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PREPARATION AND CHARACTERIZATION OF ANTIBODIES AGAINST 6-*O*-α-D-XYLOPYRANOSYL-β-D-GLUCOPYRANOSE (β-ISOPRIMEVEROSE), THE DISACCHARIDE UNIT OF XYLOGLUCAN IN PLANT CELL-WALLS

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ABSTRACT

The *p*-aminophenyl β-glycoside of 6-*O*-α-D-xylopyranosyl-D-glucopyranose (isoprimeverose), the disaccharide unit of plant xyloglucan, was coupled to bovine serum albumin, and the resulting glycoconjugate was used as an immunogen for the immunization of a rabbit. The immunochemical specificities of the rabbit antiserum raised against the glycoconjugate were characterized by immunodiffusion, quantitative precipitation, and hapten inhibition. After removal of anti-bovine serum albumin antibodies, the antiserum exhibited a specificity for the introduced disaccharide unit of the artificial antigen. The antibody-combining site was also shown to recognize the aglycon portion of the introduced hapten. The antiserum interacted with some xyloglucans, such as those from tamarind seed and the cell wall of pea stem. β-Isoprimeverose and α-D-xylopyranosides were good inhibitors of the xyloglucan–antibody precipitation system, indicating that the antibodies recognize the β-isoprimeverose unit of the xyloglucan.

INTRODUCTION

Xyloglucans are present in the primary cell-walls of most dicots and also some monocots[1]. It has been established that their basic structure consists of a β-(1→4)-linked D-glucopyranosyl backbone, with side chains of α-D-xylopyranosyl groups attached at the O-6 atoms of many of the D-glucopyranosyl residues. Some of the D-xylopyranosyl side-chains are terminated with single β-D-galactopyranosyl or 2-*O*-α-L-fucopyranosyl-β-D-galactopyranosyl groups attached at the O-2 positions. It has been shown that xyloglucans firmly associate with cellulose microfibrils to form an essential framework for the plant cell-wall[1,2]. In addition, their various regulatory

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functions have recently been reported[3,4]. To illuminate the role of xyloglucan, it is imperative to elucidate its precise structural features and its arrangement in the plant cell-wall. In connection with this, Moore *et al.*[5] reported the histological localization of xyloglucan and pectic rhamnogalacturonan I in the cell walls of suspension-cultured sycamore cells by the immunogold staining technique, using polyclonal antibodies to these polysaccharides.

In the course of studies on the structure and biosynthesis of plant cell-wall polysaccharides, we prepared anti-α-L-arabinofuranose antibodies by immunization of rabbits with *p*-azophenyl α-L-arabinofuranoside–bovine serum albumin conjugate. The purified antibodies were successfully applied to the histochemical detection of α-L-arabinofuranose-containing polysaccharides in the cell walls of various plants[6,7].

In a previous study[8], we prepared *p*-nitrophenyl 6-*O*-α-D-xylopyranosyl-β-D-glucopyranoside in order to provide a hapten for the preparation of specific antibodies that recognize the characteristic building-unit of the xyloglucan. We report here the preparation and characterization of antibodies raised against *p*-azophenyl 6-*O*-α-D-xylopyranosyl-β-D-glucopyranoside–bovine serum albumin conjugate.

RESULTS

Antibodies specific for β-isoprimeverose. — *p*-Nitrophenyl 6-*O*-α-D-xylopyranosyl-β-D-glucopyranoside [α-D-Xyl*p*-(1→6)-β-D-Glc*p*-*O*-*p*NP], synthesized in our previous study[8], was reduced to the *p*-aminophenyl derivative, and this was coupled to bovine serum albumin (BSA) as before[6]. The resulting *p*-azophenyl 6-*O*-α-D-xylopyranosyl-β-D-glucopyranoside–BSA conjugate (Xyl-Glc–BSA) was used to immunize a rabbit, and the corresponding antiserum was obtained. Fig. 1 shows the result of immunodiffusion of the antiserum; when diffused against its homologous antigen and BSA, the antiserum gave an intense precipitation band

