Studies on the Structure of Gluco-oligosaccharides Obtained from the Partial Acid Hydrolyzate of Sclerotia of *Sclerotinia libertiana*

Part I. Separation of Oligosaccharides from the Acid Partial Hydrolyzate of Defatted Sclerotia

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The defatted sclerotia powder was partially hydrolyzed with dilute acid, and the material obtained was fractionated by carbon column chromatography, separated into two disaccharides, three trisaccharides and three tetrasaccharides, respectively. In these hydrolyzates, α , α -trehalose, laminaribiose, and gentiobiose were identified.

In the course of studying the chemical components of sclerotia of *Sclerotinia libertiana*, an alkali-soluble glucan was isolated from sclerotia. This glucan was partially hydrolyzed with dilute acid, and the hydrolyzate was fractionated by carbon column chromatography (Carbon CC) to give several oligosaccharides composed of D-glucose, laminaribiose and gentiobiose as disaccharides; and presumably 3-O- β -gentiobiosylglucose, 3,6-O- β -diglucosylglucose and laminaritriose as trisaccharides were also isolated. On an acid hydrolysis the fully methylated glucan give 2,3,4,6-tetra-O-methylglucose, 2,4,6-tri-O-methylglucose and 2,4-di-O-methylglucose. The above results shows that this glucan has 1,3- β glucosidic linkage in its main chain, with a D-glucose residue linked with C 6 of intermediate D-glucose residue in the main chain. This glucan with a branched structure of new type was named "Sclerotan" by authors.^{1,2)}

In the previous report,¹⁾ the structure of

glucotrioses was determined only by paper chromatography (PPC) on the hydrolyzates of their sugars and sugar alcohols, as the quantity of glucotrioses from the hydrolyzate of sclerotan was small. Consequently, this method was never satisfactory for a determination of the structure. The structure of a polysaccharide has usually been determined by the chemical behavior of the fragmentary oligosaccharide of polysaccharide.

If the fractionation of the oligosaccharides in the hydrolyzate of sclerotia are same with the partial hydrolyzate of sclerotan, and the fractionation of the oligosaccharides in the partial hydrolyzate of sclerotia is not difficult, the direct partial acid hydrolysis of sclerotia is advantageous for simplification of the fractionation or increased yield of the oligosaccharides. From this standpoint, first, the hydrolyzate of sclerotia was compared with that of sclerotan by PPC. Accordingly three of the hydrolyzate of sclerotia were not detected in the hydrolyzate of sclerotan, and other sugars were the same. Defatted sclerotia was hydrolyzed with 0.02N sulfuric acid at

1) M. Kitahara and Y. Takeuchi, *J. Agr. Chem. Soc. Japan*, **35**, 468 (1961).

2) M. Kitahara and Y. Takeuchi, *ibid.*, **35**, 474 (1961).

150°C for 30 minutes, and the hydrolyzate was fractionated with Carbon CC. The oligosaccharides isolated were gluco-oligosaccharides composed of D-glucose, and their degree of polymerization (DP) was determined by Barker's³⁾ method. Three kinds of di-, tri-, and tetrasaccharides were obtained. It was presumed that a certain regularity exists among the R_F value, the DP and the elution order of Carbon CC of their gluco-oligosaccharides.

Various reports have described a certain regularity between R_F value [$\log(1/R_F)$ ⁴⁾, $\log(1/R_F - 1)$ ⁵⁾ and $\log\{R_F/(1 - R_F)\}$ ⁶⁾] and the DP of the homologous oligosaccharides. Aso et al.⁷⁾, having tested many homologous oligosaccharides series, found that 1,4- α series showed a relation of nearly a straight line between logarithm of a partition function α' , which was defined as $\log[R_F/(1 - R_F)]$ by French⁶⁾, and the molecular size of the oligosaccharides. They also suggested that R_F value can be calculated by the equation, and that the chemical structure of oligosaccharide can be estimated from R_F value. For our part $\log(1/R_F - 1)$ of the oligosaccharides that was separated before by Carbon CC were calculated.

