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PRODUCTION AND CHARACTERIZATION OF ANTISERA RAISED TO THE MANNINOTRIONATE GROUP [α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-D-Gluconate]*

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ABSTRACT

Manninotrionate [α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-D-gluconate] was coupled to bovine serum albumin (BSA), and antisera were raised in rabbits to this carbohydrate–protein conjugate. The antisera were studied by quantitative precipitation and hapten inhibition. Antisera against the manninotrionate–BSA conjugate showed the greatest reactivity with the homologous manninotrionate–BSA antigen, but also cross-reacted extensively with a manninotrionate–ovalbumin conjugate. The antisera gave weak precipitin reactions with beef-lung galactan, guaran, gum arabic, and larch arabinogalactan. Quantitative hapten-inhibition studies with a series of galactosyloligosaccharides indicated that the antisera possess a high degree of specificity toward terminal α-D-Gal*p*-(1→6)-α-D-Gal*p* units. The antisera did not agglutinate Ehrlich ascites tumor-cells, which have been demonstrated to contain cell-surface glycoproteins having α-D-galactosyl end-groups.

INTRODUCTION

Currently, we are studying animal glycoproteins whose oligosaccharide chains terminate in α-D-galactopyranosyl groups[1–3]. The tentative structure we postulated for this family of glycoproteins contains both α-D-Gal*p*-(1→3)-D-Gal*p* and α-D-Gal*p*-(1→6)-D-Gal*p* units[2]. We have been using in our studies a highly specific α-D-galactosyl-binding lectin (*Griffonia simplicifolia* I-B_4) as a probe for the detection of these carbohydrate groups[4,5]. Although the GS I-B_4 isolectin exhibits a slight preference for the α-(1→3)-D-galactosidic linkage[5], it is not highly specific. We wished, therefore, to assemble a battery of proteins that could distinguish in a more precise manner the nature of the intergalactosidic linkages which occur in cell-surface oligosaccharides. We now describe antisera raised

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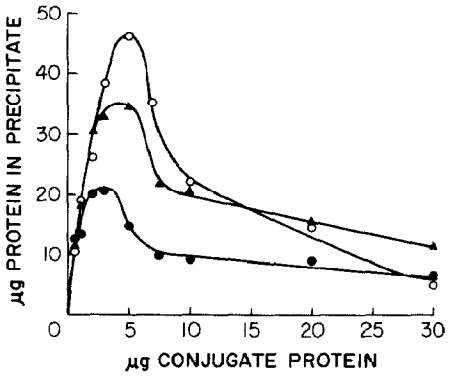

Fig. 1. Quantitative precipitin curves of the manninotrionate–ovalbumin conjugate with manninotrionate–BSA antiserum. The reaction mixture contained 40 μL of antiserum in a total volume of 90 μL. Key: (○), R-1A; (●), R-2A; and (▲), R-3A.

against a hapten containing a terminal 6-*O*-α-D-galactopyranosyl-D-galactopyranosyl group.

RESULTS AND DISCUSSION

Quantitative precipitin determinations. — Quantitative precipitin curves obtained from the individually pooled antisera (from three successive bleedings) of three different rabbits are presented in Fig. 1. The maximum amount of protein precipitated from anticonjugate antisera (40 μL) by heterologous antigen

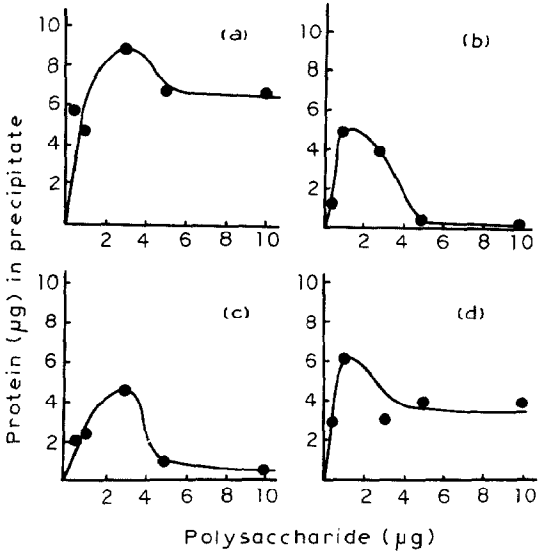

Fig. 2. Quantitative precipitin curves of polysaccharides with antiserum against manninotrionate–BSA conjugate. (a) Beef-lung galactan, (b) guaran, (c) gum arabic, and (d) arabinogalactan from wheat flour. Reaction mixtures contained 40 μL of antiserum R-1A in a total volume of 90 μL.

