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Compositional Sugar Analysis of Antitumor Polysaccharides by High Performance Liquid Chromatography and Gas Chromatography

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Carbohydrate analysis is important in studying structure and activity of complex polysaccharides. New analytical method was applied to get an information on the composition of polysaccharides showing antitumor activity. Monosaccharides were labeled with 7-amino-1,3-naphthalenedisulfonic acid (7-AGA) by reductive amination and separated by HPLC. Five kinds of polysaccharides from *Basidiomycetes* were hydrolyzed and analyzed in combination with electrophoresis and HPLC. At the same time, alditol acetate derivatives were prepared and analyzed by gas chromatography. Two different techniques using different derivatization methods showed very similiar results. The monosaccharides from *Coriolus versicolor* and *Lentinus eodes* were mainly composed of D-glucose, while those from *Ganoderma lucidium* and *Cordyceps militaris* were glucose and galactose. *Phellinus linteus* composed of glucose, galactose, mannose, arabinose and fucose. The HPLC method with fluorescence detector was very sensitive compared to other methods.

Key words: Carbohydrate analysis, Reductive amination, HPLC, GC

INTRODUCTION

The structural change of carbohydrate on cell surface has been attracted to many scientists. The major role of carbohydrate includes the interaction and growth of cells in transforming normal cells to malignant cells (Rademacher *et al.*, 1988). The polysaccharides from plants and fungi are thought to be immunomodulators showing antitumor activity (Toshio, 1990). The compositional analysis is the first step for the determination of sugar structures. The common technique which has been widely used until now is a gas chromatography. In this case sugars are derivatized in a volatile form after acid hydrolysis or methanolysis of samples.

Last decade high-performanc liquid chromatography (HPLC) has been accepted as one of the major techniques for the analysis of sugars. Early studies relied on refractometry, although the introduction of high sensitivity, pulsed amperometric detectors have largeley replaced all other methods in the detection of underivatized sugars (Townsend *et al.*, 1988). Recently, precolumn derivatization of reducing sugars has been widely used for the separation of sugars using reversed-phase HPLC. Pyridylaminated sugars are often prepared not only to overcome little hydrophobicity and but also increase the sensitivity of sugars in detection (Hase *et al.*, 1984).

We have focused on preparing sugar derivatives that are both visibly fluorescent and charged. Such derivatives are easily prepared by reductive amination with tags containing sulfonated aromatic amines such as 7-amino-1,3-naphthalene disulfonic acid (Jackson *et al.*, 1990; Lee *et al.*, 1991). Sugar-AGA conjugates can be fractionated by anion-exchange HPLC as well as by other charge-based methods, such as polyacrylamide gel electrophoresis (PAGE) (Lee *et al.*, 1991; Kim *et al.*, 1991), lectin-affinity electrophoresis (Lee *et al.*, 1992) and capillary electrophoresis (CE) (Al-Hakim *et al.*, 1991; Lee *et al.*, 1991; Linhardt, 1993). Ion-pairing HPLC to separate monosaccharides and oligosaccharides containing sulfonated sugars has been tried very recently (Kim, 1994). This technique was initially applied to analyze the sugar composition of plant polysaccharides (Kim *et al.*, 1993a; Kim *et al.*, 1993b). This

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study is concentrated on the application of new technique to analyze the composition of fungal polysaccharides. At the same time the result was compared with that from gas chromatography after preparing alditol acetate derivatives.

