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Major Transglycosylation Products of β-Glucosidase and Their Induction Effects on Cellulase Biosynthesis *

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Abstract: Using growth conditions selectively favouring β-glucosidase synthesis, we have used ceβ-free preparations of extracellular, plasmβ-membranβ-bound and intracellular β-glucosidase from *T. reesei* and *Aspergillus niger* to comparatively investigate their ability to form transglycosylation products from cellobiose by gas chromatographβ-mass spectrometβ-analysis. The results showed that only the plasmβ-membranβ-boundβ-glucosidases from both fungi exhibited significant transglycosylating activity, and that β-gentiobiose rather than sophorose was the main detectable transglycosylation product containing a β-linked diglucoside. When gentiobiose was added to washed mycelia of *T. reesei* or *A. niger*, it induced cellulase formation although with a lower efficiency as sophorose, the most potent cellulaβ-inducer so far known. These results support the assumption that the plasmβ-membrane bound β-glucosidase forms transglycosylation products from cellulose oligomers, which may act as inducers of cellulase biosynthesis.

Keywords: β-glucosidase (EC 3. 2. 1. 21); transglycosylation; cellobiose; gentiobiose; *Trichoderma reesei*; *Aspergillus niger*; cellulase induction

CLC number: Q 93 1

Many imperfect fungi form an inducible cellulase system, yet the mechanisms by which an insoluble polymer like cellulose can trigger the induction of cellulase formation in microorganism are not fully understood. In *Trichoderma reesei*, there is evidence that conidiaβ-bound cellulases enable a first attack on the cellulose molecule, but the product of this attack (i. e. cellobiose) is only a poor cellulase inducer[1]. Since the most potent cellulase inducer currently known is sophorose (2-*o*-β-D-glucopyranosyl-β-D-glucose), it is commonly believed that inducer formation depends on transglycosylation[1]. Formation of sophorose along with other β-linked diglucosides by a crude cellulase preparation and a broken cell suspension (containing β-glucosidase activity only) has been demonstrated[2], but the enzyme synthesizing sophorose has not yet been identified. Transglycosylating activity is a property of several components of the cellulolytic enzyme system (cf. [1] β-glucosidase; cellobiohydrolase; endoglucanase). Theoretically, the formation of the cellulase inducer would be most efficient if it occurs close to or already within the cell. Umile et al[3] described a constitutive β-glucosidase activity,

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bound to the plasma membrane of *T. reesei*, which they considered a good candidate for the formation of the inducer by transglycosylation. However, *T. reesei* also contains a cellobiose permease and an intracellular β-glucosidase[1], and inducer formation may therefore take place intracellularly as well. The transglycosylating ability of the plasma-membrane bound- and the intracellular β-glucosidases has never been experimentally proven. This paper presents results from such an investigation of comparisons among the different cell-free preparations from different cell locations from two typical strains of *T. reesei* and *Aspergillus niger*, which was initiated to clarify the above points.

1 Materials and methods

1.1 Materials

D (+)-cellobiose ($[\alpha]_n^{(20+35)^\circ}$) was obtained from Fluka, Inc. while glucose and glucose-derived disaccharides (sophorose, cellobiose, gentiobiose, etc.) from Sigma Co.. All other chemicals used were reagent grade and obtained from various suppliers.

1.2 Organism and conditions for cultivation

Trichoderma reesei QM 9414 was obtained from ATCC (No. 26921). *Aspergillus niger* AMS11, was isolated from soil. Both fungi were maintained on malt agar slants. They were grown in mineral salts medium[1], using cellobiose (final conc. 15 mmol/L) selectively favouring β-glucosidase synthesis. A final concentration of 10^7 conidia/mL was inoculated and incubated on a rotary shaker at 180 r/min at 30 ℃ for periods as indicated. To study the induction of cellulase formation by sophorose or gentiobiose, the replacement technique of Sternberg et al[3] was used.