In sugars of each first order of elution in each DP, a relation of straight line was formed between their DP and $\log(1/R_F - 1)$. Similarly, a relation of straight line was formed on sugars of the second order. The results are shown in Fig. 2. Each spots of I, II-2 (glucobiose-2) and III-2 (glucotriose-2) correspond to D-glucose, laminaribiose and laminaritriose, respectively. Therefore, the line (L-2) is presumed to be a line of 1,3- β homologous series. L-1 and L-3 were nearly parallel with

FIG. 1. Correlation among $\log(1/R_F - 1)$, the Elution Order and DP.

R_F value: given in developing by *n*-butanol-pyridine-water (6:4:3).

L-2, and therefore the oligosaccharides on L-1 and L-3 are presumed to have 1,3- β homologous series in each of their molecule. The spot II-1 (glucobiose-1) on the line of the first elution order corresponds to gentiobiose. And spot III-1 (glucotriose-1) on the L-1 exists on the line drawn from spot of II-2 (laminaribiose) parallel to the line combined spot of glucose with spot II-1 (gentiobiose). Thus, glucotriose-1, which shows a properties peculiar to laminaribiose and gentiobiose, corresponds to 3,6- β -diglucosylglucose (6¹- β -glucosyllaminaribiose). Actually, glucotriose-1 has been presumed to be 3,6- β -diglucosylglucose by authors on paper chromatography. From above results, it is presumed that the sugars of the first elution order have a unit of gentiobiose on the reducing terminal side of their molecule, and are a series of gentiobiose, 6¹- β -glucosyllaminaribiose and 6¹- β -glucosyllamin-

3) S. A. Barker, E. J. Bourne, P. M. Grant and M. Stacey, *J. Chem. Soc.*, 1957, 2067.

4) A. Jeanes, C. S. Wise and R. J. Dimler, *Anal. Chem.*, 23, 415 (1951).

5) E. C. Bate-Smith and R. G. Westall, *Biochim. Biophys. Acta*, 4, 427 (1950); R. J. Bayly and E. J. Bourne, *Nature*, 171, 385 (1953).

6) D. French and G. M. Wild, *J. Am. Chem. Soc.*, 75, 2612 (1953).

7) K. Aso and F. Yamauchi, *J. Ferm. Tech., Japan*, 33, 194 (1955).

TABLE I. THE PRESUMED STRUCTURE OF GLUCO-OLIGOSACCHARIDES

Elution order of Carbon CC	Disaccharide	Trisaccharide	Tetrasaccharide
1	○ ↓	○ ↓	○ ↓
2	○ ○→○	○→○ ○→○→○	○→○→○ ○→○→○→○
3	—	○ ↓ ○→○	○ ↓ ○→○→○

○: D-glucose unit →: 1,3-β glucosidic linkage ↓: 1,6-β glucosidic linkage.

aritriose. Oligosaccharide of III-3 (gluco-triose-3) on a line of L-3 corresponds to 3-O-β-gentiobiosylglucose (6²-β-glucosyllaminaribiose), and the line of L-3 is parallel to that of 1,3-β series (L-2). This glucotetraose-3 (III-4) therefore, was presumed 6²-β-glucosyllaminaritriose which has a unit of 6²-β-glucosyllaminaribiose on its reducing terminal side in the molecule. The presumed structures of gluco-oligosaccharides are shown in Table I.

D-Glucose, fucose and rhamnose as monosaccharide were obtained from the partial hydrolyzate of sclerotia. But the forms of fucose and rhamnose in sclerotia are uncertain. α,α-Trehalose, gentiobiose and laminaribiose as disaccharide were isolated and identified by [α]_D, m. p. and their β-acetate. α,α-Trehalose has been isolated from sclerotia.⁸⁾ It is uncertain whether this free trehalose is an unchanged sugar on the partial hydrolysis, or the one linked on the reducing unit of polysaccharide, which was obtained by the hydrolysis.

EXPERIMENTALS

Material

The sclerotia were gathered from the stalks and the roots of rape that tainted by *Sclerotinia libertiana*, in the suburb of Kagamigahara city on June 1962. The sclerotia were washed with water, dried, powdered and defatted with diethyl ether.

Determination of the proper condition of the partial hydrolysis

The defatted sclerotia powder (100 g) added in dilute sulfuric acid solution (20 ml) was hydrolyzed in autoclave at 150°C for 30 minutes. The concentration of sulfuric acid solution used were 0.01 N,

8) M. Kitahara, *Res. Bull. Gifu Coll. Agr.*, No. 68, 64 (1950).