MATERIALS AND METHODS

Materals

D-Glucose (Glc), D-Mannose (Man), D-Galactose (Gal), D-Arabinose (Ara), L-Fucose (Fuc), L-Rhamnose (Rham), D-Xylose (Xyl), *N*-acetyl-D-glucosamine (GlcNAc), D-Glucuronic acid (Glc-U) and D-Galacturonic acid (Gal-U) were from Sigma Chemical Co., St Louis, MO. 7-AGA and sodium cyanoborohydride were obtained from Aldrich Co (USA). The monopotassium salt of 7-AGA was used after recrystallization from distilled water. The polysaccharides from *Coriolus versicolor, Ganoderma lucidium, Phellinus linteus, Cordyceps militaris* and *Lentinus eodes* were donated from Kwangdong Pharm Co. (Seoul), Ilyang Pharm Co.(Seoul), Hankook Shinyak Co (Taejeon), and National Institute of Health (Seoul), respectively. The 16×18 cm vertical slab gel unit and Bio-Gel P2 were from Bio-Rad, Richmond, CA. The TE70 semi-dry electrophoresis transer unit and Nylone 66 plus (positively charged nylone membrane) were obtained from Hoefer Scientific Instruments, San Fransisco, CA. The hand-held UV lamp were from Spectronic Co., Wesbury, NY. All other reagents were HPLC grade or reagents grade.

Methods

Preparation of fluorescently labeled sugars by reductive amination: Each mono- and oligosaccharides (300 μg) dissolved in 15 μl water was mixed with 30 μl of 1 M $NaCNBH_3$ and 15 μl of 0.2 M AGA (acetic acid : water=15 : 85). They were incubated at 37℃ overnight. Sugar-AGA conjugates were desalted and much of the excess AGA was removed using a 2.0× 65 cm Bio-Gel P2 eluted with water. After the reaction was complete, the samples were electrophoresed. The products could then be analyzed without further purification despite the presence of small amounts of residual reactants. In cases where it was necessary to completly remove AGA, the sugar-AGA conjugates were purified by preparative gel electrophoresis (Lee *et al.*, 1991).

High performance liquid chromatographic (HPLC) analysis: HPLC was performed to analyze the sugar-AGA conjugates (Kim, 1994). The system was equipped with a SP 8860 ternary HPLC pump (Spectra Physics), fixed volume loop Rheodyne (Cocati, CA) #7125 injector and Spectra 100 variable wavelength detector. The data were processed using SP4270 integrator (Spectra Physics). The reaction mixture was injected on a Spheri-5 RP 18 of dimension 4.6 mm×22 cm from Applied Biosystem or phenyl column (Alltech, Deerfiled, IL) and eluted with 0.1 M triethylamine/acetic acid (pH 4.0) at 1.0 ml/min. The wavelength was fixed at 250 nm for detection. Alternatively, a single pump and a variable wavelength RF-535 fluorescence detector (Gilson) were equipped. Fluorescence detection was set at λ_{ex}=255 nm, λ_{em}=450 nm.

Preparation of alditol acetate derivatives: Sugars were reduced and acetylated according to the previous report (Blakeney, 1983). In brief, samples were dissolved in 0.1 M ammonia solution and incubated with reagent B (prepared with 2 g of sodium borohydride in dry DMSO) at 40℃ for 90 min. Excess $NaBH_4$ was removed by adding 0.1 ml glacial acetic acid. Reduced saccharides were acetylated by adding 2 ml acetic anhydride and 0.2 ml 1-methyl imidazole at room temperature for 10 min. Excess acetic anhydride was decomposed by adding water. Sugar derivatives were extracted with dichloromethane.

Hydrolysis of polysaccharides: A sample (10 nmol of polysaccharide) was placed in a screw-capped tube and dried. To the residue 40 μl of 2-4 M trifluoroacetic acid added, the tube was heated at 100℃ for 3 hr. To the residue, the water was added and freeze-dried twice. Free amino groups were reacetylated by adding 55 μl of a mixture of methanol-pyridine-water (30 : 15 : 10) and 2 μl of acetic anhydride. The solution was left for 30 min at room temperature with occasional stirring. The solution was freeze-dried, and to the residue the coupling reagents were added as above. The excess AGA was removed by gel-electrophoresis.

Gas chromatography: Acetylated alditols of sugars were separated on a glass-capillary column (15 m× 0.25 mm, i.d., SP 2330) in a Hewlett-Packard 5730A chromatograph equipment with a flame ionization detector (Blakeney *et al.*, 1983). The oven temperature was maintained at 180℃ and then gradually increased to 220℃ at a rate of 8℃/min. The temperature of detector and injector was set at 270℃ and 240℃, respectively.

