Pyridylamination is a fluorescence-tagging method for oligosaccharides, widely used in structural analysis and the measurement of glycans. Although this method has many advantages, including high sensitivity, high resolution, and chemical stability, it has also a major difficulty: contaminants that originate in natural fluorescent materials in the samples as well as by-products of the tagging reaction interfere with subsequent HPLC analysis. This problem is especially serious in analyzing small samples. The results are not definitive even though several purification methods have been devised.1–5) Because the yield of small size carbohydrates is not high using these purification methods, they are not suitable for PA-monomosaccharide purification. A purification method for PA-monomosaccharides with borate resin chromatography has been reported.6) Under basic conditions, cis-diol of sugar binds to boric acid to form a complex compound. In contrast, fluorescent contaminants without cis-diol can be washed out. Since the complex of sugar and boronic acid dissociates under acidic conditions, PA-monomosaccharides can be collected with an acidic solution. But reproducibility and convenience of this method are not good. Hence I attempted to develop a new purification method for PA-monomosaccharides.

A MonoSpin PBA spin column (GL Science, Tokyo) that contained monolithic silica bound phenylboronate was treated with 0.5 mL of 1% acetic acid, and then equilibrated with 0.5 mL of 0.2 M ammonia solution. To examine purification efficiency against the by-products of the tagging reaction, PA-monomosaccharides produced by a reported method7) were purified by MonoSpin PBA spin column. Five hundred picomoles of rhamnose, which is usually used as an internal standard in sugar component analysis, and 25 pmol each of N-acetylglactosamine, N-acetylgalactosamine, glucose, mannose, fucose, and galactose, were pyridylaminated. Excess reagents were removed by co-evaporation with toluene, by the reported method.7) The reaction mixture was dissolved in 0.5 mL of 0.2 M ammonia solution and loaded into the column. Contaminants were washed out with 0.5 mL of 0.2 M ammonia solution, and then the PA-monomosaccharides were eluted with 0.5 mL of 1% acetic acid. The eluate was lyophilized and dissolved in 50 μL of water, followed by borate-chelating anion-exchange HPLC analysis7) (Fig. 1). HPLC was performed on a TSKgel Sugar AX-I column (0.46 × 15 cm, Tosoh, Tokyo) at a flow rate of 0.3 mL/min using a Waters 515 HPLC pump (Waters, Milford) and a Hitachi L-7485 fluorescence spectrophotometer (Hitachi, Tokyo). The column was equilibrated with 10% acetonitrile in 0.8 M potassium borate pH 9.0 at temperature of 74°C in a column oven (CTO-10ASvp, Shimadzu, Kyoto). The sample was separated by isocratic elution and detected with a fluorescence spectrophotometer with an excitation wavelength of 310 nm and an emission wavelength of 380 nm. Although the sample labeled by the reported method carried significant amounts of obstructive con-
taminants, detected on HPLC analysis (Fig. 1A), the purification process with the MonoSpin PBA spin column efficiently removed the contaminants (Fig. 1B). The yield of PA-rhamnose through the purification process was 64%.

To examine purification efficiency against glycoprotein-derived materials, PA-monoosaccharides prepared from bovine γ-globulin were purified with the MonoSpin PBA spin column. Fifteen μg of bovine γ-globulin was hydrolyzed with 0.5 mL of 5 M TFA at 100 °C over 4 h. After removing the acid, 500 pmol of rhamnose was added as internal standard, and then the amino sugars were re-N-acetylated with 200 μL of methanol-pyridine (9:1, v/v) and 50 μL of acetic anhydride at room temperature over 30 min. Pyridylamination and reagent-removal were carried out by the reported method.7) Half of the sample was further purified with the MonoSpin PBA spin column. Equivalent amounts of the samples with and without spin column purification were analyzed by borate-chelating anion-exchange HPLC, as described above (Fig. 2). The method is summarized in Scheme 1. The sample labeled by the reported method carried significant amounts of obstructive contaminants, detected on HPLC analysis (Fig. 2A). Especially, a major contaminant was eluted close to PA-rhamnose, which was used as internal standard, on HPLC. The purification process with the MonoSpin PBA spin column efficiently removed the contaminants (Fig. 2B). The sugar composition of the γ-globulin calculated from the

Sample
↓ Hydrolyze, 0.5 mL of 5 M TFA, 100°C, 4 h
↓ Dry in vacuo
↓ Dry in vacuo, 200 μL of MeOH, twice
↓ Add 10 μL of 50 μM rhamnose (as an internal standard)
↓ Dry in vacuo
↓ Re-N-acetylation with 200 μL MeOH/pyridine (9:1, v/v), 50 μL Ac2O, room temperature, 30 min, occasionally stirring
↓ Dry in vacuo
↓ Pyridylamination
  1. 10 μL of PA-reagent, 90°C, 20 min
     \[
     \begin{align*}
     &100 \text{ mg 2-aminopyridine} \\
     &47 \mu\text{L AcOH} \\
     &60 \mu\text{L MeOH}
     \end{align*}
     \]
  2. Evaporate at 60°C, 20 min, under N2 stream
  3. 10 μL of reduction reagent, 80°C, 35 min
     \[
     \begin{align*}
     &10 \mu\text{g Me2NH · BH3} \\
     &170 \mu\text{L AcOH}
     \end{align*}
     \]
     Evaporate with 20 μL MeOH, 40 μL toluene, 50°C, 10 min, under N2 stream
     Evaporate with 50 μL toluene, 50°C, 10 min, under N2 stream, 4-times
     ↓ Dissolve in 0.5 mL of 0.2 M ammonia solution
     ↓ Load on pre-treated MonoSpin PBA spin column
       ↓ cfg., 5,000 rpm, 2 min
       ↓ Wash with 0.5 mL of 0.2 M ammonia solution, 10,000 rpm, 1 min
       ↓ Elute with 0.5 mL of 1% AcOH, 10,000 rpm, 1 min
     Eluate
     ↓ Lyophilize
     ↓ Dissolve in 100 μL water
     PA-monoosaccharides
     ↓ Analyze by borate-chelating anion-exchange HPLC

Scheme 1. Procedure for Sugar Component Analysis with Purification Steps by MonoSpin PBA Spin Column. Purification steps are typed in bold letters.

Fig. 2. Sugar Component Analysis of Bovine γ-Globulin by Borate Chelating Anion-Exchange HPLC.
A. Sample before purification by MonoSpin PBA spin column. B. Sample after purification by MonoSpin PBA spin column. Arrowheads indicate elution positions of standard PA-monosaccharides. Asterisks indicate contaminant peaks largely removed by purification by MonoSpin PBA spin column.
peak areas of the PA-monosaccharides (Fig. 2B) by comparison with those of the internal standard, PA-rhamnose, and external standards of various PA-monosaccharides (Fig. 1B). The measured values for sugar composition were 7.4 mol of N-acetylglucosamine, 1.2 mol of glucose, 8.1 mol of mannose, 1.9 mol of fucose, and 3.5 mol of galactose per mol of bovine γ-globulin. Since bovine γ-globulin has two complex-type N-glycans per molecule, the detected amounts of PA-monosaccharides are plausible. The glucose was probably a contaminant from the sample or reagents.

Based on these results, the purification method with the MonoSpin PBA spin column is useful for monosaccharide analysis, in particular when the amount of a sample is very small. Moreover, the method is very simple and easy, since the operation consists only of spin-downs.

References