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Title	<Original>Preparation and Characterization of the Antibody against the Aldobiouronic acid Unit of Xylan
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Citation	Wood research : bulletin of the Wood Research Institute Kyoto University (1990), 77: 8-17
Issue Date	1990-12-28
URL	http://hdl.handle.net/2433/53274
Right	
Type	Departmental Bulletin Paper
Textversion	publisher

Kyoto University

Original

Preparation and Characterization of the Antibody against the Aldobiouronic acid Unit of Xylan

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(Received August 31, 1990)

Abstract——The aldobiouronic acid, 2-O-(4-O-methyl-α-D-glucuronic acid)-D-xylose, which was obtained by partial acid hydrolysis of xylan was coupled to methylated bovine serum albumin (BSA). The resulting sugar-protein conjugate was used as an antigen. Antibodies were raised by immunizing a rabbit with the antigen. The specificities for the antiserum obtained were examined by immunodiffusion analysis and enzyme-linked immunosorbent assay (ELISA). These results indicate that the antiserum contains antibodies against both BSA and the antigen. After removal of anti-BSA antibody by affinity chromatography, the specificity of the purified antiserum to wood cell wall polysaccharides and their hydrolysates was investigated by ELISA. The antiserum preferentially reacted with the conjugates containing xylan and aldobiouronic acid, which suggests that the antiserum recognizes the aldobiouronic acid unit of xylan.

Key words: aldobiouronic acid, sugar-protein conjugate, antibody, immunodiffusion analysis, enzyme-linked immunosorbent assay

1. Introduction

Wood components have been analyzed by chemical methods and the chemical structure have been reported after their isolation from wood materials. The loca lization of these components within a cell wall is studied a little by such chemical analyses. However, it is difficult to visualize three-dimensional arrangements of the cell wall polymers by the hitherto used chemical methods. A complete understanding of the cell wall structures at a molecular level requires more information of the polymer to polymer interactions within the cell wall. Extensive investigations have been carried out on the interactions among wood components, especially on the interaction between lignin and carbohydrate, and the presence of chemical linkages of carbohydrate to lignin has been demonstrated[1~3]. A series of our previous papers also reported the occurrence of the lignin-carbohydrate complexes containing chemical linkages between lignin and carbohydrate and the properties of its complexes[4~9]. On the other hand, various histochemical techniques have been used in order to investigate the localization of polysaccharide components

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within plant cell walls[10~13], but are not always specific for the polysaccharides. Recently, the antibodies specific to various oligosaccharides and polysaccharides have been receiving much attention to investigate the localization of a variety of polysaccharides within the plant cell wall[14,15].

This paper reports preparation of the antiserum against aldobiouronic acid unit of xylan, 2-O-(4-O-methyl-α-D-glucuronic acid)-D-xylose, which has never been tried and the evaluation of its immunochemical specificities.

2. Materials and Methods

2.1 Materials

2.1.1 Polysaccharides

Glucomannan was isolated from alkaline extracts of pine wood and purified by chromatography on a DEAE-Sephadex A-50 (carbonate form) column. It contained residues of mannose, glucose, galactose in a molar ratio of 25:9.7:1.0. Arabinogalactan was isolated from larch wood (branches) and purified by chromatography in a similar manner used for purification of glucomannan. The molar ratio of arabinose, galactose, and rhamnose contained in arabinogalactan was found to be 11:50:1.0. Xyloglucan was prepared from the cambial tissues of aspen wood (a 15-year-old trunk) according to the method of SIMSON and TIMELL[16]. It consisted of fucose (5.6%), arabinose (2.9%), xylose (32.0%), mannose (trace), galactose (12.1%) and glucose (47.5%). Xylan was prepared from beech wood[9]. It contained 4-O-methylglucuronic acid and xylose in a molar ratio of 1:11.6. Pectin from citrus (peel) used is from commercial source.

2.1.2 Oligosaccharides

Xylo-oligosaccharides were prepared from beech xylan by its partial hydrolysis in 0.5 M trifluoroacetic acid for 3 hr at 100°C. The hydrolysate was applied to a DEAE-Sephadex G-50 column, and neutral and acidic sugars were eluted with water and 10% acetic acid, respectively. The neutral and acidic fractions were separately applied to activated charcoal powder columns. After the columns were washed thoroughly with water to remove the unadsorbed monosaccharides, the adsorbed oligosaccharides were eluted with 15% ethanol. The oligosaccharides obtained were analyzed by thin layer chromatography, paper chromatography and gas chromatography. The experimental conditions were the same as those previously described[4,9]. The analytical results showed that the oligosaccharides eluted from the neutral and acidic fractions are xylobiose and aldobiouronic acid, respectively. Similarly, galactobiose was obtained from the hydrolysate of arabinogalactan. Mannobiose was also obtained from the hydrolysate of glucomannan. Neutral xylo-oligosaccharides with more than three degree of polymerization were eluted

from the same charcoal column with 30% ethanol. Cellobiose used was from commercial source.

