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Carbohydrate structures of the cell adhesion molecule, contact site A, from *Dictyostelium discoideum*

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We determined the carbohydrate structures of contact site A from *Dictyostelium discoideum*. The carbohydrate moieties of contact site A were released by hydrazinolysis. Fractionation of the deacidified oligosaccharide mixture by Bio-Gel P-4 column chromatography revealed that it was composed of four major oligosaccharides. Their respective structures were determined by sequential exoglycosidase digestion. It is known that contact site A consists of two kinds of carbohydrates, I and II. Taking together the previous and the present results, it was deduced that carbohydrate I comprises N-linked oligosaccharides and carbohydrate II O-linked ones. Furthermore, the relative molar contents of GalNAc and GlcNAc in reducing terminal suggested that contact site A contains 67% of N-linked and 33% of O-linked oligosaccharides.

Cell-to-cell interaction; Cell adhesion molecule; Carbohydrate recognition; Carbohydrate structure; *Dictyostelium discoideum*

1. INTRODUCTION

Cell adhesion molecules play an important role in morphogenesis [1]. The aggregation process of *Dictyostelium discoideum* is a useful system to study the mechanisms of cell adhesion. Gerisch et al. indicated that EDTA-resistant cell contact newly appears at the aggregation-competent stage, and that contact site A with an apparent molecular weight of 80 kDa is involved in EDTA-resistant cell contact [2,3]. It has been shown by several researchers that carbohydrate moieties might be involved in EDTA-resistant cell contact [4–6]. Loomis et al. reported that carbohydrate moieties defective in a *mod*B mutant might be essential to EDTA-resistant cell contact [7,8]. Moreover, we indicated that carbohydrate moieties of contact site A might be functional in EDTA-resistant cell contact [9–11]. Therefore, it is necessary to determine the carbohydrate structures of contact site A in order to understand the molecular mechanism of cell adhesion molecule, contact site A.

2. MATERIALS AND METHODS

2.1. *Purification of contact site A*

Purified contact site A was prepared as described previously [9]. Purified contact site A showed up as a single band by staining SDS-polyacrylamide gels with silver according to Oakley et al. [12].

2.2. *Release of oligosaccharides from contact site A*

Analysis of oligosaccharides was carried out with a modification of the method described by Ashford et al. [13]. Briefly, the oligosaccharides from contact site A were liberated by treatment with fresh double-vacuum-distilled anhydrous hydrazine in sealed glass tubes under argon, maintaining strictly anhydrous conditions. Hydrazinolysis was carried out at 95°C for 4 h. Under these conditions hydrazinolysis led to the release of more than 85% of N-linked oligosaccharides and of more than 60% of O-linked oligosaccharides from three glycoproteins: bovine serum fetuin, human serum plasminogen and human serum IgA [14]. The hydrazinolysates were N-acetylated. The desalted sample was applied to Whatman 3MM chromatography using *n*-butanol/ethanol/water (4:1:1). After 2 days the first 5 cm from the origin was eluted with water. The sample was radiolabeled using 5-fold molar excess of 6 mM NaB^3H_4 (12 Ci/mmol, New England Nuclear) in 50 mM NaOH adjusted to pH 11 with saturated boric acid (30°C, 4 h). An equivalent volume of 1 M NaB^2H_4 in NaOH/boric acid (pH 11) was then added and the incubation was continued for 2 h. After drying, the sample was applied to Whatman 3MM chromatography for 2 days using *n*-butanol/ethanol/water (4:1:1). The radioactivity remaining at the origin was eluted with water. The isolated oligosaccharides were subjected to high-voltage paper electrophoresis in pyridine/acetic acid/water (pH 5.4, Whatman 3MM, 80 V/cm) in order to determine the relative proportions of their neutral and acidic components. The reduced ^{3}H-labeled oligosaccharides were subjected to exhaustive neuraminidase digestion (*Arthrobacter ureafaciens* neuraminidase 10 units/ml) in 50 μl of 0.1 M acetate buffer pH 5.0 for 18 h at 37°C and furthermore methanolysis was carried out as follows: reduced desialylated oligosaccharide was lyophilized and suspended in 500 μl of 0.05 N HCl anhydrous methanol. The sample was incubated in a sealed tube under an atmosphere of anhydrous argon at 37°C for 6 h, dried under vacuum, and N-acetylated. After these treatments, an aliquot of the samples was applied to high-voltage paper electrophoresis. The samples subjected to neuraminidase-treatment and methanolysis were desalted, evaporated to dryness and resuspended in 10 μl of a 20 mg/ml partial dextran hydrolysate solution and applied to a Bio-Gel P-4 (-400 mesh) gel permeation chromatography column (1.5 × 200 cm). The column was maintained at 55°C and eluted with water. The eluant was monitored for radioactivity using an in-line radioactivity flow detector and an in-line differential refractometer. Analog signals from the monitors were digitized and the digital values were analyzed by computers. The elution position of glucose oligomers detected by an refractometer was represented in

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glucose units according to the methods described by Yamashita et al. [15].

