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PURIFICATION AND SOME PROPERTIES OF β-TRANSGLUCOSYLASES OF *Sclerotinia libertiana*, HAVING THE ABILITY TO SYNTHESIZE HIGHER CELLO-OLIGOSACCHARIDES FROM CELLOTRIOSE OR CELLOTETRAOSE

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ABSTRACT

Two specific transglucosylases were extracted from the culture of *Sclerotinia libertiana*. The enzymes were separated from cellulases and cellobiases. Each of the enzymes moved as a single band in poly(acrylamide)-gel electrophoresis. The molecular weights of the enzymes were estimated to be 188,000 by electrophoresis, and 165,000 and 175,000, respectively, by chromatography on Bio Gel P-200. The optimum pH of the reaction mediated by the enzymes was 5.0. The enzymes synthesized a tetrasaccharide from cellotriose and a pentasaccharide from cellotetraose, without production of D-glucose; they also synthesized larger oligosaccharides, in a long-term action, from either substrate.

INTRODUCTION

During the course of a study of the action of *Sclerotinia* exo-β-(1→3)-glucanase on oligosaccharides, we found[1] that the exo-β-(1→3)-glucanase preparation has the ability to produce a tetrasaccharide from 3-*O*-β-cellobiosyl-D-glucose or from cellotriose, and we suspected that the *Sclerotinia* fungus produces a specific transglucosylase that might be separated from the exo-β-(1→3)-glucanase. In fact, we actually isolated two β-transglucosylases from *Sclerotinia libertiana*.

Some saccharide hydrolases have been reported[2–7] to exhibit transglucosylase activity, and to synthesize oligosaccharides without the participation of the glycosyl esters of nucleotides ("nucleotide sugars"). This type of reaction may be regarded as the reverse of hydrolysis, and it has been hypothesized that a reaction the reverse of hydrolysis is the ability to transfer a glycosyl group to the hydroxyl group of an acceptor other than water when the concentration of the acceptor is not negligible compared with that of the water. Thus, the reverse synthesis of oligosaccharides by hydrolases usually requires a high concentration of the substrates.

It is also known that, although β-D-glucosidase exhibits trans-D-glucosylase activity with a high concentration of substrate, this transfer reaction is usually accom-

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panied by hydrolysis of the substrate into D-glucose, and that, once synthesized, the oligosaccharides are degraded after consumption of the substrate[2,3]. We had observed[1] that the trans-D-glucosylase activity in the *Sclerotinia* exo-β-(1→3)-glucanase preparation is effective at low substrate concentrations, *e.g.*, <1mM. In addition, the reaction was not accompanied by production of a significant amount of D-glucose: that is, there was no hydrolytic activity, in which the trans-D-glucosylase acts on 3-*O*-β-cellobiosyl-D-glucose or on cellotriose. The trans-D-glucosylation mediated by the *Sclerotinia* enzyme does not seem to be a reaction the reverse of hydrolysis, and thus the enzyme appears to be a specific β-D-trans-D-glucosylase. A procedure for the purification, and some properties, of the β-trans-D-glucosylase are described herein.

EXPERIMENTAL

Measurement of enzyme activity. — *Transglucosylase.* The *Sclerotinia* transglucosylase transfers a D-glucosyl group from one cellotriose molecule to another cellotriose molecule, with the production of cellobiose and cellotetraose. Thus, the trans-D-glucosylase activity is proportional to the amount of cellobiose or cellotetraose produced, as the enzyme lacks hydrolytic activities. In this study, the amount of cellobiose was tentatively used as the measure of trans-D-glucosylase activity. The reaction mixture (100 μL) consisting of 1.0% cellotriose solution (50 μL), 20 μL of 0.05M acetate buffer, pH 6.0, and enzyme solution (30 μL) was incubated for 5 h at 30°. Products of the enzyme reaction were placed on plates of silica gel 60 (Merck). The chromatograms were developed in solvent system *1*, 8:1:1 1-butanol–pyridine–water, by two ascents of the solvent during 5 h. The components on the plate were detected by a quick dip of the plate into ethanol containing 10% of sulfuric acid, followed by heating for 10 min at 120°. The amount of cellobiose on a chromatogram was determined with a densitometer (Toyo Densitorol DMU-2). Enzyme activity was referred to as 1 unit when 1 μmol of cellobiose was produced under these conditions. For analysis of trans-D-glucosylase reaction-products, the thin-layer chromatogram was developed in solvent system *2*, *viz.*, 6:4:3 1-butanol–pyridine–water, for 5 h.