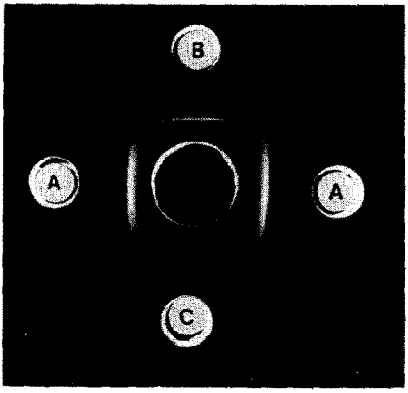

Fig. 1. Immunodiffusion of anti-Xyl-Glc-BSA serum. Center well: original antiserum. Peripheral wells: A, Xyl-Glc–BSA (0.9 mg/mL); B, BSA (1 mg/mL); C, borate-buffered saline (BBS).

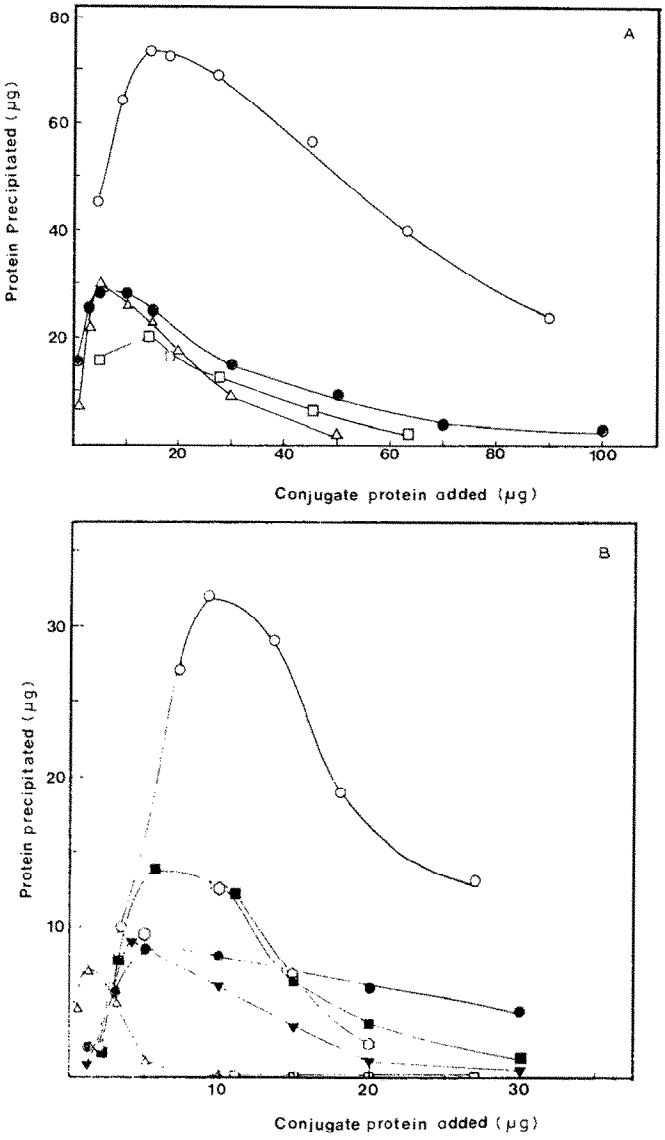

Fig. 2. Quantitative precipitation curves of (A) original anti-Xyl-Glc–BSA serum, and (B) antiserum free from anti-BSA antibodies with various glycoconjugates. ○-○, Xyl-Glc–BSA; △-△, Xyl–BSA; ●-●, Glc–BSA; □-□, BSA; ■-■, BSA conjugate of heptasaccharide fragment of xyloglucan; ▼-▼, BSA conjugate of octasaccharide fragment; ⬡-⬡, BSA conjugate of nonasaccharide fragment.

