Isolation and Characterization of Oligosaccharides from Purified Cellulase Digest of Jojoba (*Simmondsia chinensis*) Seed Xyloglucan[†]

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Treatment of xyloglucan from jojoba seeds with purified cellulase from *Trichoderma viride*, followed by gel filtration on Bio-Gel P-2, yielded higher and lower oligosaccharide fractions. From the latter fraction, six kinds of pure oligosaccharides were obtained by preparative paper chromatography. Examination of the partial acid hydrolyzates of the oligosaccharides, the molar ratio of the component monosaccharides, treatment with partially purified β -glucosidase from *Aspergillus oryzae* enzyme preparation and the degree of polymerization by gel filtration on Bio-Gel P-2 showed them to be: (a) isoprimeverose; (b)

In a previous paper,¹⁾ a xyloglucan was isolated from the 24% potassium hydroxide extract of jojoba seed. It was homogeneous on zone electrophoresis and by sedimentation analysis. The xyloglucan has cellulosic main chains in which three of every four D-glucose residues carry side chains of a single α -D-xylosyl, a galactosyl-galactosyl-xylosyl, and a fucosyl-galactosyl-xylosyl group.

A trisaccharide, galactosyl-galactosyl-xylose was obtained from the xyloglucan by treatment with cellulase and β -glucosidase followed by acetolysis.²⁾

The presence of consecutive galactose residues in the side chains have not been found in the xyloglucans isolated from any other sources.³⁻⁶⁾

Treatment of the xyloglucan with cellulase yielded a small amount of lower oligosaccharides in addition to higher oligosaccharides (deca-, nona- and heptasaccharides).¹⁾ In this study, the isolation and characterization of six kinds of lower oligosaccharides were conducted in order to confirm the structure of the side chain of the xyloglucan accurately.

MATERIALS AND METHODS

Plant material. The powdered defatted jojoba seed was prepared as described previously.¹⁾

Enzymes. *Aspergillus oryzae* enzyme preparation was a generous gift of Dr. A. Endo (Tokyo Noko University). The purified cellulase from *Trichoderma viride*, purchased from Seikagaku Kogyo Co., Ltd., was entirely free from β -glucosidase, although it contained a small activity of xylanase, amylase and (1 $\rightarrow$ 3)- β -D-glucanase. Its specific activity was 15,000 units/

[†] Carbohydrates of Jojoba Seed. Part II. See Ref. 1)

mg. The purified protease (Pronase E, 70,000 PUK/g) from *Streptomyces griseus* was purchased from Kakenkagaku Co., Ltd. The β -glucosidase from *Aspergillus oryzae* enzyme preparation was partially purified by DEAE-Sephadex A-50 column chromatography and gel filtration on Sephadex G-100 and Sephadex G-200.⁷⁾

General methods. All evaporations were conducted under diminished pressure below 40°C. Paper chromatography was performed on Toyo No. 50 filter paper by multiple ascending method with the following solvent systems (A) 6:4:3 (v/v) 1-butanol-pyridine-water, and (B) 6:4:2 (v/v) 1-propanol-ethyl acetate-water. Preparative paper chromatography was carried out on Toyo No. 514 thick filter paper with the former solvent system. Aniline hydrogen phthalate⁸⁾ or silver dip method⁹⁾ was used for the detection of sugars. Gas-liquid chromatography was performed with a Yanagimoto Model G-80 gas chromatograph fitted with a flame ionization detector. The glass column (200 $\times$ 0.4 cm) was packed with 1.5% QF-1 on Chromosorb W (AW-DMCS) (mesh 60~80). Analysis was performed at 120°C at a nitrogen flow rate of 15 ml/min. Total sugars and reducing sugars were determined by the phenol sulfuric acid¹⁰⁾ and the Somogyi-Nelson method,¹¹⁾ respectively.

