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# FINE STRUCTURE OF (1→3),(1→4)-β-D-GLUCAN FROM *Zea* SHOOT CELL-WALLS*

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ABSTRACT

The insoluble material that remains after extraction of *Zea* shoots with cold buffer was treated successively with 3M LiCl and hot water. The polysaccharides solubilized by these treatments were mostly (1→3),(1→4)-β-D-glucans. The β-D-glucan from the hot-water-soluble fraction was hydrolyzed by *Bacillus subtilis* (1→3),(1→4)-β-D-glucan 4-glucanohydrolase. The oligosaccharides were characterized by methylation analysis of the enzymic fragments and by methylation analysis of secondary fragments generated by treatment of the isolated oligosaccharides with *Streptomyces* QM B814 cellulase. The results demonstrate that the native polysaccharide consists mainly of cellotriosyl and cellotetraosyl residues joined by single (1→3) linkages. Evidence is presented to show that certain other glucosyl sequences are also present in the native polysaccharide including (*a*) two, three, or four contiguous (1→3)-linkages; (*b*) blocks of more than four (1→4)-linked glucose residues; (*c*) regions having alternating (1→3)- and (1→4)-linkages.

## INTRODUCTION

Important structural features of cell walls derived from monocots remain unresolved. In an effort to learn more about these wall polysaccharides, we have examined *Zea* shoot excised at the coleoptile node 96 h after inhibition of the caryopses. In a previous paper[1], we reported that three water-soluble polysaccharide fractions (WSP-I, -II, and -III) were obtained from *Zea* shoots: WSP-I was obtained from the soluble fraction of the buffer-homogenate of *Zea* shoots, and WSP-II and -III were obtained from the insoluble fraction of the buffer-homogenate upon treatment with 3M LiCl and hot water, respectively. We also reported that the major polysaccharide in WSP-I was a glucuronic acid-containing arabinogalactan[1].

Studies on WSP-II and -III revealed that both fractions were comprised mainly of (1→3),(1→4)-β-D-glucans. Although extensive structural studies on β-D-

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glucan from ceral endosperm have been reported, there are relatively few detailed analyses of the β-D-glucan derived from non-endospermic primary cell-walls. Therefore, we initiated the present study on the fine structure of the soluble β-glucan fraction prepared from WSP-III, relying on a *Bacillus subtilis* (1→3),(1→4)-β-D-glucan 4-glucanohydrolase and a *Streptomyces* QM B814 cellulase for specific hydrolysis of glucosidic linkages.

## RESULTS AND DISCUSSION

*Characterization of WSP-II and WSP-III.* — WSP-II and WSP-III, obtained from the insoluble fraction of the buffer homogenate of *Zea* shoots by successive treatment with 3M LiCl and hot water, contained glucose, arabinose, xylose, galactose, and mannose in the molar ratio of 89.0:4.8:3.1:3.1:0 and 94.6:2.1:1.1:2.1:0.1, respectively, as reported previously[1]. WSP-II and WSP-III were separately methylated by the method of Hakomori[2]. G.l.c. of the alditol acetates obtained from the acid hydrolyzate of the methylated WSP-II and WSP-III revealed 2,3,6-tri- and 2,4,6-tri-*O*-methyl-D-glucose as major structural components. The molar ratios of 3-linked glucose and 4-linked glucose residues were about 1:2.4 and 1:2.1 for WSP-II and WSP-III, respectively.

Hydrolysis of WSP-II or WSP-III with purified β-D-glucanase from *B. subtilis* followed by separation of the products by gel-filtration on Bio-Gel P-2 yielded two predominant oligosaccharides, 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose in the molar ratio of 3.2:1.0. This value is in agreement with previous results in which the glucan component in the cell walls of *Zea* shoots was investigated[3].

Although the iodine test and hydrolysis of WSP-II and WSP-III by alpha amylase indicated that a small quantity of starch contaminated both polysaccharide fractions (<3%), the foregoing results clearly show that WSP-II and WSP-III are mainly comprised of (1→3),(1→4)-β-D-glucans. Small proportions of arabinose, xylose, and galactose residues in WSP-II and WSP-III are considered to be derived from an arabinogalactan[1] and an arabinoxylan.

*Hydrolysis of a β-D-glucan preparation by* B. subtilis *β-D-glucanase and structural analyses of the products. — Isolation of a β-D-glucan from WSP-III.* A large quantity of WSP-III (~2 g as glucose equivalents) was prepared from 3800 g of *Zea* shoots. Starch in WSP-III was hydrolyzed upon incubation with alpha amylase, and polysaccharides in WSP-III were resolved into seven fractions by the graded ammonium sulfate precipitation-method[4,5]. Table I shows the yields, neutral-sugar content and composition of these fractions. Fraction 40P-P, which accounts for ~40% of total recovered carbohydrate, consisted mostly of glucose. Fraction 40P-P was insoluble in cold water but was soluble in hot water. It gave no color in the presence of iodine reagent and was not degraded by alpha amylase. Chromatography of fraction 40P-P on a calibrated column of Bio-Gel A-1.5m indicated an average molecular weight of $7.0 \times 10^4$ (Fig. 1). This estimate of

molecular weight is based on a calibration curve generated from the elution characteristics of dextrans, which may behave somewhat differently from that of the extended conformations exhibited by the (1→3),(1→4)-β-D-glucans[6]. This fraction was used for additional experiments as a β-D-glucan preparation.

*Hydrolysis of fraction 40P-P by β-D-glucanase and separation of the hydrolyzate.* Fraction 40P-P was exhaustively hydrolyzed with purified *B. subtilis* β-D-glucanase. The extent of hydrolysis of the fraction in the presence of the enzyme was estimated to be 28% of the potential glucose residues (inset of Fig. 2). The enzyme hydrolyzate was first separated into a fraction (A), which was rendered insoluble during sample concentration, and a soluble fraction. The latter was fractionated by gel-filtation on Bio-Gel P-2 (Fig. 2). Tubes 26–33 corresponding to the void volume of the column were designated fraction B. Tubes 34–50 were combined, concentrated and centrifuged to give insoluble (fraction M) and soluble fractions. The soluble fraction was rechromatographed on a Bio-Gel P-2 column (Fig. 3A), and fractions C, D, E, F, G, and H, which correspond to d.p. (degree of polymerization) values of 10 and higher, 9, 8, 7, 6, and 5 respectively, were obtained. The main fractions in Fig. 2, 52–54 and 56–59 were separately purified by rechromatography on Bio-Gel P-2 to give fractions I (d.p. = 4) and J (d.p. = 3), respectively. Tubes 60–80 obtained as in Fig. 2 were rechromatographed on a column of Bio-Gel P-2 and fractions K and L, corresponding to d.p. = 2 and 1, respectively, were resolved (Fig. 3-B). The yields of fractions A–M are shown in Table II with their neutral-sugar composition.