2.2 Preparation of sugar-protein conjugate

Aldobiouronic acid was coupled to methylated BSA by use of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimidehydrochloride (EDC) according to the method of LÖNNGREN et al[17], which was modified as follows. Aldobiouronic acid (60 mg) was dissolved in 1 ml of water. To this solution, EDC (50 mg) in water (1 ml) was added dropwise over a period of 10 min. The mixture was stirred for 10 min. Then, methylated BSA (30 mg) in 1 ml of water was added dropwise to the mixture, pH of which was maintained at 4.75 with 0.1 N hydrochloric acid until the addition of the methylated BSA solution was completed. The reaction mixture was kept with stirring for 6 hr at room temperature, and then dialyzed against water. The dialysate was lyophilized to collect the aldobiouronic acid-methylated BSA conjugate as dried matters. The conjugate was purified by gel filtration through a Sephadex G-25 column with water as an eluant. Fractions (1.2 ml each) were collected, and the aldobiouronic acid and the protein contained in each fraction were monitored by measurement of the absorbances at 490 nm and 280 nm, respectively, the former component was subjected to the coloring reaction with phenol-sulfuric acid. The fractions containing the conjugate were combined and used as an antigen. Contents of sugar and protein in the conjugate were determined by use of the same spectrophotometric method. Alternatively, xylose, xylobiose, xylo-oligosaccharide, cellobiose, galactobiose, mannobiose and xylan were used for preparation of the corresponding sugar-BSA conjugates by the method of GRAY[18]. The reactions with both mono- and oligosaccharides, and the polysaccharide were carried out at 25°C for 10 days and for 20 days, respectively.

2.3 Preparation of antiserum

The antiserum was raised in a 1-year-old rabbit. The rabbit was immunized with the aldobiouronic acid-methylated BSA conjugate which had been emulsified in Freund's complete adjuvant diluted with 0.05 M phosphate-buffered saline (PBS) (pH 7.3) as follows. One ml of the emulsion containing 3 mg of the antigen was injected into the back of the rabbit at the intervals of two or three weeks over a period of four months. After nine injections, blood which was drawn from the rabbit ear was centrifuged to remove the clot. The antiserum obtained was stored at −84°C. The preimmune serum was obtained from the rabbit before the first injection for the control experiments.

2.4 Purification of antiserum

A portion of the antiserum stored was subjected to the BSA affinity chroma-

tography as follows. One ml of the antiserum was applied to a column (1×5 cm) of BSA-Affi gel 10 (Bio Rad) which had been prepared by use of a coupling buffer (pH 7.8) containing 0.1 M 3-(N-morpholine) propanesulfonic acid and 0.08 M $CaCl_2$ according to FROST et al[19]. Protein content in each fraction (0.5 ml) was monitored by measurement of the absorbance at 280 nm. The desired antibody was eluted with 10 ml of PBS and collected. The adsorbed proteins mainly consisting of BSA-specific antibody were eluted with 5 M $MgCl_2$ and discarded. The first fraction with the purified antibody was used for ELISA, as immunoassay samples.

2.5 Immunodiffusion assay

Double diffusion was carried out by use of 1% agar gel containing 3% polyethylene glycol 4000 in PBS. The original antiserum (2 μl) was placed in the center well in an agar plate. The sugar and BSA samples (2 μl, 2~3 mg/ml) were placed in the peripheral wells, respectively. The plate was kept for 24 hr at room temperature.

2.6 Enzyme-linked immunosorbent assay (ELISA)

Each ELISA sample (1 μl, 2~3 mg/ml) was dotted on nitrocellulose paper, which was soaked in the solution of 0.05% Tween 20 in 0.02 M Tris-buffered saline (TTBS) (pH 7.5) with 5% milk for 1 hr at room temperature. The soaked paper was taken out and then incubated in the antiserum for 2 hr at room temperature. After washing with the solution of TTBS with 5% milk, the paper was further incubated in the second antibody (alkaline phosphatase conjugated goat anti-rabbit IgG) obtained commercially for 1 hr at room temperature. After washing the incubated paper with TTBS and barbital buffer (pH 9.6), the paper was subjected to the color reaction. The color was developed in barbital solution containing 0.01% nitro blue tetrazolium, 0.005% 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and 0.04 M $MgCl_2$.