RESULTS AND DISCUSSION

Precolumn derivatization of sugars is one of the popular technique in analytical chemistry. Our initial studies on the separation of monosaccharides was done by isocratic PAGE. The migration of each monosaccharide on the gel was not significant, but hexose and pentose could be recognized each other (data not shown). Owing to a lack of quantitation of the electrophoretic method, HPLC was complemented for the

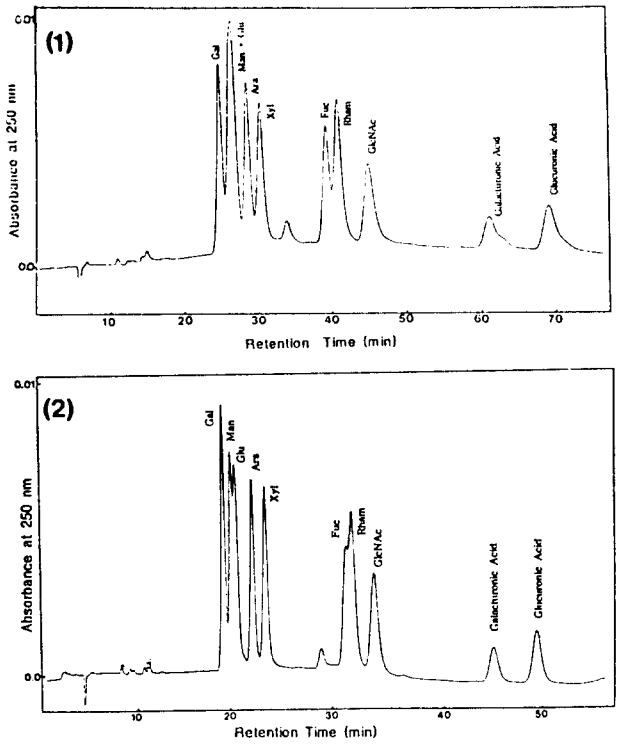

Fig. 1. Separation of monosaccharide-AGA conjugates using C_{18} and phenyl columns. The eluant was directed from phenyl to C_{18} columns 1) and C_{18} column to phenyl columns 2). Each peak was assinged with standard conjugates. From the left, galactose, mannose, glucose, arabinose, xylose, fucose, rhamnose, N-acetylglucosamine, galacturonic acid and glucuronic acid were eluted in order.

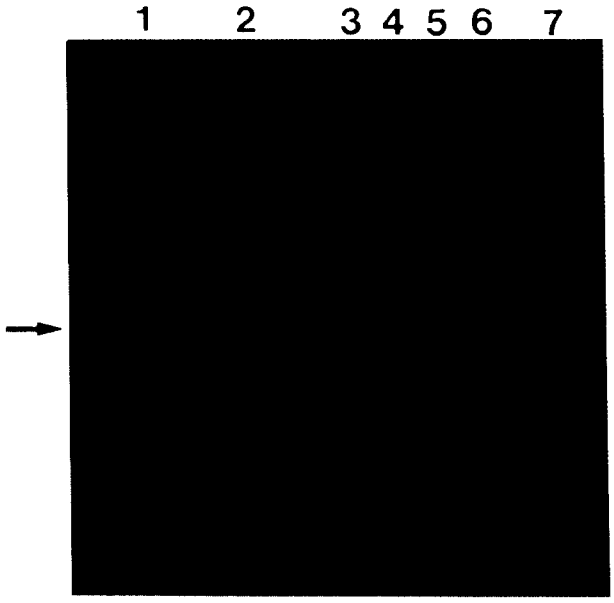

Fig. 2. Hydrolysates of *Coriolus versicolor* and *Ganoderma lucidium* were electrophoresed. The main bands indicated were cut and analyzed. Lane 1: *Coriolus versicolor*, Lane 2: *Ganoderma lucidium*, Lane 3: Glucose-AGA, Lane 4: N-acetylchitobiose, Lane 5: N-acetylchitotriose-AGA Lane 6: N-acetylchitotetraose.