Cellulase. Filter paper (Toyo No. 51 A) homogenized in M/30 acetate buffer, pH 3.5, was used as the substrate for measurement of cellulase activity. Eight mL of the substrate suspension (1.25%) was incubated with the enzyme solution (1 mL) for 10 min at 40°, and the reaction was stopped by adding M NaOH (1 mL). The mixture was then centrifuged, and the sugar content in the supernatant liquor was determined by the Nelson–Somogyi method[8]. Cellulase activity was referred to as 1 unit when reducing sugar equivalent to 1 μmol of D-glucose was produced in 1 min under these conditions.

Cellobiase. A reaction mixture (1 mL) containing 0.5 mL of 0.02M acetate buffer, pH 4.5, cellobiose (100 μg), and the enzyme solution (0.5 mL) was incubated at 40°. After 10 min, the content of reducing sugar in the mixture was determined by the

Nelson–Somogyi method[8]. Cellobiase activity was referred to as 1 unit when a reducing equivalent of 1 μmol of D-glucose was liberated in 1 min under these conditions.

Preparation of cellotriose and cellotetraose. — Filter paper (Toyo No. 2; 80 g) was acetolyzed with 10:10:1 acetic acid–acetic anhydride–sulfuric acid (800 mL) for 4 days at room temperature. The acetolyzed sample was washed with water, and then deacetylated with 0.05M sodium methoxide for 3 h. The deacetylation products were dissolved in water, and the base neutralized with Amberlite IR-120B (H^+) resin, to obtain a mixture of cello-oligosaccharides. The mixture was subjected to chromatography on a column packed with charcoal (Wako Pure Chem.). The column was washed with water and 5% ethanol, and then cellotriose and cellotetraose were successively eluted with 10% and 14% ethanol.

Electrophoresis. — Electrophoresis of proteins on poly(acrylamide) gel was conducted according to the method of Weber and Osborn[9]. Proteins were incubated with 1.0% of SDS and 1% of 2-mercaptoethanol for 24 h at 20°, or with 2.5% of SDS and 5% of 2-mercaptoethanol for 2 h at 60°, and the respective solution placed on a column filled with 5% of poly(acrylamide) gel. Proteins on the column were separated at a current of 8 mA/column for 3.5 h, and detected with 0.25% Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

Purification of trans-D-glucosylase. — The purification procedure and the results are summarized in Table I.

The fungus was inoculated on wheat bran (250 g) moistened with water (400 mL), and incubated for 2 weeks at 25°. The medium was then extracted with water (1 L), and solid ammonium sulfate was added. Proteins precipitated at 65%

TABLE I

SUMMARY OF PURIFICATION OF TRANSGLUCOSYLASE

Treatment		*Volume (mL)*	*Absorbance at 280 nm*	*Activity*			*Specific activity*
				Cellulase units	*Cellobiase units*	*Trans-D-glucosylase units*	
Crude extract		325	2405	286.0	55.7	—	—
Bio Rex 70		780	725	0.0	39.5	—	—
DEAE-cellulose	I	170	80	—	15.3	—	—
	II	140	66	—	5.3	—	—
Bio Gel P-150	I	13.2	4.5	—	0.0	2.9	0.64
(1st)	II	19.8	4.6	—	0.0	2.9	0.63
Bio Gel P-150	I	23.1	2.4	—	—	1.7	0.70
(2nd)	II	16.5	3.2	—	—	2.1	0.65