3. Results and Discussion

3.1 Preparation of antigen

Xylan has a backbone of β (1→4) linked xylose residues which are partially substituted by 4-O-methylglucuronic acid residues at C-2 of the xylose residues with α-linkage. Therefore, the aldobiouronic acid with the 4-O-methylglucuronic acid is one of the most characteristic units contained in xylan.

In order to obtain the aldobiouronic acid-specific antibody, aldobiouronic acid was isolated from the hydrolysate of beech xylan. The aldobiouronic acid isolated was identified as a 2-O-(4-O-methyl-α-D-glucuronic acid)-D-xylose mainly by methylation analysis (Fig. 1). The purified aldobiouronic acid was coupled to methylated

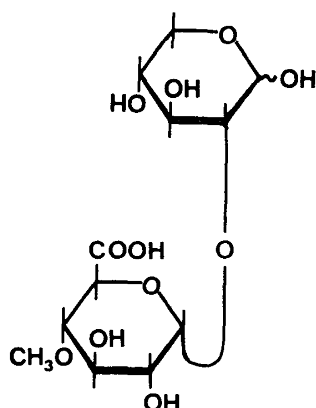

Fig. 1. The structure of an aldobiouronic acid obtained by partial acid hydrolysis of xylan.

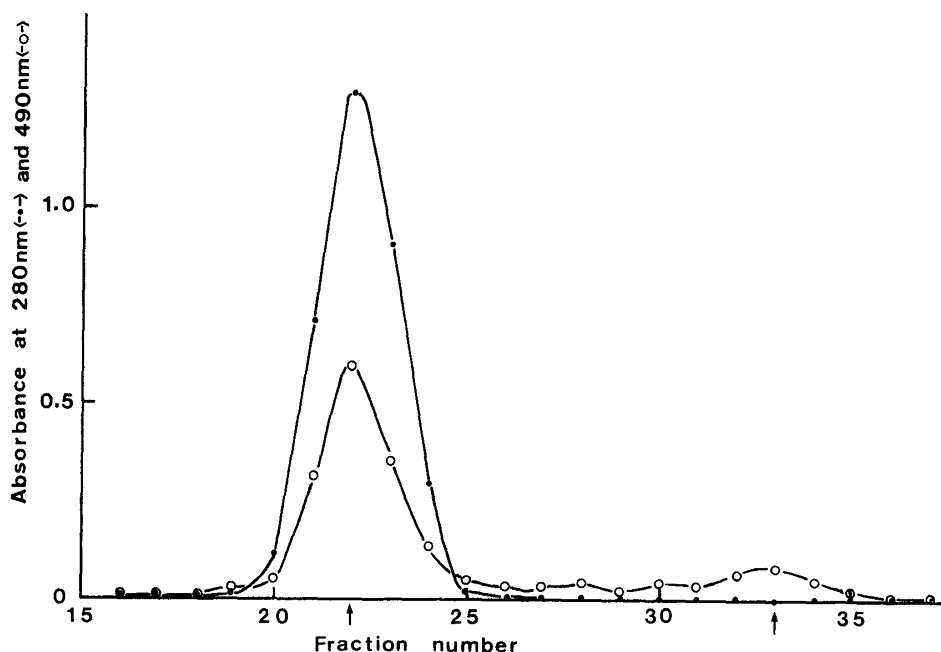

Fig. 2. Gel filtration of the aldobiouronic acid-methylated BSA conjugate on Sephadex G-25 column. The arrows represent the void volume and the elution volume of the aldobiouronic acid, respectively.

BSA. After dialysis, ther esulting conjugate was purified by gel filtration through the column of Sephadex G-25 (Fig. 2). The elution profile for carbohydrate in Fig. 2 indicates that aldobiouronic acid is bound to methylated BSA. The desirable sugar -protein conjugate was eluted at the void volume of the column. The cojugate was collected and lyophilized. The conjugate thus obtained was used as an antigen. The sugar content of the antigen was 7.5% and its protein content was 94%. This value of the sugar content was about half that of the antigen used by NORTHCOTE et al.[15] and LONNGREN et al.[17].

3.2 Characterization of antiserum

3.2.1 Immunodiffusion assay

The original antiserum obtained from the rabbit was analyzed by gel double diffusion. The antiserum gave respective intense precipitin lines both with BSA and antigen, but an obvious spur between their precipitin lines was not detected (Fig. 3). This indicates that the antiserum contains antibodies against BSA and/or aldobiouronic acid.

