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SHORT COMMUNICATION

Large scale preparation of PA-oligosaccharides from glycoproteins using an improved extraction method

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We have developed a new method for the large scale preparation of pyridylaminated (PA-) oligosaccharides from glycoproteins. Phenol/chloroform extraction was adapted for the removal of protein and excess 2-aminopyridine, improving the efficiency of preparation. From a 2.5 g sample of human apo-transferrin, 25–30 μmol of agalacto biantennary PA-oligosaccharide could be obtained. By increasing the concentration of PA-oligosaccharide substrate, we were able to detect a very low level of *N*-acetylglucosaminlytransferase IV activity in CHO cell extracts.

Keywords: Oligosaccharide labelling, pyridylamination, glycosyltransferase

Abbreviations: PA, 2-aminopyridine; SDS, sodium dodecyl sulfate; GlcNAc, *N*-acetylglucosamine; GnT, *N*-acetylglucosaminyltransferase; Gn,Gn-bi-PA, GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-2-aminopyridine; Gn,Gn,Gn-tri-PA, GlcNAcβ1-2(GlcNAcβ1-4)Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-2-aminopyridine; Gn,Gn,Gn-tri′-PA, GlcNAcβ1-2Manα1-3({GlcNAcβ1-2(GlcNAcβ1-6)Manα1-6})Manβ1-4GlcNAcβ1-4GlcNAc-2-aminopyridine; Gn,(Gn),Gn-bi-PA, GlcNAcβ1-2Manα1-3(GlcNAcβ1-4)(GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-2-aminopyridine.

Introduction

The labelling of mono or oligosaccharides is a technique essential to carbohydrate research. For this purpose, fluorescence labelling using 2-aminopyridine was developed by Hase *et al.* [1]. Many PA-oligosaccharides are now commercially available as standards for the structure analysis of sugar chains. PA-oligosaccharides are also useful as specific substrates for glycosyltransferases. Nishikawa *et al.* developed a sensitive assay for *N*-acetylglucosaminyltransferases (GnT) III, IV and V using *N*-acetyllactosamine type, agalacto biantennary PA-oligosaccharide as a substrate [2]. The GnT assay requires several nanomoles of PA-oligosaccharide per sample. However, as commercial PA-oligosaccharides are only available in small amounts (usually 100–500 pmol per vial), it is economically impossible to use a commercial PA-sugar chain as a substrate.

As the pyridylamination methods and the protocols for oligosaccharide(s) release were orginially designed for analytical work [1, 3], we have modified the analytical scale methods for use in large scale preparation. Here we report on a new procedure for the large scale (over 10 μmol) preparation of PA-oligosaccharides from glycoproteins using an improved extraction method.

Materials and methods

Release of oligosaccharides from glycoprotein(s)

Human apo-transferrin (2.5 g, Sigma) was denatured for 2 h at 60 °C in 100 ml of 50 mM Tris-HCl, pH 7.2, containing 1.0% β-mercaptoethanol and 2.5% SDS. After cooling to room temperature, 4.0% (final concentration) of Nonidet P-40 (Nakarai), 75 mU of N-glycosidase F (Böeringer) and 100 μl of toluene were added and the sample was then incubated at 37 °C overnight. The digested sample was extracted with an equal volume of

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a phenol/chloroform solution (as used in DNA manipulations; TE-Buffer saturated phenol:chloroform:isoamyl alcohol = 25:24:1) to remove the proteins. The resulting sample was then centrifuged at 8000 rpm, 20 min at room temperature, and the aqueous layer was lyophilized and desalted with a Biogel P-100 (Bio-Rad) column (2.5 × 80 cm, equilibrated with 50 mM NH_4HCO_3-HCl, pH 7.2). Aliquots of collected 2 ml fractions were stained for carbohydrate determination with Phenol/H_2SO_4. Carbohydrate concentrations were quantified using mannose as a standard.

Pyridylamination of oligosaccharides

The carbohydrate-positive fractions obtained by gel filtration were pooled and lyophilized. The sample was then dissolved in 13 ml of CH_3COOH with 27 g of 2-aminopyridine (Wako) and incubated at 90 °C for 1 h. After addition of 57 g of borane-dimethylamine complex (Wako), 23 ml of CH_3COOH and 14 ml of water, the solution was incubated at 80 °C for 1 h. After cooling to room temperature, 100 ml of water was added and the excess reagent was removed by phenol/chloroform extraction. The sample was extracted twice with equal volumes of phenol/chloroform and once with chloroform. The aqueous phase was then lyophilized and the sample was further purified by Toyopearl HW-40F (Tosoh) gel filtration (5 × 20 cm, equilibrated with 10 mM CH_3COONH_4, pH 6.0; Flow rate: 5 ml min^{-1}) using an FPLC system (Pharmacia).

