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Aspects of Cellulase Induction by Sophorose in *Trichoderma reesei* QM9414

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Trichoderma reesei QM9414의 sophorose에 의한 섬유소 분해효소 유도현상에 관하여

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The aim of this investigation was to resolve the contradiction between the results of Sternberg and Mandels (1980, 1982) and those of Nisizawa *et al.*, (1971) in cellulase induction by sophorose, and furthermore to study the conditional effects on sophorose-induced cellulase induction in *Trichoderma reesei* QM 9414. Sophorose could induce the synthesis of CMCase and β-glucosidase simultaneously. Optimal induction medium by sophorose had the potassium citrate buffer solution of pH 3.0-4.0 for CMCase, but one of pH 5.0-6.0 for β-glucosidase. At this time, two different types of β-glucosidases could be induced by sophorose: one was extracellular and had maximum at pH 5.0, the other was intracellular and had maximum activity at pH 6.5.

Induction study showed that methyl-β-glucoside was not a true inducer of β-glucosidase and that large β-glucosidase induction could be obtained only by the addition of sophorose into the induction medium. Glucose repressed the induction of cellulase by sophorose. The repression of glucose could not be overcome by the addition of cyclic AMP into the induction medium.

Trichoderma is the best known source of an extracellular cellulase capable of solubilizing crystalline cellulose. Several investigators have suggested that a group of enzymes are responsible for the stepwise saccharification of cellulose into glucose. Well-known enzymes of these cellulase are exoglucanase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). Cellulase synthesis in *Trichoderma* is inducible and can be affected by cellulose, lactose, cellobiose, β-methyl-glucoside, sophorose, and glucose (Mandels *et al.*, 1962; Nisizawa *et al.*, 1971a, 1971b, 1972; Sternberg and Mandels, 1977, 1980, 1982; Zhu *et al.*, 1982; Loewenberg, 1984). Of these, sophorose (2-O-β-D-glucopyranosyl-D-glucose) is by far the most potent soluble inducer of cellulase in *Trichoderma*.

The mechanism and physiology of induction of cellulase have been the subject of several investigators. However, knowledge of cellulase synthesis and regulation is still limited and there are some contradictions concerned the role of sophorose in synthesis of β-glucosidase in *Trichoderma*. Nisizawa *et al.* (1971) claimed that sophorose stimulated β-glucosidase formation, yet Sternberg and Mandels (1980) reported that it actually

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repressed the enzyme level and β-methyl-glucoside increased the β-glucosidase level associated with mycelium (1982). While, Zhu *et al.* (1982) reported that total extracellular cellulase were strongly induced by sophorose. Recently, Loewenberg (1984) claimed that two different β-glucosidase were associated with the mycelium of *Trichoderma*: one of these is soluble and has an optimum pH of 6.5 in crude extracts, the other is associated with the pelleted-mycelial fraction of homogenates and has an optimum pH of 4.8. While sophorose stimulates the appearance of the former, it depresses the latter.

The aim of this investigation was to study the effects of sophorose on cellulase induction and to resolve the conflicting results of several investigators.

MATERIALS AND METHODS

Strain and media

Trichoderma reesei QM9414 was maintained on a slope of potato dextrose agar. The culture was grown at 28 °C for 5 days before storing at 4 °C. For preparing early exponential mycelia, malt extract medium containing malt extract 20 g, peptone 1 g, yeast extract 1 g, and dextrose 10 g in one liter of distilled water was used. For induction experiments, induction medium according to the mineral component of Medium C (Mandels *et al.*, 1962) in varied concentration (0.01 M, 0.05 M, 0.1 M) of various buffer solution (potassium phosphate, potassium citrate and sodium acetate buffer) was used. Sophorose (10^{-4} M) or β-methyl-D-glucoside (5 mM) was used as an inducer.

Induction of cellulase

Conidia on slope of potato dextrose agar were suspended in an appropriate volume of distilled water and the spore suspension was passed through a sintered glass filter to remove from large particles including mycelia. The filtered conidia suspension was used as an inoculum into a 100 ml conical flask containing 40 ml of malt extract medium (ca. 5.0×10^5 conidia/ml).