TABLE I

YIELDS AND NEUTRAL-SUGAR COMPOSITION OF FRACTIONS OBTAINED FROM WATER-SOLUBLE POLYSACCHARIDE FRACTION-III OF *Zea* SHOOT BY THE GRADED AMMONIUM SULFATE-PRECIPITATION METHOD[a]

| *Faction* | *Yield (Total carbohydrate content, mg)* | | *Neutral-sugar composition (mol%)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | [b] | [c] | *Rha* | *Fuc* | *Rib* | *Ara* | *Xyl* | *Man* | *Gal* | *Glc* |
| OP | 9.4 | 3.5 | | | | 22.3 | 24.9 | 4.8 | 7.7 | 40.3 |
| 20P-S | 308 1 | 21.2 | 8.1 | | | 1.2 | 9.1 | | 46.3 | 25.3 |
| 20P-P | 5.2 | 3.3 | 0.5 | | 2.5 | 1.1 | 1.3 | tr. | 4.4 | 90.2 |
| 40P-S | 249.0 | 145.7 | 0.1 | | 0.6 | 5 8 | 8.0 | tr | 1.6 | 83 9 |
| 40P-P | 585.8 | 506.5 | | | | 0.6 | 0.2 | | tr. | 99.2 |
| 40S-S | 214.2 | 229.1 | 8.6 | 1.0 | 4.0 | 10.6 | 7.0 | | 4.8 | 63.9 |
| 40S-P | 34.5 | 26.7 | 3.0 | 2.2 | 1.3 | 1.3 | 0.7 | | 1.3 | 90.2 |

[a]Water-soluble polysaccharide fraction-III (~2 g as a glucose equivalents) in sodium-phosphate buffer (pH 6.9) was treated with alpha amylase and centrifuged to remove insoluble material (fraction OP). The supernatant was made 20% (w/v) with respect to $(NH_4)_2SO_4$ and centrifuged to collect the precipitate (fraction 20P). The supernatant was next made 40% with respect to $(NH_4)_2SO_4$ and centrifuged to separate insoluble (fraction 40P) and soluble (fraction 40S) materials. Fractions 20P, 40P, and 40S were separately dialyzed against water and then centrifuged. The precipitate and the supernatant from fractions 20P, 40P, and 40S were designated 20P-P and -S, 40P-P and -S, and 40S-P and -S, respectively. [b]Determined by the phenol–sulfuric acid method and expressed as glucose equivalents. [c]Determined by g.l.c. analysis of the alditol acetates of the acid hydrolyzate.

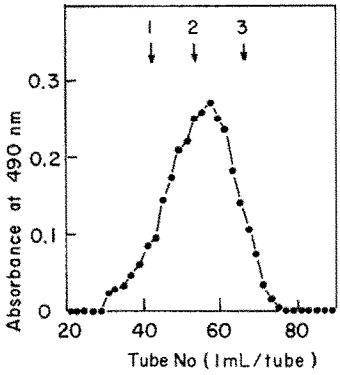


Fig 1. Chromatographic profile of fraction 40P-P as resolved on a column of Bio-Gel A-1.5m. Fraction 40P-P (4 mg in 25mM sodium phosphate buffer, pH 6.0) was applied to a column (1.3 × 65 cm) of Bio-Gel A-1.5m pre-equilibrated with 0.1 M sodium phosphate buffer (pH 6.0) containing 0.1% $NaH_3$, followed by filtration through the column with the same buffer. The arrows in the Figure index the elution positions of, left to right, dextrans of molecular weight $1.7 \times 10^5$, $7.0 \times 10^4$, and $1.0 \times 10^4$.

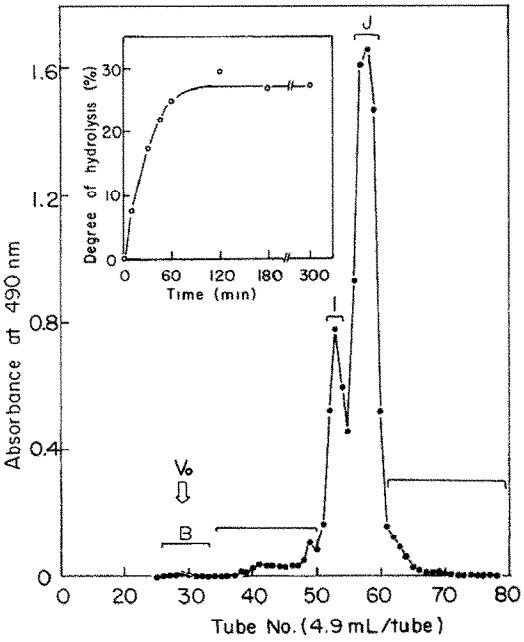


Fig. 2. Separation by gel filtration on Bio-Gel P-2 of the *B. subtilis* β-D-glucanase-hydrolyzate of fraction 40P-P. Fraction 40P-P in sodium phosphate buffer was incubated with *B. subtilis* β-D-glucanase At intervals, the degree of hydrolysis was calculated (inset) After 315 min of incubation, the hydrolyzate was concentrated and centrifuged to remove insoluble materials (fraction A). The supernatant was resolved by gel filtration on Bio-Gel P-2 (3.5 × 45 cm). Tubes 26–33 (fraction B), 34–50, 52–54 (fraction I), 56–59 (fraction J) and 60–80 were separately combined and concentrated to low volume Fractions I and J were separately rechromatographed on the same column

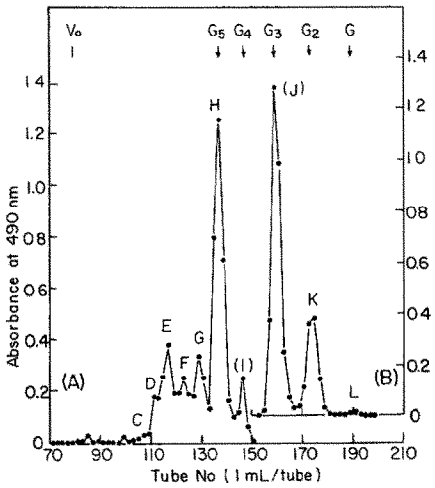


Fig. 3. Rechromatography of fractions from tubes 34–50 and 60–80 obtained in Fig. 2. These fractions were separately rechromatographed on a column (1.5 × 150 cm) of Bio-Gel P-2. A: Tubes 34–50 were resolved into fractions C (101–113), E (114–119), F (121–125), G (127–132), and H (134–141). B: Tubes 60–80 were resolved into fractions K (171–179) and L (185–195).

