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Note

Studies on the linkage specificity of a β-transglucosylase of *Sclerotinia libertiana**

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This work shows that transglucosylase of *Sclerotinia libertiana* yields oligosaccharides from cellotriose or 3-*O*-β-cellobiosylglucose, but does not act on laminaratriose, 3-*O*-β-gentiobiosylglucose, 4-*O*-β-laminarabiosylglucose, and several disaccharides of D-glucose. β-(1→4)-Linked D-glucose at the nonreducing end of the substrate appears to serve as the glucosyl donor. Tetra- and penta-saccharides arising through transglucosylase action on cellotriose are exclusively β-(1→4)-linked. The tetrasaccharide is degraded to D-glucose by a β-D-glucosidase but not by an α-D-glucosidase. The transglucosylase is judged to produce (1→4)-β-D-glucosidic linkages, and appears to be able to produce cellulose molecules of high molecular weight from cellotriose.

Enzymes mediating transglycosylation without participation of nucleotide sugars have already been reported[1–6]. Thus, bacterial levansucrase synthesizes levan from the D-fructosyl moiety of sucrose and releases D-glucose[1]. However, much less has been reported concerning β-transglucosylases[7,8]. Previous papers[9,10] reported that *Sclerotinia libertiana* produces β-transglucosylases that synthesize a tetrasaccharide from cellotriose and a pentasaccharide from cellotetraose. The enzyme also produces higher oligosaccharides, whose d.p. values were larger than 7, from cellotriose or cellotetraose[9].

In general, saccharide hydrolases have the capacity for transglucosylation, but D-glucosidases generally transfer much more readily than D-glucanases. The reaction mediated by D-glucosidase is thought to be a simple reversible hydrolysis and thus accompanied by hydrolysis activity[11,12]. However, the transglucosylase of

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Sclerotinia libertiana did not show hydrolytic activity as it mediates transglucosylation[10], and it may thus be a specific transglucosylase.

This work examines the action of transglucosylase on several substrates and the linkages synthesized by the enzyme. The enzyme has the ability to synthesize a form of cellulose from cellotriose without the participation of nucleotide sugars.

RESULTS AND DISCUSSION

The action of the transglucosylase on the following substrates was examined; cellotriose (**1**), 3-*O*-β-cellobiosyl-D-glucose (**2**), laminaratriose (**4**), 3-*O*-β-gentiobiosyl-D-glucose (**3**), and 4-*O*-β-laminarabiosyl-D-glucose (**5**) (200 μg of each). The profiles observed on Bio-Gel P-2 chromatography of each digest are shown in Fig. 1. A tetrasaccharide and a pentasaccharide from **1** and a tetrasaccharide from **2** were produced. The enzyme transfers a D-glucosyl group from one trisaccharide to another, yielding tetra- and di-saccharide molecules. The D-glucosyl group to be transfered is at the nonreducing end of the substrate, as only laminarabiose was detected as the disaccharide product of the transglucosylase acting on **2**. Production of a pentasaccharide from cellotriose indicates that the tetrasaccharide product of the transglucosylase can also be an acceptor of D-glucosyl groups. The enzyme did not appear to act on substrates having β-(1→3)or β-(1→6) linkages at their nonreducing ends (Fig. 1). Thus, (1→4)-β-D-glucosidic linkages at the nonreducing end of the substrate appeared to be required for the action of the enzyme.

Action of the transglucosylase on disaccharides was examined by using cellobiose, laminarabiose, gentiobiose, maltose, and isomaltose as substrates. The transglucosylase products in the enzyme digests were separated by chromatography

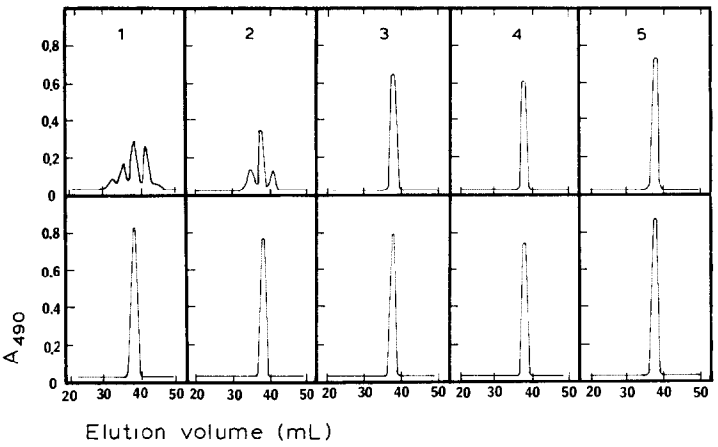


Fig. 1. Elution profiles on Bio-Gel P-2 of cellotriose (**1**), 3-*O*-β-cellobiosyl-D-glucose (**2**), 3-*O*-β-gentiobiosyl-D-glucose (**3**), laminaratriose (**4**), and 4-*O*-β-laminarabiosyl-D-glucose (**5**) treated with the transglucosylase. Top row: Products of the transglucosylase reaction Bottom row: Respective substrate profiles.