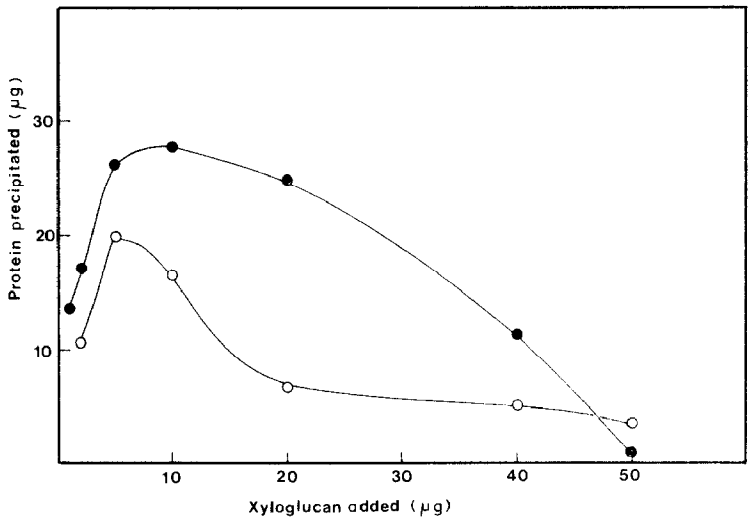

Fig. 3. Quantitative precipitation curves of original anti-Xyl-Glc–BSA serum with xyloglucans. ●-●, Tamarind xyloglucan; ○-○, pea xyloglucan.

that spurred over the band given by BSA, indicating that the antiserum contained antibodies specific for the disaccharide unit, as well antibodies for BSA (anti-BSA antibodies). The antiserum also gave a faint precipitation band with the xyloglucan of tamarind seed (photograph not shown), indicating that the antiserum raised against Xyl-Glc–BSA is capable of interacting with the native xyloglucan. After removal of anti-BSA antibodies by passage of the serum through a column of BSA–Sepharose immunoadsorbent, the eluate (partially purified antiserum) gave a precipitation band with the homologous antigen but not with *p*-azophenyl β-D-glucopyranoside–BSA conjugate (Glc–BSA), *p*-azophenyl α-D-xylopyranoside–BSA conjugate (Xyl–BSA), or BSA (photograph not shown).

The antiserum was examined, with six glycoconjugates containing different haptens and two plant xyloglucans, by quantitative precipitation; the results are shown in Figs. 2-A, 2-B, and 3. In these precipitation reactions, when 20 μL of the original antiserum was used (see Fig. 2-A), 74 μg of protein was precipitated by 14 μg of Xyl-Glc–BSA, whereas 20 μg of protein was precipitated by 15 μg of BSA. Under the same conditions, Xyl–BSA (which possesses the nonreducing, terminal group of isoprimeverose) precipitated 30 μg of protein at the maximum point of precipitation, and Glc–BSA (which contains the reducing-terminal site of the disaccharide) precipitated 28 μg of protein at the maximum. As shown in Fig. 2-B, when 50 μL of partially purified antiserum was used, Xyl-Glc–BSA (9 μg) precipitated 35 μg of protein at the maximum point of precipitation, whereas Xyl–BSA (1 μg) and Glc–BSA (5 μg) respectively precipitated 8 μg and 9 μg of protein, at their maximum points. Under the same conditions, no precipitation was observed with BSA only. These results confirmed those obtained by immuno-

diffusion, namely, that the antiserum contained two antibody populations, anti-BSA antibodies and antibodies specific for the introduced 4-(β-isoprimeverosyl-oxy)phenylazo groups (anti-Xyl-Glc antibodies), and also suggested that both of the component sugar residues of isoprimeverose contribute to the immunochemical specificity of anti-Xyl-Glc antibodies. Fig. 2-B also shows the quantitative precipitation curves of the partially purified antiserum with the BSA conjugates of the oligosaccharide fragments prepared from tamarind xyloglucan. The result demonstrated that those conjugates precipitated ~9–14 μg of protein, corresponding to about one-third of the amount precipitated by the homologous antigen.

Fig. 3 shows the quantitative precipitation curves of the original antiserum with the galactoxyloglucan of tamarind seed (*Tamarindus indica* L.) and the fucogalactoxyloglucan isolated from pea (*Pisum sativum* L. var. Alaska) stem. Twenty eight micrograms and 20 μg of antibody protein were precipitated by 10 μg of tamarind xyloglucan and 5 μg of pea xyloglucan, respectively, with 50 μL of the antiserum. These results confirmed that antibodies raised to Xyl-Glc–BSA are able to bind to β-isoprimeverose units in the xyloglucan.

