TRANSGLYCOSYLATION OF EXTRACELLULAR β -GLUCOSIDASE OF *TRICHODERMA PSEUDOKONINGII* S38 AND ITS FUNCTION IN REGULATION OF CELLULOSE BIOSYNTHESIS

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Two β -glucosidase (β G) components, β G1 and β G2, purified from extracellular culture filtrate of *Trichoderma pseudokoningii* S38 by chromatography techniques, showed transglycosylation activity, the character of which was previously believed to belong to the membrane-bound β G. β G1 also hydrolyzed cellobiose (and other β -linked disaccharides) to produce glucose, and could hydrolyze *p*-nitrophenyl- β -D-glucoside (PNPG). However, the activity of hydrolysis of disaccharides and PNPG of β G2 was lower than that of β G1, but it had higher transglycosylation activity, showing different substrate specificities. The transglycosylation products originating from cellobiose by β Gs, such as all of the other β -linked disaccharides and even cellotriose, were determined by HPLC. Among them, gentiobiose was believed to be the most effective inducer of cellulose biosynthesis in this strains. The induction ability of a certain disaccharide had a relationship with its amount in the traction mixture which was controlled by the dual activities of β G, hydrolysis and transglycosylation.

Trichoderma is one of the most potent cellulase producers known (1). The cellulase of fungi mainly includes endoglucanase (EC 3.2.1.4, EG), cellobiohydrolase (EC 3.2.1.91, CBH) and β -glucosidase (EC 3.2.1.21, β G) (4,20). Among them, EG and CBH are believed to be subject to control by induction and carbon catabolite repression coordinatively (2). β G is regulated by a relatively independent mechanism, and plays an important role in the synthesis of cellulase by insoluble cellulose (10,16,21). Cellulose seems to be a true inducer in the synthesis of cellulase. As insoluble cellulose can not enter the fungal cell directly, it has been suggested that cellulose must be degraded by the combined action of EG and β G

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to form smaller, soluble molecular products which can enter the cell. Then the true inducers will be formed by the transglycosylation of β G (13, 14, 17). This postulate is proved to be true by the inhibition of β G. Two inhibitors of β G, nojirimycin and glucono- β -lactone, can prevent EG induction by cello-oligosaccharides and not by sophorose, implicating β G in de novo cellulase induction (3).

β G is constitutively synthesized in fungi, and distributed in different cellular regions, that is extracellular, plasma membrane-bound (8), conidial-bound (9) and intracellular. β G is believed to have various functions (6, 7), such as hydrolysis of β -linked disaccharides, aryl-alkyl- β -D-glucosides, and transglycosylation, and the functions vary with the locations of β G. The extracellular β G is believed to mainly catalyse hydrolysis; however, membrane-bound and conidial-bound β G have the transglycosylation activity which produce the inducers to increase the level of cellulase biosynthesis (15). In our present paper, we report two extracellular β Gs obtained from *T. pseudokoningii* S38 (19), β G1 and β G2, which still have transglycosylation activity. Their functions in the regulation of cellulase biosynthesis in *T. pseudokoningii* S38 are also discussed in this paper.

MATERIALS AND METHODS

Organism and seed development. *T. pseudokoningii* S38 is a wild type isolated from soil and defined in our laboratory (11). The seed development was done using a medium of wheat bran extract added with 2% (w/v) agar with cultivation for 5 days at 30°C.

Purification of extracellular cellulase and β -glucosidase. Fermentations were carried out in several 250-ml flasks containing 8 g corn straw powder and 2 g wheat bran added with 30 ml Mandels' salt solution (12). Into each of the flasks was inoculated 2 ml (10^7 of spores per milliliter) of seed culture. After 7 days of inoculation at 30°C, 270 ml of 0.1 M sodium acetate buffer (pH 4.8) was poured into each flask and soaked 2 h at 25°C. Then, the whole suspended substance was centrifuged at 3,000 rpm for 10 min. The supernatant was recentrifuged at 10,000 rpm for 5 min. The supernatant was the extracellular crude cellulase. Then the extracellular cellulase filtrate was fractionated successively on Sephadex G50, DEAE-Sephadex A25 and HPLC-DEAE-8HR. Two purified β -glucosidase components, β G1 and β G2, were obtained (19).