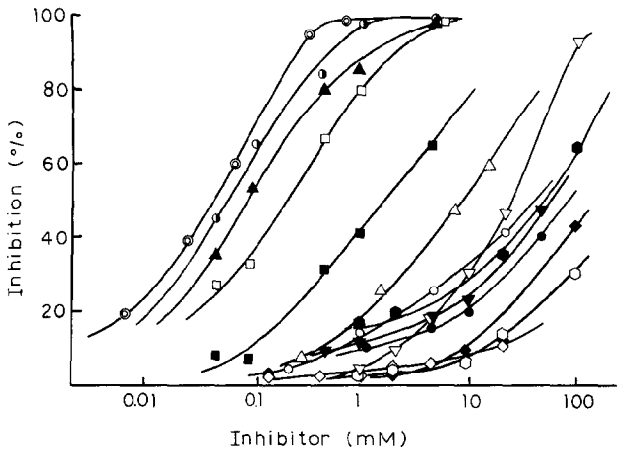

Fig. 3. Quantitative hapten inhibition of the manninotrionate–anti-manninotrionate system. Each tube contained manninotrionate–ovalbumin (5 μg of protein), antiserum R-3A (40 μL), and carbohydrate hapten in a total volume of 90 μL. Symbols for carbohydrate haptens are provided in Table I.

(manninotrionate–ovalbumin) varied with individual specimens of rabbit serum, and was found to be 46, 21, and 35 μg of protein, respectively, for antisera R-1A, R-2A, and R-3A. This is equivalent to 1.15, 0.525, and 0.875 mg/mL, respectively. Quantitative precipitin curves for the interaction of antiserum with four different polysaccharides (beef-lung galactan, guaran, gum arabic, and larch arabinogalactan) containing D-galactose residues are shown in Fig. 2.

Guaran is a galactomannan that consists of a linear chain of (1→4)-linked β-D-mannopyranosyl residues, one half of which carry single α-D-galactopyranosyl stubs linked α-(1→6) to the D-mannose residues[6]. This molecular architecture undoubtedly poses steric hindrance to interaction with the anti-manninotrionate antibodies, resulting in the limited precipitation reaction observed. Both the lung galactan[7] and the larch arabinogalactan[8] contain multiple β-D-Gal*p*-(1→6)-D-Gal*p* end-groups, accounting for their limited interaction with the manninotrionate antiserum. Finally, gum arabic is known to contain some α-D-galactosyl end-groups linked to L-arabinose[9]. All of these polysaccharides reacted poorly with anti-manninotrionate–BSA antiserum. The maximum amount of protein precipitated with 40 μL of anticonjugate antiserum was found to be 8.8, 5.0, 4.5, and 6.2 μg of protein for the respective polysaccharides.

Hapten inhibition studies. — The specificity of the anticonjugate antisera, as well as an evaluation of the importance of the α-(1→6)-linked digalactosyl group for interaction with antibody, were examined by hapten inhibition. Quantitative, hapten-inhibition data for the manninotrionate anti-manninotrionate system employing rabbit antiserum R-3A are shown in Fig. 3 and Table I. As expected, potassium manninotrionate [α-D-Gal*p*-(1→6)-α-D-Gal*p*(1→6)-$OCH_2(CHOH)_4$-$CO_2^-K^+$] was the best inhibitor of the manninotrionate–anti-manninotrionate