Hapten inhibition. — The immunochemical specificity of the anti-Xyl-Glc antibodies was examined by the quantitative hapten-inhibition technique using 50 μL of the partially purified antiserum in a reaction total-volume of 150 μL. Fig. 4 shows the inhibitory capacities of several derivatives in the antiserum and Xyl-Glc–BSA precipitation reaction. Of these haptens tested, α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-*p*NP was the most potent inhibitor, 2.4 nmol giving 50% inhibition. The importance of the α configuration (of the anomeric linkage of the D-xylopyranosyl group in the

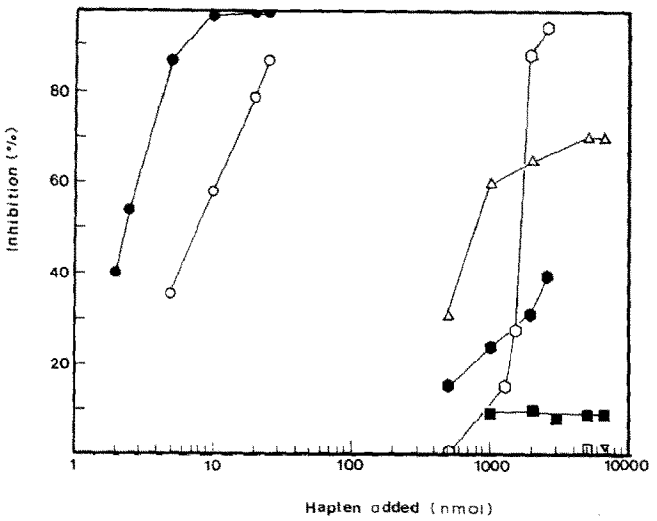

Fig. 4. Inhibition of precipitation between Xyl-Glc–BSA and the partially purified antiserum with various sugar derivatives. ●-●, α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-*p*NP; ○-○, α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-Me; ◐-◐, β-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-*p*NP; ◯-◯, β-D-Glc*p*-O-*p*-NP; ■-■, β-D-Glc*p*-O-Me; △-△, α-D-Xyl*p*-O-Me; □-□, α-D-Glc*p*-O-Me; and ▽-▽, β-D-Xyl*p*-O-Me.