1.3 Preparation of extracellular, intracellular and plasma-membrane-bound β-glucosidases from *T. reesei* and *A. niger*

An 48 h old culture was centrifuged (15 000 r/min, 10 min, 4 ℃), 1 mL of the supernatant dialyzed (24 h, 4 ℃) against distilled water (containing 3 mmol/L NaN_3), lyophilized, taken up in 1.0 mL 10 mmol/L sodium acetate buffer (pH 5.0) and stored at 4 ℃ and used as a source of extracellular β-glucosidase. The mycelia were washed five times with distilled water, and used to prepare protoplasts as described in [3]. The protoplasts were then carefully washed five times with 0.6 mol/L sodium citrate buffer, pH 5.0, and resuspended in the same buffer to yield a final concentration of 10^8 protoplasts/mL. To prepare the cytoplasmic and the plasma membrane-bound β-glucosidase, 1.0 mL of this suspension was centrifuged in an Eppendorf centrifuge (5 min), 1.0 mL of distilled water was added, and the protoplasts were lyzed by incubation with occasional gentle stirring at 4 ℃ for 3 h. Following further centrifugation (25 000 r/min, 4 ℃, 30 min), the supernatant as well as the protoplast membrane debris were collected. The former was treated as above and dialyzed against distilled water containing 3 mmol/L NaN_3 for 24 h. It was then stored at 4 ℃ and used as a source of intracellular β-glucosidase. The protoplast membrane debris was washed five times with distilled water, dialyzed as described above and finally purified by the concanavaline A technique[3]. Alkaline phosphatase (AP, EC 3.1.3.1) and pyruvate kinase (PK, EC 2.7.1.40) were used as marker enzymes for the plasma membrane and the cytoplasm, respectively[4,5]. Only when the membrane preparation exhibited the highest specific activities of AP and BGL, but were almost completely devoid of PK, they were resuspended in 1.0 mL of 10 mmol/L sodium acetate buffer pH 5.0, and stored at 4 ℃, and used as a source of plasma-membrane bound β-glucosidase.

1.4 Assay of transformation products by GC-MS

1.0 mL 17.5 mmol/L D (+) cellobiose ($[\alpha]_n^{(20+35)^\circ}$) was incubated in 10 mmol/L sodium acetate buffer, pH 5.0 with the different β-glucosidase-preparations on a rotary shaker operating at 180 r/min for 48 h at 37 ℃. The mixtures were then centrifuged (25 000 r/min, 4 ℃, 30 min), the supernatants lyophilized, and the carbohydrates converted into triemethylsilyl derivatives[6]. Aliquots of 1

μL were analyzed by gas chromatography-mass spectrometry (GC-MS) on a Finnigan MAT-SSQ 710 unit, using a dbwax/0.18×25 capillary column, and a temperature increase of 15～25 ℃, 35 min. Mass spectrometry was carried out at 70 eV EI and 0.6 sec scanning-time. Data were automatically collected 4～6 min after the sample was injected in order to exclude the interference by the pyridine peak, and ULTRIX 4.2 and ICIS 7.0 software were used for data processing. The identification of individual sugars was carried out automatically using P.A. sugars as calibration standards.

1.5 Cellulase enzyme assays

For demonstration of *T. reesei* CBH Ⅰ in culture supernatants, samples from the culture broth were subjected to SDS-PAGE, followed by Western blotting to nitrocellulose, and immunological detection by a monoclonal antibody against CBH Ⅰ as described previously[7]. β-Glucosidase activity was assayed with esculin (6, 7-dihydrocoumarin-β-1, 4-glucoside) as a substrate. To this end, 1.0 mL of enzyme preparation were mixed with each 1.0 mL of $w=1\%$ solution of esculin and ferriammonium-citrate, both in 50 mmol/L acetate buffer pH 5.0, and incubated at 37 ℃ for 30 min. The reaction was stopped by boiling for 5 min, then kept on ice for 30 min and finally its absorbancy read at 375 nm. Cellulase activity was assayed with carboxymethyl-cellulose (Hercules, Wilmington, DE) as a substrate[1]. Alkaline phosphatase was assayed as described in [4], and pyruvate kinase as in [5].

