**Note**

**Method for Purification of Fluorescence-Labeled Oligosaccharides by Pyridylamination**

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Received December 10, 2001; Accepted January 19, 2002

We developed a convenient method for purification of PA-oligosaccharides to remove contaminants originating from natural fluorescent materials, and excess reagents as well as by-products of tagging reactions in glycan analysis. The method, using a C18-cartridge, is simple and powerful to remove them. Several examples of experiments that showed the usefulness of this purification method are described in this report.

Key words: pyridylamination; oligosaccharide; fluorescence; glycome

Pyridylation, which is a fluorescence-tagging method for oligosaccharides, is widely used for structural analysis and measurement of glycans. Although this method has many advantages, such as high sensitivity, high resolution, and chemical stability, it also has a major difficulty; namely contaminants, source of which originate from natural fluorescent materials in the samples as well as by-products of the tagging reaction, interfere with the subsequent HPLC analysis. The problem is especially serious in analyzing small samples. They are not decisive although several purification methods have been devised.†-4) The samples prepared by these purification steps still contain considerable amounts of impurities that had similar retention times as the PA-oligosaccharides in the HPLC. Therefore we attempted to develop a new purification method for PA-oligosaccharides. The method is especially effective in analyzing extremely small amounts of samples including many kinds of components, for example a glycome assay, because it is easy, time saving, and has high yields.

A C18 cartridge (Sep-Pak Plus, Waters) was treated with methanol, and then equilibrated with 0.1% acetic acid. To set up adsorption as well as elution conditions for PA-oligosaccharides, PA-IMO (DP = 4 – >20) were loaded into the cartridge, and eluted by stepwise addition of 0.1% acetic acid containing various concentrations of methanol. The eluates were analyzed by size-fractionation HPLC (Fig. 1). A Shimadzu LC-6A liquid chromatograph with a Shimadzu RF-530 fluorescence spectrophotometer (excitation 310 nm, emission 380 nm) was used. Size-fractionation HPLC was done on a Shodex NH2P-50 column (ϕ 0.46 × 15 cm, Showa Denko) at a flow rate of 0.8 ml/min. The column was equilibrated with 50 mM ammonium acetate, pH 7.0, with 86.5% acetonitrile. After a sample was injected, the acetonitrile concentration decreased linearly from 86.5 to 37.5% for 50 min. Most of PA-IMO larger than trisaccharide eluted with less than 20% methanol, although the unadsorbed fractions contained a trace of them. Thus, 0.1% acetic acid and 20% methanol in 0.1% acetic acid were used as the adsorption and elution solvent, respectively.

A mixture of IMO (DP = 4 – >20, Honen Corporation) was pyridylaminated, and purified with a C18 cartridge. The pyridylamination reaction of the oligosaccharides was done by the ordinary method† with 20 µl of PA-reagent and 70 µl of reduction reagent, followed by lyophilization of the reactant. The reactant was re-dissolved in 100 µl of 0.1% acetic acid and then put onto the C18 cartridge. After unadsorbed materials were flushed out with 3 ml of 0.1% acetic acid, PA-oligosaccharides were eluted with 2 ml of 20% methanol in 0.1% acetic acid. Samples were concentrated by evaporation, and then analyzed by size-fractionation HPLC (Fig. 2). PA-oligosaccharides were successfully purified to remove excess reagents and by-products of the reaction.

Hydrazinolysis is an excellent method to eliminate oligosaccharides from glycoproteins, because each glycan is easily taken out. In this reaction, some polypeptides also decompose to amino acid derivatives containing fluorescent materials, which disturb the analysis of PA-oligosaccharides. One milligram of a glycoprotein, bovine fetuin (Sigma) was treated with 200 µl of anhydrous hydrazine at 100°C for 10 h to eliminate N-glycans, followed by acetylation of free amino groups as reported before.† Briefly, after removing of excess hydrazine in vacuo, 200 µl of saturated sodium bicarbonate solution and 8 µl of...
acetic anhydride were added to the residue. Five minutes later, another 200 µl of the bicarbonate solution and 8 µl of acetic anhydride was added. Sodium ion was removed by a Dowex 50 X 2 (H+ form) cation exchanger. The released oligosaccharides were tagged with 2-aminopyridine, and then purified by the same procedure described above. After desialylation with 25 mM HCl at 80°C for 60 min, the sample was analyzed by size-fractionation HPLC (Fig. 3). Peaks corresponding to bianntenary and trianntenary complex type N-glycans were clearly detected at 30.6 and 31.6 min of retention time in the chromatogram, respectively. This result coincided with a previous report in which a trianntennary complex type was shown as a dominant N-glycan of bovine fetuin.

We could develop the new purification step in the analysis of pyridylaminated oligosaccharides as shown above. This should be is better than the previous method in the aspects of simplicity and ease, as well as effectiveness. Furthermore, the method will be applicable to an automatic analyzers of oligosaccharides, which will be useful for glycome analysis.

References