Note

Improved Method for the Component Sugar Analysis of Glycoproteins by Pyridylamino Sugars Purified with Immobilized Boronic Acid

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Component sugar analysis is one of the most important methods for a structural analysis of the sugar chains in glycoproteins. In our previous paper,1 we reported a sensitive method which involves acid hydrolysis of glycoproteins, pyridylamination of the sugars liberated, and separation of the pyridylamino (PA-) sugar chains by anion exchange HPLC. In this procedure, the pyridylaminated sugars were directly analyzed by HPLC; however, several contaminating peaks probably derived from the reagents disturbed the highly sensitive analysis. Because of these peaks, the amount of a sample that can be analyzed was often limited to 100-500 pmol. This sensitive component analysis was further improved by the use of immobilized boronic acid or phenyl boronic acid,2 which was introduced by Stoll and Hounsell. The present paper describes the use of the resin which could eliminate a small amount of the contaminating peaks. The principle is based on the finding that immobilized boronic acid can bind a sterically unhindered planar array of vicinal hydroxy groups,3 and other minor changes were also made.

Glass test tubes for hydrolysis were heated with 6 M HCl at 100°C for 3 hr, washed well with water, and dried, before being heated overnight at 500°C. Immobilized boronic acid was purchased from Pierce (Rockford, IL).

Procedure

Hydrolysis: A sample (0.05-10 nmol of sugars or glycoproteins) was placed in a test tube (10 x 100 mm) tapered at the bottom, and then dried. To the residue was added 40 μl of 4 M trifluoroacetic acid, before the tube was sealed in vacuo and heated at 100°C for 3 hr. The solution was freeze-dried. To the solution was added a ribose solution (100-500 pmol) as an internal standard, and the solution was again freeze-dried, before acetylaying the free amino groups.4

N-Acetylation: To the residue was added 80 μl of a freshly prepared saturated sodium bicarbonate solution and 4 μl of acetic anhydride at room temperature for 30 min. To the solution was then added Dowex 50W-X2 (H+(100-200 mesh, about 0.2 ml) to bring the pH of the solution to about 3. The resin and the solution were placed in a small column, and the resin was washed with a 5-bed volume of water. The pass-through fraction and the washings were combined, concentrated, and freeze-dried.

Pyridylamination:1 To the residue in a test tube tapered at the bottom was added 7 μl of a coupling reagent prepared by mixing 1.00 g of 2-amino-pyridine, 0.47 ml of acetic acid, and 0.60 ml of methanol. The tube was sealed and heated at 90°C for 15 min. The excess reagents were removed at 150 mmHg under a stream of nitrogen (300 ml/min) at 60°C for 20 min (this procedure can be done by using a Pallstation from Takara, Kyoto). Then, 10 μl of a reducing reagent, which had been prepared just before use by mixing 59 mg of borane-dimethylamine complex in 1 ml of acetic acid, was added. The tube was re-sealed and heated at 90°C for 30 min. The reaction mixture was dried three times at 150 mmHg under a stream of nitrogen gas (300 ml/min) with 30 μl of toluene at 40°C for 10 min each time to remove the excess reagents.

Purification of PA-sugars: The residue was dissolved in a small amount of 0.2 M ammonia water and placed in an immobilized boronic acid column (0.3 x 2 cm), the column size being measured when the resin had been washed with acid. The column had been activated before use by successively washing with 0.55 ml of 0.1 M HCl and 0.85 ml of 0.2 M ammonia water. The column was washed with 0.42 ml of 0.2 M ammonia water with 0.14 ml of water. PA-sugars were then eluted with 0.85 ml of 0.2 M aqueous acetic acid. The solution was evaporated to dryness, and the residue was dissolved in a small amount of water.

HPLC analysis:11 A part of the solution (1/10) was analyzed with a

Fig. 1. Effects of Immobilized Boronic Acid on the Purification of PA-Sugars.

One hundred pmol each of sugars was combined, and the mixture was hydrolyzed, N-acetylated and pyridylaminated as described in the text. One tenth of the product was analyzed (A), or the product was purified with immobilized boronic acid and one tenth of the PA-sugar fraction was analyzed (B). using a 0.7 M potassium borate buffer as described in the text. 1 PA-GalNac; 2 PA-Xyl; 3 PA-GlcNac; 4 PA-Rib; 5 PA-Glc; 6 PA-Man; 7 PA-Fuc; 8 PA-Gal.

Fig. 2. Sugar Component Analysis of Taka-α-amylase A.

Taka-α-amylase A (50 pmol) was treated as described in the text, and one tenth of the product was analyzed by HPLC, using a 0.8 M potassium borate buffer. Ribose was used as an internal standard. See the legend to Fig. 1 for the key.

Abbreviations: PA-, pyridylamino; HPLC, high-performance liquid chromatography.

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Component Sugar Analysis by Pyridylamination

TSKgel Sugar AXI column (0.46 × 15 cm). The elution buffer was 0.7 M or 0.8 M boric acid, which had been adjusted to pH 9.0 with potassium hydroxide, containing 10% acetonitrile. The flow rate was 0.3 ml/min, and the column was operated at 73°C. For detection of the PA-sugars, a Shimadzu RF-535 HPLC fluorescence monitor with an excitation wavelength of 310 nm and emission wavelength of 380 nm was used.

The effectiveness of immobilized boronic acid is shown in Fig. 1. Most of the contaminating peaks indicated by arrowheads could be removed. Under the conditions used, PA-galactose, PA-glucose, PA-mannose, PA-N-acetylgalactosamine, PA-N-acetylgalactosamine, PA-fucose, PA-rhamnose, PA-ribose, and PA-xylose were recovered quantitatively in the fraction described above; however, PA-2-deoxyribose was found in the fraction eluted with water (data not shown). The sensitivity of this method was greatly improved especially for determining galactosamine (Fig. 1). The modified method was used for a sugar component analysis of Taka-amyrase A, which contains one mole of an oligomannose type sugar chain, and PA-N-acetylgalactosamine (2.0 mol/mol) and PA-mannose (8.4 mol/mol) were detected.\(^1\) PA-glucose and a trace amount of PA-galactose, probably from the glycoprotein, were also seen (Fig. 2). When a component analysis of PA-sugar chains was done, reducing-end PA-sugars were bound to Dowex 50. The reducing-end PA-sugars are, therefore, eluted from the resin with a 5-bed volume of 0.2 M ammonia water.

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References