Culture conditions and cellulase induction. Mandels' salt solution added with 0.5% (w/v) glucose was used as the basal medium. Batches of this medium were inoculated with *T. pseudokoningii* S38 seed and incubated on a gyratory shaker at 160 rpm for 48 h. Then, the mycelium was harvested on a filter paper by suction filtration and washed with 200 ml of 0.01 M phosphate buffer (5.0). The washed mycelium was resuspended in 50 ml of 0.01 M phosphate buffer (5.0). Then, the mycelium suspension (20 mg dry weight) was transferred to induction medium (Mandels' salt solution) containing proper inducers, and incubated in a reciprocating shaker (160 rpm) at 28°C. The culture filtrate was harvested at different times

of incubation for the cellulase assay.

Transglycosylation. Purified β G components were transferred to transglycosylation medium which is 0.01 M phosphate buffer (pH 5.0) containing 5 mg/ml cellobiose (and 50 mg/ml glucose), and incubated at 30°C for 48 h. Then, the culture filtrate was centrifuged at 10,000 rpm for 10 min. The transglycosylation products in the supernatant were determined by HPLC (Waters Co., Bedford, MA, U.S.A.) with an ammonia column, eluted with acetonitrile–water (80/20, v/v) at the rate of 1 ml/min, using commercially available disaccharides (Sigma Chemical Co., St. Louis, MO, U.S.A.) as the standard.

Enzyme assay. Filter paper hydrolyzing activity (FPA), which was used as the representative of the cellulase, was measured as follows: with 50 mg Whatman no. 1 filter paper (6 $\times$ 1 cm) as the substrate, the cellulase activity was measured under the conditions: pH 5.0, 0.01 M phosphate buffer, 60 min of incubation at 50°C. The reducing sugars released were determined by the dinitrosalicylic acid (DNS) method, where a unit of enzyme activity represents 1 μ mol of glucose released per minute.

Chemicals. Sephadex used in enzyme purification was obtained from Pharmacia Co., Uppsala, Sweden. Chemicals used in the seed development and enzyme culture were obtained from Sigma Chemical Co. Other various commercial saccharides were also obtained from Sigma Chemical Co.

RESULTS AND DISCUSSION

Comparison of the characteristics of β G1 and β G2

After three steps of purification (19), two β -glucosidase, β G1 and β G2, were obtained from *T. pseudokoningii* S38. The enzyme characteristics and substrate specificities of these two β G components were determined and the results are shown in Table 1. The isoelectric points (pIs) of these components were similar to that of other mycelial fungi reported by many authors (1, 10, 13). The molecular weights (MW) of β G1 and β G2 obtained by SDS-PAGE were similar to the results from gel filtration chromatography (data not shown), indicating that these two β G components were composed of a single subunit. β G1 was the main β -glucosidase, its protein yield was almost 10 times than that of β G2 (19). β G1 had the higher activity to act on cellobiose and *p*-nitrophenyl- β -D-glucoside (PNPG). These results showed that there were some differences between β G1 and β G2.

Transglycosylation of β G1 and β G2

β G1 and β G2 were added to the glucose (50 mg) and cellobiose (5 mg) mixture solution to a final concentration of 10 mg/ml, respectively. Then, the solution was incubated at 30°C for 48 h. HPLC with an ammonia column was used to determine the transglycosylation products (see Fig. 1A and B). The results showed that all other β -linked disaccharides besides cellobiose, such as sophorose, laminaribiose, gentiobiose, trehalose, and even cellotriose, appeared in the reaction

Table 1. The characteristics and substrate specificities of β G components from *Trichoderma pseudokoningii* S38.

Contents	β G1	β G2
Characteristics		
<i>MW</i>	86,000	68,000
<i>pI</i>	5.9	5.7
Substrates	Relative activity (%)	
Cellobiose	100	18
PNPG	120	0.5
PNPC	0.5	0.5
CMC-Na	0	0
Avicel	0.05	0
FPA	0	0
Xylan	0.1	0.1

PNPG, *p*-nitrophenyl- β -D-glucoside; PNPC, *p*-nitrophenyl- β -D-cellobioside; CMC-Na, carboxymethyl cellulose sodium salt; FPA, Whatman no. 1 filter paper hydrolyzing activity.

Fig. 1. Transglycosylation products of β Gs with cellobiose and glucose as the substrates.