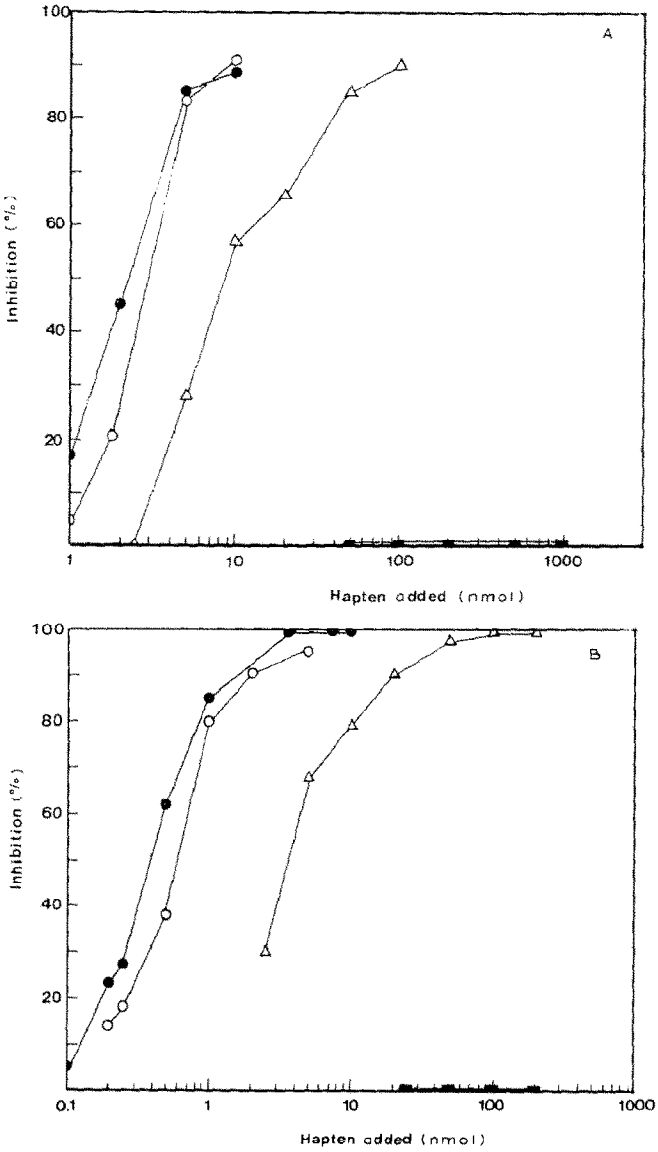

Fig. 5. Inhibition of (A) the original antiserum–tamarind xyloglucan, and (B) the original antiserum–pea xyloglucan precipitation reaction with some sugar derivatives. ●-●, α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-*p*NP; ○-○, α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-Me; ■-■, β-D-Glc*p*-O-Me; and △-△, α-D-Xyl*p*-O-Me.

disaccharide) for binding to antibodies was indicated on comparison of the inhibitory potency of methyl α-D-xylopyranoside (α-D-Xyl*p*-O-Me, 50% at 800 nmol) with that of methyl β-D-xylopyranoside (β-D-Xyl*p*-O-Me, no inhibitory activity at 7000 nmol). The contribution of the aglycon moiety of the introduced hapten to interaction with the antibodies was indicated by comparison of the in-

inhibitory potency of α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-*p*NP (50% at 2.4 nmol) with that of methyl 6-*O*-α-D-xylopyranosyl-β-D-glucopyranoside [α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-Me, 50% at 8 nmol]. The fact that, the inhibitory activity of *p*-nitrophenyl β-D-glucopyranoside (β-D-Glc*p*-O-*p*NP, 86% at 2000 nmol) is stronger than that of methyl β-D-glucopyranoside (β-D-Glc*p*-O-Me, 10% at 2000 nmol) also supports the conclusion that the antibodies recognize the aglycon moiety of the hapten.

The recognition of β-isoprimeverose groups in the xyloglucan by the anti-Xyl-Glc antibodies was examined by quantitative hapten inhibition in the antiserum-xyloglucan system. Fig. 5-A shows the inhibitory potency of the several sugar derivatives to the precipitation reaction between the antiserum and tamarind xyloglucan. α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-*p*NP and α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-Me showed similar inhibitory potency (50% at 2.3 and 2.8 nmol, respectively). The strong inhibitory potency of α-D-Xyl*p*-O-Me (50% at 8 nmol) confirmed the decision that the nonreducing-terminal residues play an important role in the immunochemical reaction between the anti-Xyl-Glc antibodies and xyloglucan. Similar results of hapten inhibition were obtained when the pea xyloglucan was used as an antigen (see Fig. 5-B).

DISCUSSION

Well characterized antibodies raised against the oligosaccharide fragments of the polysaccharides in plant cell-walls would constitute useful probes for investigations on the histological localization and function of these polysaccharides. In the present study, we prepared antibodies specific to 6-*O*-α-D-xylopyranosyl-β-D-glucopyranose (β-isoprimeverose), the disaccharide unit of the xyloglucan of plant cell-wall, by immunizing a rabbit with Xyl-Glc–BSA. We have characterized the immunochemical specificities of the resulting antibodies. The immunogen, Xyl-Glc–BSA, was prepared by diazo-coupling of *p*-aminophenyl 6-*O*-α-D-xylopyranosyl-β-D-glucopyranoside to BSA. About 10 disaccharide residues were incorporated per molecule of BSA, corresponding to a coupling efficiency of 17%, assuming that BSA has 57 L-lysyl residues[9].

The result of gel diffusion (see Fig. 1) and quantitative precipitation (see Figs. 2-A and 2-B) showed that the antiserum obtained contained anti-BSA antibodies and anti-Xyl-Glc antibodies. Anti-BSA antibodies were selectively removed from the original antiserum by a BSA-Sepharose immunoadsorbent column.