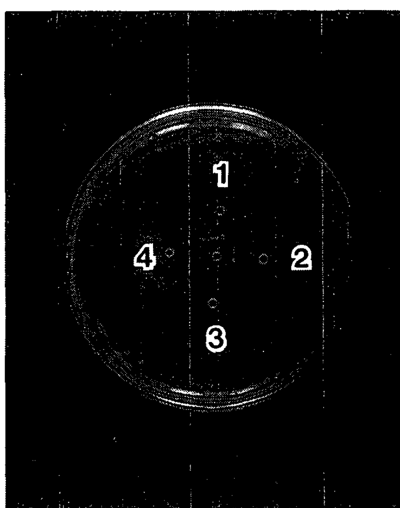

Fig. 3. Immunodiffusion analysis of antiserum in agar gel. Central well: the original antiserum, Peripheral wells: 1, BSA; 2, antigen; 3, aldobiouronic acid; 4, saline.

3.2.2 Enzyme-linked immunosorbent assay (ELISA)

MOORE et al.[20] have tested the specificity of the antiserum by placing polysaccharides on nitrocellulose paper. We also tested the specificity of the antiserum against the polysaccharides by ELISA on nitrocellulose paper (Table 1 and Fig. 4). The experiment was performed at different concentrations of the antiserum. The original antiserum reacted with BSA, antigen and xylan, and its reactivity decreased with increasing dilution of the antiserum. The antiserum also reacted faintly with pectin only at the high concentration (25 fold dilution). Since the preimmunization serum did not react with all of the sample examined (Fig. 4C), it is clear that the antiserum contains at least another antibody besides anti-BSA antibody. KAKU et al.[21] have succeeded in removing the anti-BSA antibody by use of affinity chromatography. Therefore, in order to remove the anti-BSA antibody, the original antiserum was applied to the BSA-Affi gel 10 column and divided into two fractions (Fig. 5). The serum protein contained in the fraction corresponding to the first peak (Fraction number 1～20) was demonstrated to react with only the

Table 1. Enzyme-linked immunosorbent assay for the specificity of the original antiserum against cell wall polysaccharides

Polysaccharides	Original antiserum			
Dilution	1:25	1:50	1:100	1:200
BSA	+	+	+	+
antigen (BSA-aldobiouronic acid)	+	+	+	+
xylan	+	+	−	−
xyloglucan	−	−	−	−
arabinogalactan	−	−	−	−
pectin	(+)	−	−	−
glucomannan	−	−	−	−

A + sign indicates a positive reaction, (+) sign faintly, − sign no reaction.

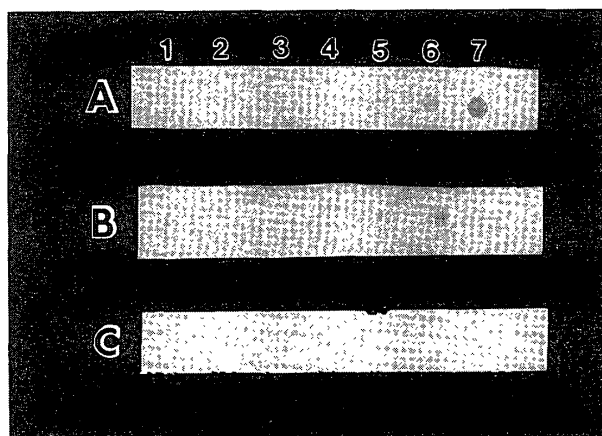

Fig. 4. Enzyme-linked immunosorbent assay for the specificity of the antiserum against cell wall polysaccharides. A: the original antiserum (1:50 dilution), B: the purified antiserum (1:25 dilution), C: the preimmunization serum (1:25 dilution), 1: glucomannan, 2: pectin, 3: arabinogalactan, 4: xyloglucan, 5: xylan, 6: antigen, 7: BSA.

antigen and xylan (Fig. 4B), whereas the original antiserum reacted with all samples including BSA (Fig. 4A). The samples corresponding to the second peak was found to contain anti-BSA antibody (data not shown). The results obtained by the ELISA indicate that the first peak is free of anti-BSA antibody.

