TRANSGLYCOSYLATION PRODUCTS OF CELLULASE SYSTEM OF *TRICHODERMA REESEI*

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SUMMARY

From cellulose and cellobiose the formation of sophorose, laminaribiose, and gentiobiose was catalyzed by *Trichoderma reesei* culture filtrate containing exo- and endoglucanase and β-glucosidase activity and from cellobiose by a broken cell suspension from *T.reesei* with β-glucosidase activity. The results indicate that β-glucosidase is the component responsible for transglycosylation reaction catalyzed by *T.reesei* cellulase enzyme complex.

The normal enzymatic degradation products of cellulose by *Trichoderma reesei* are glucose and cellobiose. However, the cellulase enzyme complex has been shown to posses also transglycosylation activity. Toda *et al.* (1968) have found that a mixture of pentasaccharides and higher oligosaccharides were formed from cellotetraose by purified cellulase preparation. According to Okada *et al.* (1975) endoglucanase of the cellulase complex catalyzes the synthesis of cellotetraose from cellobiose. In both studies the sugars were analyzed and identified by paper chromatography, which reveals only the number of glucose units, but not the exact structure of oligosaccharides. Crook *et al.* (1957) have shown that *Aspergillus niger* β-glucosidase catalyzes the formation of a β(1,6)-β(1,4)glucose trimer, gentiobiose (glucosyl-β(1,6)-glucose), laminaribiose (glucosyl-β(1,3)-glucose), and presumably sophorose (glucosyl-β(1,2)glucose) in small amounts.

In analogy with these studies and other known transglycosylation reactions catalyzed by hydrolases /Vaheri and Kauppinen, 1978/ it is conceivable that also the *T.reesei* cellulase complex may give rise to new oligosaccharides and even disaccharides with other than β(1,4)-glycosidic bonds during cellulose hydrolysis.

The true induction mechanism of *T.reesei* cellulase complex is not known but in addition to cellulose at least sophorose, cellobiose, and gentiobiose are known to enhance the cellulase production /Mandels, 1960; Nisizawa, 1971/. It has been suggested that cellobiose liberated from cellulose is the inducer of the enzyme complex in natural conditions /Mandels, 1960; Reese *et al.*, 1972/. The induction caused by cellobiose is, however, relatively weak compared to the effect brought about by sophorose /Mandels, 1960/. Therefore it has been proposed that a glucosyl transfer reaction is responsible for the formation of the inducer from cellobiose /Mandels, 1974/.

In the present study the enzymatic formation of these potential inducers by *T.reesei* cellulase complex was studied using methylation analysis combined with gas chromatography - mass spectrometry.

MATERIALS AND METHODS

Enzyme assays: Filter paper hydrolyzing activity (= endo- and exoglucanase) was measured and expressed as "filter paper" units (FPU) according to /Ghose *et al.*, 1975/. The activity unit was an inverse of the dilution that liberated 0,5 mg reducing sugars in one hour.

The modified method of Mandels and Weber (1969) with 10 min reaction time was used to measure the endoglucanase activity of cellulase enzyme complex with carboxymethylcellulose as substrate ("CMCase"). One unit liberates 1 mg reducing sugars in 10 minutes.

β-glucosidase activity was determined with p-nitrophenyl-β-D-glucoside as substrate according to Berghem *et al.* (1973). One unit of the enzyme liberates 5 μmol p-nitrophenol in 10 minutes.

Determination of reducing sugars: The reducing sugars were determined with the dinitrosalisylic acid method /Miller, 1959/ using glucose as standard.

Conditions for transglycosylation reaction: Two enzyme preparations were used: 1) An ultrafiltrate from *T.reesei* culture medium /Mandels, 1978/ with CMCase (100 U/cm^3), filter paper hydrolyzing (20 FPU/cm^3) and β-glucosidase (0.35 U/cm^3) activity. 2) A broken cell suspension of *T.reesei* cells grown with glycerol as the only carbon source and indicating only β-glucosidase activity (0.35 U/cm^3). As substrates for the enzymatic reaction were used: cellobiose (Fluka A.G.), gentiobiose (Merck A.G.), sophorose (a gift from Biotechnical Laboratory of Technical Reseach Centre of Finland), laminaribiose (isolated from a partial acid hydrolysate of laminaran) and Avicel cellulose pH (Serva).

The reaction mixture consisted of equal volumes of enzyme preparation and acetate buffer (0.05 M, pH 4.8). Substrate concentration in the final mixture was 10 % (w/v) unless otherwise stated. The reaction was allowed to proceed at 50 °C for three days with occasional sampling for the analysis of disaccharides.

Gas chromatography of sugars as trimethylsilyl derivatives: The sugars were separated as trimethylsilyl derivatives by gas chromatography /Brobst, 1972/(Varian Aerograph 1520 fitted with stainless steel columns, 2 mm I.D., length 6 m filled with 3 % OV-17 on 80-100 mesh Chromosorb W-AW).