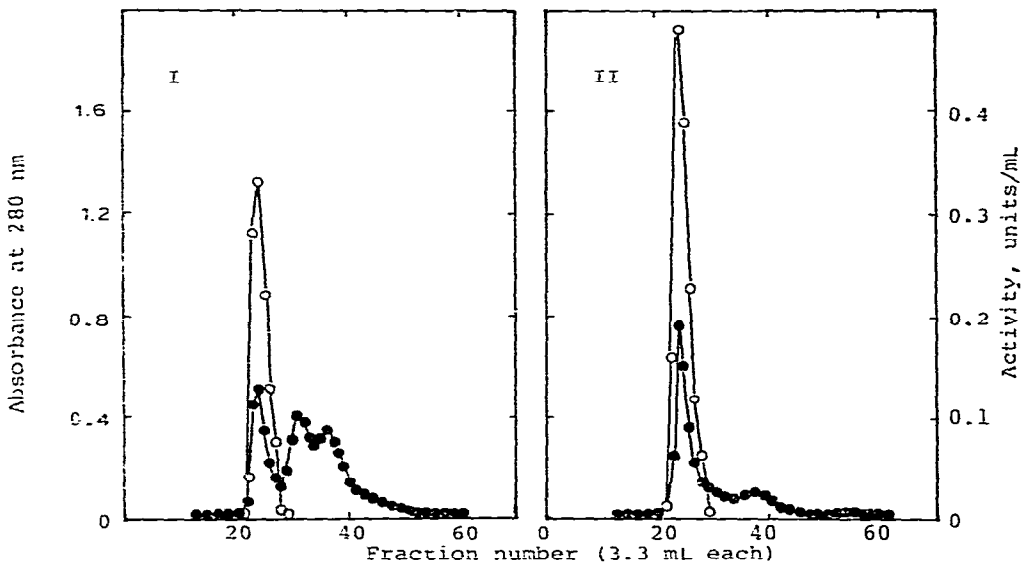

Fig. 1. Gel filtration of trans-D-glucosylase on Bio Gel P-150. [Transglucosylase T-I (I) and T-II (II) were applied to the column (2.5 × 67 cm) packed with Bio Gel P-150. (○), enzyme activity; (●), absorbance at 280 nm.]

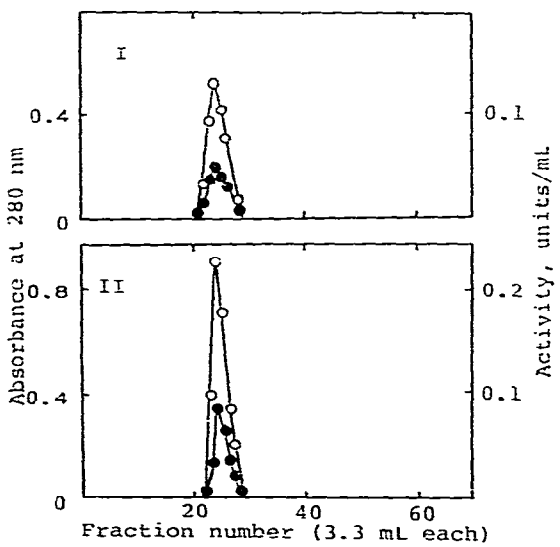

Fig. 2. Rechromatography of trans-D-glucosylase on Bio Gel P-150 (2.5 × 67 cm). [(○), enzyme activity; (●), absorbance at 280 nm.]

saturated ammonium sulfate were collected, and dialyzed overnight against running tap-water. The dialyzed sample was acidified to pH 3.5 with acetic acid, and the precipitate formed was filtered off, to afford crude enzyme solution.

The solution (325 mL) was applied to a column (2.5 × 25 cm) packed with

Bio Rex 70 resin equilibrated with 0.1M acetate buffer, pH 3.5. The column was rinsed with 0.1M acetate buffer, pH 3.5, and pH 4.5 (1 L each). Fractions eluted with 0.1M acetate buffer, pH 6.0, were precipitated with 65% saturated ammonium sulfate. The proteins obtained were dissolved in 0.02M acetate buffer, pH 6.0, and dialyzed against the same buffer. The dialyzed solution was then applied to a column (2.5 × 10 cm) packed with DEAE-cellulose equilibrated with 0.02M acetate buffer, pH 6.0, and the column was eluted with 0, 0.1, 0.2, and 0.5M NaCl in 0.02M acetate buffer, pH 6.0. Trans-D-glucosylase activities found in fractions eluted at 0.2 and 0.5M NaCl are referred to as T-I and T-II, respectively. As these fractions had cellobiase activities (see Table I), the total trans-D-glucosylase activity could not be determined. Cellobiase would degrade products of the trans-D-glucosylase. Fractions comprising transglucosylases, T-I and T-II were separately collected, concentrated (collodion bag) and subjected to chromatography in a column (2.5 × 67 cm) of Bio Gel P-150. T-I and T-II were eluted at fractions 21–28 (see Fig. 1), and fractions 21–26 were combined, and rechromatographed in the same column (see Fig. 2). By chromatography on Bio Gel P-150, cellobiase activity was separated from the transglucosylase activities, and thus their activities were determined (see Table I). The trans-D-glucosylases purified by repeated chromatography with Bio Gel P-150 were used in the following experiments.