(A) β G2, (B) β G1. Glu, glucose; Lam, laminaribiose; Cb, cellulose; Sop, sophorose; Gen, gentiobiose; Tre, trehalose; Cellotri, Cellotriose.

solution. However, the activity of transglycosylation was different between β G1 and β G2, and the relative amount of each disaccharide was also different. The most abundant disaccharide was gentiobiose among all of the transglycosylation products. As shown in Fig. 1, the presence of excessive glucose in the reaction mixture increased the amount of transglycosylation products compared with that of the absence of glucose. Glucose inhibited the hydrolysis of cellobiose by β Gs. Thus, the excessive glucose could affect the action of β Gs in this process. Comparison of the effect of the excessive glucose on transglycosylation of β G1 and β G2 (Fig. 1)

showed that the hydrolyzing activity of the former was more susceptible to the presence of glucose owing to its higher activity of hydrolysis. The presence of cellotriose might indicate that the β Gs had the β -glucoside condensation activity. There might be an equilibrium among above activities of β Gs.

Effect of transglycosylation on cellulase synthesis

The washed mycelium of S38 was cultured with the induction medium with the addition of 0.5% (w/v) sophorose, gentiobiose, cellobiose or 5 ml of the incubated solution of cellobiose and β G2 (the concentration of each enzyme was 10 mg/ml), respectively. The cellulase (FPA) synthesis was determined at various culture times (Fig. 2). The FPA synthesis was obviously increased by the addition of commercial gentiobiose and the transglycosylation products of β G2, and the effect of the above two treatments was similar. This indicated that the transglycosylation of extracellular β G also had the function of increasing the cellulase synthesis.

Effect of the rate of hydrolysis of β -linked disaccharides on the induction of cellulase

The rate of hydrolysis of various β -linked disaccharides by β G1 was different (Fig. 3). Among them, the rate of hydrolysis of gentiobiose was the lowest. On the other hand, the amount of gentiobiose was the greatest among the transglycosylation products. So the amount of a kind of disaccharide in the reactive mixture was controlled by the two activities of β Gs, hydrolysis and transglycosylation. The high level of gentiobiose seems to be why it plays an important role in cellulase synthesis. Although the mechanism by which native cellulose induces the synthesis of cellulase in *Trichoderma* is far from clear, more and more researchers believe the hypothesis that the basal levels of EGs may be sufficient to initiate attack on crystalline

Fig. 2. Effect of transglycosylation on cellulase synthesis of *Trichoderma pseudokoningii* S38.

◇, gentiobiose; □, cellobiose + β G2; △, cellobiose; ×, sophorose; ○, glucose (control).

Fig. 3. The rate of hydrolysis of β -linked disaccharides by β G1.

◇, laminaribiose; *, sophorose; □, trehalose; △, cellobiose; ○, gentiobiose.

cellulose, thereby forming the smaller, soluble products, such as oligosaccharides and cellobiose. Then, the inducers are formed during the process in which the soluble degradation products are transferred from extracellular to intracellular (1, 2). The transglycosylation by β Gs, especially those located at the plasma membrane and conidial surface, play an important role in this process. However, the function of intracellular and extracellular β Gs, to some extent, is more hydrolyzing disaccharides to glucose than producing transglycosylation products which induce the synthesis of cellulase.

In our present paper, we discovered that two extracellular β Gs, β G1 and β G2, which were purified from *T. pseudokoningii* S38, also had transglycosylation activity. Fairly speaking, the component of β G2 has more transglycosylation than hydrolysis of disaccharides. From our results, we confirm the extracellular β Gs have two kinds of activities, hydrolysis and transglycosylation, and that there may be some kind of equilibrium between the above activities. The equilibrium is affected by the reaction condition, or we may control the reaction to produce more transglycosylation products which can increase the synthesis of cellulase effectively.

Sophorose, which is only effective in a few of *Trichoderma* strains, can be hydrolyzed quickly by β G1 of S38. That may be the reason why sophorose is ineffective in most cellulose degradation microorganisms (5). Gentiobiose, which is the most abundant among the transglycosylation products, can increase the synthesis of FPA. This suggests that the amount of certain disaccharides is an important factor influencing its role in the regulation of cellulase synthesis. Using the resting mycelia, we may produce artificially a great quantity of more effective inducers from soluble and cheaper substances, such as from cellobiose. The soluble inducers may be used in cellulase production by immobilized cells of mycelial fungi (18). So the obstacle of adding insoluble cellulose into continuous bioreactors can

be overcome. This improvement may increase cellulase production significantly.

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