TABLE II

YIELDS AND NEUTRAL-SUGAR COMPOSITION OF FRACTIONS OBTAINED FROM *Bacillus subtilis* β-D-GLUCANASE-HYDROLYZATE OF FRACTION 40P-P

| *Fraction* | *Yield*[a] *(μg)* | *%*[b] | *D.p.*[c] | *Neutral sugar composition (Mol%)* | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *Glc* | *Gal* | *Ara* | *Xyl* | *Man* | *Ul*[d] |
| A | 1550 | 0.7 | | 98.4 | trace | 0.7 | 0.9 | trace | |
| B | 588 | 0.3 | >~15 | 43.3 | 2.8 | 32 8 | 16.5 | 4.7 | |
| C | 196 | 0.1 | ~10 | 95.8 | trace | 2 1 | 2.1 | | |
| D | 396 | 0.2 | 9 | 96.4 | trace | 2.4 | 1.2 | | |
| E | 1350 | 0.6 | 8 | 99.3 | trace | 0.4 | 0.3 | | |
| F | 756 | 0.4 | 7 | 98.4 | trace | 1.0 | 0.6 | | |
| G | 1220 | 0.6 | 6 | 100 | | | | | |
| H | 3400 | 1.6 | 5 | 100 | | | | | |
| I | 56935 | 26.8 | 4 | 100 | | | | | |
| J | 139120 | 65.5 | 3 | 100 | | | | | |
| K | 6100 | 2.9 | 2 | 100 | | | | | |
| L | 300 | 0.1 | 1 | 45 | | | | | 55 |
| M | 518 | 0.2 | | 100 | trace | trace | trace | | |

[a]From 250 mg of fraction 40P-P. Determined by the phenol–sulfuric acid method and expressed as glucose equivalent. [b]Recovered carbohydrate (%) from fractions A–M. [c]Degree of polymerization. [d]Unidentified sugar.

*Susceptibility of several authentic oligosaccharides to* Streptomyces *cellulase preparation.* Anderson and Stone[7] have reported that lichenan, oat glucan, and *O*-(carboxymethyl)cellulose are attacked by *Streptomyces* QM B814 cellulase, but β-D-glucan with alternating (1→3) and (1→4) linkages, prepared by reduction of the uronic acid residues of SIII pneumococcal polysaccharide, is not. In order to further establish the characteristics of this enzyme-preparation for structural analysis of (1→3),(1→4)-β-D-gluco-oligosaccharides in this study, the cellulase was assayed using several kinds of authentic oligosaccharides. Table III shows substrate specificities of the enzyme preparation.

The enzyme preparation does not hydrolyze cellobiose or 3-*O*-β-cellobiosyl-D-glucose, but it can hydrolyze 3-*O*-β-cellotriosyl-D-glucose, forming as products cellobiose and laminarabiose. Therefore, the enzyme preparation hydrolyzes oligosaccharides having certain sequences of (1→4)-linked glucosyl residues and (1→3)-linked glucosyl residues, for example *O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→3)-D-glucose to cellobiose and laminaratriose.

*Characterization of fractions A–M derived from fraction 40P-P by* β-D-*glucanase with* B. subtilis *hydrolysis.* Fractions L, K, J, I, and H, having d.p. values of one, two, three, four, and five respectively, were first subjected to paper chromatography (p. c.) to check their homogeneity. Factions K and I appeared homogeneous in p. c. whereas fractions L, K, and H were found to be composed of two kinds of oligosaccharides. Compounds K-a and K-b, and compounds H-a and H-b were prepared from fractions K and H by preparative p. c., respectively. The isolated oligosaccharides were subjected to methylation analysis before and after reduction with $NaBH_4$, and to hydrolysis by *Streptomyces* cellulase followed

TABLE III

SUBSTRATE SPECIFICITIES OF *Streptomyces* QM B814 CELLULASE

| *Substrate*[a] | *Hydrolysis products* |
|---|---|
| $Gl\xrightarrow{\beta}3G1\xrightarrow{\beta}3G$ | Not hydrolyzed |
| $G1\xrightarrow{\beta}3G1\xrightarrow{\beta}3G1\xrightarrow{\beta}3G$ | Not hydrolyzed |
| $G1\xrightarrow{\beta}3G1\xrightarrow{\beta}3G1\xrightarrow{\beta}3G1\xrightarrow{\beta}3G$ | Not hydrolyzed |
| $G1\xrightarrow{\beta}4G1\xrightarrow{\beta}4G$ | Not hydrolyzed |
| $G1\xrightarrow{\beta}4G1\xrightarrow{\beta}4G1\xrightarrow{\beta}4G$ | $G1\xrightarrow{\beta}4G$ |
| $G1\xrightarrow{\beta}4G1\xrightarrow{\beta}4G1\xrightarrow{\beta}4G1\xrightarrow{\beta}4G$ | $G1\xrightarrow{\beta}4G$ $G1\xrightarrow{\beta}4G1\xrightarrow{\beta}4G$ |
| $G1\xrightarrow{\beta}4G1\xrightarrow{\beta}3G$ | Not hydrolyzed |
| $G1\xrightarrow{\beta}4G1\xrightarrow{\beta}4G1\xrightarrow{\beta}3G$ | $G1\xrightarrow{\beta}4G$ $G1\xrightarrow{\beta}3G$ |

[a]G denotes D-glucose (Glc) and the numerals the linkage positions

by analysis of the oligosaccharide products. The results are summarized in Table IV, and each oligosaccharide was assigned the structure as shown in the right column.

Although fractions B–G were considered to be mixtures of oligosaccharides having the same d.p., no attempt was made to resolve them further. It is exceedingly difficult to separate β-D-linked gluco-oligosaccharides having higher degrees of polymerization. Methylation analysis of fractions B–G and of fractions A and M indicates that the ratios of (1→3) to (1→4) were 1:0.7, 1:0.7, 1:1.3, 1:1.7, 1:1.2, 1:1.3, 1:1.3, and 1:2.1 for fractions A, B, C, D, E, F, G, and M, respectively. These results suggest that oligosaccharides having more than one (1→3)-linkages are present in such oligosaccharide fractions. Accordingly, fractions G (d.p. = 6), F (d.p. = 8) and M (insoluble in water), which are obtained in higher yields among the fractions, were studied further.