The culture was incubated at 28 °C on a rotatory shaker (200 rpm) for 27 hr. The mycelia were harvested, washed two times with buffer solution, and resuspended in a same buffer. After standing the mycelial suspension for 1 hr at room temperature, mycelia were harvested and transferred into a 100 ml conical flask containing 25 ml of induction medium. The final mycelial dry weight in the induction medium was ca. 1.9 mg/ml. The sample was taken and centrifuged at a given interval. Supernatant was used for an extracellular enzyme preparation.

Preparation of intracellular and pellet-associated enzyme

The mycelia incubated in an induction medium were pelleted and washed two times with the buffer solution same as for induction medium. The washed mycelia were resuspended in 10 ml of the same buffer solution and sonicated (Lab-line ultratip Labsonic system, Lab-line Industries, Inc., USA) three times at 120 watt for 5 sec. The sonicated mycelial suspension were centrifuged and supernatant was used as an intracellular enzyme preparation. The remaining pelleted mycelia were washed two times and resuspended in buffer. The resuspended mycelia were sonicated under the same condition described above. These sonicated mycelial fraction were washed two times, resuspended in 5 ml of buffer, and used as an insoluble pellet-associated enzyme preparation.

Enzyme assay

Carboxymethylcellulose(CMC)-saccharifying (CMCase) activity; Each reaction mixture was composed of 0.8 ml of 0.5 % CMC in 0.05 M sodium acetate buffer (pH 5.0), where did not note, and 0.2 ml of enzyme solution. After incubation at 40 °C for 30 min., the reducing sugar concentration was determined according to the method of Somogyi (1952) and Nelson (1944).

β-Glucosidase activity; β-Glucosidase activity was assayed by measuring the amounts of p-nitrophenol (PNP) liberated from *p*-nitrophenyl-β-D-glucopyranoside (PNPG). The reaction mixture was composed of 0.8 ml of 1 mM PNPG in 0.05 M sodium acetate buffer (pH 5.0), where did not note, and 0.2 ml of enzyme solution. After incubation at 40 °C for 30 min., 2 ml of 1 M sodium carbonate

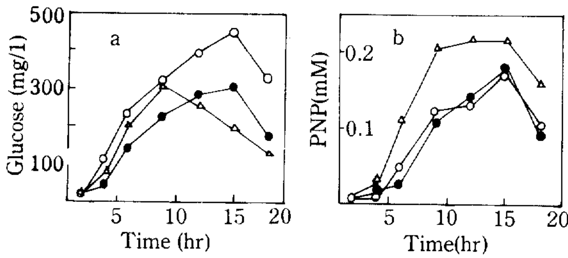

Fig 1. *Induction of extracellular CMCase (a) and extracellular β-glucosidase (b) in Trichoderma reesei QM9414 by sophorose. Induction media were buffered with potassium phosphate buffer (0.01 M, pH 5.0) according to Nisizawa et al. (1971a): ○, phosphate buffer + sophorose + minimal component of Medium C lacking urea and KH_2PO_4; ●, phosphate buffer + sophorose; △, phosphate buffer + sophorose + minimal component of Medium C.*

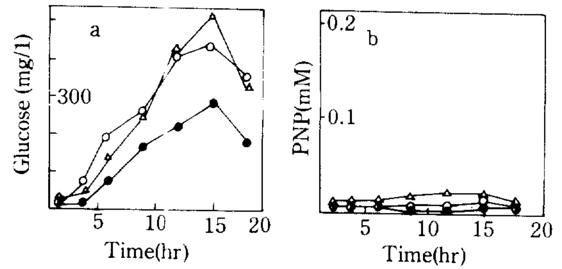

Fig 2. *Induction of extracellular CMCase (a) and extracellular β-glucosidase (b) in Trichoderma reesei QM9414 by sophorose. Induction media were buffer with potassium citrate buffer (0.05 M, pH 3.0): ○, potassium citrate buffer + sophorose + mineral component of Medium C lacking urea and KH_2PO_4; ●, potassium citrate buffer + sophorose; △, potassium citrate buffer + sophorse + mineral component of Medium C.*

solution was added to the mixture. The mixture was diluted with 10 ml of distilled water and the absorbance at 420 nm was measured.