Electrophoresis of T-I and T-II. — The purity of enzyme preparations for T-I and T-II was examined by SDS–poly(acrylamide) gel electrophoresis. Fig. 3 shows that T-I and T-II moved as a single band on SDS–poly(acrylamide) gel. Contaminating proteins in the preparations were not detected.

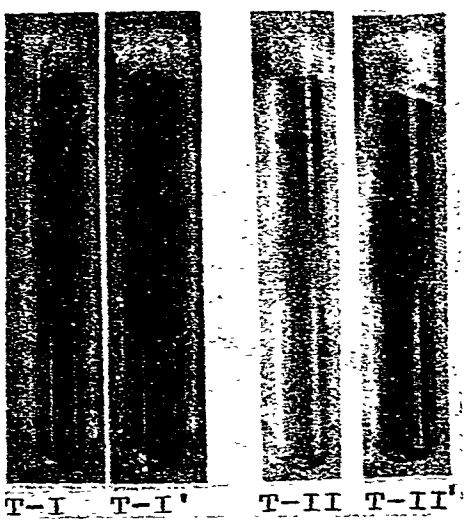

Fig. 3. SDS–poly(acrylamide) gel electrophoresis of trans-D-glucosylase. [Transglucosylase T-I and T-II, 10 μg each, were subjected to electrophoresis for 3.5 h at 8 mA per column. The proteins were stained with 0.25% Coomassie Brilliant Blue. T, incubated with 1.0% of SDS and 1% of 2-mercaptoethanol for 24 h at 20°; T', incubated with 2.5% of SDS and 5% of 2-mercaptoethanol for 2 h at 60°.]

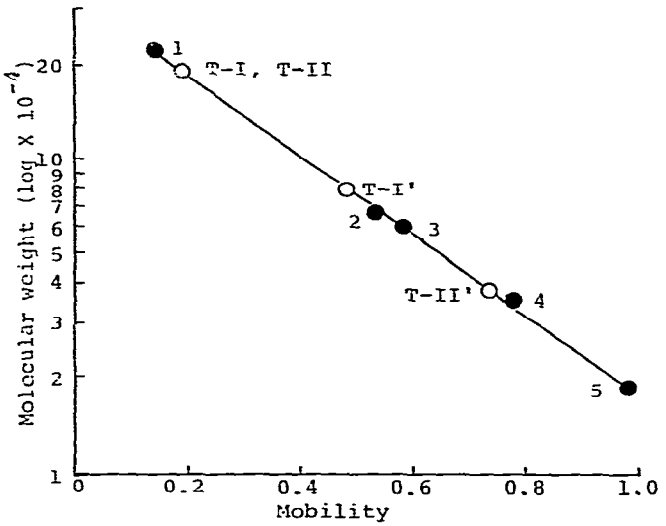

Fig. 4. Determination of molecular weight of trans-D-glucosylase by SDS–poly(acrylamide) gel electrophoresis. [Markers: 1, ferritin (half unit); 2, albumin; 3, catalase; 4, lactate dehydrogenase; and 5, ferritin.]

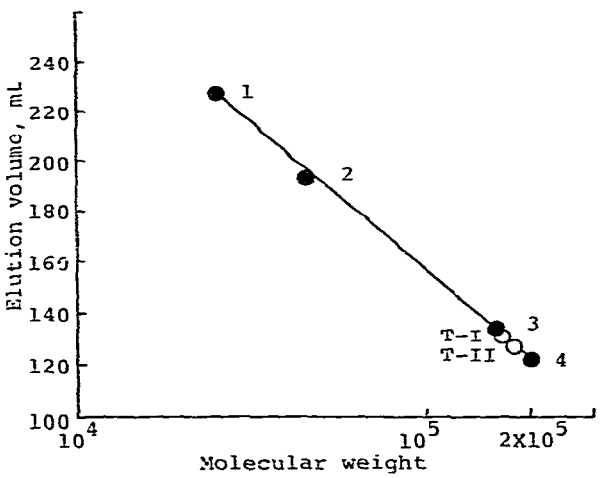

Fig. 5. Determination of molecular weight of trans-D-glucosylase by gel filtration on Bio Gel P-200 (2.5 × 67 cm). [Markers: 1, chymotrypsinogen A; 2, ovalbumin; 3, γ-globulin; and 4, Blue Dextran 2000.]