2.3. *Analysis of reducing terminal sugar*

The identity of the reducing terminal monosaccharide was determined as follows. An aliquot of ^{3}H-labeled oligosaccharide was hydrolyzed by treatment with 2 N HCl at 100°C for 6 h, the excess acid was removed and the sample was N-acetylated by the addition of acetic anhydride. Following N-acetylation, the sample was separated by HPLC on a Shodex sugar SP1010 column according to the method described by Takeuchi et al. [16]. The column was maintained at 80°C and run with 20% ethanol.

2.4. *Sequential exoglycosidase digestion*

Sequential exoglycosidase digestion was performed as follows. The oligosaccharides eluted from Bio-Gel P-4 (-400 mesh) were evaporated to dryness and resuspended in the appropriate volume of sterile buffer containing the desired amount of glycosidase. Digestion with each exoglycosidase was carried out under the following conditions: β-galactosidase (*Streptococcus pneumoniae*), 20 μl of 0.2 units/ml in 0.1 M citrate-phosphate, pH 6.0; β-galactosidase (bovine testes), 20 μl of 5 units/ml in 0.1 M citrate-phosphate, pH 4.0; β-*N*-acetylhexosaminidase (*S. pneumoniae*), 20 μl of 0.1 units/ml in 0.1 M citrate-phosphate, pH 6.0; α-mannosidase (jack bean), 20 μl of 10 units/ml in 0.1 M sodium acetate, pH 4.5; β-mannosidase (*Helix pomatia*), 20 μl of 3 units/ml in 0.1 M sodium acetate, pH 4.0; β-*N*-acetylhexosaminidase (jack bean), 20 μl of 10 units/ml in 0.1 M citrate-phosphate, pH 4.0. The exoglycosidase digestion was carried out at 37°C for 18 h in the presence of toluene, according to the conditions described by Parekh et al. [17]. After the mixture was desalted and the glycosidase removed, it was evaporated to dryness, suspended in an aqueous solution of a partial acid hydrolysate of dextran, and applied to a Bio-Gel P-4 (-400 mesh) gel permeation chromatography column (1.5 × 100 cm). The eluant was detected as described above.

3. RESULTS AND DISCUSSION

3.1. *Oligosaccharides isolated from contact site A*

The oligosaccharides from contact site A were released and labeled by reduction with NaB^3H_4. Labeled oligosaccharides were separated into three components by high-voltage paper electrophoresis (N1, A1 and B1, in Fig. 1a). N1 comigrating with lactose was neutral, whereas A1 and B1 were acidic. From measurement of radioactivity in the relevant region of the paper, the ratio of neutral and acidic oligosaccharides was determined to be 34 and 66%, respectively. Next, labeled oligosaccharides were treated by exhaustive neuraminidase digestion. The results showed that neuraminidase treatment converted acidic components A1 and B1 into a neutral one, N2 (Fig. 1b). This indicated that contact site A contained sialic acids, although it had been believed that no sialic acids existed in *D. discoideum* [18,19]. The results concerning sialic acids in *D. discoideum* were reported in detail elsewhere [20]. However, the incomplete conversion to a neutral structure suggested that other acidic groups were present (see A2 and B2 in Fig. 1b). The acidic groups were thought to be sulfate [21,22]. In fact, methanolysis completely converted the acidic component B2 into a neutral one, N3 (Fig. 2). A2 was also converted into a neutral component by methanolysis (data not shown). It is, therefore,