Determination of protein concentraion

Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Chemicals

CMC, PNP, PNPG, methyl-β-D-gulcoside, and bovine serum albumin were supplied by Sigma Chemical Co., St. Louis. Missouri, USA. α-D-sophorose monohydrate was purchased from Koch-Light Laboratories Ltd. Colnbrook Bucks, England.

RESULTS AND DISCUSSION

Effect of induction medium

As an induction medium, Nisizawa *et al.* (1971) used 0.017 M phosphate buffer of pH 6.0, Sternberg and Mandels (1982) used 0.25 strength Medium C (Sternberg and Mandels, 1979) lacking urea and KH_2PO_4 in 0.05 M potassium citrate buffer of pH 3.0, and Zhu *et al.*, (1982) used 0.5 strength Medium C in 0.05 M phosphate buffer (ca. pH 5.0), respectively. To examine the effect of induction medium on the sophorose-induced synthesis of cellulase, various mineral composition of induction medium in 0.01 M potassium phosphate buffer (pH 5.0) and 0.05 M potassium citrate buffer (pH 3.0) were investigated. Sophorose was always added into induction medium as an inducer. As the results, the synthesis of extracellular CMCase and β-glucosidase were induced by sophorose using all of the induction medium in 0.01 M phosphate buffer (Fig. 1). On a while, hardly any synthesis of β-glucosidase (Fig. 2b), but extracellular CMCase (Fig. 2a) was induced by sophorose in the three induction media in 0.05 M potassium phosphate buffer.

These results agree with those of Nisizawa *et al.*, (1971), but differ from those of Sternberg and Mandels (1980). The present data suggest that the contradiction between the results of Nisizawa (1971) and those of Sternberg and Mandels (1980) may partly due to the different buffer system used. These results also indicate that the mineral composition of induction medium does not significantly affect on sophorose-induced synthesis of extracellular cellulases in *Trichoderma* and that an exogenous supply of nitrogen and minerals was not necessary for induction, even though the presence of nutrient salts increased the yield slightly over buffer only.

The idea that the kind of buffer system for induction medium could affect on the sophorose-induced synthesis of cellulases was supported by the experiment in which the effect of various buffer was tested on sophorose-induced synthesis of extracellular CMCase. As shown in Fig. 3, sophorose could induce the synthesis of extracellular

CMCase in potassium phosphate buffer and potassium citrate buffer, but not in acetate buffer.

Effect of pH

Although the synthesis of extracellular CMCase was induced in phosphate buffer (Nisizawa *et al.*, 1971; Sternberg and Mandels, 1979; Zhu *et al.*, 1982) or potassium citrate buffer (Sternberg and Mandels, 1982) by sophorose, the pH of induction buffer was different from each other. In this respect, effect of pH on the sophorose-induced synthesis of extracelluar CMCase was investigated.

When washed-mycelia were incubated in 0.05 M potassium citrate buffer with varied pH from 3.0 to 7.0, the synthesis of extracellular CMCase, with concomitant increase of extracellular protein, was successfully induced by sophorose at pH 3.0 and 4.0. Above 4.0, however, the yield of cell-free CMCase decreased sharply (Fig. 4).

