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Effect of D-glucono-1,4-lactone on the production of CMCase, pNPCase and true cellulase by *Clostridium thermocellum*

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The effect of varying concentrations of D-glucono-1,4-lactone (2.6-8.4 mM), a potent inhibitor of β-D-glucosidase, on the production of carboxymethylcellulase (CMCase), 4-nitrophenylcellobiosidase (pNPCase) and true cellulase by *Clostridium thermocellum* strain YS was studied in the presence of cellobiose and Avicel. It was found that D-glucono-1,4-lactone up to 8.4 mM had no effect on the production of CMCase, pNPCase and true cellulase by *C. thermocellum* grown either on cellobiose or on Avicel. Thus, it was concluded that cellobiose rather than its transglycosylated product is the inducer of the cellulase system in *C. thermocellum*.

INTRODUCTION

Many fungi and bacteria are known to produce high levels of cellulase when grown either on pure cellulose or lignocellulosic substrates (Stewart and Leatherwood, 1976; Ryu and Mandels, 1980; Wood, 1985). However, soluble sugars such as cellobiose, lactose and sophorose (2-*O*-β-D-glucopyranosyl-D-glucose) are also reported to induce the production of cellulase by some micro-organisms (Mandels *et al.*, 1962; Mandels, 1975). Since cellulose is an insoluble substrate and cannot enter the microbial cell, it is rather surprising that insoluble cellulose induces the production of high levels of cellulase. This has been the subject of considerable debate in recent years.

Some glycosidases, in addition to hydrolysis, catalyse transglycosylation reactions during which either a sugar or an alcohol acts as an acceptor of a sugar moiety (Umezurike, 1971; Meyer and Canevacini, 1981; Bhat *et al.*, 1993a). As a result, the product of transglycosylation reaction could be either a disaccharide, oligosaccharide or an alkylglycoside (Bhat *et al.*, 1993a; Christakopoulos *et al.*, 1994a, b, c, 1995a, b). It has generally been accepted that a transglycosylated product of cellobiose could be the true inducer of cellulase in *Trichoderma reesei* (Beguin and Aubert, 1993). Also, sophorose, a contaminant found in glucose preparations, has been shown to be an effective inducer of cellulase in *T. reesei* (Sternberg and Mandels, 1979). However, sophorose did not induce the production of cellulase in other fungi and in a mutant of *T. reesei* QM 9414 (Moloney *et al.*, 1983; Messner *et al.*, 1988). In contrast, Fritscher *et al.* (1990) demonstrated that cellobiose induced the production of cellulase in *T. reesei*, either in the presence of β-D-glucosidase inhibitor or when a β-D-glucosidase defective mutant was used. This suggested that cellobiose could be the true inducer of cellulase in *T. reesei*. Recently, using three strains of *C. thermocellum*, we have demonstrated the production of a qualitatively similar multienzyme complex, termed cellulosome, on cellobiose and Avicel (Bhat *et al.*, 1993b). In the present paper, we confirm that cellobiose rather than its transglycosylated product is the inducer of the cellulase system in *C. thermocellum*.

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MATERIALS AND METHODS

Materials

C. thermocellum strain YS was a kind gift from Prof. R. Lamed, Tel Aviv University, Israel. Cellobiose, Avicel, carboxymethylcellulose (CM-cellulose), 4-nitrophenyl β-D-cellobioside (pNPC) and D-glucono-1,4-lactone were from Sigma. All other chemicals used were either from Sigma or BDH.

Growth and production of cellulase by *C.thermocellum*

For the production of cellulase, the *C. thermocellum* strain YS was grown anaerobically at 60°C for 48 and 96 h using GM medium (Garcia-Martinez *et al.*, 1980) with cellobiose and Avicel respectively. The production of cellulase by *C. thermocellum* on cellobiose and Avicel was checked in the presence of varying concentrations of D-glucono-1,4-lactone (2.8–8.4 mM). In all cases, samples were withdrawn at different time intervals aseptically and assayed for CMCase and pNPCase as described below. Also, the culture filtrates were tested for true cellulase activity as described below.