Methylation analysis, before and after reduction by $NaBH_4$, showed that fraction G contained a C-3 substituted, reducing terminal glucose residue. Fraction G was comprised of non-reducing terminal glucosyl groups, 4-substituted glucose residues, and 3-substituted glucose residues present within the chain and 3-substituted glucose groups at the reducing terminal in the molar ratio of approximately 17:53:13:17. Fraction G was hydrolyzed with *Streptomyces* cellulase followed by resolution of the products by gel-filtration on Bio-Gel P-2 (Fig. 4-A). Oligosaccharides comprising the disaccharide fraction (G-2) and trisaccharide fraction (G-3) were examined by p.c. Fractions G-2 and G-3 were composed of laminarabiose and cellobiose and of laminaratriose, 4-*O*-β-laminarabiosyl-D-glucose, 3-*O*-β-cellobiosyl-D-glucose and cellotriose, respectively. The tetrasaccharide fraction (G-4) had two components (G-4-a, $R_{Glc}$ 0.38 and G-4-b, $R_{Glc}$ 0.26, method *A*). The major component, G-4-a was isolated by preparative p.c. The G-4-a and fractions G-5 (pentasaccharide) and G-6 (hexasaccharide) were separately subjected to methylation analysis, and their structures were tentatively proposed from these results and from the analysis of the products of cellulase treatment (Table V). On the basis of structures of oligosaccharide fragments derived from cellulase treatment, the tentative structures of glucohexaoses present in fraction G were proposed as shown in the right column of Table V.

Methylation analysis after reduction by $NaBH_4$ showed that fraction E contained oligosaccharide(s) having 3-substituted glucose at the reducing terminal and those having 4-substituted glucose at the reducing terminal in the ratio of ~1:1. Fraction E was hydrolyzed with *Streptomyces* cellulase followed by resolution of the products by gel-filtration on Bio-Gel P-2 (Fig. 4-B). Disaccharide (E-2) and trisaccharide (E-3) fractions were separately subjected to paper-chromatographic examination, and tetrasaccharide (E-4), pentasaccharide (E-5), and hexasaccharide (E-6) fractions were separately subjected to methylation analysis. Fractions E-7 and E-8 did not contain glucose. On the basis of structures of oligosaccharide fragments derived from cellulase treatment, the tentative structures of glucooctaoses present in fraction E were proposed as shown in the right column of Table V.

TABLE IV

ANALYSIS OF OLIGOSACCHARIDES IN FRACTIONS H–L OBTAINED FROM *Bacillus subtilis* β-D-GLUCANASE-HYDROLYZATE OF FRACTION 40P-P

| *Fraction* | *Paper chromatography* | | *Methylation analysis*[a] | *Cellulase-hydrolysis product* | *Proposed structure* | |
|---|---|---|---|---|---|---|
| L | L-a | $R_{Glc}$ 1.34 (method *A*) | | | Unidentified | |
| | L-b | $R_{Glc}$ 1.0 | | | G (glucose) | |
| K | K-a | $R_{Glc}$ 0.88 (method *B*) | t-Glc:3-Glc = 1:1 | (G)[c] | $G3G_R$ | K-a/K-b = 1/4 |
| | K-b | $R_{Glc}$ 0.71 | t-Glc:4-Glc = 1:1 | (G)[c] | $G4G_R$ | |
| J | | $R_{Glc}$ 0.42 (method *B*) | t-Glc:4-Glc:3-Glc = 1:1:1<br>t-Glc:4-Glc 3-Glc(OH)[b] = 1:1:1 | Not hydrolyzed | $G4G3G_R$ | |
| I | | $R_{Glc}$ 0.19 (method *B*) | t-Glc:4-Glc:3-Glc = 1:2:1<br>t-Glc:4-Glc:3-Glc(OH)[b] = 1:2:1 | $G3G_R$ and $G4G_R$ | ↓[e]<br>$G4G4G3G_R$ | |
| H | H-a | $R_{Glc}$ 0.14 (method *B*) | t-Glc.4-Glc:3-Glc = 1:2:2<br>t-Glc:4-Glc:3-Glc:3-Glc(OH)[b] = 1:2:1:1 | $G3G_R$ and $G3G4G_R$[d] ($R_{Glc}$ 0.55, method *A*) | ↓<br>$G3G4G4G3G_R$ | H-a/H-b = 1/3 |
| | H-b | $R_{Glc}$ 0.05 (method *B*) | t-Glc.4-Glc:3-Glc = 1.3.1<br>t-Glc:4-Glc:3-Glc(OH)[b] = 1:3:1 | $G3G_R$, $G4G_R$, $G4G3G_R$ and $G4G4G_R$ | ↓<br>$G4G4G4G3G_R$<br>↑ | |

[a]The ratio of t-Glc (non-reducing terminal glucose), 4-Glc (4-linked glucose), 3-Glc (3-linked glucose), and 3-Glc(OH) (3-linked glucitol). [b]After reduction by $NaBH_4$. [c]K-a and K-b were subjected to treatment with almond β-glucosidase. [d]This structure was proposed from analysis of the partial acid-hydrolyzate of the sample before and after reduction by $NaBH_4$. [e]Hydrolysis by *Streptomyces* cellulase. $G_R$ = D-glucose as reducing group.