Determination of molecular weight. — Using the results obtained by electrophoresis, the molecular weight of T-I and T-II was estimated to be ~188,000. Ferritin, ovalbumin, catalase, and lactate dehydrogenase were used as standards for determination of molecular weights (see Fig. 4). On the other hand, after treating the enzymes with 2.5% of SDS and 5% of 2-mercaptoethanol for 2 h at 60°, T-I and T-II were detected as a single band in the region of lower molecular weight on SDS-poly(acryl-

amide) gel (see Fig. 3). T-I and T-II seemed to dissociate into subunits, referred to as T-I′ and T-II′. The molecular weights of T-I′ and T-II′ were determined to be 80,000 and 38,000, respectively, as shown in Fig. 4. T-I and T-II may be a dimer of T-I′, and a tetramer of T-II′, respectively.

The molecular weights of T-I and T-II were also examined by chromatography on Bio Gel P-200. Fig. 5 shows that they are 165,000 and 175,000. Chymotrypsinogen A, ovalbumin, γ-globulin, and Blue Dextran 2000 were used as standards of molecular weights. The molecular weights of T-I and T-II as determined by gel chromatography appeared to be different from each other.

Optimum pH. — To examine the pH dependency of transglucosylase activities, T-I and T-II were incubated with acetate or phosphate buffer of various pH values for 5 h at 30° in the presence of 0.5% of cellotriose. The activities were expressed by the amount of cellobiose formed, as described in the Experimental section. Fig. 6 shows that these enzymes had maximum activities at pH ~5.0. This result agreed

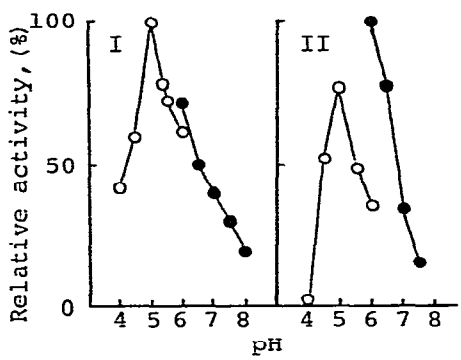

Fig. 6. Dependence of activity of trans-D-glucosylase on pH. [Transglucosylase T-I (I) and T-II (II) were incubated with 0.5% of cellotriose for 5 h at 30°. 0.05M acetate buffer (○) and 0.05M phosphate buffer (●) of various pH values were used.]

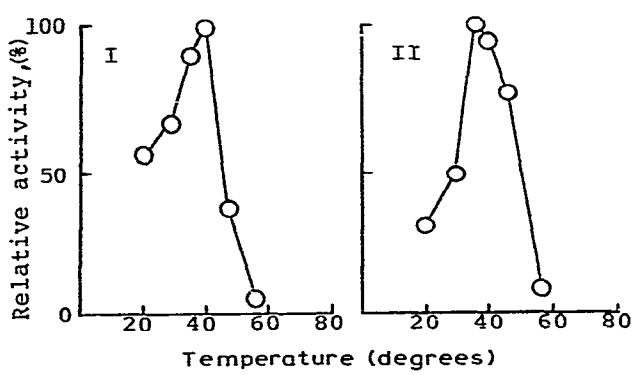

Fig. 7. Thermal activity of trans-D-glucosylase. [Trans-D-glucosylase T-I (I) and T-II (II) were incubated with 0.5% of cellotriose for 5 h at pH 6.0, at various temperatures.]