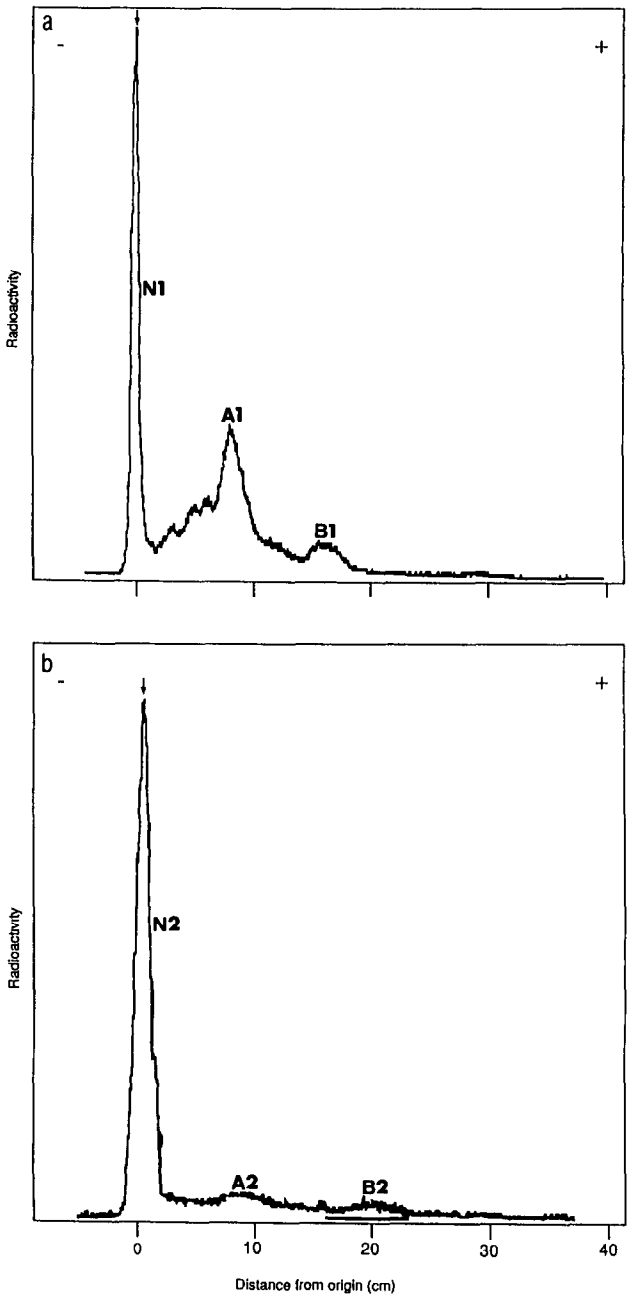


Fig. 1. High-voltage paper electrophoresis of contact site A oligosaccharides. An aliquot of ^{3}H-labeled oligosaccharides before (a) and after (b) neuraminidase treatment was applied to high-voltage paper electrophoresis in pyridine/acetic acid/water (3:1:387, by volume), pH 5.4. The arrows indicate the position of [^{3}H]lactitol. The component that comigrated with lactitol was neutral and the other components were negatively charged.

concluded that the oligosaccharides contain two kinds of acidic sulfate and sialic acid residues. Deacidified oligosaccharides were applied to gel-permeation chromatography on Bio-Gel P-4. This procedure, in which oligosaccharides were separated by differences in hydrodynamic volumes, yielded four components (A–D in Fig. 3). Peak A was eluted at 13.3 glucose units (gu)

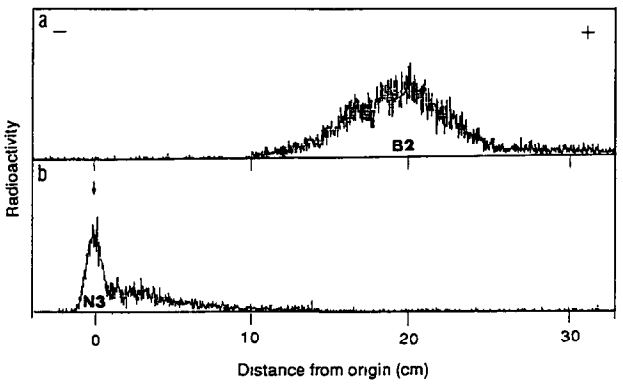
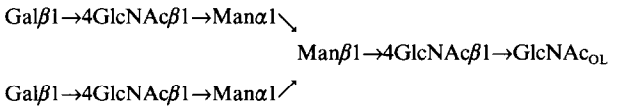


Fig. 2. High-voltage paper electrophoresis of neuraminidase-treated oligosaccharides. An aliquot of ^{3}H-labeled oligosaccharides in peak B2 of Fig. 1b before (a) and after (b) metanolysis was applied to high-voltage paper electrophoresis in pyridine/acetic acid/water (3:1:387, by volume), pH 5.4. The arrow indicate the position of [^{3}H]lactitol.

compared to internal standard glucose oligomers. Peak B was eluted at 10.6 gu, peak C at 3.2 gu, and peak D at 2.2 gu.