Sialidase/β-galactosidase treatment of PA-oligosaccharides and HPLC purification

The lyophilized PA-oligosaccharides were dissolved in 10 ml of 0.4 M sodium acetate buffer, pH 5.0. Ten units of sialidase derived from *Arthrobactor ureafacience* (Nakarai), 10 mg of β-galactosidase derived from *Aspergillus* sp. (TOYOBO) and one drop of toluene were added to the solution, which was then incubated at 37 °C overnight. The PA-sugar chain was purified by HPLC (Vydac 218TP152010, 10 × 250 mm; eluent, 100 mM ammonium acetate, pH 4.0, 0.15% *n*-butanol; flow rate, 2.5 ml min^{-1}; detection, UV 300 nm). Purification was performed by repeating a 1 ml sample injection ten times.

Confirmation of the purity of the PA-oligosaccharide

The homogeneity of purified PA-oligosaccharide was checked by HPLC (TSKgel ODS-80 TM, 4.6 × 150 mm; eluent, 100 mM ammonium acetate, pH 4.0, 0.15% *n*-butanol; flow rate, 1.2 ml min^{-1}; fluorescence detection, ex. 320 nm, em. 400 nm). The homogeneity and molecular weight of the sample were analysed by MALDI-TOF MS (Finnigan MAT, LASER MAT 2000; Matrix: 2,5-dihydroxybenzoic acid).

Results and discussion

Table 1 shows the carbohydrate amount at each step of the protocol. Figure 1 shows typical chromatographic patterns for PA-sugar chain purification. From 2.5 g of human apo-transferrin, 25–30 μmol of agalacto biantennary PA-oligosaccharide was obtained. The yield was approximately 25–40% when the protein was fully glycosylated (2 mol sugar chain per 1 mol protein). HPLC chromatography showed that the purity of the PA-oligosaccharide obtained was very high (more than 99.8%), and the molecular weight determined by MALDI-TOF MS corresponded to the calculated value (calculated value, 1396.3; determined value, 1394.5; Fig. 2).

We were unable to detect GnT-IV activity in CHO cell extracts with the standard assay protocol for GnT-III, IV and V [2]. In the GnT assay, the apparent K_m value for the PA-sugar chain was relatively high (3.4 mM) [2]. The concentration of PA-sugar chain (80 μM) was not saturated enough in the standard assay protocol. As a result of our large scale preparation, the GnT-IV activity in CHO cell extracts could be quantitatively determined by increasing the concentration of PA-sugar substrate (20 pmol h^{-1} per mg of protein, Fig. 3).

Phenol/chloroform extraction was highly effective in this preparation. Waard *et al.* removed proteins by gel filtration after N-glycosidase F digestion in their analytical scale protocol [3], but it is very difficult to apply this method to the large scale preparation. Phenol/chloroform extracted proteins effectively (Fig. 1) and loss of the sugar moiety was insignificant (Table 1). Hase described an extraction method using chloroform for the removal of excess 2-aminopyridine [4]. We compared the extraction efficiency of chloroform and phenol/chloroform. After pyridylamination, the sample was extracted with chloroform or phenol/chloroform, and the fluorescence of the aqueous phase was measured. The highest extraction efficiency was obtained using phenol/chloroform, with an acidic pH (Table 2). In our protocol, phenol/chloroform removed excess 2-aminopyridine effectively. Any residual 2-aminopyridine was removed by Toyopeal HW-40 chromatography (Fig. 1B).

Our method may be applied to the preparation of other types of PA-oligosaccharides, and native oligosaccharides (without PA-) released from glycoproteins. Large amounts

Table 1. Purification of Gn,Gn-bi-PA from human apo-transferin.

	Carbohydrate (mg)	*Yield (%)*
Human apo-transferin	70	100
BioGel P-100	65	93
Phenol/chloroform extraction	38	54
Vydac C18	30	43