TABLE I

HAPTEN INHIBITION OF THE ANTI-MANNINOTRIONATE-BSA ANTISERUM–MANNINOTRIONATE-OVALBUMIN CONJUGATE SYSTEM

Symbol	*Carbohydrate*	*Concentration for 50% inhibition (mM)*
◎	Potassium manninotrionate [α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-O-$CH_2(CHOH)_4CO_2^-K^+$]	0.05
◑	Manninotriose [α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-D-Glc]	0.06
▲	Stachyose [α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*]	0.09
□	α-D-Gal*p*-(1→6)-D-Gal	0.2
■	α-D-Gal*p*-(1→6)-(1→1)-glycerol	1.5
△	β-D-Gal*p*-(1→6)-D-Gal	9.0
●	Raffinose [α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*]	40% inhibition at 50mM
	α-D-Gal*p*-(1→3)-D-Gal	inactive at 50mM
	α-D-Gal*p*-(1→2)-D-Glc	inactive at 50mM
	α-D-Gal*p*NAc-(1→3)-D-Gal	inactive at 50mM
	α-D-Gal*p*NAc-(1→6)-D-Gal	inactive at 50mM
▼	Melibiose [α-D-Gal*p*-(1→6)-D-Glc]	48% inhibition at 50mM
	Lactose [β-D-Gal*p*-(1→4)-D-Glc]	20% inhibition at 50mM
◇	*p*-Nitrophenyl α-D-galactopyranoside	16% inhibition at 50mM
○	*p*-Nitrophenyl β-D-galactopyranoside	40% inhibition at 20mM
	Methyl α-D-galactopyranoside	45
◆	Methyl β-D-galactopyranoside	34% inhibition at 50mM
▽	D-Galactose	25
	Isomaltose [α-D-Glc*p*-(1→6)-D-Glc]	inactive
	Gentiobiose [β-D-Glc*p*-(1→6)-D-Glc]	inactive
	Panose [α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc]	inactive

precipitation reaction, requiring 0.05mM to give 50% inhibition. All saccharides examined containing a nonreducing, α-(1→6)-linked digalactosyl group (potassium manninotrionate, manninotriose, stachyose, and α-D-Gal-(1→6)-D-Gal) were relatively good inhibitors. However, α-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→1)-glycerol, which contains a β-D-galactopyranosidic linkage to glycerol required a higher concentration to give 50% inhibition than the other sugars (see Table I). This strongly suggests that the antibody specificity extends to include an α-linkage, as indicated, at the reducing end of the α-D-Gal*p*-(1→6)-α-D-Gal unit.

An evaluation of the effect of the anomeric nature of the intergalactosidic linkage was made by comparing the relative molar inhibitory concentrations of 6-*O*-α- and 6-*O*-β-D-galactopyranosyl-D-galactose required in order to give 50% inhibition (see Table I). Because the α-(1→6)-linked digalactose required 0.2mM, the former was ~50-fold more effective as an inhibitor of precipitation than the latter (9mM). The α-(1→3)-linked disaccharide α-D-Gal-(1→3)-D-Gal was a non-inhibitor.

Additional oligosaccharides containing nonreducing galactosyl end-groups, namely, lactose [β-D-Gal*p*-(1→4)-D-Glc], melibiose [α-D-Gal*p*-(1→6)-D-Glc], and raffinose [α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*] were relatively poor inhibitors of precipitation. As may be seen in Table I, melibiose and raffinose, which contain a terminal α-D-Gal*p*-(1→6)-D-Glc group, were more effective as inhibitors of precipitation than the β-linked disaccharide lactose. Although D-galactose was ~2-fold more effective as an inhibitor than melibiose, its inhibitory capacity was nevertheless poor, requiring 25mM in order to give 50% inhibition. Other derivatives of galactose (*p*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl β-D-galactopyranoside, methyl α-D-galactopyranoside, and methyl β-D-galactopyranoside) were significantly poorer in molar inhibitory ability. In addition, the (1→6)-linked diglucosyl isomers, isomaltose [α-D-Glc*p*-(1→6)-D-Glc] and gentiobiose [β-D-Glc*p*-(1→6)-D-Glc], and the D-glucose trisaccharide panose [α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc] did not inhibit the precipitation of the manninotrionate–anti-manninotrionate system.

The three anti-manninotrionate antisera (50 μL) failed to agglutinate murine Ehrlich ascites tumor-cells after incubation for 2 h at 25°. This is, perhaps, not surprising, for, although we have isolated and characterized an α-(1→3)-galactosyltransferase[10], we have been unable to detect any α-(1→6)-galactosyltransferase activity from Ehrlich tumor cells.

EXPERIMENTAL

Materials. — Bovine serum albumin (BSA) was purchased from Miles Laboratories, Kankakee, IL, and ovalbumin from Worthington Biochemical Corp., Freehold, NJ. *p*-Nitrophenyl α- and β-D-galactopyranoside were obtained from Vega Biochemicals, Tucson, AZ, stachyose and 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (DEC) from Sigma Chemicals Co. St. Louis,

MO, and lactose, melibiose, raffinose, methyl α- and β-D-galactopyranoside from Pfanstiehl Laboratories, Waukegan, IL. α-D-Gal*p*-(1→6)-β-D-Gal*p*-glycerol was a gift of Dr. D. V. Myhre, Procter and Gamble Co., Cincinnati, OH. The disaccharides 6-*O*-α- and β-D-galactopyranosyl-D-galactose were synthesized in this laboratory. The rest of the oligosaccharides and the polysaccharides were available from previous studies. All chemicals used were of reagent grade or the best quality available. Phosphate-buffered saline (PBS) consisted of 0.15M NaCl, 0.01M sodium phosphate, pH 7.2, containing 0.1mM $CaCl_2$ and 0.02% of sodium azide. Precoated thin-layer plates of silica gel G-60 were from Brinkmann Instruments, Inc. Bio-Gel P-2 was purchased from Bio-Rad, Richmond, CA.