3.2. *Structural analysis of oligosaccharides of peak A*

Peak A oligosaccharides (13.3 gu) were incubated with β-D-galactosidase (*S. pneumoniae*) specific for β-1,4-linkage. The product was subjected to Bio-Gel P-4 gel filtration and a single product at elution position 11.3 glu was obtained. This product was then incubated with β-*N*-acetyl-D-hexosaminidase (*S. pneumoniae*). The product subjected to gel filtration gave a single product at elution position 7.3 gu. The 7.3 gu product digested with α-D-mannosidase (jack bean) was applied to gel filtration and a 5.5 gu product was obtained. Digestion of the 5.5 gu product with β-D-mannosidase (*H. pomatia*) specific for β-1,4-linkage and subsequent gel filtration yielded a 4.5 gu product. The 4.5 gu product treated with β-*N*-acetyl-D-hexosaminidase (jack bean) was applied to gel filtration, and the 2.5 gu product obtained was identified as *N*-acetyl-D-glucosaminitol by HPLC on a Shodex sugar SP1010 column. From these results, the structure of oligosaccharide alditol peak A was deduced as follows:



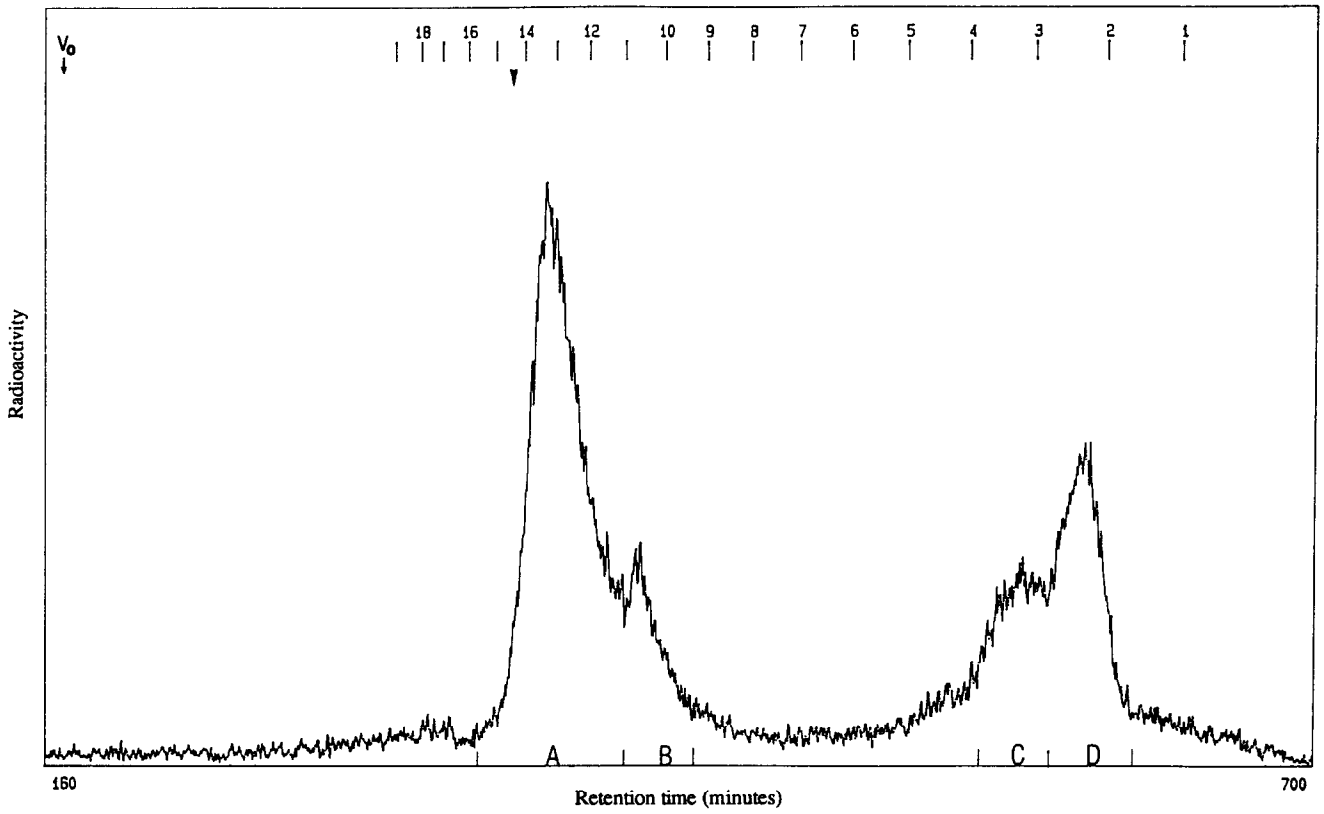


Fig. 3. Gel permeation chromatogram of deacidified oligosaccharides derived from contact site A. Deacidified oligosaccharides were separated by a Bio-Gel P-4 (-400 mesh) gel permeation chromatography column as described in section 2. Each fraction (A–D) was pooled for further analysis. V_0 indicates the position of void volume. The numbered arrows refer to the elution position of the internal standard glucose oligomer. The arrowhead indicates the elution position of Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc$_{OL}$ prepared from human serum IgG.

3.3. *Structural analysis of oligosaccharides of peaks B and C*

Peak B oligosaccharides (10.6 gu) were incubated with β-D-galactosidase (*S. pneumoniae*). The product was subjected to Bio-Gel P-4 gel filtration and a single product at elution position 9.6 gu was obtained. Digestion of the 9.6 gu product with β-*N*-acetyl-D-hexosaminidase (*S. pneumoniae*) gave a single product at elution position 