FIG. 2. Paper Chromatograms of the Partial Hydrolyzate of Sclerotia and Sclerotan.

Solvent: *n*-butanol-acetic acid-water (4:1:2); development: five times ascending development.

0.015 N, 0.02 N and 0.025 N respectively. Each hydrolyzate thus obtained was filtered, neutralized with barium carbonate, then concentrated to a syrup. The syrup was dissolved in water, and made up to 10 ml. An aliquot of each solution (0.05 ml) was placed on a sheet of Toyo No. 50 filter paper (15×40 cm) and developed five times with *n*-butanol: acetic acid: water (4:1:2) as the developing solution. The guide strips were cut off from both sides of the chromatogram as shown in Fig. 2 and the position of the sugars were located by aniline hydrogen phthalate (AHP). The corresponding zones to the sugar were cut off from the unsprayed central portion of chromatogram, and eluted with water, made up to 10 ml.

TABLE II. DETERMINATION OF THE PARTIAL HYDROLYZATE OF SCLEROTIA

No. of spot	Concentration of sulfuric acid		
	0.025N	0.02N	0.015N
3	64.8	26.9	7.8
4,4'	9.0	9.9	5.0
5,6	6.9	12.0	8.2
7,8,9	6.2	9.2	7.0
10	4.1	6.3	6.2
11	2.5	5.7	5.1
12	6.5	30.0	60.7

Each fraction is shown as a quantity of glucose, and each number of fraction is a ratio when the total is assumed as 100.

A mixture of 2.5 ml of this sugar solution and 5 ml of anthrone reagent (0.2 g of anthrone in 100 ml of concentrated sulfuric acid) was heated in the boiling water bath for 7 minutes; after cooling, the intensity of color was determined by Hitachi EPB-A spectrophotometer. The concentration of sugars was determined by reference to the standard curve. The results are shown in Table II. The sugars in the hydrolyzate by 0.01 N sulfuric acid were detected very weakly on PPC, therefore the separative determination was not possible. The results of Table II seem to show that the condition by 0.02 N sulfuric acid is most suitable for the separation of oligosaccharides from the partial hydrolyzate of sclerotia.

Preparation of the partial hydrolyzate of sclerotia

The defatted sclerotia powder was hydrolyzed in fifty volumes of 0.02 N sulfuric acid solution in autoclave at 150°C for 30 minutes. After removal of the insoluble substances by filtration, the filtrate was neutralized with sodium hydroxide to pH 6. Saturated basic lead acetate solution was added to the above neutralized solution. The precipitated Pb-complexes were filtered off and the filtrate was then treated with hydrogen sulfide to remove the excess lead acetate. The filtrate was further purified by passing through a column of Amberlite IR-120 and 411 to remove the ionic substances. The purified solution was concentrated in vacuo to a syrup. In the above partial hydrolysis of the defatted sclerotia powder (500 g), 200 g of syrup was obtained. By developing five times with a mixture of *n*-BuOH: acetic acid: water (4:1:2), a chromatogram, Fig. 2 was obtained. It shows about 13 spots of partial hydrolyzate. The spot No. 4' was not detected with AHP but with alkaline silver nitrate.

Separation of sugars in the acid partial hydrolyzate of sclerotia by Carbon CC

The sugars contained in the above acid partial hydrolyzate were each chromatographically separated on Carbon CC by the method of Whistler and Durso.⁹⁾

TABLE III. FRACTIONATION OF THE PARTIAL HYDROLYZATE OF SCLEROTIA BY CARBON COLUMN CHROMATOGRAPHY

Fraction No.	Solvent	Volume of eluate (l)	Sugar*	Yield(g)
1~2	water	6		
3~5	"	6	No. 3	63.3
6~7	5% ethanol	6	No. 3, No. 1	0.5
8	"	3	No. 1, No. 2	3.7
9	10% "	3	No. 4'	6.8
10~11	"	6		
12~13	"	6	No. 5	10.7
14~15	15% "	6	No. 4	7.9
16~20	"	15		
21~22	20% "	4	No. 8	3.3
23~25	"	6		
26~27	25% "	4	No. 6	2.0
28	"	2	No. 6, No. 7	3.5
29~31	"	6	No. 7	4.9
32~35	25~30% "	8		
36~37	30% "	4	No. 11	0.5
38~40	30~35% "	8		
41	35% "	2	No. 9	0.3
42	"	2	No. 9, No. 10	1.7
43~48	"	10	No. 10	4.5
49~50	40% "	4	No. 10, No. 12	0.2

* Numbers shown here are the same as shown in Fig. 1.