This result suggest that the pH of induction medium also affect on the sophorose-induced synthesis of extracellular CMCase. Therefore, it can be inferred that the conflicting roles of sophorose on induction of β-glucosidase synthesis might result from the different pH of induction medium used. To approach that presumption, the washed-mycelia were incubated in various pH of 0.05 M potassium citrate buffer with sophorose and the activity of β-glucosidase in each filtrate was determined with PNPG solutions in 0.05 M potassium citrate buffer of different pH. With potassium citrate buffer of pH 3.0, hardly any extracellular (Fig. 5a), intracellular (Fig. 5b), and pellet-associated β-glucosidase activities (Fig. 5c) could be detected, regardless of the pH of substrate PNPG solution. With potassium citrate buffer of pH 4.0 and 5.0, extracellular β-glucosidase with an optimum pH at 5.0, however, was induced by sophorose. With potassium citrate buffer of pH 6.0, two types of β-glucosidase with optima pH at 5.0 and 6.5 were induced by sophorose (Fig. 5a). Intracellular β-glucosidase with an optimum pH at 6.5 and two types of pellet-associated β-glucosidases with optima pH at 5.0 and 6.5 were induced in potassium citrate buffer of pH 4.0, 5.0, and 6.0 by sophorose (Fig. 5b,c). Recently, Inglin *et al.*, (1980) reported that intracellualr β-glucosidase with an optimum pH at 6.5 may function on regulation of cellulase induction and/or serve as proenzyme. Loewenberg (1982) reported that the β-glucosidase released into the medium and pellet -associated both have optima at 4.8; intracellular β-glucosidase has an optimum at pH 6.5.

The results reported here agree with those of Inglin *et al.*, (1980) and Loewenberg (1982). The extracellular β-glucosidase with optimum pH at

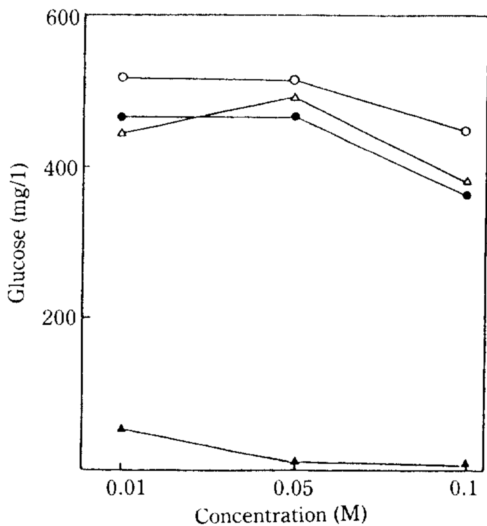

Fig. 3. *Effect of various buffer on extracellular CMCase induction by sophorose: ▲, sodium acetate buffer (pH 5.0); ●, potassium citrate buffer (pH 5.0); △, potassium phosphate buffer (pH 5.0); ○, potassium citrate buffer (pH 3.0).*

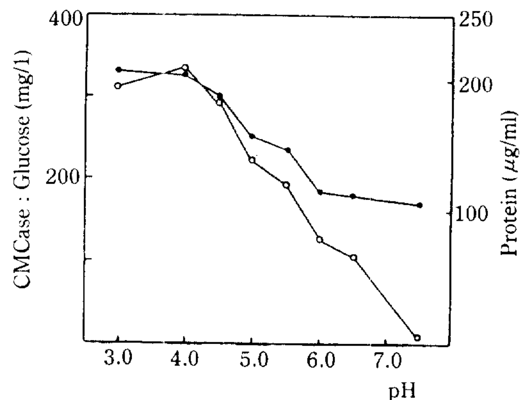

Fig. 4. *Effect of pH on extracellular CMCase induction by sophorose. Washed-mycelia were incubated in induction medium for 14 hr: ●, protein (μg/ml); ○, extracellular CMCase.*