7.6 gu, α-D-mannosidase (jack bean) treatment converted 7.6 gu into 5.6 gu, and β-D-mannosidase (*H. pomatia*) treatment converted 5.6 gu into 4.6 gu. β-*N*-acetyl-D-hexosaminidase (jack bean) treatment converted 4.6 gu into 2.6 gu. The 2.6 gu product was identified as *N*-acetyl-D-glucosaminitol by HPLC. These results indicated that the structure of oligosaccharide alditol peak B was as follows:

$$\text{Gal}\beta1\rightarrow4\text{GlcNAc}\beta1\rightarrow \begin{cases} \text{Man}\alpha1\searrow \\ \text{Man}\alpha1\nearrow \end{cases} \text{Man}\beta1\rightarrow4\text{GlcNAc}\beta1\rightarrow\text{GlcNAc}_{\text{OL}}$$

Peak C oligosaccharides (3.2 gu) were treated with sequential exoglycosidase. β-D-galactosidase (*S. pneumoniae*) specific for β-1,4-linkage was not functional, but β-D-galactosidase (bovine testes) for β-1,3-linkage was effective for the release of galactose, and a 2.5 gu product was obtained. This was identified as *N*-acetyl-D-galactosaminitol by HPLC. These results indicate that the structure of oligosaccharide alditol peak C was as follows:

$$\text{Gal}\beta1\rightarrow3\text{GalNAc}_{\text{OL}}$$

Finally, peak D was identified as *N*-acetyl-D-galactosaminitol by HPLC (data not shown). The structural analysis of peaks A–D showed that the oligosaccharides of contact site A consisted of N- and O-linked oligosaccharides. Muller et al. [23] reported that contact site A contained fucose. However, we could not detect fucose in any of the oligosaccharides. Although we cannot rule out the possibility of the release of fucose, Ashford et al. [13] pointed out that strict anhydrous conditions in hydrazinolysis and reduction reaction at pH 11, as used in this study, would prevent the release of fucose. It is known that contact site A contains two kinds of carbohydrates, I and II [24], and that carbohydrate I is cotranslationally transferred into contact site A, whereas carbohydrate II is posttranslationally transferred into contact site A, although the glycosylation of both carbohydrates I and II is sensitive to tunicamycin