⁹⁾ R.L. Whistler and D.F. Durso, *J. Am. Chem. Soc.*, **72**, 677 (1957).

The partial hydrolyzate of sclerotia (200 g) was dissolved in 2 liters of water. The solution was poured on the column (11×75 cm) composed of the same amount of carbon (Takeda, Shirasagi, 1 Kg) and Celite (No.545, 1 Kg), eluted with water (12 l), and ethanol, 5%(9 l), 10%(15 l), 15%(21 l), 20%(16 l), 30%(12 l), 35%(18 l) and 40%(4 l) successively. Each eluate was concentrated to a syrup under reduced pressure and sugars were detected by PPC, and further dried. The results are shown in Table III. Each oligosaccharides were isolated almost as a simple fraction, and the complete acid hydrolyzate gave only D-glucose.

Determination of the degree of polymerization of oligosaccharides by paper ionophoresis

The degree of polymerization (DP) of oligosaccharides separated with Carbon CC were determined by the method of Barker et al.³⁾ The separation was made by paper ionophoresis (PI) as their N-benzylglycosylammonium ion, and by their mobilities the DP were presumed. Each isolated sugar and reference sugar were spotted on a sheet (16×40 cm) of Toyo No. 50 paper and a benzylamine-methanol-10 N formic mixture (1:9:5.5 μ l) was superimposed. After the papers had been kept at 95°C for 5 minutes, the N-benzylglycosylammonium ions were separated by PI at 600 v for 6 hours. As the electrolyte a mixture of 5% aqueous sodium hydroxide (600 ml) and formic acid (400 ml) was used. After drying, the sugars and their complexes were detected with alkaline

silver nitrate. The mobility of component was expressed as the ratio of the distances of the N-benzylglycosylammonium ion from the unchanged sugar, and of the N-benzylglycosylammonium ion from unchanged glucose. The results are shown in Table IV.

Correlation of R_F and the degree of polymerization among each elution order of column chromatography

$\log(1/R_F - 1)$ of the oligosaccharides that separated before with Carbon CC were calculated. It was found that oligosaccharides showed a relation of nearly a straight line between $\log(1/R_F - 1)$ and DP, in the each elution order of Carbon CC. The results are shown in Fig. 1.

Identification of sugars

i) **D-Glucose.** When the concentrated eluted fraction by water (No. 3-5) which contained D-glucose alone was kept at the room temperature, abundant crystalline substances were obtained, which was recrystallized from absolute methanol, m.p. 146°C, $[\alpha]_D^{20} = +110.2 \rightarrow +52.3^\circ (c=5, l=2, \text{water})$. Melting point of this substance was unchanged on admixture with authentic D-glucose anhydride.

ii) **Eluted fraction by 5% ethanol.** The fraction eluted (No. 8) by 5% ethanol contained two kinds of monosaccharides, and their R_F values were in good agreement with those of rhamnose and fucose, respectively, though the authors have completely not identified it yet.

iii) **α, α -Trehalose.** The first eluted fraction No. 5 by 10% ethanol presented one spot by alkaline silver nitrate on the paper chromatogram, but no spot was detected by AHP. This syrup showed no reducing power against Fehling's reagent, but when hydrolyzed with dilute sulfuric acid at 100°C for 1 hour, the sugar was detected as D-glucose by PPC. The R_F value of this substance was in agreement with that of trehalose. This fraction was concentrated under reduced pressure, dissolved in hot methanol, and the insoluble materials were filtered off. The excess methanol was removed by evaporation under reduced pressure, and thus the concentration and filtering were repeated until a white amorphous powder was obtained, the yield was 4.7 g. The amorphous substance was crystallized from 80% ethanol. m.p. 96~97°C, $[\alpha]_D^{20} = +179^\circ (c=1, l=1, \text{water})$. Melting point was unchanged on admixture with α, α -trehalose dihydrate (m.p. 96~97°C).