accurate analysis. Chromatography of neutral and acidic sugars by HPLC on two kinds of reverse phase columns (C_{18} and phenyl packings) was tried at the same condition (data not shown). Most of sugar-AGA conjuagates are well separated, but Man-AGA and Glu-AGA are overlapped each other in using C_{18} column (Kim, 1994). Phenyl column gave a moderate separation between Man-AGA and Glu-AGA, but Gal-AGA and Man-AGA were overlapped. When two kinds of reverse phase columns (C_{18}→phenyl) were connected in series, the separation was fairly good. A typical chromatogram for the separation of monosaccharides conjugated with 7-AGA using the reverse phase columns are shown in Fig. 1. The mobile phase was modified for the selection of experimental parameters. For instance, pH was adjusted (4 to 4.5) and organic modifier (butanol and acetonitrile) was added for the selectvity.

Table I. Compositional sugar analysis of three kinds of antituomr polysaccharides from various basidiomycetes (area %)

Sample \ Sugar	Galactose	Glucose	Mannose	Arabinose	Xylose	Fucose
Coriolus versicolor		>97[1)]				
		>98[2)]				
Ganoderma lucidium	13.3	>80.6		2.2	3.8	
	11.3	75.1	5.7	7.8		
Phellinus linteus	7.0	61.8	12.3	8.2	2.8	2.9
	18.6	46.1	15.2	12.8	6.4	
Cordyceps militaris	19.1	78.6		2.2		
	6.3	92.6				
Lentinus eodes		96.7		2.7		
		99.4				

[1)] Analyzed by HPLC
[2)] Analyzed by GC

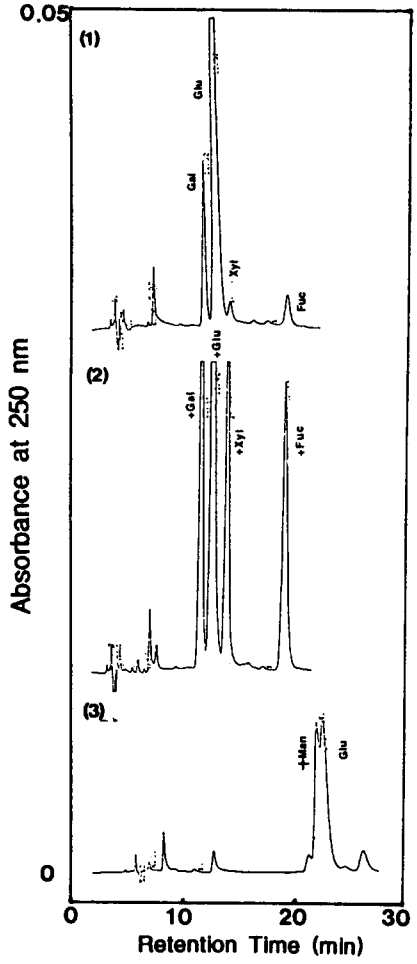

Fig. 3. HPLC analysis of hydrolysate-AGA conjugates from *Ganoderma lucidum*. (1) It was run using C_{18} column. (2) The peaks were identified with standard mixtures. (3) Mannose-AGA conjugate was added to the hydrolysate-AGA conjugate and separated using C_{19} and phenyl column in series. An increase of new peak was due to the additon of Man-AGA.