Acetylation¹²⁾ of the xyloglucan. A suspension of the purified xyloglucan (42 mg) in formamide (3 ml) was shaken overnight at room temperature. After addition of pyridine (4 ml), acetic anhydride (4 ml) was added dropwise with stirring. The temperature of the reaction mixture was maintained at 20°C until all of the acetic anhydride was added. The mixture was agitated for 16 hr at 37°C and then poured into ice water. After the whole mixture was allowed to stand overnight at room temperature, the precipitated product was collected by filtration and washed successively with methanol and ether, and finally air-dried. Sixty-seven mg of the acetylated xyloglucan was obtained.

Acetolysis of the acetylated xyloglucan. The acetylated xyloglucan (67 mg) was treated with a mixture of acetic anhydride, acetic acid and sulfuric acid (10:10:1, v/v)⁵⁾ (10.5 ml) for 22 hr at 20°C and the reaction mixture was poured into ice water. After being allowed to stand for 24 hr at room temperature, the whole mixture was extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to a syrup, yield 63 mg. The acetolyzate of the acetylated xyloglucan was subjected to deacetylation with 0.05 M sodium methoxide, treated with Amberlite IR-120 (H⁺) resin, evaporated, and examined by paper chromatography in solvent system A.

Acetolysis of the pentasaccharide. Acetolysis of the pentasaccharide (L-OS-6) was performed by treating

the sample with a mixture of acetic anhydride, acetic acid and sulfuric acid (10:10:1, v/v) for 3 hr at 40°C. The mixture was poured into ice water, and after being kept for 24 hr, the acetolysis product was extracted with chloroform. The chloroform layer was dried and evaporated to a syrup. The acetolyzate was subjected to deacetylation with 0.05 M sodium methoxide, treated with Amberlite IR-120 (H⁺) resin, and evaporated.

Estimation of molar ratio. Complete acid hydrolysis was performed by heating 300 μ g of oligosaccharide with 2 ml of 1 N hydrochloric acid for 2 hr at 100°C, and the hydrolyzate was evaporated under diminished pressure. To remove hydrochloric acid, the residue was repeatedly evaporated with small amounts of water and methanol. After acid hydrolysis of the oligosaccharides, the monosaccharides in the hydrolyzates were converted into their corresponding alditol trifluoroacetates and quantitative estimation by gas chromatography was performed.

Treatment of oligosaccharides (L-OS-2~L-OS-6) with partially purified β -glucosidase from *Aspergillus oryzae* enzyme preparation. Each oligosaccharide (L-OS-2~L-OS-6, 2 mg) was dissolved in 1 ml of deionized water. To the oligosaccharide solution, 0.2 ml of partially purified β -glucosidase solution was added. After incubation at 40°C for 15 hr, the enzyme digest was examined by paper chromatography.

Partial acid hydrolysis. Partial acid hydrolysis of L-OS-4 and 6 was performed by heating 500 μ g of the sample with 3 ml of 0.1 N hydrochloric acid for 60 min at 100°C, and to remove hydrochloric acid, the hydrolyzates were repeatedly evaporated with small amounts of water and methanol. The hydrolyzate was examined by paper chromatography.

RESULTS AND DISCUSSION

Purification of the xyloglucan

The polysaccharides of jojoba seeds were extracted as described previously.¹⁾ HC-II (24% potassium hydroxide extract, 4.31 g) was obtained from 100.02 g of defatted jojoba seeds.

Fraction HC-II was separated into subfraction A (precipitated by acidification) and B (not precipitated by acidification) by neutralization of the alkali solution followed by precipitation. Only the latter fraction was strongly stained with iodine. Fraction HC-IIB was further separated into three subfractions, HC-IIB-CP, HC-IIB-IS and HC-IIB-IP by the procedure of Gaillard.¹³⁾ The procedure

FIG. 1. Fractionation of HC-II.

for the separation of fraction HC-II is shown in Fig. 1. (Total yield of fraction HC-IIB-IP, 1488.5 mg).