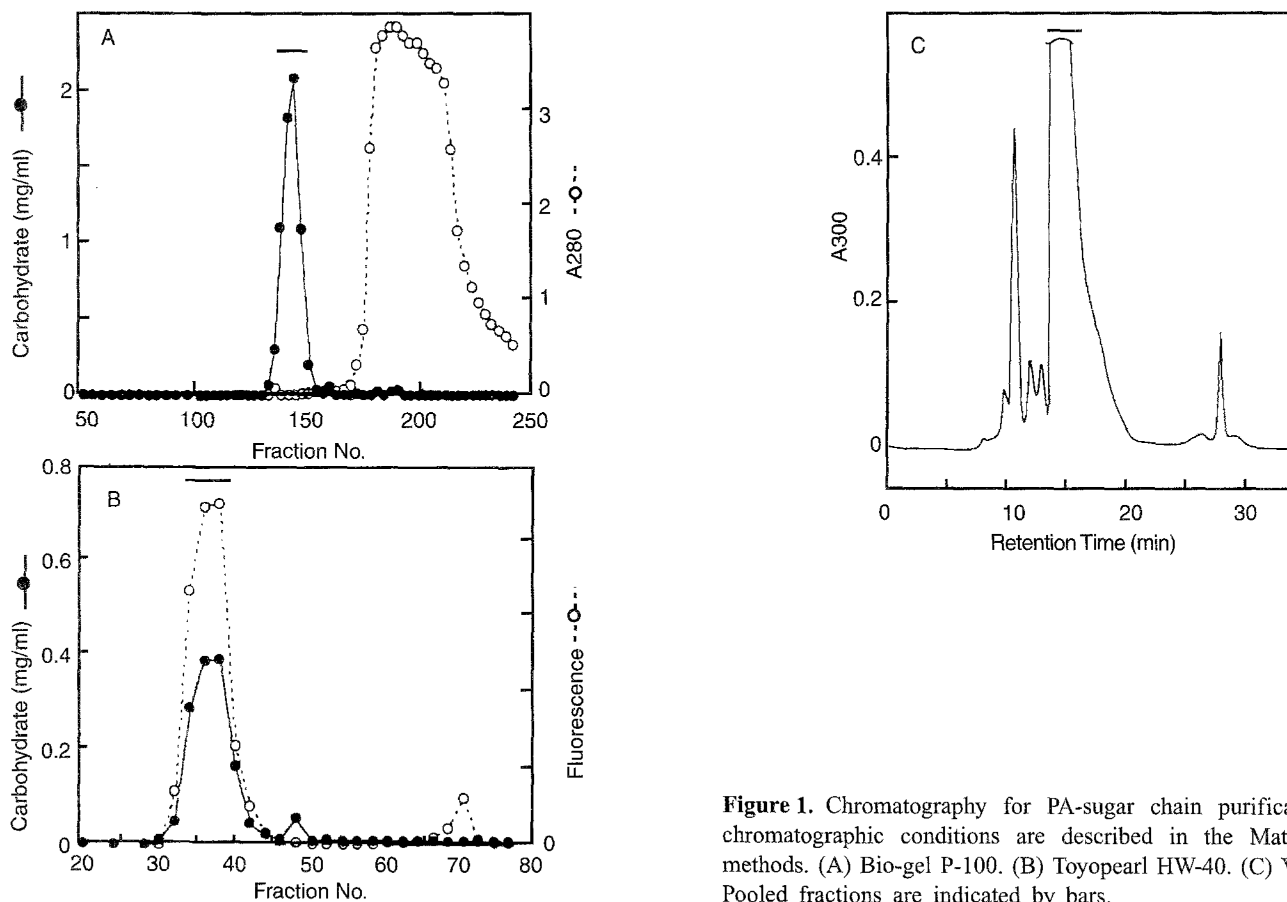

Figure 1. Chromatography for PA-sugar chain purification. The chromatographic conditions are described in the Materials and methods. (A) Bio-gel P-100. (B) Toyopearl HW-40. (C) Vydac C_{18}. Pooled fractions are indicated by bars.