TABLE V

ANALYSIS OF THE CELLULASE-DERIVED FRACTION G OR E OLIGOSACCHARIDE AND TENTATIVE STRUCTURES OF ORIGINAL OLIGOSACCHARIDES PRESENT IN FRACTIONS G AND E

| *Oligosaccharide fraction* | % | *Identified oligosaccharide or the ratio of t-Glc, 4-Glc and 3-Glc* | *Tentative structures of oligosaccharides present in fraction G or E* |
|---|---|---|---|
| *G (D.p. = 6)* | | | |
| Disaccharide (G-2) | 38.6 | $G3G_R$ and $G4G_R$ | $G4G4G4G4G3G_R$ |
| Trisaccharide (G-3) | 29.8 | $G3G3G_R$, $G3G4G_R$, $G4G3G_R$, and $G4G4G_3$ | $G3G4G4G3G3G_R$ |
| Tetrasaccharide (G-4) | 20.7 | $G3G4G4G_R$ ($R_{Glc}$ 0.38, method *A*) and unidentified oligosaccharide ($R_{Glc}$ 0.26) | $G3G4G4G4G3G_R$ |
| Pentasaccharide (G-5) | 5.0 | t-Glc:4-Glc:3-Glc = 22:34:44 | $G4G4G4G3G3G_R$ |
| Hexasaccharide (G-6) | 6.0 | t-Glc:4-Glc:3-Glc = 22:33:45 | |
| *E (D.p. = 8)* | | | |
| Disaccharide (E-2) | 33.4 | $G3G_R$ and $G4G_R$ | $G4G4G4G4G4G3G3G_R$ |
| Trisaccharide (E-3) | 8.2 | $G3G3G_R$, $G3G4G_R$, $G4G3G_R$, and $G4G4G_R$ | $G3G3G4G4G4G4G3G_R$ |
| Tetrasaccharide (E-4) | 13.8 | t-Glc:4-Glc:3-Glc = 1:1:2 [$G3G3G4G_R$ and/or $G4G3G3G_R$] | $G3G4G4G4G4G3G3G_R$<br>$G3G4G4G4G4G4G3G_R$ |
| Pentasaccharide (E-5) | 17.3 | t-Glc:4-Glc:3-Glc = 1:2:2 [$G3G3G4G4G_R$] | $G4G4G4G4G4G4G3G_R$<br>$G3G4G4G4G4G4G4G_R$ |
| Hexasaccharide (E-6) | 16.1 | t-Glc:4-Glc:3-Glc = 1:1:4 [$G3G3G3G3G4G_R$ and/or $G4G3G3G3G3G_R$ ?] | $G3G3G4G4G4G4G4G_R$ |
| Heptasaccharide (E-7) | 7.3 | | |
| Octasaccharide (E-8) | 3.9 | | |

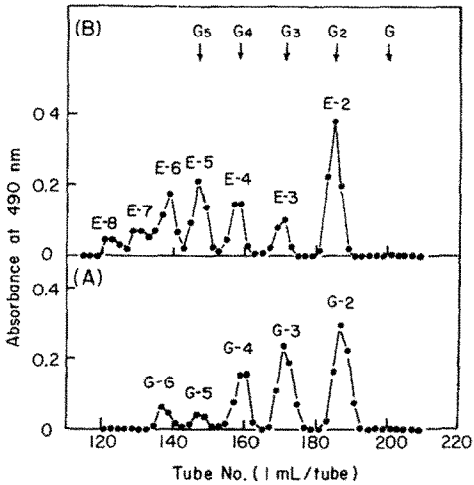


Fig. 4. Separation by gel filtration on Bio-Gel P-2 of *Streptomyces* cellulase-hydrolyzate of fractions E and G. A: The cellulase hydrolyzate of fraction G (d.p. = 6, 805 μg as glucose equivalent) was resolved by gel filtration on Bio-Gel P-2. Tubes 135–141 (fraction G-6), 145–151 (fraction G-5), 155–163 (fraction G-4), 168–175 (fraction G-3), and 184–191 (fraction G-2) were separately combined and concentrated B: The cellulase hydrolyzate of fraction E (d p. = 8, 1130 μg as glucose equivalent) was resolved by gel filtration on Bio-Gel P-2. Tubes 120–125 (fraction E-8), 127–133 (fraction E-7), 135–142 (fraction E-6), 144–150 (fraction E-5), 155–161 (fraction E-4), 165–173 (fraction E-3), and 181–189 (fraction E-2) were separately combined and concentrated

Methylation analysis of fraction M, which was obtained in an insoluble form as a consequence of concentration of oligosaccharides, showed that the ratio of (1→3) to (1→4) was 1:5.4. The estimated d.p. values of M ranged from 5 to ~14. Upon cellulase hydrolysis, fraction M gave mainly laminarabiose, cellobiose, 3-*O*-β-cellobiosyl-D-glucose, cellotriose, and 3-*O*-β-cellotriosyl-D-glucose in the approximate molar ratio of 1.8:10.5:2.6:2.2:1. It is well known that (1→4)-β-D-gluco-oligosaccharides of d.p. >7 have very low solubility in water. Therefore, fraction M is likely to consist of a mixture of oligosaccharides containing mainly six or more adjacent (1→4)-linkages and a single (1→3)-linkage at the reducing terminal.

*Isolation of (1→3)-β-D-gluco-oligosaccharides from the enzymic hydrolyzate of WSP-III.* — In order to establish whether or not contiguous (1→3)-linked glucose residues existed in the β-D-glucan of *Zea* shoot cell-walls, an attempt was made to isolate (1→3)-β-D-gluco-oligosaccharides from fraction WSP-III.

WSP-III, which was prepared from 3800 g of *Zea* shoots, was treated with *B. subtilis* β-D-glucanase followed by separation of the products by chromatography on Bio-Gel P-2. A mixture of oligosaccharides (58 mg) having d.p. values ranging from 5 to ~14 was obtained. The mixture was then hydrolyzed with *Streptomyces* cellulase. The enzymic hydrolyzate was resolved into di- (yield 4.4 mg), tri- (4.7 mg), tetra- (2.1 mg), penta- (2.7 mg), and hexa-saccharide (1.2 mg) fractions by chromatography on Bio-Gel P-2. Oligosaccharide a ($R_{Glc}$ 0.84, method *A*),

TABLE VI

CHARACTERIZATION OF LAMINARA-OLIGOSACCHARIDES OBTAINED FROM ENZYMIC HYDROLYZATE OF WSP-III

| *Oligosaccharide* | *Yield (μg)* | *D.p.* | $R_{Glc}$ | $R_F$ | *Product of complete acid hydrolysis* | *Methylation analysis* t-Glc:3-Glc[a] |
|---|---|---|---|---|---|---|
| a | 2495 | 2 | 0.84 | 0.56 | Glucose | 1:1 |
| b | 376 | 3 | 0.66 | 0.45 | Glucose | 1:2 |
| c | 288 | 4 | 0.54 | 0.34 | Glucose | 1:3 |
| d | 356 | 5 | 0.40 | 0.27 | Glucose | 1:4 |

[a]t-Glc and 3-Glc indicate non-reducing terminal glucosyl groups and 3-linked glucose residues, respectively.

b ($R_{Glc}$ 0.66), c ($R_{Glc}$ 0.54) and d ($R_{Glc}$ 0.40) which have the same $R_{Glc}$ values as authentic (1→3)-β-D-gluco-oligosaccharides were isolated from di-, tri-, tetra-, and penta-saccharide fractions by preparative p.c. Neutral-sugar analysis and methylation analysis of each oligosaccharide were performed and the results are summarized in Table VI. Log $R_F/(1 - R_F)$ of glucose and oligosaccharides, when plotted against d.p., yielded a linear regression. These results indicate that isolated oligosaccharides are β-(1→3)-linked gluco-oligosaccharides.