The fraction HC-IIB-IP (641.2 mg) recovered from the precipitated iodine complex was dissolved in 0.01 M phosphate buffer (pH 6.0) and insoluble material was removed by centrifugation. The supernatant was applied to a column (10.5 × 3.7 cm) of DEAE-Sephadex A-25 (phosphate type), which was equilibrated with 0.01 M phosphate buffer (pH 6.0). The column was eluted stepwise with 0.01 M and 0.5 M phosphate buffer (pH 6.0) followed by 0.5 N and 1 N sodium hydroxide solution. Each eluate was dialyzed three times against deionized water for 24 hr, and freeze-dried after concentration by evaporation. The eluate with 0.01 M phosphate buffer was examined further as the purified xyloglucan; yield 499.8 mg.

Properties of the purified xyloglucan

The purified xyloglucan was soluble in water. It had $[\alpha]_D^{+47.3^\circ}$ (c 0.95, water), and, on acid hydrolysis, yielded glucose, xylose, galactose and fucose in the approximate molar ratio of 4.0:2.5:1.8:0.1. The xyloglucan gave glucose, xylose, galactose, fucose and isoprimeverose after treatment with crude β -glucosidase. Xylose, glucose, galactose, cellobiose, galactosyl-xylose and galactosyl-galactosyl-xylose were detected in the deacetylated acetylate of xyloglucan by paper chromatography.

Molecular weight. The purified xyloglucan (8.5 mg) was dissolved in 1 ml of 0.1 N NaOH solution. The xyloglucan solution was applied to a Sepharose CL-6B column (94 × 1.5 cm). The column was eluted with 0.1 N NaOH solution, and the eluate was separated into tubes containing 1.5 ml each. The column was calibrated with standard dextrans (T 500,

FIG. 2. Determination of the Molecular Weight of Xyloglucan by Gel filtration on Sepharose CL-6B.

The column was calibrated with standard dextrans [T 500 (M_w 500,000), T 250 (M_w 267,000), T 110 (M_w 110,000), T 70 (M_w 70,000) and T 40 (M_w 40,000)].

T 250, T 110, T 70 and T 40). As shown in Fig. 2, the molecular weight (M_w) of the xyloglucan was estimated to be 174,000.

Iodine staining of the xyloglucan. To a solution (1 ml) of purified xyloglucan (245 μ g), 0.5% I_2 ~ 1% KI solution (5 ml) was added. After being allowed to stand for 1 hr in the dark (4°C), the optical density was measured at 500~750 nm. The solution of the purified xyloglucan gave a reddish violet color with iodine staining reagent and the maximum absorption was observed at 645 nm.

Preparation of lower oligosaccharides

The purified xyloglucan (320 mg) was dissolved in 34 ml of deionized water. To the xyloglucan solution, 3.6 ml of purified cellulase was added and the whole solution was covered with a few drops of toluene to prevent microbial infection. After incubation for 48 hr at 40°C, the enzyme reaction was stopped by heating in a boiling water bath for 10 min. The digest was applied to a column of Bio-Gel P-2 (114 $\times$ 1.9 cm), then the column was eluted with water at 30°C, and collected in every 1.3 ml (Fig. 3).

As can be seen from the elution profile, the

FIG. 3. Elution Profile of Purified Cellulase Digest of Purified Xyloglucan on Bio-Gel P-2.

xyloglucan digest was mainly composed of higher oligosaccharide fractions (No. 104~149). A small amount of lower oligosaccharide fractions (No. 150~180) and void fractions (No. 75~103) were also detected. The amounts of V_0 fraction, higher oligosaccharide fractions and lower oligosaccharide fractions were 3.9%, 82.6% and 13.5%, respectively.

Isolation and purification of oligosaccharides

The pooled lower oligosaccharide fraction was evaporated to dryness, and was dissolved in 1 ml of deionized water. The lower oligosaccharide fraction was separated and purified by repeated preparative paper chromatography. Six kinds of paper chromatographically pure oligosaccharides were isolated, as shown in Table I. The content of these oligosaccharides was estimated by paper chromatography (Table I).