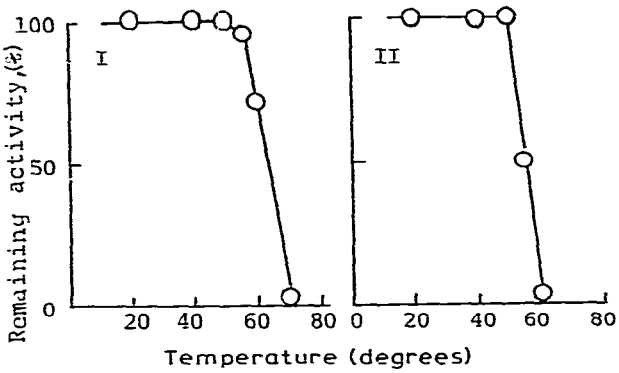

Fig. 8. Thermal stability of trans-D-glucosylase. [Trans-D-glucosylase T-I (I) and T-II (II) were heated for 40 min at the temperature indicated. Then, the enzymes were incubated with 0.5% of cellotriose for 5 h at pH 6.0.]

TABLE II

EFFECT OF METAL IONS ON ACTIVITY OF TRANS-D-GLUCOSYLASE

Metal ion (2 mM)	*Trans-D-glucosylase activity*[a]	
	T-I	T-II
None	100	100
Ca^{2+}	70	78
Mn^{2+}	7	7
Cu^{2+}	8	21
Pb^{2+}	32	100
Hg^{2+}	0	0
Ag^{+}	10	12
Ni^{2+}	77	50
Co^{2+}	61	28
Fe^{2+}	115	250
Fe^{3+}	27	37
Sr^{2+}	129	112
Zn^{2+}	155	14
Ba^{2+}	134	81
Mg^{2+}	136	50
pCMB	64	40

[a]% of the trans-D-glucosylase activity with no metal ions added, as 100%.

with that of previous work[1], in which the amount of cellotetraose formed was measured as the trans-D-glucosylase activity.

Effect of temperature. — The dependence of the enzyme activities on temperature was examined. The activity of T-I was maximal at 40° when it was incubated with cellotriose at pH 6.0 for 5 h, whereas that of T-II was maximal at 37° under the same conditions (see Fig. 7). The thermal stability of the enzyme was also examined

(see Fig. 8). After incubation for 40 min at 60°, T-I retained 70% of the initial activity whereas T-II lost its activity. Both T-I and T-II were stable below 50°, and decay of the activities could not be detected when the enzymes were stored in a refrigerator for several months.

Effect of various, metal ions. — An examination was made as to whether or not some metal ions would modify the activities of the trans-D-glucosylases. Table II shows that Ag^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+}, and Fe^{3+} are inhibitory to the activities of both T-I and T-II. However, Ba^{2+}, Mg^{2+}, and Zn^{2+} showed differential actions on T-I and T-II, being promotive for T-I, but inhibitory to T-II. The Fe^{2+} ion caused a remarkably large promotion of the T-II activity.

Analysis of products. — To analyze trans-D-glucosylase reaction products, cellotriose was incubated with T-I or T-II at 30°, or 40°, for various intervals of time. The reaction mixture was then placed on a silica gel plate, and developed in solvent system *2* (see the Experimental section). The results of the tests at 30° are shown in Fig. 9, and those at 40°, in Fig. 10. Cellobiose and cellotetraose were the major new