Isolation of laminara-triose, -tetraose, and -pentaose from the cellulase hydrolyzate of *B. subtilis* β-D-glucanase-derived WSP-III oligosaccharides shows that the β-D-glucan in WSP-III has two, three, and four contiguous linkages in addition to isolated (1→3)-linkages.

*Fine structure of a β-D-glucan preparation.* — Hydrolysis of a β-D-glucan preparation (fraction 40P-P) isolated from WSP-III with *B. subtilis* β-D-glucanase followed by separation of the products afforded thirteen fractions, A–M (Table II). Oligosaccharides in major fractions, I and J were assigned the structures 3-*O*-β-cellotriosyl-D-glucose and 3-*O*-β-cellobiosyl-D-glucose, respectively. It is clear from the enzyme specificity[7] that the tetra- and the tri-saccharides are derived from cleavage at the arrow in sequences*, ----4G3G4↓G4G4G3G4↓G4---- and ----4G3G4↓G4G3G4↓G4----, respectively: the β-D-glucan preparation mainly consists of cellotriosyl and cellotetraosyl residues separated by single (1→3)-linkages.

Fraction H contained two oligosaccharides, $G3G4G4G3G_R$ (H-a) and $G4G4G4G3G_R$ (H-b). The latter oligosaccharide is derived from cleavage at the arrow in sequences, ----4G3G4↓G4G4G4G3G4↓G4G4----: cellopentaosyl residues separated by single (1→3)-linkages are present in the β-D-glucan preparation.

Anderson and Stone[7] have reported the *B. subtilis* β-D-glucanase also slowly hydrolyzes the (1→3), (1→4)-β-D-glucan with alternating (1→3)- and (1→4)-

*The abbreviations G for D-glucose and $G_R$ for D-glucose as a reducing group are used here and in Figs. 3 and 4 to permit compact designation.

linkages, producing G3G4G3G$_R$ and G3G$_R$, and that the former is further hydrolyzed to G3G$_R$. They have concluded that, although the *Bacillus* enzyme has a requirement for a --G3G$_R$-- arrangement in the glycosyl portion of its substrate, it prefers a glycosyl sequence of --G4G3G$_R$-- over G3G4G3G$_R$. Therefore, oligosaccharide H-a must arise as a consequence of incomplete hydrolysis. Apart from incomplete hydrolysis of the oligosaccharide by the β-D-glucanase, this oligosaccharide might originate from portions of the molecule having alternating (1→3) and (1→4) linkages: with cleavage at the arrow in the indicated sequences, ---4G4G3G4↓G3G4G4G3G4↓G4---. Another possibility is that H-a originates from the non-reducing end of the glucan: G3G4G4G3G4↓G---.

Presence of oligosaccharides having the structures of G3G--------G3G$_R$ in fractions G and E (Table V) suggests incomplete hydrolysis by the *B. subtilis* β-D-glucanase treatment as described here. The origin of this fragment is considered to be the same as that of oligosaccharide H-a.

Characterization of fractions M, G, and E also indicates that cellohexaosyl residues and longer (1→4)-β-D-gluco-oligosaccharide associations are separated by single (1→3)-linkage or two contiguous (1→3)-linkages present in the glucan. Presence of such longer sequences of (1→4)-β-D-gluco-oligosaccharides in a glucan preparation may explain the insolubility of some β-D-glucans (including fraction 40P-P) obtained after resolution of WSP-III by the ammonium sulfate-precipitation method. The regions associate non-covalently with corresponding sequences in other β-D-glucan molecules to render them insoluble in water.

It is expected that the *B. subtilis* β-D-glucanase will yield oligosaccharides derived from (1→3),(1→4)-β-D-glucans having 3-substitution at the reducing-terminal glucose group if they are derived from internal cleavage of (1→3),(1→4)-β-D-glucans. However, cellobiose and oligosaccharides having a 4-substituted, reducing-terminal glucose group in the structure were found in fractions K and E, respectively. It is reasonable to consider that such oligosaccharides originate from the reducing-end portion of the native β-D-glucan preparation (fraction 40P-P).

Many structural studies on non-endospermic cell-wall β-D-glucans using *B. subtilis* β-D-glucanase (E.C. 3.21.73) or *Rhizopus* laminaranase (E.C. 3.21.6) have revealed that the polysaccharides are predominantly repeating sequences of the oligosaccharide units 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose[8–13]. Most recently, Woodward *et al.*[14] studied the structure of water-soluble β-D-glucans from barley endosperm using a (1→3),(1→4)-β-D-glucan 4-glucanohydrolase (E.C. 3.21.73) purified from germinating barley and reported that 90% of barley-endosperm glucans consisted of cellotriosyl and cellotetraosyl residues separated by single (1→3)-linkages, but blocks of 5–11 (1→4)-linked glucosyl residues were also present in significant proportions. In that study, they did not detect blocks of two or more adjacent (1→3)-linkages. Luchsinger *et al.*[15] and Dais and Perlin[16] also failed to resolve contiguous (1→3) linkages in barley β-D-glucans. There are, however, some reports[17–21] showing the presence in oat and barley endosperm β-D-glucans of regions consisting of contiguous (1→3) linkages. Until

this current work, it remained unresolved whether non-endospermic cell-wall β-D-glucans have such regions.

The present structural studies of *Zea* shoot cell-wall β-D-glucan using *B. subtilis* β-D-glucanase and *Streptomyces* cellulase clearly show that ~92% of the β-D-glucan is made up of 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose in the molar ratio of 3.2:1, joined mainly by single (1→4) linkages. The glucan also contains blocks of more than four contiguous (1→4)-linkages and regions of two, three, or four contiguous (1→3)-linkages- the latter accounts for 0.2–0.3% of the linkages in the glucan.