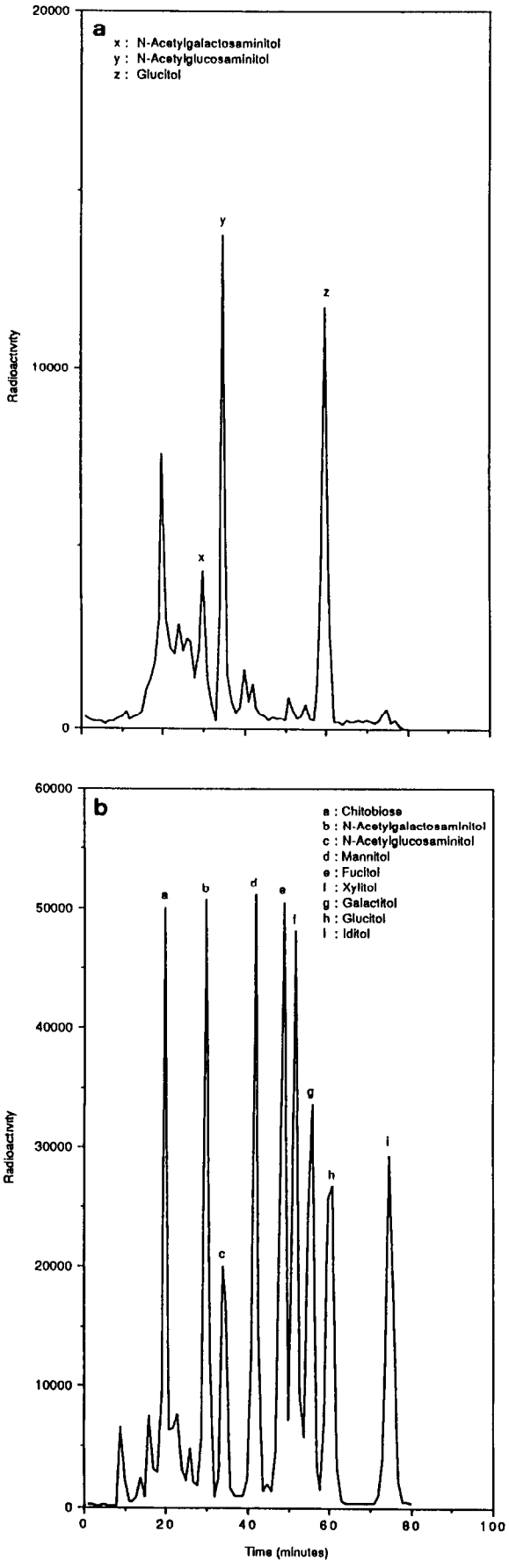


Fig. 4. HPLC chromatograms of alditols from contact site A oligosaccharides. After hydrolysis, the reducing terminal monosaccharides were detected by HPLC as described in section 2. (a) Alditols from contact site A oligosaccharides; (b) standard alditols. Abscissa, retention time (minutes); ordinate, radioactivity.

[9,10,25]. Therefore, it was necessary to determine whether carbohydrate II is comprised of N-linked oligosaccharides or not. Taking together the previous and the present results, it was deduced that carbohydrate I is comprised of N-linked oligosaccharides and carbohydrate II is of O-linked ones.

3.4. *The relative molar contents of N- and O-linked oligosaccharides*

The ratio of N- and O-linked oligosaccharides at contact site A was determined by identification of the terminal monosaccharide. The chromatogram of alditols by HPLC is shown in Fig. 4a. Each alditol was identified by reference to standard monosaccharide alditols in Fig. 4b. These results indicated that *N*-acetyl-galactosaminitol, *N*-acetylglucosaminitol and glucitol were present in the reducing termini of contact site A oligosaccharides. It is probable that *N*-acetylglucosaminitol reflects the attachment of N-linked oligosaccharides, and that *N*-acetylgalactosaminitol reflects the attachment of O-linked ones. However, the detection of glucitol was unexpected. Assuming that glucose could be a contamination of contact site A samples, it was determined that the ratio of N-linked oligosaccharides was 67%, while that of O-linked oligosaccharides was 33%. These ratios were unpredictable from the previous results, which suggested that two kinds of carbohydrates, I and II at contact site A, contributed equally to the molecular weight of the 80-kDa contact site A [9,10]. It seemed that the amount of O-linked oligosaccharides was remarkably less than that expected. This might be due to the recovery method of O-linked oligosaccharides. Although the hydrazinolysis condition used in this study was applicable to release O-linked oligosaccharides of more than 60% from glycoproteins, its efficacy might be dependent on each glycoprotein [14,26]. It is possible that glucose is *N*-glycosidically linked to asparagine or that it is O-glycosidically linked to serine (cf. [27,28]). Therefore, it is of interest to investigate whether in *D. discoideum* asparagine- or serine-linked glucose exists or not.

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