Quantitative precipitation of the partially purified antiserum, free from anti-BSA antibodies, with several glycoconjugates (see Fig. 2-B) showed that anti-Xyl-Glc antibodies could interact not only with the homologous antigen but also with the BSA-conjugates of oligosaccharide fragments of the xyloglucan, namely, Xyl-BSA and Glc-BSA. From the quantitative hapten-inhibition studies of the precipitin reaction of the partially purified antiserum with Xyl-Glc–BSA, we obtained information on the specificities of the anti-Xyl-Glc antibodies. Almost complete inhibition of the precipitation reaction was obtained with α-D-Xyl*p*-

(1→6)-β-D-Glc*p*-O-*p*NP, indicating that the specificities of the antibodies are directed against the introduced haptenic group. Less inhibitory potency of α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-Me than that of the *p*-nitrophenyl glycoside indicated the contribution of the aglycon moiety of the hapten to the activities of antibodies; this was also indicated on comparing the inhibitory potencies, which are in the order β-D-Glc*p*-O-*p*NP > β-D-Glc*p*-O-Me. The fact that the inhibitory activity of α-D-Xyl*p*-O-Me, corresponding to the nonreducing-terminal residue of isoprimeverose, was stronger than that of β-D-Glc*p*-O-Me, the reducing-terminal site, indicates the major contribution of the nonreducing-terminal sugar in determining the specificity of the antibodies to this artificial antigen.

One of the most important properties of the present antibodies for their practical utilization is their ability to interact with natural plant polysaccharides containing this haptenic group as a part of their building units. As anticipated from the reactivity of the antibodies with the BSA-conjugates of oligosaccharide-fragments of xyloglucan, gel diffusion and quantitative precipitation (see Fig. 3) demonstrated that the antibodies raised against Xyl-Glc–BSA are able to interact with the xyloglucans from tamarind seed and the cell wall of pea stem, whose chemical structures are very similar, containing a backbone of β-(1→4)-linked D-glucopyranosyl residues, most of which are substituted at their O-6 atoms with single D-xylopyranosyl groups, forming isoprimeverose groups. The D-xylopyranosyl groups are in terminal positions, or substituted with β-(1→2)-linked D-galactopyranosyl groups in tamarind xyloglucan[10,11], and have additional L-fucopyranosyl groups α-(1→2)-linked to the D-galactopyranosyl groups in pea xyloglucan[12]. Quantitative hapten-inhibition studies of the precipitation reaction between antiserum and xyloglucans revealed the binding specificities of the antibodies to the xyloglucans. In both cases, *p*-nitrophenyl and methyl α-D-xylopyranosyl-(1→6)-β-D-glucopyranoside gave similar inhibitory potencies. This indicated that the anti-Xyl-Glc antibodies recognize the isoprimeverose units of the xyloglucan molecule. The strong inhibitory activity of α-D-Xyl*p*-O-Me on the precipitation reactions indicates that the nonreducing-terminal α-D-xylopyranosyl groups of the isoprimeverose units of xyloglucan are the dominant binding sites to the antibodies.

The foregoing findings suggested that the antibodies against α-D-Xyl*p*-(1→6)-β-D-Glc*p* will interact with the isoprimeverose units of the xyloglucan in the primary cell-wall both of monocots and dicots. Our preliminary studies by indirect immunofluorescence staining, using FITC-labeled goat anti-rabbit IgG as a secondary antibody showed[12a] that the antibodies bind to the cell-wall tissues of soy-bean cotyledon and coleoptile of avena.

EXPERIMENTAL

Materials. — Syntheses of *p*-nitrophenyl 6-*O*-α-D-xylopyranosyl-β-D-glucopyranoside and 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside were conducted as

described[8]. Methyl 6-*O*-α-D-xylopyranosyl-β-D-glucopyranoside was synthesized by a method similar that used for α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-*p*NP, except that methyl 2,3,4-tri-*O*-benzoyl-β-D-glucopyranoside was used as a nucleophile. Its purity and structure were confirmed by ^{1}H- and ^{13}C-n.m.r. spectroscopy. All other sugar derivatives used in the hapten-inhibition studies were purchased from Wako Pure Chemical Industries, Ltd., Osaka, and Sigma Chemical Company, St. Louis, MO, U.S.A.

