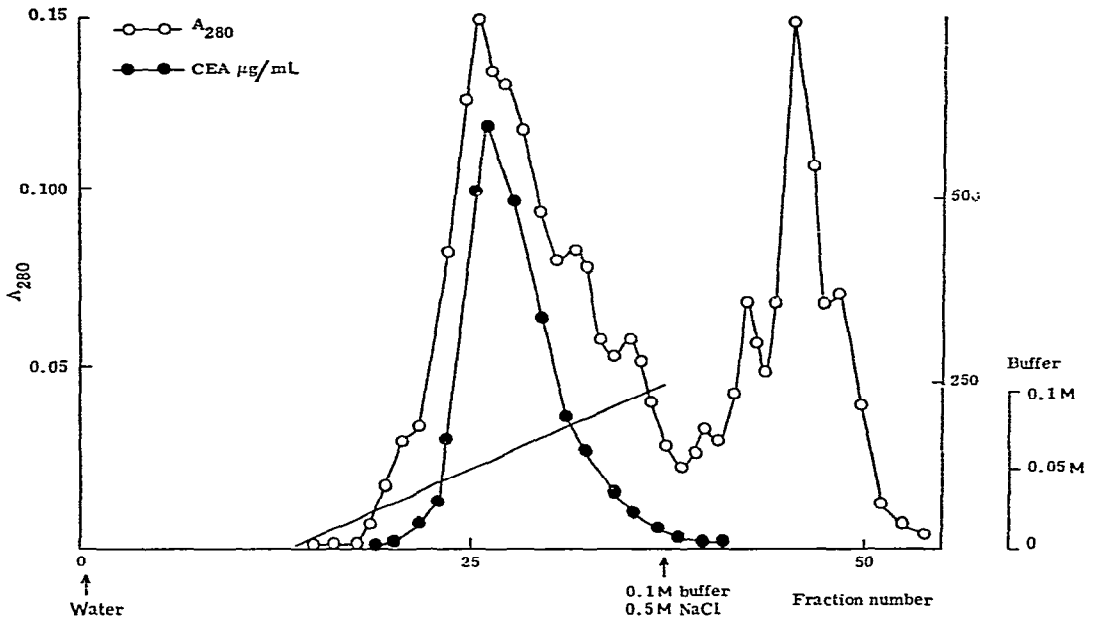

Fig. 2. Fractionation of sialoglycoproteins from tumour extracts. Glycoproteins were eluted successively with water, a linear buffer gradient (0→100mM phosphate, pH 7.0), and phosphate buffer (100mM) containing sodium chloride (500mM) (fraction vol., 2.0 mL).

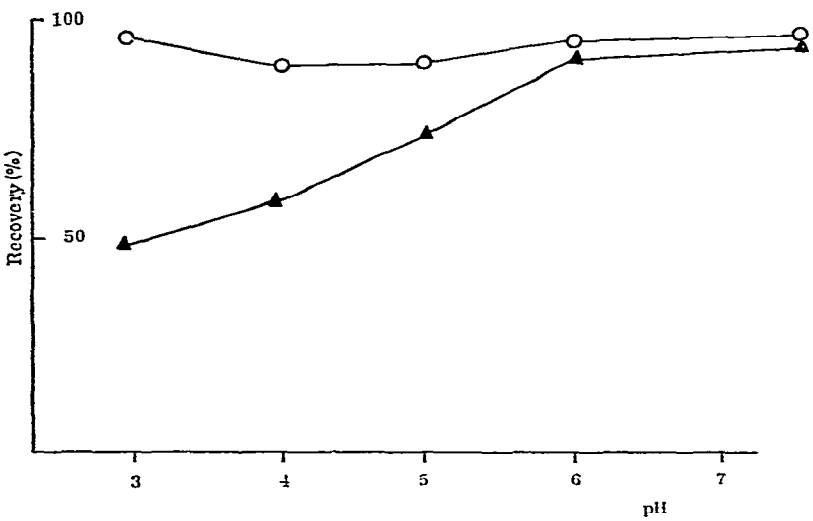

Fig. 3. ^{125}I-Prostatic acid phosphatase and ^{125}I-CEA (~100,000 c.p.m. each) recovery from immobilised serotonin. Samples were separately adsorbed on the columns and eluted with phosphate buffer (100mM) containing sodium chloride (500mM). Recoveries were determined as percentages of the total counts loaded: —○—, ^{125}I-CEA; —▲—, ^{125}I-prostatic acid phosphatase.

The only lectin with absolute specificity for neuraminic acid is that from *Limulus polyphemus*. However, the difficulty in obtaining large amounts of the lectin limits its use for large-scale work. Many workers have compromised by using immobilised wheat-germ agglutinin, although it is not specific for neuraminic acid[6-8]. Monsigny *et al.*[8] have shown that wheat-germ agglutinin also binds to 2-acetamido-2-deoxy-D-glucosyl residues in glycoproteins. 2-Acetamido-2-deoxy-D-galactosyl residues are responsible for the binding of ovine submaxillary mucin to wheat-germ agglutinin[7].

Immobilised serotonin has been applied to the fractionation of mixtures of sialoglycoproteins. On subjecting a human-liver tumour extract to gradient elution from the affinity matrix, a three-fold purification of CEA was achieved (Fig. 2).

The recovery of glycoproteins from affinity-chromatography columns may be low, especially from columns of some immobilised lectins. When CEA and prostatic acid phosphatase were chromatographed on columns of immobilised serotonin, recoveries of 95 and 90%, respectively, were obtained (Fig. 3). The difference in binding of acid phosphatase observed at low pH may be a result of conformational changes in the enzyme molecule.

When compared with existing methods of purification of sialoglycoproteins, the method now reported is advantageous in a number of respects. Preparation of the matrix is a simple procedure that requires only reagents that are readily available. The matrix can be washed rapidly and re-used following each purification. Rapid and quantitative recovery of glycoproteins can be achieved by using immobilised serotonin, which appears to have a high degree of specificity for neuraminic acid. The immobilised preparations may be used repeatedly over a period of months without apparent loss of binding activity. Also, the method has considerable potential for the separation of sialoglycoconjugates and for the separation of complex mixtures of sialoglycoproteins.

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