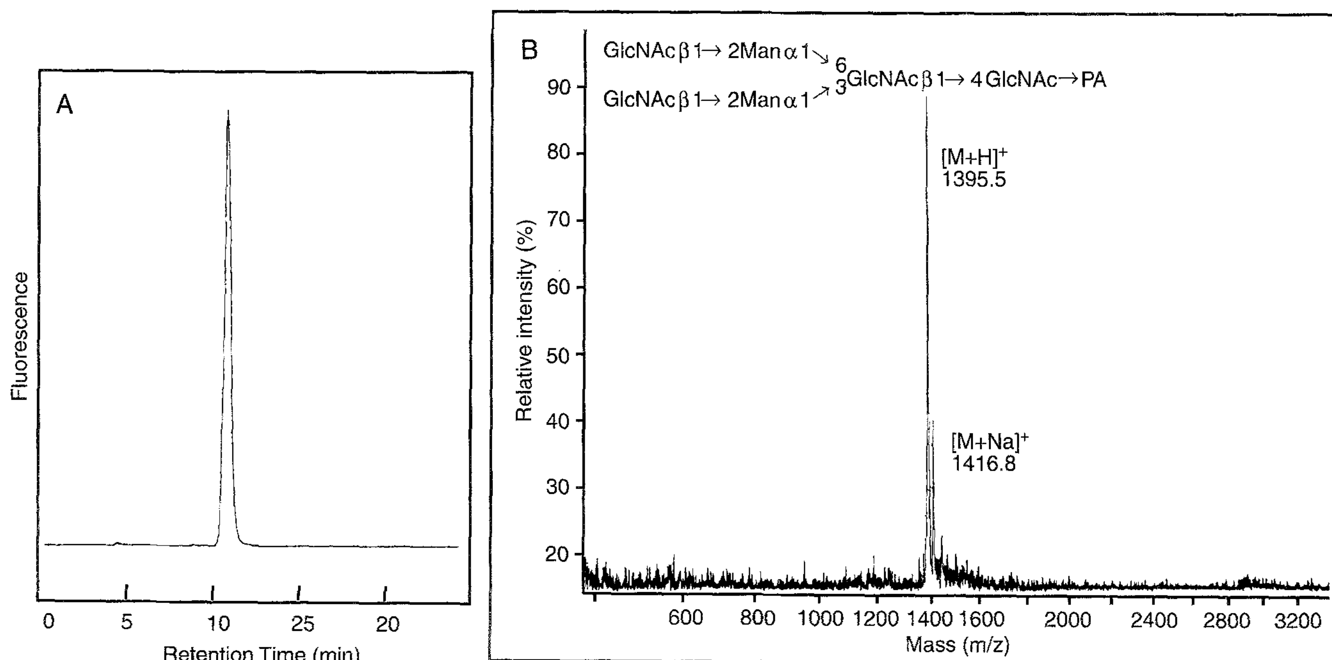

Figure 2. The HPLC chromatogram (A) and MALDI TOF-MS spectra (B) of purified PA-sugar chain. Experimental details are described in the Materials and methods. (A) The retention time of the sample corresponds to that of Gn,Gn-bi-PA. No contaminating peaks were observed in sample injections of up to 2 nmol. (B) The molecular weights of the main peaks corresponded to the calculated values. The structural formula of Gn,Gn-bi-PA is shown in the panel.

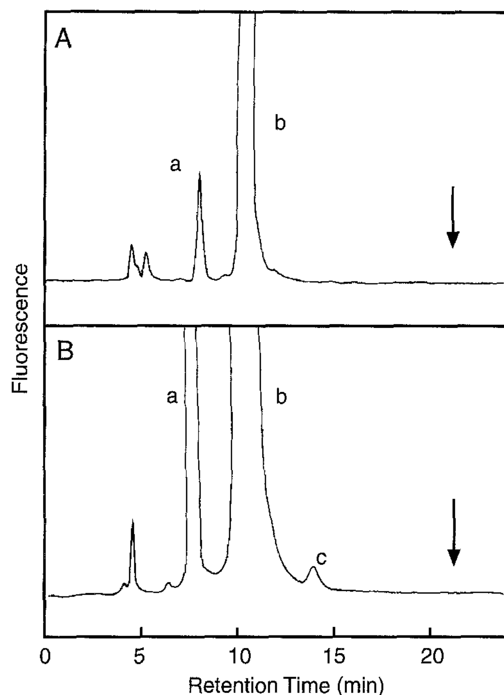

Figure 3. The HPLC elution pattern for the simultaneous assay of the GnT-III, IV and V enzymes at different substrate concentrations. A crude enzyme extract from CHO-K1 cells was treated according to the GnT-IV assay method [2]. The substrate concentrations were 80 μM (panel A) and 800 μM (panel B), respectively. The GnT-V product, Gn,Gn,Gn-tri′-PA, the substrate, Gn,Gn-bi-PA and the GnT-IV product, Gn,Gn,Gn,-tri-PA are indicated as (a), (b) and (c), respectively. The elution positions of the GnT-III product, Gn,(Gn),Gn-bi-PA are indicated by arrows.

Table 2. Comparison of the extraction efficiency of chloroform and phenol/chloroform.

Solvents	*Chloroform*	*Phenol/ chloroform*	*Phenol/ chloroform*
pH	*9.5*	*9.5*	*5.0*
Fluorescence*	>770	77	0.2

*Figures represent relative fluorescence values.

of PA-oligosaccharides and native oligosaccharides are useful, not only as substrates for glycosyltransferases, but also in the NMR analysis of sugar structure and other studies in glycobiology. Further refinement of this protocol is being studied.

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References

1. Hase S, Ibuki T, Ikenaka T (1984) *J Biochem* **95**: 197–203.
2. Nishikawa A, Gu J, Fuji S, Taniguchi N (1990) *Biochim Biophys Acta* **1035**: 313–18.
3. Waard PD, Koorevaar A, Kamerling JP, Vliegenthart JFG (1991) *J Biol Chem* **266**: 4237–43.
4. Hase S (1994) *Methods Enzymol* **230**: 225–37.