The purified antiserum was used for the further examination of its specificity. Since the dotted mono- and oligosaccharides is probably diffused out from the paper under the experimental condition used for the ELISA, all of the sugars were converted to the corresponding sugar-BSA conjugates. Thus, the specificity of the purified antiserum against the series of xylo-sugars with a different degree of

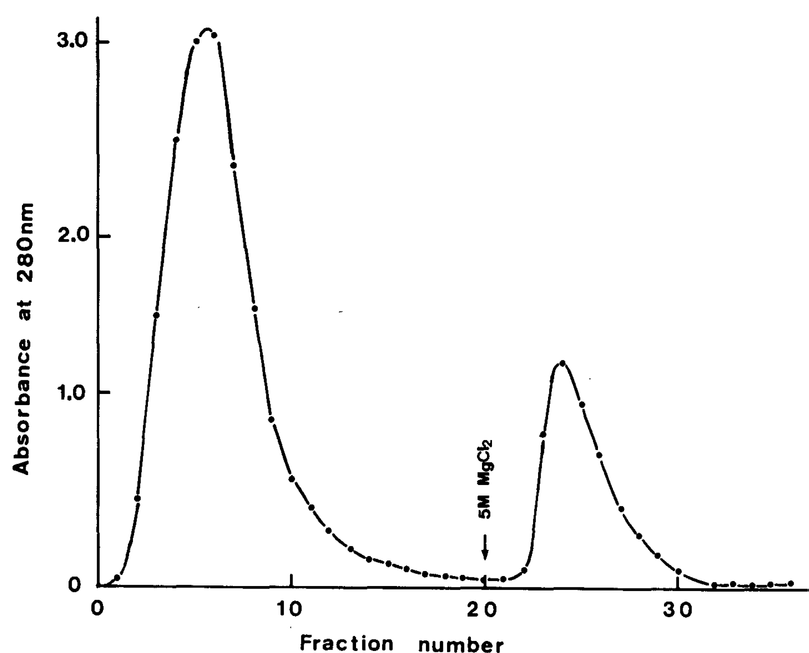

Fig. 5. Elution profile of the antiserum on a BSA-Affi gel 10 affinity column.

polymerization and various oligosaccharides was tested (Table 2 and Fig. 6). Both Table 2 and Fig. 6 show that the purified antiserum reacts with both the antigen and xylan, but does not react with any of xylo-sugars without 4 O-methylglucuronic acid and any of the other oligosaccharides. These results suggest that the antiserum recognize the aldobiouronic acid unit of xylan. In conclusion, since our aldobio-

Table 2. Enzyme-linked immunosorbent assay for the specificity of the purified antiserum against the series of xylo-sugars with a different degree of polymerization and various oligosaccharides

BSA-sugar conjugates	Purified antiserum			
Dilution	1:25	1:50	1:100	1:200
BSA	−	−	−	−
antigen (BSA-aldobiouronic acid)	+	(+)	−	−
BSA-xylan	+	−	−	−
BSA-xylose	−	−	−	−
BSA-xylobiose	−	−	−	−
BSA-xylo-oligosaccharide	−	−	−	−
BSA-xylobiose	−	−	−	−
BSA-cellobiose	−	−	−	−
BSA-galactobiose	−	−	−	−
BSA-mannobiose	−	−	−	−

Signs are as in Table 1.

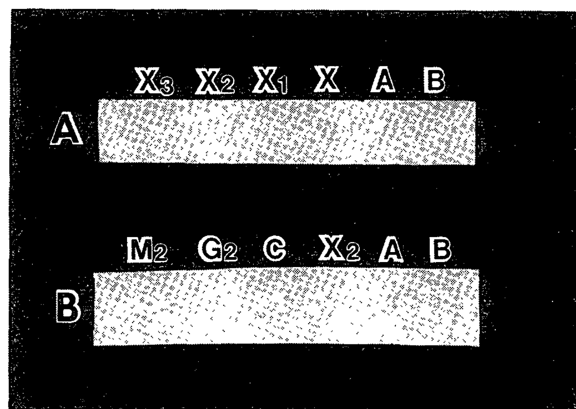

Fig. 6. Enzyme-linked immunosorbent assay for the specificity of the purified antiserum against the series of xylo-sugars with a different degree of polymerization (A) and various oligosaccharides (B). A: antigen, B: BSA, C: cellobiose, X: xylan, X_1: xylose, X_2: xylobiose, X_3: xylo-oligosaccharides with more than three degree of polymerization, G_2: galactobiose, M_2: mannobiose. (1:25 dilution)

uronic acid-specific antibody does not react with pectin at the lower concentration, it might be used as a probe to examine the localization of xylan selectively under the specified experimental conditions.

Acknowledgments

The authors would like to thank Dr. Mikio SHIMADA, Lignin Chemistry, Wood Research Institute, Kyoto University for critical reading of this manuscript and also Mr. Masahiko OHMINAMI, Rakken, Uji, Kyoto for proofreading of this manuscript. The authors thank Mr. Tomoaki OGAWA, Wood Biology, Wood Research Institute for his assistance in the preparation of the antiserum.

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