2 Results

The cell-free preparations of β-glucosidase from the three different locations were adjusted to the same activity of about 8.335×10^{-9} mol/(s·mL), and incubated with 17.5 mmol/L cellobiose. Then the products were analyzed by GC-MS (Fig. 1). As seen from Fig. 1, upon incubation with cellobiose and the β-glucosidase preparations, α- and β-glucose and α- and β-cellobiose (4-*o*-β-D-glucopyranosyl-β-D-glucose) were the major peaks observed in all incubations. From the anomeric mixture of α- and β-D-cellobiose, the β-D-form was hydrolysed to a higher percentage. The key point was that besides the cellobiose and glucose peaks, one (at 2 475) and two (at 2 175 and 2 490) additional peaks were observed in the case of the plasma-membrane β-glucosidase preparations of *A. niger* and *T. reesei*, respectively. The one occuring in the reaction mixture from both fungi (at 2 475～2 490) was identified by MC-analysis as β-gentiobiose (6-*o*-β-D-glucopyranosyl-β-D-glucose), and was also proven by the analysis of reference compounds. The relative amounts of β-D-gentiobiose in the assay were 0.124 and 0.131 mmol/L, corresponding to 0.71% and 0.75% of the initially supplied cellobiose. In addition, other paint peaks at 2 850 and 2 870 for α- and β-cellutriose, resepectively, were detectable in the case of plasma-membrane preparations of both strains. These findings strongly indicates the activities of enzymes which are capable of transfering α- or D-glucose monomer to a second glucose or cellobiose molecule by forming a β-linkage.

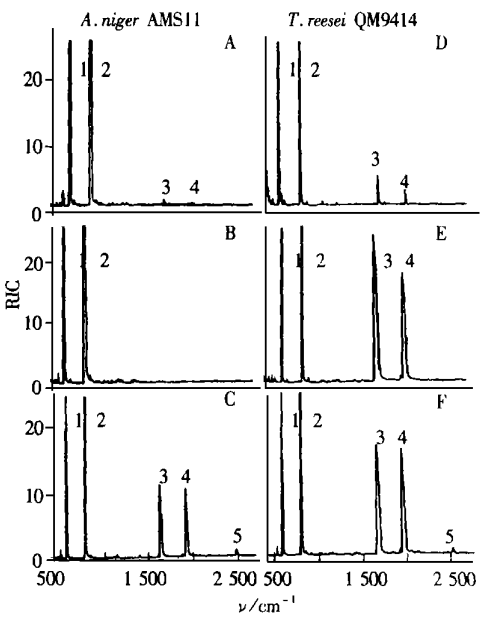

Fig 1 GC-MS RIC graph of sugar-TMS derivatives formed in the reaction of extracellular(A, D), intracellular(B, E) and plasma membrane-bound β-glucosidase(C, F), respectively, with D(+)-cellobiose. Individual identified sugars are as follows: 1 α-D-glucose; 2 β-D-glucose; 3 α-D-cellobiose; 4 β-D-cellobiose; 5 β-D-gentiobiose.

On the other hand, one paint detectable peak at 2 175 only occuring in the reaction mixture of the *T. reesei plasma membrane preparations was identified by the same approach as isomaltose* (6-*o*-β-D-glucopyranosyl-β-D-glucose). This indicates that *T. reesei*- but not *A. niger*- membranes contain an enzyme which is capable of transfering glucose to a second glucose molecule by forming an β-linkage. The nature of this enzyme is not known and requires more investigations.

We have added gentiobiose at a final concentration of 1 ~ 5 mmol/L to resting mycelia of *T. reesei* to investigate whether gentiobiose is actually capable of inducing cellulase formation in both of two typical strains, (Fig. 2). Although gentiobiose induces lower cellulase activities (and cellobiohydrolase I titers as well, data not shown) than sophorose, its inducing ability is clearly documented. The time course of formation as well as the concentration required for half-maximal induction were both the same as with sophorose. In a similar experiment, 2 mmol/L gentiobiose also induced 2.0 mol/(s·mL) cellulase activity in *A. niger* after 20 h of incubation, whereas sophorose yielded 7.0 mol/(s·mL). We conclude from these results that the major β-linke-

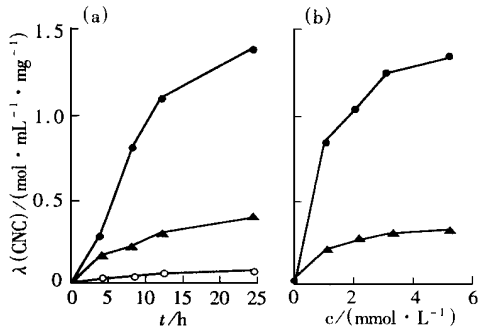

Fig 2　Induction of cellulase activity (CMC-ase) in *T. reesei* by sophorose (●) or gentiobiose (▲)
(a) time course of induction;
(b) effect of disaccharide concentration on the activity obtained after 24 h;
(○) in (a) indicates control without inducer addition

diglucose transglycosylation product gentiobiose detected in this investigation does induce cellulase biosynthesis in both fungi.