Molecular size of oligosaccharides

The oligosaccharides (L-OS 2, 3, 4, 5 and 6, 1 mg each), 1 mg of blue dextran and 1 mg of glucose were dissolved in 1 ml of deionized water. This solution was applied to a column of Bio-Gel P-2 (115 $\times$ 1.9 cm), then the column was eluted with water at 30°C, and collected in 1.5 ml fractions. The column was calibrated with a manno-oligosaccharide mixture,¹⁴⁾ which was obtained from the acetolizate of

TABLE I. YIELDS AND PROPERTIES OF OLIGOSACCHARIDES FROM PURIFIED CELLULASE DIGEST OF JOJOBA SEED XYLOGLUCAN

Oligosaccharide	Yield (%)	DP	R _G	Sugar composition	Sugar composition after treatment with β -glucosidase
L-OS 1	3.4	2	0.78 (Solvent A) 0.57 (Solvent B)	Glucose : Xylose 1.0 : 1.1	Not cleaved
L-OS 2	20.1	3	0.62 (Solvent A)	Glucose : Xylose 1.8 : 1.0	Glucose, isoprimeverose
L-OS 3	23.0	4	0.44 (Solvent A)	Glucose : Xylose 1.1 : 1.0	Isoprimeverose
L-OS 4	10.0	4	0.34 (Solvent A)	Glucose : Xylose : Galactose 1.8 : 1.0 : 1.2	Glucose, galactose, isoprimeverose
L-OS 5	17.2	5	0.28 (Solvent A)	Glucose : Xylose 3.3 : 2.0	Glucose, isoprimeverose
L-OS 6	26.3	5	0.22 (Solvent A)	Glucose : Xylose : Galactose 1.7 : 1.0 : 2.1	Not cleaved

FIG. 4. Elution Profile of Lower oligosaccharides (L-OS 2, 3, 4, 5 and 6) on Bio-Gel P-2.

Peak I: L-OS-5, L-OS-6

Peak II: L-OS-3, L-OS-4

Peak III: L-OS-2

Candida sp. mannan. The results are shown in Fig. 4.

As can be seen from the elution profile, lower oligosaccharides (L-OS-2 ~ L-OS-6) were eluted at the position of DP 5, 4 and 3. Each fraction was evaporated and examined by paper chromatography. Degree of polymerization of each oligosaccharide is summarized in Table I.

From the results of gel filtration and paper

chromatography, L-OS-2 had DP 3, L-OS-3 and 4 had DP 4 and L-OS-5 and 6 had DP 5.

Characterization of the oligosaccharides

The characterization of the oligosaccharides was performed by the examination of their partial acid hydrolyzates, determination of the molar ratio of component monosaccharides, examination of their digest with partially purified β -glucosidase. The results are summarized in Table I.

1) *L-OS-1* (disaccharide). The molar ratio of glucose and xylose residues of L-OS-1 was 1.0:1.1. The R_G values of L-OS-1 was identical with those of authentic isoprimeverose,¹⁵⁾ R_G 0.78 (solvent A) and R_G 0.57 (solvent B). From these results, L-OS-1 was characterized as isoprimeverose.

2) *L-OS-2* (trisaccharide). The molar ratio of glucose and xylose residues of L-OS-2 was 1.8:1.0. Glucose and isoprimeverose were liberated by action of β -glucosidase on L-OS-2. From these results, L-OS-2 was characterized as follows:

3) *L-OS-3* (tetrasaccharide). The molar ratio of glucose and xylose residues of L-OS-3 was 1.1:1.0. L-OS-3 was converted into isoprimeverose exclusively by β -glucosidase. From these results, it was characterized as

follows:

4) *L*-OS-4 (tetrasaccharide). The molar ratio of glucose, xylose and galactose residues of *L*-OS-4 was 1.8:1.0:1.2. Degradation of the oligosaccharide with β -glucosidase gave rise to glucose, galactose and isoprimeverose. The liberation of galactose from *L*-OS-4 might be due to the contamination of galactosidase in the β -glucosidase preparation. *L*-OS-4, on partial acid hydrolysis, yielded xylose, glucose, galactose, cellobiose and *L*-OS-2. From these results, *L*-OS-4 was characterized as follows:

5) *L*-OS-5 (pentasaccharide). The molar ratio of glucose and xylose residues of *L*-OS-5 was 3.3:2.0. This oligosaccharide was converted into glucose and isoprimeverose by β -glucosidase. From these results, *L*-OS-5 was characterized as follows:

6) *L*-OS-6 (pentasaccharide). The molar ratio of glucose, xylose and galactose residues of *L*-OS-6 was 1.7:1.0:2.1. The oligosaccharide was not degraded by β -glucosidase. The oligosaccharide, on partial acid hydrolysis, yielded isoprimeverose and cellobiose in addition to the component monosaccharides. Paper chromatography of the deacetylated acetolyzate of *L*-OS-6, revealed the presence of galactosyl-xylose (R_G 0.76, solvent A), cellobiose, galactosyl-galactosyl-xylose (R_G 0.46, solvent A) in addition to the component monosaccharides.

The pentasaccharide (*L*-OS-6) was also isolated from the β -glucosidase digests of the nonasaccharide which was obtained from cellulase digests of the xyloglucan.¹⁾ A trisaccharide was isolated from the deacetylated

acetolyzate of this pentasaccharide by preparative paper chromatography. The structure of the trisaccharide was determined to be $\text{Galp}_1 \rightarrow_2 \text{Galp}_1 \rightarrow_2 \text{Xylp}_1$.²⁾

From these results, *L*-OS-6 was characterized as follows:

Acetolysis of the xyloglucan

Both the disaccharide (galactosyl-xylose) and the trisaccharide (galactosyl-galactosyl-xylose) were isolated from the deacetylated acetolyzate of the xyloglucan by preparative paper chromatography.

The disaccharide consisted of xylose and galactose in the molar ratio of 1.0:0.9. It showed R_G 0.76 (solvent A). The reducing end of this oligosaccharide was determined to be xylose by the examination of its corresponding alditol. The disaccharide was not detected on a paper chromatogram by spraying with triphenyl tetrazolium chloride (TTC).¹⁶⁾ From these results, this disaccharide was characterized as 2-*O*-galactosyl-xylose.

The trisaccharide consisted of xylose and galactose in the molar ratio of 1.0:1.8. It showed R_G 0.46 (solvent A). The reducing end of this trisaccharide was determined to be xylose in the same manner as described before. This trisaccharide was not detected on a paper chromatogram by spraying with TTC. The trisaccharide, on partial acid hydrolysis, yielded 2-*O*-galactosyl-xylose, xylose, galactose and an unidentified oligosaccharide (R_G 0.60). From these results, the trisaccharide was characterized as $\text{Gal} \rightarrow \text{Gal}_1 \rightarrow_2 \text{Xyl}$.

Treatment of the xyloglucan from jojoba seed with cellulase yielded a small amount (13.5%) of lower oligosaccharides in addition to 82.6% of higher oligosaccharides (deca-, nona- and heptasaccharides).¹⁾

In a previous paper,¹⁾ the structure of the higher oligosaccharides was characterized and the repeating units of the xyloglucan molecules were proposed. In this study, isolation and characterization of the lower oligosaccharides

(di- ~pentasaccharide) were carried out.

The presence of galactosyl-galactosylxylose residues is shown in the lower oligosaccharide (L-OS-6) obtained from the digests of the xyloglucan with cellulase. The trisaccharide (Gal→Gal→Xyl) is also derived from the xyloglucan by acetolysis.

These results show that some galactose residues are located consecutively in the side chains of the xyloglucan. This structural feature has not been found in the xyloglucan molecules from any other sources.^{3~6)}

Although the fine structure of the xyloglucan molecule is yet unknown, it is very useful for the study of the action of purified cellulase from *Trichoderma viride* on the xyloglucan to elucidate the mode of the liberation of the lower oligosaccharides.

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