## EXPERIMENTAL

*Materials.* — Cellulase from *Streptomyces* QM B814 was kindly provided by Dr. E. T. Reese, Pioneering Research Laboratory, U. S. Army Natick Laboratories, Natick, MA, U.S.A. β-D-Glucosidase from almond and alpha amylase (Type I-A) from porcine pancreas were purchased from Sigma. (1→3),(1→4)-β-D-Glucan 4-glucanohydrolase (β-D-glucanase) was purified from Novo Ban 120 (an enzyme preparation from *Bacillus subtilis*, Novo Industri A/S, Copenhagen, Denmark). The purification of this enzyme is described in a separate paper of this series[22]. 3-*O*-β-Cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose were prepared from a *B. subtilis* β-D-glucanase-hydrolyzate of *Avena* glucan. (1→3)-β-D-Gluco-oligosaccharides and (1→4)-β-D-gluco-oligosaccharides were prepared from the partial acid-hydrolyzate of pachyman and from the acetolyzate of cellulose, respectively. Bio-Gel A-1.5m (100–200 mesh) and Bio-Gel P-2 (~400 mesh) were purchased from Bio-Rad.

*General methods.* — Concentration of carbohydrate solutions by rotary evaporation was performed under diminished pressure at 35–40°. Paper chromatography (p.c.), both analytical and preparative, was performed on Whatman No. 1 filter paper by the multiple ascending method with 6:4:3 (v/v/v) butanol–pyridine–water (method *A*) or the descending method with 5:3:1:3 (v/v/v/v) butanol–pyridine–benzene–water (method *B*). Neutral sugars on the chromatogram were detected with alkaline silver nitrate[23]. Total carbohydrate was determined by the phenol–sulfuric acid method[24]. Reducing potential was measured by the Nelson–Somogyi method[25,26]. Enzymic hydrolysis of poly- and oligo-saccharides was performed in the presence of a few drops of toluene to prevent microbial contamination. Gas–liquid chromatography (g.l.c.) was performed with a Varian Model 3700 gas chromatograph.

*Analysis of neutral sugars in oligo- or poly-saccharides.* — Polysaccharide (100 μg as glucose equivalent) was hydrolyzed with 2M HCl for 5–6 at 100°, and oligosaccharide (20 μg as glucose equivalent) was hydrolyzed with M HCl for 3–4 h at 100°. After hydrolysis, *myo*-inositol (10 μg, internal standard for g.l.c.) was introduced into the hydrolyzate, which was then evaporated to dryness with a stream of air. Sugars were reduced with 200 μg of $NaBH_4$ in 0.5 mL of 0.5M

$NH_4OH$ overnight at room temperature. The solution was acidified with acetic acid, evaporated to dryness with a stream of air and methanol was distilled from the residue to remove borate. The reduced sugars were then dissolved in 0.3 mL of 1:1 pyridine–acetic anhydride and heated at 100°. After 2 h, a small volume of water was added to the solution, and the resulting solution was evaporated to dryness with a stream of air. The resulting alditol acetates corresponding to polymer components were analyzed by g.l.c. on a glass column (0.2 × 190 cm) packed with 3% SP-2340 (Supelco) at 210° with a helium flow-rate of 35 mL/min.

*Methylation analysis of poly- and oligo-saccharides.* — Polysaccharide (100–200 μg as glucose equivalent) or oligosaccharide (20–100 μg as glucose equivalent) in dimethyl sulfoxide (0.2 mL) was methylated with methylsulfinyl carbanion (0.1 mL) and methyl iodide (0.1 mL) by the method of Hakomori[2]. The methylated poly- or oligo-saccharide, which was extracted into chloroform followed by evaporation of the extract, was hydrolyzed with 90% $HCO_2H$ for 1 h at 100°. The acid was removed with a stream of air, and the residue treated with 0.5M HCl for 4 h at 100°. The acid was removed with a stream of air. Oligosaccharide (~100 μg in 100 μL of 0.05M $NH_4OH$) was also reduced with 200 μg of $NaBH_4$ at room temperature. After 2–3 h, the solution was made acidic with $CH_3CO_2H$, evaporated to dryness with a stream of air, and methanol was distilled from the residue to remove borate. The reduced oligosaccharide was then methylated as just described and hydrolyzed with 90% $HCO_2H$–0.25M $H_2SO_4$. The methylated sugars were converted into their corresponding alditol acetates[27] followed by g.l.c. analysis on a 10-m glass WCOT capillary column (SP-2330 Supelco) or a 30-m fused-silica capillary column (DB-1, J & W Scientific Inc.) with a linear increase in temperature of from 150 to 230° and a split ratio of 50:1. The helium carrier flow was 1 mL/min.

*Hydrolysis of WSP-II or WSP-III by β-D-glucanase and separation of the hydrolyzate.* — WSP-II (40 mg) and WSP-III (40 mg) were obtained from the insoluble fraction of the buffer-homogenate of *Zea* shoots by treatment with 3M LiCl and hot water[1], respectively. The individual fractions were suspended in 20 mL of 20mM sodium phosphate buffer (pH 6.0) and incubated with a *B. subtilis* β-D-glucanase (7.9 units) for 44 h at 25°. The incubation mixture was then heated in a boiling-water bath for 10 min and centrifuged. The supernatant was concentrated to low volume and applied to a column (1.5 × 150 cm) of Bio-Gel P-2 operated at 50°. Fractions (1 mL) eluted with water were collected and assayed for carbohydrate. Fractions corresponding to tri- and tetra-saccharide were separately combined and concentrated to give oligosaccharide-1 and oligosaccharide-2.

*Characterization of oligosaccharide-1 and oligosaccharide-2.* — Oligosaccharides-1 and -2 were separately characterized in a manner almost identical to that described later for fractions I and K which were obtained from the β-D-glucanase hydrolyzate of fraction 40P-P.

*Confirmation of starch contamination.* — *Method A.* A sample containing 5 mg of carbohydrate was dissolved in 1 mL of 10mM sodium phosphate buffer (pH 6.7), and incubated at 25° with 0.2 units of alpha amylase from porcine pan-

creas (Sigma Type I-A). At intervals, a portion of the incubation mixture was assayed for reducing power and the total carbohydrate content.

*Method B.* A few drops of iodine reagent (0.02% of $I_2$ in 0.2% of KI) was added to a 2-mg sample of carbohydrate in water, and the color of the solution was compared with that of potato starch–iodine complex.