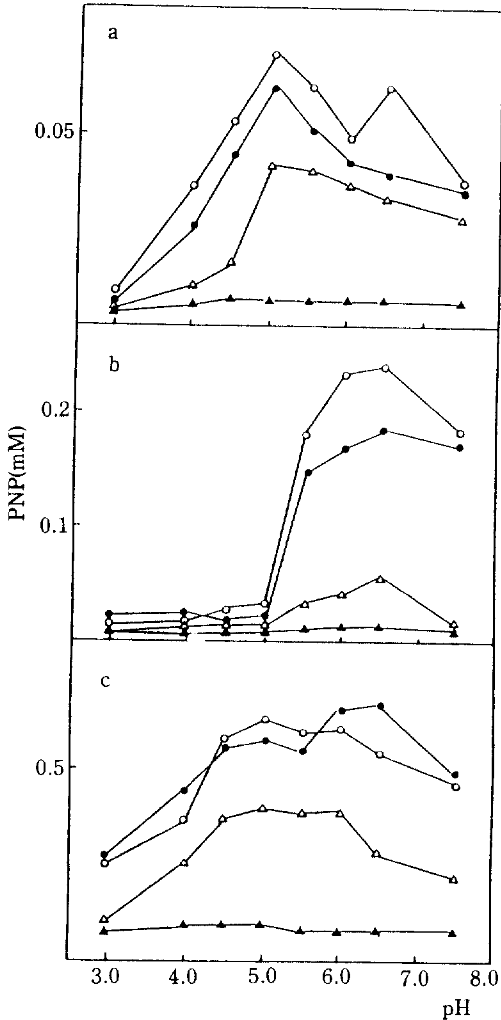

Fig. 5. *Effect of pH on extra (a), cytoplasmic (b), and pellet-associated β-glucosidase induction by sophorose. Washed-mycelia were incubated in induction medium for 14 hr: ▲, pH 3.0; △, pH 4.0; ●, pH 5.0; ○, pH 6.0.*

6.5 seems to be the intracellular β-glucosidase secreted to the induction medium of pH 6.0 and intracellular β-glucosidase is essentially inactive at pH 5.0.

The cellulases are secreted enzymes, with more than 90% of the activity being in the medium at the end of the induction period, however, β-glucosidase, whether constitute (at a low basal level) or induced, remains associated with the mycelium (Sternberg and Mandels, 1980). The result in Fig. 5 shows that level of β-glucosidase activity associated with mycelium was higher than that of extracellular one of which was one tenth of pellet-associated one.

The above results indicate that the pH condition of induction medium greatly affect on the sophorose-induced synthesis of cellulase especially β-glucosidase. The optimum pH of induction medium range between 5.0 and 6.0. At 6.0, intracellular β-glucosidase with optimum pH of 6.5 can be secreted into induction medium.

Loewenberg (1982) claimed that the conflicting results of Sternberg and Mandels (1980) were caused by the examination of β-glucosidase activity only 4.8. The present data, however, suggest that the conflicting results of Sternberg and Mandels (1980) were basically caused by no induction of β-glucosidase in induction medium of pH 3.0, and the synthesis of, at least, two types of β-glucosidases with optima of 5.0 and 6.5 can be induced in induction medium in potassium citrate buffer of pH 6.0 by sophorose.

Effect of methyl-β-D-glucoside

Sternberg and Mandels (1980) reported that sophorose induced the synthesis of CMCase, but actually repressed that of β-glucosidase. They also reported that β-glucosidase could be induced by methyl-β-D-glucoside (MβG) (Sternberg and Mandels, 1982).

Present data, however, showed that sophorose could induce the synthesis of β-glucosidase in buf-

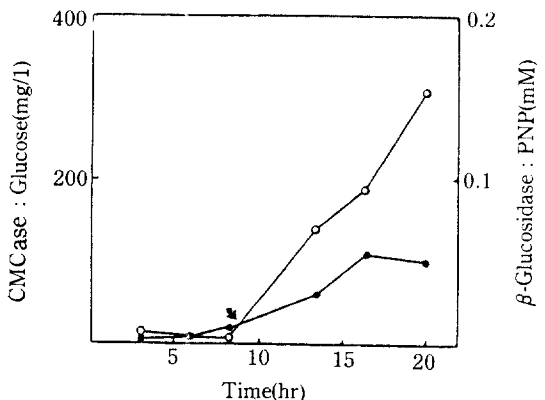

Fig. 6. *Effect of methyl-β-D-glucoside and sophorose on induction of extracellular CMCase and β-glucosidase. Arrow indicated that sophorose was added into induction medium containing 5 mM methyl-β-D-glucoside: ●, extracellular β-glucosidase; ○, extracellular CMCase.*