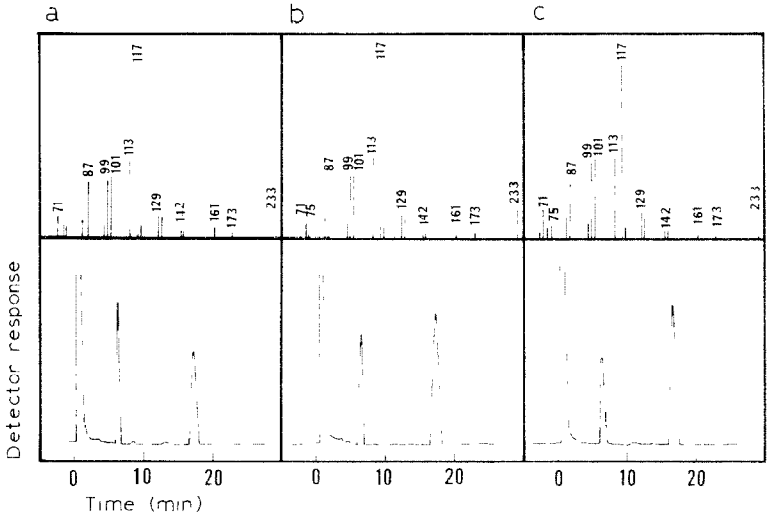


Fig. 2 Gas–liquid chromatograms of methylated sugars derived from tri- (a), tetra- (b), and penta-(c)-saccharide of the transglucosylase products, and mass spectrograms of the second component separated by g.l.c.

on Bio-Gel P-2, and no trisaccharide was detected, nor any sugar components except substrate. The transglucosylase thus appeared inactive on disaccharides. In general, exoglycanases have a strong affinity for long-chain oligosaccharides, but disaccharides are resistant to the enzymes. The substrate specificity of the transglucosylase thus seems to resemble that of an exoglucanase. Moreover, nojirimycin, an inhibitor for both D-glucosidase and exoglucanase, hindered the transglucosylase reaction, although D-glucono-1,5-lactone, an inhibitor for D-glucosidase did not affect it[10].

For analysis of the linkages synthesized, cellotriose (7 mg) was treated with the transglucosylase and the product subjected to chromatography on Bio-Gel P-2. Tri- (1.7 mg), tetra- (1.2 mg), and penta-saccharides (0.7 mg) were obtained, and methylated by the method of Hakomori[13], and subjected to formolysis and hydrolysis. Hydrolyzates were successively reduced, acetylated, and subjected to g.l.c. Partially methylated sugars derived from tri-, tetra- or penta-saccharides were separated on g.l.c. (Fig. 2) Retention times on gas chromatograms of the methylated sugars indicate that they are 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol. The molar ratios of these methylated sugars were ~1:2, 1:3, and 1:4 for tri-, tetra-, and penta-saccharides, respectively. As tri-*O*-2,3,6-tri-*O*-methyl-1,4,5-glucitol is coeluted with 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol on ECNSS-M or SE-30 columns, the identity of the former was confirmed by g.l.c.–m.s. The mass-fragmentation spectra (Fig. 2) indicate that the products of the action of the transglucosylase on cellotriose contained exclusively β-(1→4) linkages.