A mixture of crystal (50 mg), acetic anhydride (2 ml) and newly fused sodium acetate anhydrous (50 mg)

TABLE IV. DETERMINATION OF THE DEGREE OF POLYMERIZATION OF SEPARATED OLIGOSACCHARIDES BY PAPER IONOPHORESIS

(Barker's Method)			
Sugar	Mobility	DP	
D-glucose	1.00	1	
No. 4	0.69	2	(glucobiose-2)
No. 5	0.70	2	(glucobiose-1)
maltose	0.68	2	
No. 6	0.56	3	(glucotriose-2)
No. 7	0.55	3	(glucotriose-3)
No. 8	0.55	3	(glucotriose-1)
maltotriose	0.55	3	
No. 9	0.40	4	(glucotetraose-2)
No. 10	0.42	4	(glucotetraose-3)
No. 11	0.39	4	(glucotetraose-1)

Electrolyte: 5% aqueous sodium hydroxide-90% formic acid (6:4), pH 1.8; ionophoresis: 600v for 6hr; mobility: ratio of the distances separating (a) the N-benzylglycosylammonium ion from the unchanged sugar and (b) the N-benzylglycosylammonium ion from unchanged glucose.

was heated at 120°C for 20 minutes, then kept at 100°C for 1 hour on the oil bath. After cooling, the resulted acetylated material was poured into 10 ml of ice water. After the hydrolysis of residual acetic anhydride, the sugar acetate was extracted with chloroform. The extract was washed with aqueous solution of sodium carbonate and subsequently with water. After removal of solvent by evaporation under reduced pressure, the amorphous material was dried in a vacuum desiccator. Yield 100 mg. The crude acetate was crystallized from 95% ethanol after being kept at room temperature; Recrystallized from the same solvent, m.p. 72~73°C. By Matsuda's method¹⁰⁾ this acetate was dried under reduced pressure over phosphorous pentoxide at 65°C for 2 hours; m.p. was 96~97°C, unchanged on admixture with the authentic specimen dried in the same way. $[\alpha]_D^{20} = +168^\circ$ ($c=1.25$ l=1, chloroform). Its infrared spectrum was very close to that of α, α -trehalose octaacetate.

iv) **Gentiobiose.** The R_F latter half (glucobiose-1) of eluted fraction of 10% ethanol agreed with that of gentiobiose on PPC, which was evaporated under reduced pressure to give 10.7 g of a syrup. This syrup was dissolved in hot methanol, filtered and evaporated to a syrup. Methyl cellosolve¹¹⁾ was used as solvent in crystallization of gentiobiose. A portion (5 g) was dissolved with hot methyl cellosolve (150 ml), filtered, kept in oven at 80°C for overnight, then left at room temperature for a week. Yield 2.5 g. This crystal was recrystallized from

the same solvent. Treatment of a portion (100 mg) with acetic anhydride and sodium acetate at 100~120°C for 2 hours gave crystalline β -gentiobiose octaacetate. Yield 190 mg. After recrystallization from ethanol, m.p. and mixed m.p. were 192~193°C. $[\alpha]_D^{20} = -5.2^\circ$ ($c=1$, l=1, chloroform) Wolfrom et al.¹²⁾ gave m.p. 191.5~192.5°C and $[\alpha]_D^{20} = -4.6^\circ$ ($c=3.3$, chloroform).

v) **Laminaribiose.** The elution fraction (glucobiose-2) of 15% ethanol (No. 14~15, 61), in which laminaribiose was detected by PPC, was evaporated to a syrup under reduced pressure, yield 7.9 g. The syrup (3 g) was dissolved with methanol, filtered off the impurity, and the filtrate was evaporated to a syrup. Resulted syrup was again dissolved with hot methanol, and kept at room temperature to give the crystal, yield 1.9 g m.p. 195~198°C. After recrystallization from methanol, m.p. rised to 204~205°C, which was unchanged on admixture with anhydrous α -laminaribiose obtained from laminarin. The crystal (100 mg) was acetylated with sodium acetate-acetic anhydride as above described to give a crystalline β -laminaribiose octaacetate 180 mg, m.p. and mixed m.p. 160~161°C. $[\alpha]_D^{20} = -27.8^\circ$ ($c=1$, l=1, chloroform) Peat et al.¹³⁾ gave m.p. 160~161°C and $[\alpha]_D = -28.6^\circ$ (in chloroform).

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10) K. Matsuda, *J. Agr. Chem. Soc. Japan*, **30**, 119 (1956).

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