3　Discussion

Transglycosylation products were commonly observed with β-glycosidases, and occured whenever the supply of H_2O as the OH-donor was limited (e. g. under conditions of high substrate concentrations, high temperature, low water activity)[8]. The present finding of formation of transglycosylation products by the plasma-membrane bound β-glucosidases of *T. reesei* and *A. niger* would agree well with this rule if it is assumed that the active center of these enzymes faces a hydrophobic environment. The mode of β-glucosidase binding to the membrane is unknown. However, the *T. reesei* enzyme can be released from the membrane by treatment with soft detergents but not by proteases[3], suggesting an embedding of at least part of the protein within the membrane. The absence of transglycosylation products from the mixture of either the extracellular or the intracellular β-glucosidases does not argue against a transglycosylating activity of these preparations, yet show that under the conditions employed are superior for the membrane-bound enzymes. These data therefore support the assumption that the plasma-membrane-bound β-glucosidase is responsible for the transglycosylating activity in vivo, which is also in accordance with previous findings that the β-glucosidase, which is secreted into the extracellular medium by *T. reesei*, is beneficial to growth on cellulose, but dispensable for cellulase induction by sophorose[9]. The almost exclusive formation of gentiobiose indicates preferred transfer to C6, and gentiobiose has also been shown to be the major disaccharide formed by a broken cell preparation of *T. reesei*[2]. Gentiobiose proved to be a stable endproduct of the reaction in the present study.

The riddle as to the true inducer of cellulase formation is unsolved. Sophorose has been discussed as the most probable candidate in *T. reesei*, because it is the most active inducer *in vitro* known so far[1]. However, the identity of the true cellulase-inducer with sophorose has recently been questioned by the findings that sophorose induction of cellulase formation depends on the activity of the plasma-

membrane bound β-glucosidase[9], and is significanths reduced when the latter is inhibited. In view of the present findings, it appears conceivable that sophorose may itself be transglycosylated by the membrane-bound β-glucosidase to a very potent intracellular inducer. However, during growth on cellulose sophorose does not accumulate up to the amounts required for this induction, and its role *in vivo* is therefore rather unlikely. The facts that gentiobiose can be formed from cellobiose, the primary product of cellulase action, and acts as an inducer of cellulase formation in both *T. reesei* and *A. niger* here renders this disaccharide a very good candidate for an inducer under *in vivo* conditions. However, it should be noted that during growth on plant cellulose under natural conditions, there would be several other glycosides and monosaccharides to be available for transglycosylation, and that several cellulase inducers may be formed *in vivo*.

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β-葡萄糖苷酶的转糖基作用主产物及其对纤维素酶合成的诱导效应

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摘 要：以纤维二糖特异诱导培养里氏木霉和黑曲霉菌丝，制备含有细胞外、细胞内和细胞质膜结合态β-葡萄糖苷酶的无细胞抽提物，采用色谱持谱联用分析，比较研究他们对纤维二糖的转糖基作用，结果表明两菌株中都只有细胞质膜结合态β-葡萄糖苷酶表现出显著的转糖基活性，可检测到的含有β-糖苷键葡二糖类的主要转糖基产物是β-龙胆二糖而不是槐糖．当在里氏木霉和黑曲霉菌丝的洗脱置换培养体系中加入β-龙胆二糖时，可以诱导纤维素酶合成，但它比槐糖（迄今最有潜力的诱导物）的诱导效率低．这些结果支持假说认为是质膜结合态β-葡萄糖苷酶从纤维素寡聚物形成转糖基作用产物，作为纤维素酶合成的诱导物．

关键词：β-葡萄糖苷酶（EC3.2.1.21）；转糖基作用；纤维二糖；龙胆二糖；里氏木霉；黑曲霉；纤维素酶诱导

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