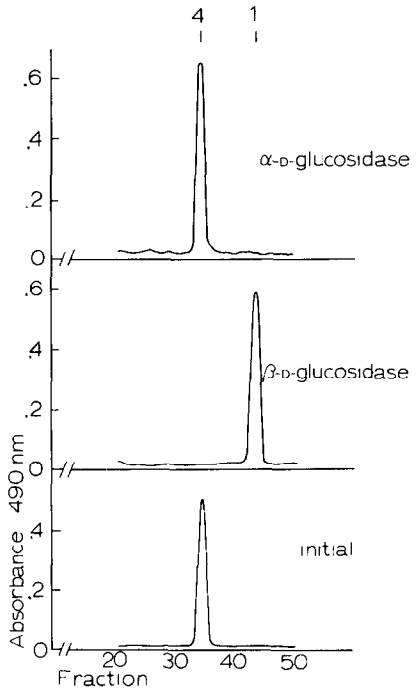
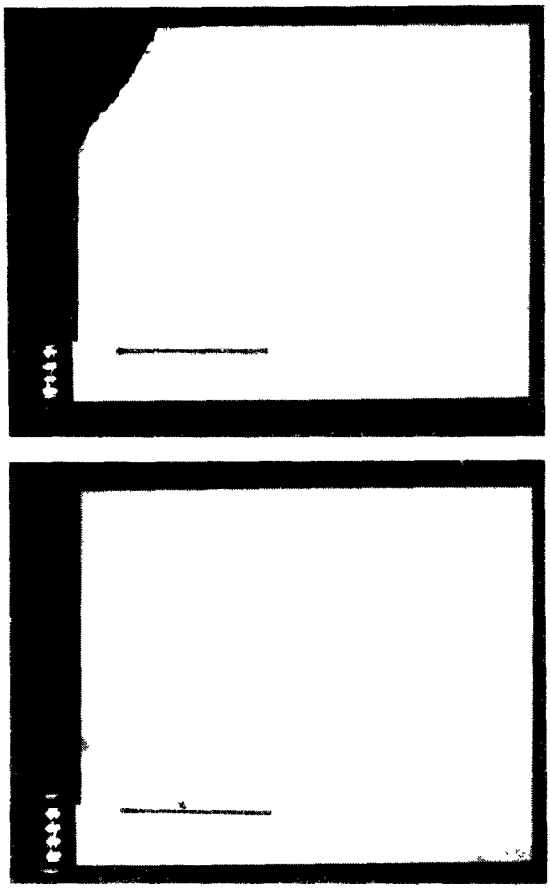


Fig. 3. Elution profiles on Bio-Gel P-2 of the tetrasaccharide of the transglucosylase products following treatment with α- or β-D-glucosidase.

The anomeric configurations of glucosidic linkages synthesized by the enzyme were examined. The tetrasaccharide produced from cellotriose by the transglucosylase was treated with α- or β-D-glucosidase. The β-D-glucosidase completely degraded the tetrasaccharide to D-glucose, whereas α-D-glucosidase was inactive (Fig. 3), indicating that the linkage synthesized is β-D. Furthermore, partial hydrolysis of the tetrasaccharide gave cellobiose as the only disaccharide.

The transglucosylase is judged to be an enzyme that cleaves a (1→4)-β-D-glucosidic linkage in the substrate and resynthesizes a (1→4)-β-D-glucosidic linkage on an acceptor molecule.

As already mentioned, the transglucosylase produced a pentasaccharide from cellotriose, and thus the tetrasaccharide synthesized also appeared to act as a D-glucosyl acceptor. If the synthesized pentasaccharide could act as a D-glucosyl acceptor, a hexasaccharide would be produced. If higher orders of synthesized oligosaccharides could continue to act as D-glucosyl acceptors, a form of cellulose should ultimately be produced. In fact, linear, extended fibers were observed in the electron micrograph of the cellotriose solution after 48 h of incubation with the transglucosylase (Fig. 4). The fibers were not observed in the mixture before incubation, and scarcely found after 24 h of incubation. When the enzyme was omitted, the fibers were not observed in the mixture. Synthesized fibers were collected and



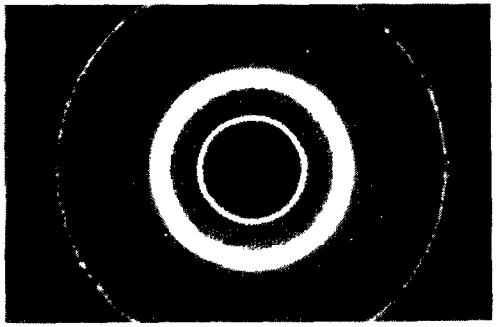


Fig. 4. Electron micrograph of cellotriose solution treated with the transglucosylase. Horizontal bars represent 1 μm.

Fig. 5. X-ray diffraction pattern of insoluble products of the transglucosylase.

subjected to X-ray diffraction analysis. A discrete diffraction-pattern (Fig. 5) was observed, indicative of crystallinity in the transglucosylase products. These facts suggest that the transglucosylase may produce a form of cellulose from cellotriose without participation of nucleotide sugars[14]. Further analysis of the X-ray diffraction pattern will be reported elsewhere. The insoluble product appeared to be soluble in M sodium hydroxide solution but partially insoluble in 0.5M sodium hydroxide and insoluble in 0.25M sodium hydroxide. A large proportion of the product may not have high molecular weight.

EXPERIMENTAL

Transglucosylase assay. — The transglucosylase, identified as T-I in the previous report[9] (16 μg/mL) was incubated with 200 μg/mL of substrate in 0.02M sodium phosphate, pH 5.0, unless otherwise mentioned. The optimum enzyme activity has been reported[9,10] to be at pH 5.0. The substrate oligosaccharides were treated with the enzyme for 60–90 min at 30° and the reaction was stopped by boiling the mixture for 10 min.