Separation was accomplished in following conditions: isothermal hold of 12 min at 165 °C followed by 2 °C/min increase to 230 °C, and a final hold of 40 min at 230 °C. Cellobiose, laminaribiose, sophorose, and gentiobiose were identified with the aid of reference disaccharides with combined gas chromatography - mass spectrometry.

The identification of gentiobiose was confirmed with gas liquid chromatography - mass spectrometry on the basis of high m/e 583 fragment /Kamerling *et al.*, 1971/. Partial overlapping of cellobiose, laminaribiose and sophorose cannot be avoided in the gas chromatography. Therefore the gas chromatographic identification of disaccharides was confirmed by methylation analysis.

Methylation analysis: The methylation analysis was carried out according to Lindberg (1972). It involved methylation of all hydroxyl groups in the sugar, hydrolysis of fully methylated oligosaccharide to a mixture of partially methylated sugars, and analysis of this mixture as alditol acetates by retention times of gas chromatograph and as peaks in mass spectra.

RESULTS

Transglycosylation during hydrolysis of CELLULOSE by crude cellulase enzyme complex:

During the enzymatic cellulose hydrolysis in addition to cellobiose and glucose, also sophorose, laminaribiose, and gentiobiose were demonstrated after one day reaction time (fig 1.).

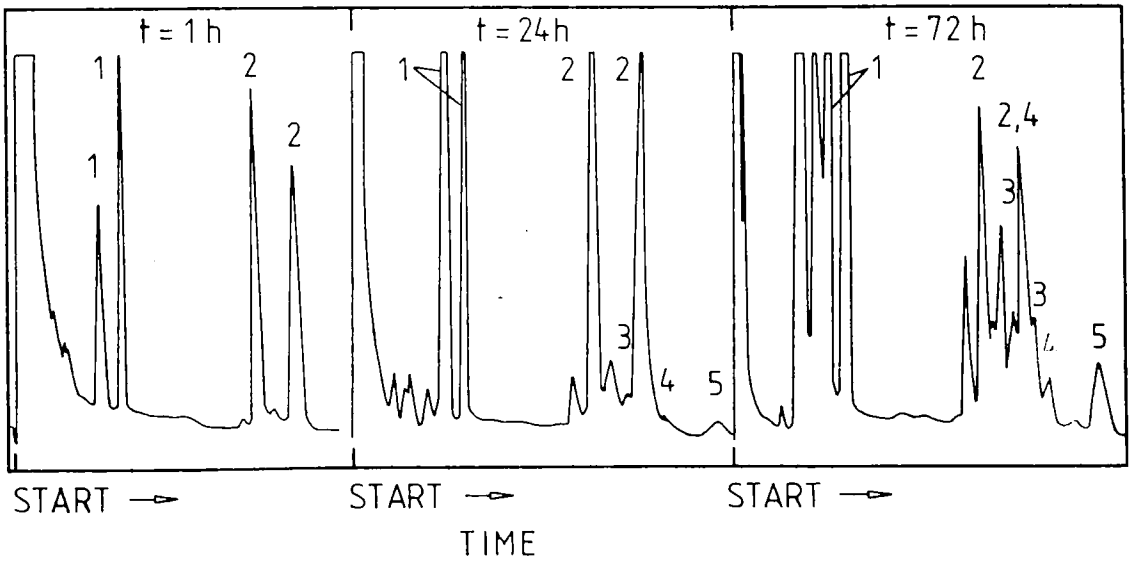
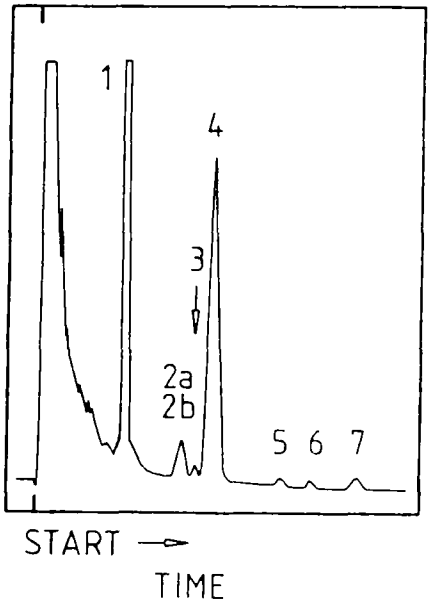


Figure 1. GLC-analysis of sugars in the hydrolysis mixture of cellulose. 1 = glucose; 2 = cellobiose; 3 = laminaribiose; 4 = sophorose; 5 = = gentiobiose.

The gas chromatogram after methylation analysis (fig 2.) showed peak no 2a,b in addition to the ones derived from glucose and cellobiose. On the basis of gas chromatographic retention times and high m/e fragments (β-1,3: m/e 45, 161, 117, 233 and β-1,2; m/e 129 and 189) in mass spectra this peak was concluded to indicate the presence of laminaribiose and sophorose /Lindberg, 1972/.