Measurement of cellulase activities

CMCase

This was measured by a reducing sugar method (Somogyi, 1952) using CM-cellulose as a substrate as described previously (Bhat and Wood, 1992). A solution of medium viscosity CM-cellulose (10 g/litre, 1 ml) was mixed with 0.5 ml of 200 mM 2-(*N*-morpholino)ethane–sulphonic acid (MES) buffer and 0.5 ml of suitably diluted culture filtrate, and incubated at 60°C for 15 min. The reaction was terminated by adding Somogyi reagent (2 ml). The reducing sugar released was estimated by the Somogyi–Nelson method (Somogyi, 1952). One unit of CMCase was expressed as μmol of reducing sugar released as glucose equivalent per ml min.

pNPCase

This was measured using pNPC as substrate in a microtitre plate as described previously (Bhat *et al.*, 1994). A reaction mixture containing 25 μl of 4 mM pNPC, 25 μl of 200 mM MES buffer, pH 6.0 and 50 μl of suitably diluted culture filtrate was incubated at 60°C for 30 min. The reaction was stopped by adding 100 μl of 0.4 M glycine buffer pH 10.8. The intensity of the yellow colour developed was read at 405 nm and the amount of pNP released was calculated using a pNP standard curve obtained under identical conditions. One unit of pNPCase was expressed as μmol of pNP released per ml. min.

True cellulase activity

This was measured by a turbidimetric method using Avicel. A reaction mixture containing 2 ml of Avicel PH 101, 5 ml of 100 mM MES buffer pH 6.0, 0.1 ml of 50 mM NaN_3, 1 ml of 70 mM $CaCl_2$, 1 ml of 100 mM DTT and 0.9 ml of diluted culture filtrate (equivalent to 0.5 IU of CMCase) was incubated over a period of 72 h at 60°C. The turbidity of the reaction mixture was measured at 660 nm at varying time periods, using a single beam spectrophotometer. The percentage solubilization of Avicel was calculated using the following equation:

$$\text{Avicel solubilization}(\%) = \frac{\text{Original turbidity } (A_{660}) - \text{Turbidity after incubation } (A_{660})}{\text{Original turbidity } (A_{660})} \times 100$$

RESULTS

Production of CMCase and pNPCase by *C. thermocellum* on cellobiose and Avicel in the presence of varying concentrations of D-glucono-1,4-lactone

It is generally believed that a transglycosylated product of cellobiose is the true inducer of cellulase in most micro-organisms. To confirm that cellobiose rather than its transglycosylated product induces the production of cellulase in *C.thermocellum*, the effect of varying concentrations of D-glucono-1,4-lactone, a potent inhibitor of β-D-glucosidase, on cellulase production was studied.

The presence of CMCase activity in culture filtrates from cellobiose cultures either with or without D-glucono-1,4-lactone was detected after 12 h and the levels of CMCase increased steadily and reached the maximum level (0.5 IU/ml) by 48 h (Fig. 1(A)). The pNPCase activity in the above culture filtrates was detected only after 24 h (Fig. 1(B)). However, the pattern of CMCase and pNPCase production by *C. thermocellum* on Avicel either with or without D-glucono-1,4-lactone was very similar (Fig. 1(C) and (D)). Both CMCase and pNPCase appeared after 24 h of incubation and increased steadily up to 72 h. Thereafter, the levels of both activities remained the same up to 96 h. The levels of CMCase and pNPCase produced in the presence of Avicel either with or without D-glucono-1,4-lactone were twofold and tenfold higher than that produced in the presence of cellobiose under identical conditions. However, there was no appreciable difference in the level of CMCase and pNPCase produced by *C. thermocellum* at different stages of growth either on cellobiose or Avicel, in the presence and absence of D-glucono-1,4-lactone. This