Bio-Gel P-2 chromatography. — The mixture was applied to a column (1.2 × 60 cm) packed with Bio-Gel P-2 (400 mesh, Bio-Rad) and eluted with distilled water at 10 mL/h at 50°. The sugar content in each fraction (1 mL) was determined by the phenol–sulfuric acid method[15].

Paper chromatography. — Oligosaccharides in the mixture were examined by descending paper-chromatography on Whatman No. 1 paper in the solvent systems: pyridine–ethyl acetate–water (2:8:1, v/v/v) for 95–135 h and butanol–pyridine–water (6:4:3, v/v/v) for 40 h at 26°. For preparative purposes, Whatman No. 3MM paper was also used. Reducing components on the paper were detected with alkaline silver nitrate reagent[16].

Treatment with glucosidases. — The tetrasaccharide arising from the action of transglucosylase on cellotriose was treated with 4 units of α-D-glucosidase (from yeast, Sigma Chemical) or β-D-glucosidase (from almond, Worthington Biochemical) in 1 mL of solution for 6 h at 30° at pH 7.0 for α-D-glucosidase or pH 4.5 for β-D-glucosidase. The mixture was heated for 10 min at 100° and subjected to chromatography on Bio-Gel P-2.

Preparation of oligosaccharides. — Cellotriose (**1**) was prepared by acetolysis of cellulose (Whatman No. 1 paper). Acetolyzed samples were deacetylated with sodium methoxide and subjected to paper chromatography on Whatman No. 3MM paper. Laminaratriose (**4**) was prepared by Bio-Gel P-2 chromatography of laminaran (*Laminaria hyperborea*) pretreated with 0.2M sulfuric acid for 2 h at 100°. 3-*O*-β-Cellobiosyl-D-glucose (**2**) was prepared from oat-endosperm glucan by hydrolysis with *Bacillus subtilis* glucanase according to the method of Huber and Nevins[17]. 4-*O*-β-Laminarabiosyl-D-glucose (**5**) was prepared from lichenan (*Usnea barbata*) treated with cellulase of *Streptomyces* QM B814. The cellulase was a kind gift from Dr. E. T. Reese of the Food Science Lab., U.S. Department of the

Army, Natick Laboratories. 3-*O*-β-Gentiobiosyl-D-glucose (**3**) was prepared from a laminaran (*Eisenia bicyclis*) treated with *Rhizopus* endo-(1→3)-β-D-glucanase which was purified from an amyloglucosidase preparation (Sigma A 7255) with a column of DEAE-cellulose. Yamamoto and Nevins[18] reported that this enzyme preparation degraded laminaran, lichenan, and oat-endosperm glucan, but not cellulose, nigeran, or pustulan.

Methylation analysis — The tri-, tetra-, and penta-saccharides (1.7–0.7 mg) derived from cellotriose treated with the transglucosylase were methylated by the method of Hakomori[13]. Methylated sugars were subjected to formolysis with 90% formic acid for 1 h at 100° and hydrolyzed with 0.2M sulfuric acid[19]. The hydrolyzates were made neutral with barium carbonate, reduced with sodium borohydride, and acetylated with 1:1 (v/v) pyridine–acetic anhydride for 1 h at 100°. Methylated sugars were then examined by g.l.c. at 180° on a glass column (0.5 × 150 cm) packed with 3% ECNSS-M or 3% SE-30 on GasChrom Q. Components on the chromatogram were subjected to mass-fragmentation spectrometry[20] (JEOL D-300). The ionization potential was 70 eV.

Electron microscopy[21,22]. — Cellotriose (5 mg/mL) was treated with 16 μg/mL of the transglucosylase and 0.02M sodium phosphate, pH 5.0, for 48 h at 30° and heated for 10 min at 100° to stop the reaction. A drop of toluene was added to the solution to supress microbial activity. The mixture was then deposited on a copper grid covered with collodium film and stained with 0.5% uranium acetate. Observations were made with a Hitachi electron microscope (h-300) at a magnification of 30,000. Dr. K. Ikenishi of Osaka City University kindly helped with the photography.

X-Ray diffraction analysis. — Cellotriose (10 mg/mL) was treated with 32 μg/mL of the transglucosylase and 0.02M sodium phosphate, pH 5.0, for 48 h at 30° and centrifuged at 1000*g* for 15 min. The pellet was then washed twice with water and dried. Powder diffraction patterns of the dried samples were obtained with CuK_{α} radiation: interplanar spacings 0.398, 0.445, and 0.718 nm.

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