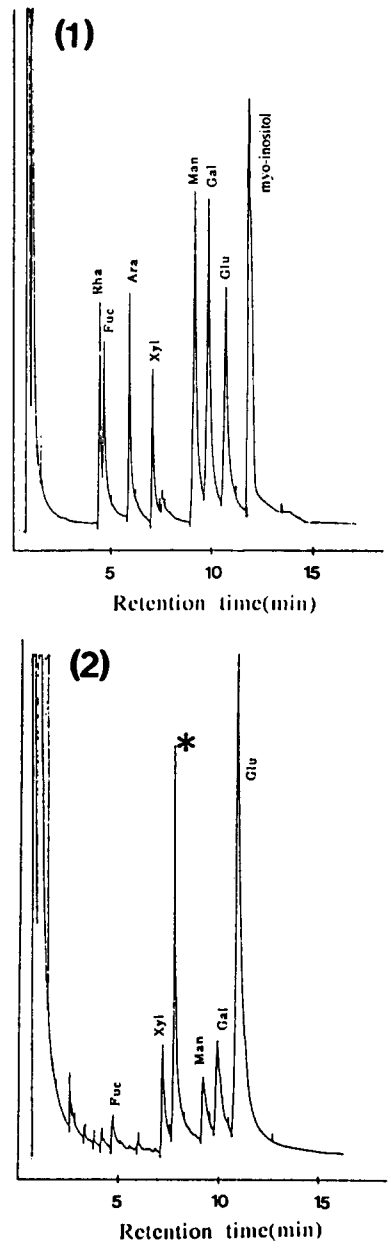

Fig. 4. GC analysis of aldiotol acetate derivatives of hydrolysate from *Ganoderma lucidum*. (1) The standards were separated under the conditions described in Methods. (2) The derivatives of hydrolysate were separated and each peak was identified with standards, respectively. A small amount of mannose were included in the hydrolysate. An unidentified peak (*) was found in the chromatogram.

The increase of pH gave a rather poor resolution. When a very small amount of butanol (0.1%) was added to the mobile phase, it could reduce retention time but the elution order was not changed. When such a technique was first used with a pyridylamino sugar derivatives for the purpose of sugar analysis, reverse phase column was used (Takemoto, 1985). Similar problems were observed in the resolution among sugars.

Five kinds of polysaccharides from *Basidiomycetes* were hydrolyzed and sugar-AGA conjugates were electrophoresed to remove excess fluorescent amine (Fig. 2). The main band which were thought to be a mixture of monosaccharides were cut and transferred to the nylone membrane. Alternatively, excess AGA and salts were removed by gel permeation chromatography on a Bio-Gel P2 column. The isocratic analysis of hydrolysate was initially done using C_{18} column. One of the typical example for the analysis of *Ganoderma lucidum* was shown in Fig. 3. The major peaks were identified as glucose and galactose and minor ones were xylose and fucose with coinjection of authentic sugar-conjugates. The serial elution using C_{18} to phenyl column was carried out to trace any mannose in the hydrolysate (Fig. 3-3). Although the isocratic elution

using phenyl column gave a moderate separation between Glu-AGA and Man-AGA, the small peak of Man-AGA were found to be overlapped with that of Glu-AGA. When chiral amine and boronic acid were added to the eluant very recently, base-line separation between two sugar conjugates could be obtained (Kim, 1994). This was not tried this time, but it should be optimized in the future. The separation of alditol acetate derivatives of hydrolysate of *Ganoderma lucidum* by gas chromatography was shown together in Fig. 4. Mannose, galactose and glucose derivatives were clearly separated each other. In comparison with using HPLC the resolution of alditol acetate derivatives was better. Actually, some amount of mannose were found to be contained in the hydrolysate of *Ganoderma lucidum* and *Phellintus linteus* (Fig. 4). The results from both analyses were shown in Table I. Fluorescence or UV intensities of pyridylaminated sugars were almost equal, indicating that the peak area ratios observed represented molar ratios of the sugars (Hase, 1993). The area differences of both techniques could result from the incomplete derivatization and response factors.

The result is consistent that chemical structure of polysaccharide from *Coriolus versicolor* and *Ganoderma lucidium* are mainly composed of β-1,3 or 1,6-D-glucose (Toshio, 1990). Interestingly, the hydrolysate from *Phellinus linteus* are composed of galactose, arabinose, xylose, fucose in addition to glucose. About fifty picomoles of the sugar-AGA conjugate could be quantified in the HPLC. This technique was applied to prove enzyme specificity against oligosaccharides. Their reducing terminals were reductively aminated and treated with β-galatosidases from green onion, it was found that a specific β1-4 linkage could be cleaved (Kim, 1993). This technique could provide an useful information on the structure of carbohydrate moiety of glycoprotein as well as polysaccharide.

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