Xyloglucan (galactoxyloglucan) of tamarind seed (*Tamarindus indica* L.), and cell-wall xyloglucan (fucogalactoxyloglucan) of pea (*Pisum sativum* L. var. Alaska) stem were gifts from Mr. K. Ohtsu, Dainippon Pharmaceutical Co., Ltd., Osaka, and Dr. R. Hayashi, the Central Research Laboratories of Ajinomoto Co., Inc., Kawasaki, respectively.

Antigens. — α-D-Xyl*p*-(1→6)-β-D-Glc*p*-O-*p*NP was converted into the *p*-aminophenyl glycoside and this was coupled to bovine serum albumin (BSA) by the conventional diazo-coupling method described by Ginsburg *et al.*[13]. After exhaustive dialysis against distilled water, the resulting α-D-Xyl*p*-(1→6)-β-D-Glc*p*-(1→4)-oxyphenylazo–BSA was purified by filtration chromatography on a column of Sephadex G-50 gel, and then lyophilized. The disaccharide–BSA contained 10 mol of disaccharide residues (determined by the phenol–sulfuric method[14]) per mol of BSA.

p-Azophenyl β-D-glucopyranoside– and α-D-xylopyranoside–BSA conjugates were prepared from the corresponding, commercial *p*-nitrophenyl glycosides and BSA by the same method as that used for the disaccharide–BSA conjugate. Glc–BSA and Xyl–BSA contained 25 mol of D-glucose residue and 13 mol of D-xylose residue, respectively, per mol of BSA. BSA conjugates of oligosaccharide fragments of xyloglucan (heptasaccharide–, octasaccharide–, and nonasaccharide–BSA) were prepared by coupling BSA with the oligosaccharides, which were obtained by the cellulase digestion of tamarind xyloglucan, according to the reductive-amination method of Gray[15]. Heptasaccharide–BSA, octasaccharide–BSA, and nonasaccharide–BSA contained 6, 6, and 4 mol of corresponding oligosaccharide per mol of BSA.

Antiserum. — A rabbit was immunized with an emulsion of Xyl-Glc–BSA and Freund's complete adjuvant. The rabbit was injected subcutaneously in the back region with 1 mg of the conjugate every week for four successive weeks. One week after the last injection, the animal was bled from a marginal ear-vein.

To prepare the antiserum free from antibodies to BSA, the antiserum was applied to a BSA-Sepharose 4B immunoadsorbent column, and the fractions unadsorbed to the column were pooled, and concentrated to the original volume by ultrafiltration. Complete removal of the anti-BSA antibodies from the antiserum was confirmed by gel diffusion and quantitative precipitation. The adsorbed anti-BSA antibodies were eluted from the immunoadsorbent column by glycine·HCl buffer (0.17M), pH 2.3, and dialyzed against borate-buffered saline (BBS: 0.05M sodium borate, pH 7.9, 0.15M sodium chloride).

Immunodiffusion. — Double diffusion was carried out by the method of Ouchterlony[16], using Gelrite gel (Kelco, Division of Merck & Co., Inc.), instead of agar gel, in BBS.

Quantitative precipitation and hapten inhibition. — Quantitative precipitation of antiserum with carbohydrate–BSA conjugates or xyloglucan was conducted by the modified method of Kabat[17]. Increasing amounts of antigen dissolved in BBS were added to duplicate tubes containing 50 μL of antiserum and BBS in a final volume of 150 μL. After preliminary incubation for 1 h at room temperature, the tubes were incubated for 4–5 d at 4°. The precipitates were washed twice with 600 μL of saline, and dissolved in 330 μL of 0.05M sodium hydroxide, and the amount of protein was determined by a semi-micro Lowry method[18].

Hapten inhibition was carried out by the following procedure. Increasing amounts of hapten in BBS were added to 50 μL of original antiserum, the reaction mixture was kept for 20 min at room temperature, and antigen (9 μg of Xyl-Glc–BSA, 5 μg of pea fucogalactoxyloglucan, and 10 μg of tamarind galactoxyloglucan, respectively) in BBS was added, to give a total volume of 150 μL. The tubes were incubated for 1 h at room temperature, and then for 4–5 d at 4°. Precipitates were washed, and the amount of protein in precipitates was determined by the method used for the quantitative precipitation analysis.

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