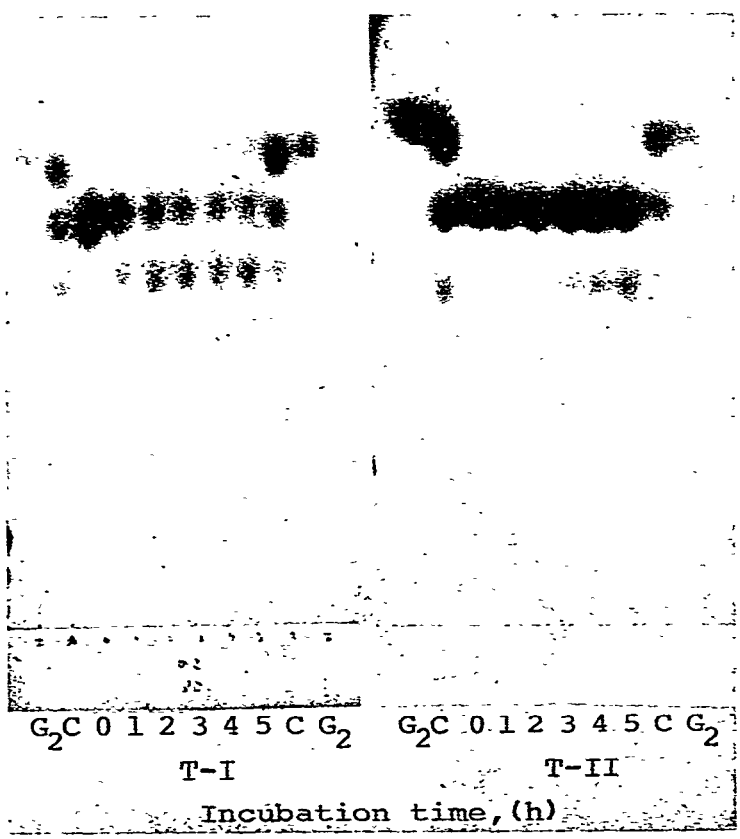

Fig. 9. Thin-layer chromatograms of oligosaccharides synthesized by trans-D-glucosylase. [Trans-glucosylase T-I (3.7×10^{-3} units) and T-II (1.3×10^{-3} units) were incubated with 0.5% of cellotriose for various intervals of time at 30° and pH 6.0. Markers: G_2, cellobiose; C, cello-oligosaccharides.]

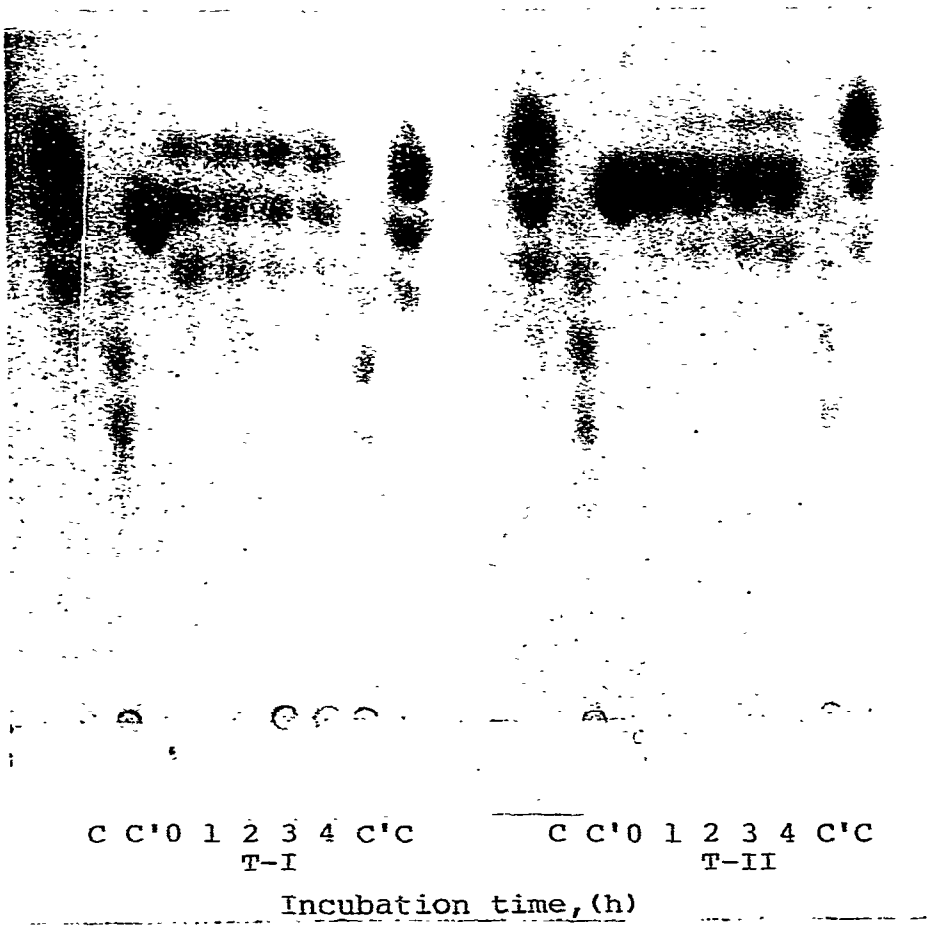

Fig. 10. Thin-layer chromatograms of oligosaccharides synthesized by trans-D-glucosylase. [Trans-glucosylase T-I (3.7 × 10^{-3} units) and T-II (1.3 × 10^{-3} units) were incubated with 0.5% of cellotriose for various intervals of time at 40° and pH 6.0. Markers: C, cello-oligosaccharides; C′, cello-oligosaccharides reduced with $NaBH_4$.]