*Preparation and fractionation of WSP-III.* — *Zea* shoots (fresh weight: 3800 g) were homogenized in ice-cold 0.3% NaCl in 10mM sodium phosphate buffer (pH 6.5) using a Waring Blendor and the resulting homogenate was filtered through Miracloth. The insoluble material was treated with 3M LiCl as described previously[1]. LiCl-pretreated *Zea* shoot cell-walls (wet weight, 310 g) were suspended in 1.5 L of boiling water and maintained for 30 min. The suspension was filtered through Miracloth. The residue was washed with 1 L of hot water. The filtrate and the washings were combined and cooled. The combined solution (~2 g as glucose equivalent) was adjusted to pH 6.9 with 0.5M sodium phosphate buffer and M NaOH, and incubated with alpha amylase (15.6 units) for 48 h at 30°. After incubation, the mixture was centrifuged. The precipitated material was washed successively with water, methanol and ether, and dried *in vacuo* to give fraction OP. To the supernatant solution (2.6 L), 520 g of $(NH_4)_2SO_4$ was added gradually at room temperature with stirring. After 5 h, the insoluble material (fraction 20P) was collected by centrifugation. Another 520 g of $(NH_4)_2SO_4$ was gradually added to the supernatant with stirring. After 14 h the mixture was centrifuged to separate insoluble (fraction 40P) and soluble (fraction 40S) materials. Fractions 20P, 40P, and 40S were separately dialyzed at 5° against four 12-h changes of 5 L of distilled water, and then centrifuged. The precipitates designated 20P-P, 40P-P, or 40S-P were washed successively with water, methanol, and ether, and dried *in vacuo*. The supernatants from 20P, 40P, and 40S were designated 20P-S, 40P-S, and 40S-S, respectively.

*Hydrolysis of fraction 40P-P by β-D-glucanase and separation of the hydrolyzate.* — Fraction 40P-P (250 mg) was suspended in 50 mL of 25mM sodium phosphate buffer (pH 6.7) and heated in a boiling water-bath until completely dissolved. After cooling, the solution was incubated at 32° with 119 units of β-D-glucanase. At intervals, a portion (25 μL) of the incubation mixture was assayed for reducing power and the total carbohydrate content determined to calculate the degree of hydrolysis. After 315 min, the incubation mixture was heated for 10 min in a boiling water-bath, and concentrated to ~5 mL. The concentrate was centrifuged. The precipitate was washed with water to give fraction A. The supernatant was applied to a column (3.5 × 45 cm) of Bio-Gel P-2 operated at 25° and eluted with water; 4.9-mL fractions were collected and assayed for carbohydrate (Fig. 2). Tubes 26–33 corresponding to the void volume of the P-2 column were combined to give fraction B. Tubes 34–50 were combined, concentrated to low volume, and centrifuged. The precipitate was washed with water to give fraction M. The supernatant was applied to a column (1.5 × 150 cm) of Bio-Gel P-2 operated at 50° and eluted with water; 1.0-mL fractions were collected and assayed

for carbohydrate. The elution profile is shown in Fig. 3-A. Tubes 101–109, 110–113, 114–119, 121–125, 127–132, and 134–141 were separately combined and concentrated to give fractions C, D, E, F, G, and H, respectively. Tubes 52–54 and 56–59 obtained from the 45-cm P-2 column were separately combined, concentrated, and rechromatographed on the same column to give fractions I and J, respectively. Tubes 60–80 in Fig. 3 were combined, concentraed, and rechromatographed on the 150-cm P-2 column (Fig. 3-B). Tubes 171–179 and 185–195 were separately combined and evaporated to give fractions K and L.

*Hydrolysis of oligosaccharides by* Streptomyces *cellulase QM B814.* — A white powder containing *Streptomyces* QM B814 cellulase (9.5 mg) was dissolved in 0.1M sodium acetate buffer (pH 5.0). Ten $\mu$L of this enzyme preparation contained 0.12 units of cellulase activity: one unit is represented by the production of 180 $\mu$g of glucose equivalent per 10 min in 0.1M sodium acetate buffer (pH 5.0) at 30°, using 0.2% *O*-(carboxymethyl)cellulose. Authentic oligosaccharide (65–70 $\mu$g) in 200 $\mu$L of 20mM sodium acetate buffer (pH 5.0) was incubated for 1 h at 32° with the cellulase (0.06 units). After incubation, the solution was heated for 10 min in a boiling water-bath, and assayed for total carbohydrate content and reducing power. The samples were hydrolyzed, treated with Dowex 50W ($H^+$) resin, concentrated to low volume, and analyzed by p.c. (method *A*). Individual oligosaccharide fractions obtained from the *B. subtilis* $\beta$-D-glucanase-hydrolyzate of fraction 40P-P were incubated for 1 h at 32° with *Streptomyces* cellulase (0.086 units/100 $\mu$g of carbohydrate). After incubation, the solution was heated for 10 min in a boiling water-bath and treated with Dowex 50W ($H^+$) resin. The hydrolyzate was then subjected to paper-chromatographic analysis or gel-filtration on Bio-Gel P-2 followed by p.c.

*Isolation of (1→3)-$\beta$-D-gluco-oligosaccharides from the* B. subtilis $\beta$-D-*glucanase hydrolyzate of WSP-III.* — WSP-III (~2 g as glucose equivalent) was prepared from *Zea* shoots (fresh weight 3800 g) and hydrolyzed with *B. subtilis* $\beta$-D-glucanase as already described. The supernatant was applied to a column (3.5 × 45 cm) of Bio-Gel P-2 and eluted with water. Oligosaccharide fractions having d.p. values ranging from 5–14 were collected; yield 58 mg. The mixture of oligosaccharides in 90 mL of 20mM sodium acetate buffer (pH 5.0) was incubated with *Streptomyces* cellulase (20 mg) for 2 h at 32°. After incubation, the mixture was heated in a boiling water-bath for 10 min, treated with Dowex 50W ($H^+$) resin, concentrated, and centrifuged. The supernatant (~5 mL) was applied to a column (1.5 × 150 cm) of Bio-Gel P-2 and eluted with water. Di-, tri-, tetra-, penta-, and hexa-saccharide fractions were separately rechromatographed on the same column. Yields of di- to hexa-saccharide fractions were 4.4, 4.7, 2.1, 2.7, and 1.2 mg, respectively. The oligosaccharide fractions were separately subjected to preparative p.c. (method *A*). Sugars having the same $R_{Glc}$ values as authentic (1→3)-$\beta$-D-gluco-oligosaccharides were eluted with water. Oligosaccharides a ($R_{Glc}$ 0.84), b (0.66), c (0.54), and d (0.40) were obtained in yields of 2495, 376, 288, and 356 $\mu$g respectively.

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