Table 1. *Repression of cellulase synthesis by glucose and effect of cAMP*

Incubation period	2		4		6		10		12		15 (hr)	
	C*	β**	C	β	C	β	C	β	C	β	C	β
Control	2.88	0.001	3.24	0.011	82.8	0.015	216	0.059	216	0.083	208	0.107
Glucose (10mM)	–	0.001	–	0.002	–	0.010	166	0.033	191	0.046	108	0.051
Glucose (10mM) +cAMP (50mM)	–	–	–	0.002	–	0.010	155	0.034	187	0.044	180	0.050

* C; Extracellular CMCase was expressed as the amounts of glucose equivalents (mg/ml).
** β; Extracellular β-glucosidase activity was expressed as the concentration of PNP (mM).

fer system of pH 5.0 and 6.0 (Fig. 5a, b, c). To examine the effect of MβG on the synthesis of β-glucosidase, the induction pattern of MβG was compared with that of sophorose. No appreciable activities of CMCase and β-glucosidase were detected in 0.05 M potassium citrate buffer of pH 5.0 containing 5 mM of MβG but the synthesis of cellulase was immediately induced by the addition of sophorose into the induction medium containing MβG (Fig. 6).

Conflicting with the results of Sternberg and Mandels (1982), the result suggest that MβG is probably not a true inducer of β-glucosidase. However, more detailed studies should be done for the resolution of those conflicting results.

Effect of cyclic AMP

The cellulases of *Trichoderma* are subjected to a number of biochemical and genetic controls. One of the most important control on those enzymes is endproduct inhibition. Furthermore, the synthesis of cellulase in *Trichoderma* is subject to catabolite repression as well as induction. Regulation of catabolite repressible enzymes in bacteria is through an additional control involving the level of cyclic adenosine monophosphate (cAMP) in the cell.

When washed-mycelia were incubated in induction medium supplemented with sophorose and glucose (10 mM), the sophorose- induced synthesis of extracellular CMCase and β-glucosidase was repressed. The catabolite repression of glucose could not overcome by the presence of cAMP in the induction medium. In the presence of glucose, hardly any appreciable activities of extracellular cellulases were detected within 4hr of induction period. After the exhaustion of glucose (may need at least 4 hr), the activities of sophorose-induced extracellular cellulase started to appear.

It was already reported that cellulase in *Trichoderma* did not appear to be under the same type of cAMP control demonstrated in bacteria (Montenecourt *et al.*, 1980) and that catabolite repression of glucose could not be overcome by cAMP (Zhu *et al.*, 1982). Therefore, it seems that the control mechanism of catabolite repressible cellulases in the eukaryote *Trichoderma* is different from the enzyme regulation in prokaryotes. However, this result is not sufficient to exclude the possible cAMP regulation of catabolite repressible cellulases in *Trichoderma*.

In this paper, several experiments were performed to resolve some conflicting results concerned the sophorose-induced synthesis of cellulases in *Trichoderma*. However, the results presented here are not conclusive and further studies should be necessary for the resolution of conflicting results and genetic regulation of cellulase synthesis in *Trichoderma*.

적 요

Sophorose에 의한 섬유소분해효소의 유도현상에 있어, Nisizawa등과 Sternberg와 Mandels가 연구보고한 상호 다른 결과들을 재규명하고, sophorose에 의한 섬유소분해효소 합성에 영향을 미치는 몇가지 요인들을 조사하고자

본 연구를 행하였다. Sophorose는 *Trichoderma reesei* QM9414에서 CMCase와 β-glucosidase의 합성을 동시에 유도하며, CMCase는 pH 3.0~4.0의 완충용액을 갖는 유도배지에서, β-glucosidase는 pH 5.0~6.0의 K-citrate완충용액을 갖는 유도배지에서 그 합성이 최대로 유도되었다. 또한, 세포내 β-glucosidase는 pH 6.5의 가실용액에 대하여, 세포외 β-glucosidase는 pH 5.0의 가실용액에 대하여, 각각 최대 활성도를 나타내었다.

Methyl-β-D-glucoside는 β-glucosidase의 진정한 유도물질이 아닌 것임이 밝혀졌다. 포도당은 sophorose 에 의한 섬유소분해효소의 유도과정을 억제하며, 이 억제효과는 cAMP의 첨가에 의해서 영향을 받지 아니하였다.

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