oligosaccharides produced from cellotriose by the action of T-I and T-II. Little or no D-glucose was detected, indicating that the enzymes do not mediate hydrolysis of the substrate. Thus, it is considered that a D-glucose unit, probably a D-glucosyl group, from the nonreducing end of cellotriose, is transferred to another molecule of cellotriose, resulting in the simultaneous production of cellobiose and cellotetraose. In prolonged incubation, T-I produced oligosaccharides larger than cellotetraose, such as cellopentaose and cellohexaose. T-II also showed a similar ability to synthesize cellopentaose and cellohexaose from cellotriose. As shown in Fig. 10, when T-I was incubated with cellotriose at 40°, it synthesized much larger oligosaccharides (remaining at the origin of the chromatogram) whose d.p. seemed to be >7. On the other hand, alpha-D-glucosyltransferases mediating a similar reaction on malto-

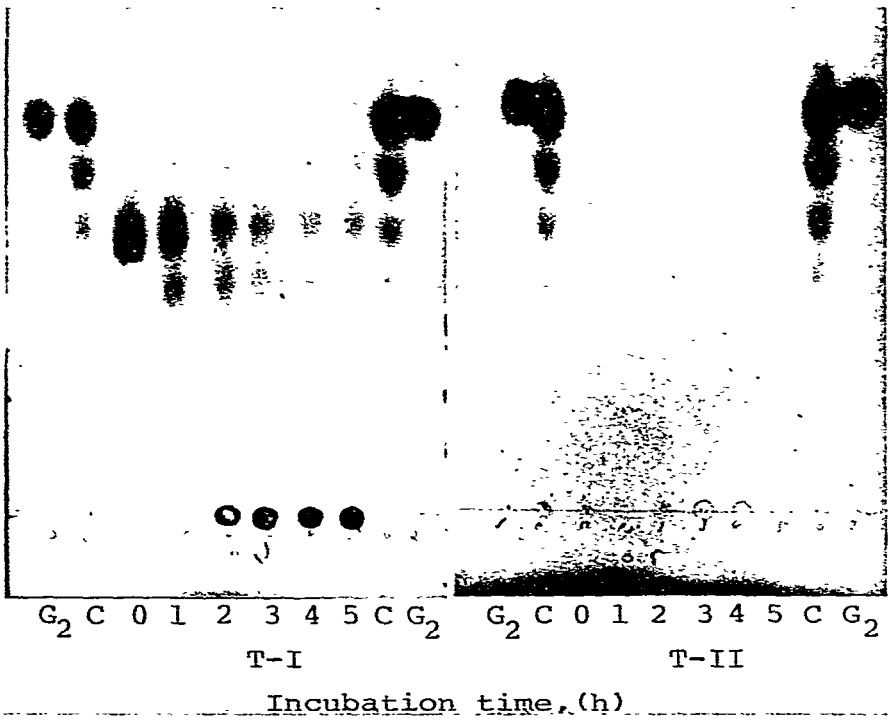

Fig. 11. Thin-layer chromatograms of oligosaccharides synthesized by trans-D-glucosylase. [Trans-glucosylase T-I (3.7×10^{-3} units) and T-II (1.3×10^{-3} units) were incubated with 0.5% of cello-tetraose for various time-intervals at 30° and pH 6.0. Markers: G_2, cellobiose; C, cello-oligosaccharides.]

oligosaccharides were reported by Walker[10,11]. The enzymes can synthesize high malto-oligosaccharides (that are stained with iodine) from maltose, maltotriose, and other malto-oligosaccharides, without the contribution of such nucleotide esters as UDP-glucose. These enzymes were reported to degrade amylose in the presence of D-[^{14}C]glucose as the acceptor, and produced a mixture of malto-oligosaccharides labeled in the (reducing) D-glucose residue. However, the β-trans-D-glucosylase of *Sclerotinia* did not transfer sugars to D-glucose as an acceptor[12].

Fig. 11 shows that T-I and T-II produced oligosaccharides (d.p. >7), that remained at the origin of chromatograms, more effectively from cellotetraose than was noted with cellotriose as the substrate. These results indicated that newly synthesized oligosaccharides would, in turn, become acceptors of the trans-D-glucosylation.

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