Analytical methods. — Hexose concentrations were determined by the phenol–sulfuric acid assay[11], using standards of suitable methyl glycosides. Protein concentrations were assayed by the Lowry procedure[12], using BSA as the standard.

Preparation of manninotriose. — Stachyose (1 g) was hydrolyzed with 20% acetic acid for 4 h at 100° as described by French[13]. After completion of the reaction, the acetic acid was removed by extraction with diethyl ether, and the solution was evaporated to a syrup from which manninotriose was purified by passage through a column (2.5 × 200 cm) of Bio-Gel P-2 at a flow rate of 5 mL/h. Fractions (2.2 mL) were collected, and the fractions having R_F 0.2 on silica-gel plates developed with 5:5:3:1 pyridine–ethyl acetate–water–acetic acid were combined and lyophilized, to give 0.46 g of manninotriose. The trisaccharide showed $[\alpha]_D$ +162° (lit.[13] +167°), gave positive silver nitrate and Fehling tests, and, on hydrolysis with Dowex-50 resin in 0.02M HCl for 48 h at 100°, gave only glucose and galactose as detected by thin-layer chromatography using 5:5:3:1 pyridine–ethyl acetate–water–acetic acid.

Preparation of manninotrionate–BSA conjugate. — Manninotriose was oxidized with iodine and potassium hydroxide in methanol–water by the procedure of Moore and Link[14] to give potassium manninotrionate. The manninotrionate (0.2 g) was coupled to BSA (0.1 g) with DEC (70 mg) at pH 4.75 by the aldonate coupling procedure[15], to give the manninotrionate–BSA conjugate (0.104 g) containing, as determined by the phenol–sulfuric acid assay[11], 16 and 14 glycosyl groups per BSA molecule in two separate preparations.

Potassium manninotrionate was also conjugated to ovalbumin, using the same procedure as for BSA. Six residues of manninotrionate were incorporated into each ovalbumin molecule.

Antisera. — A group of three adult white rabbits (R-1, R-2, and R-3) was immunized with the manninotrionate–BSA conjugate incorporated into complete Freund's adjuvant (Difco Laboratories, Detroit, MI). A total of 1 mL of emulsion containing 1 mg of conjugate was administered to each animal over the course of four weeks as multiple, subcutaneous injections (0.2 mL) into the back. Blood was drawn from the marginal ear-vein at weekly intervals beginning 1 week after the last injection. The sera (R-1A, -2A, and -3A) obtained, after separation of the blood clot, were stored frozen, without addition of preservative.

Agglutination assay. — Ehrlich ascites tumor-cells, grown in the peritoneum of mice, were harvested, and washed with PBS. Agglutination was carried out in microtiter plates as conducted previously[1].

Quantitative precipitin studies. — Precipitin studies were carried out in 3-mL centrifuge tubes. Reaction mixtures contained 40 μL of the manninotrionate–BSA antisera and 0 to 30 μg of manninotrionate–ovalbumin conjugate dissolved in 50 μL of PBS. The volume of mixture in each tube was adjusted to 90 μL with PBS. The control mixture contained 40 μL of the antiserum and 5 μg of ovalbumin. These conditions were maintained throughout the course of this work. After incubation for 48 h at 4°, the precipitates formed upon interaction of anti-manninotrionate–BSA antisera with manninotrionate–ovalbumin were centrifuged, washed, and dissolved in 0.3 mL of 0.05M NaOH for protein assay by the Lowry procedure[12]. Precipitin studies of various polysaccharides with anti-manninotrionate–BSA antiserum (R-1A) were carried out in the same way.

Quantitative hapten inhibition. — Haptens were assayed for their ability to inhibit the precipitation reaction between manninotrionate–ovalbumin (5 μg of protein) and anti-manninotrionate–BSA antiserum (R-3A; 40 μL). Various concentrations of haptens were added to the reaction mixture. The total volume of each reaction mixture was adjusted to 90 μL with PBS. The reaction mixtures were kept for 48 h at 4°, and then assayed for precipitated protein. Percentage inhibition was calculated from the difference between experimental and control tubes.

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