Identification of GLC-peaks

Peak no	Position of OCH_3	Parent Sugar
1	2,3,4,6	(1-x)glycosidic oligo-saccharides
2a	2,4,6	laminaribiose
2b	3,4,6	sophorose
3	2,3,4	gentiobiose
4	2,3,6	cellobiose
5	2,6	Glu(1→4)Glu(3→x)Glu
6	3,6	Glu(1→4)Glu(2→x)Glu
7	2,3	Glu(1→4)Glu(6→x)Glu

Conditions for gas chromatography

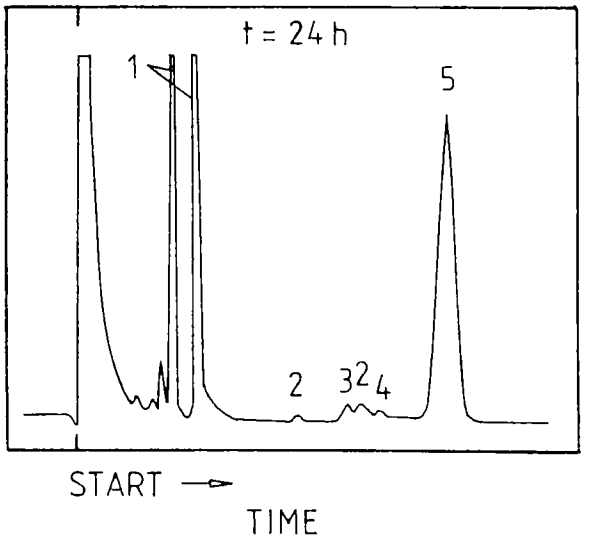
Column: 2-m glass column of 2 mm I.D. filled with 1 % OV-225 on Gas-Chrom Q, 100-120 mesh.
Temperature program: 4 °/min increase from 160 °C to 220 °C.

Figure 2. GLC for methylation analysis of hydrolysis mixture of cellulose.

Semiquantitative estimation by peak height measurement in gas chromatograms showed that sugars formed by transglycosylation reached their maximum concentration after different reaction times. Laminaribiose and sophorose were formed faster and in smaller amounts than gentiobiose.

Transglycosylation during hydrolysis of DISACCHARIDES by crude cellulase enzyme complex:

Both hydrolysis and transglycosylation reactions took place when cellobiose, laminaribiose, and sophorose or gentiobiose were used as substrates. Glucose and small amounts of the other glucosylglucose disaccharides were observed in all cases (fig 3.).



1 = Glucose
2 = Cellobiose
3 = Laminaribiose
4 = Sophorose
5 = Gentiobiose

Figure 3. Transglycosylation products during hydrolysis of gentiobiose by crude cellulase enzyme complex.

Transglycosylation during hydrolysis of CELLOBIOSE by broken cell suspension of T.reesei

When *T.reesei* was grown with glycerol as the sole carbon source the cells contained only β-glucosidase activity but no detectable endo- or exoglucanase activity. The broken cell suspension from these cells also catalyzed transglycosylation reaction with cellobiose as substrate leading to the same sugars as with crude cellulase, only much faster. With 1 % cellobiose new disaccharides were detectable already after half an hour. The maximal concentration of newly formed disaccharides were reached in a few hours and after 22 hours reaction time only traces of them were left.

DISCUSSION

In the studies on the transglycosylation reaction of *T.reesei* cellulase enzyme complex the attention has been focused mainly on endo-β-glucanase, (CMCase) /Toda *et al.*, 1975/. To our knowledge there are no earlier studies on the transglycosylation reaction catalyzed by β-glucosidase of *T.reesei* cellulase enzyme complex, although glucosidases in general transfer glycosyl units more readily than exo- or endo-glucanases. *A.niger* β-glucosidase is known to transfer the glucosyl moiety from a cellobiose unit to the sugar acceptor present most abundantly with mainly

β-1,6-linked oligosaccharides as a result /Crook *et al.*, 1957/. We were able to show that in addition to cellobiose and glucose at least laminaribiose, sophorose, gentiobiose, and some trisaccharides were formed during enzymatic hydrolysis of cellulose with crude cellulase preparation of *T.reesei*. The same disaccharides were formed also with *T.reesei* broken cell suspension containing only β-glucosidase as the enzyme and cellobiose as the substrate. Therefore we believe that transglycosylation during cellulose hydrolysis by the crude cellulase enzyme complex is catalyzed by the β-glucosidase component, which is probably a cell bound enzyme /Berg *et al.*, 1977/ and leaks into the medium during cultivation.

The cell bound β-glucosidase was formed also without induction, with glycerol as the only carbon source. On the other hand, this enzyme catalyzed the formation of the known potent inducers of cellulase complex, sophorose, and gentiobiose by transglycosylation. Since sophorose is known to be considerably more powerful inducer than cellobiose, these results bring the induction mechanism for cellulase enzyme complex into a new light.

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