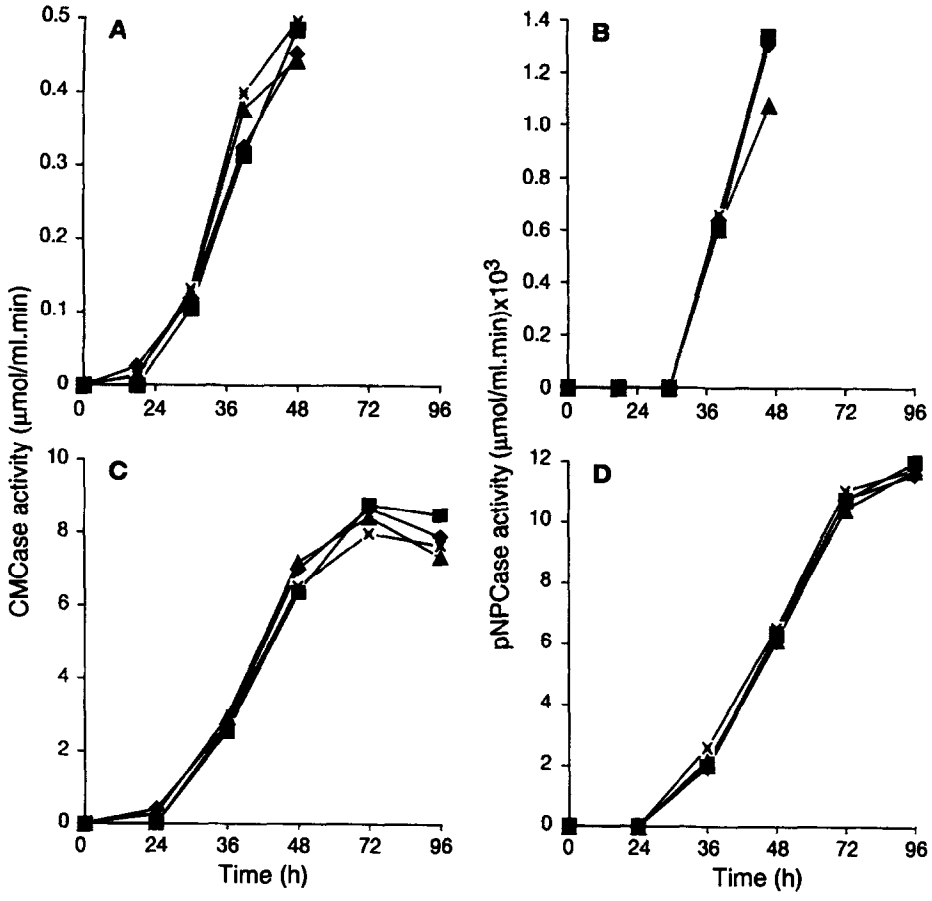

Fig. 1. Time course of CMCase and pNPCase production by *C. thermocellum* strain YS on cellobiose (A and B) and Avicel (C and D) in the presence of varying concentrations of D-glucono-1,4-lactone. Symbols ■, ▲, ◆ and × represent 0, 2.6, 5.8 and 8.4 mM D-glucono-1,4-lactone, respectively. Each reading is the mean of two determinations from two different cultures.

clearly showed that the presence of D-glucono-1,4-lactone has no effect on the production of CMCase and pNPCase by *C. thermocellum* on cellobiose and Avicel.

Effect of varying concentrations of D-glucono-1,4-lactone on the production of true cellulase by *C. thermocellum* on cellobiose and Avicel

True cellulase activity is defined as the ability of the cellulase preparation to degrade crystalline cellulose extensively (Johnson *et al.*, 1982). The effect of D-glucono-1,4-lactone on the production of true cellulase by *C. thermocellum* on cellobiose and Avicel was tested *in vitro* by using the crude cellulase of *C. thermocellum* produced on cellobiose and Avicel, in the presence and absence of D-glucono-1,4-lactone. The data presented in Fig. 2(A) and (B) show that the cellulase system of *C. thermocellum* produced on cellobiose and Avicel, either with or without D-glucono-1,4-lactone, degrades Avicel extensively. However, the cellulase system produced on Avicel in the presence and absence of D-glucono-1,4-lactone degraded Avicel faster and to a greater extent than that produced on cellobiose under identical conditions. Interestingly, the rate and extent of Avicel solubilization showed that the presence of D-glucono-1,4-lactone up to 8.4 mM did not influence the production of true cellulase by *C. thermocellum* on cellobiose and Avicel.

DISCUSSION

It has been demonstrated that most fungi produce appreciable levels of cellulase in the presence of cellobiose at concentrations as high as 1% or more (Reese *et al.*, 1969; Reese and Maguire, 1971). In contrast, Garcia-Martinez *et al.* (1980) reported that *C. thermocellum* ATCC 27405 produced CMCase on cellobiose at concentrations as low as 0.2% and the production reached the highest level at 1% of cellobiose. In addition, using three strains of *C. thermocellum*, we have demonstrated the production of CMCase, true cellulase and qualitatively similar cellulosome at different concentrations of cellobiose and Avicel (Bhat *et al.*, 1993b). These results indicated that in *C. thermocellum*, cellobiose acts as an inducer of cellulase.

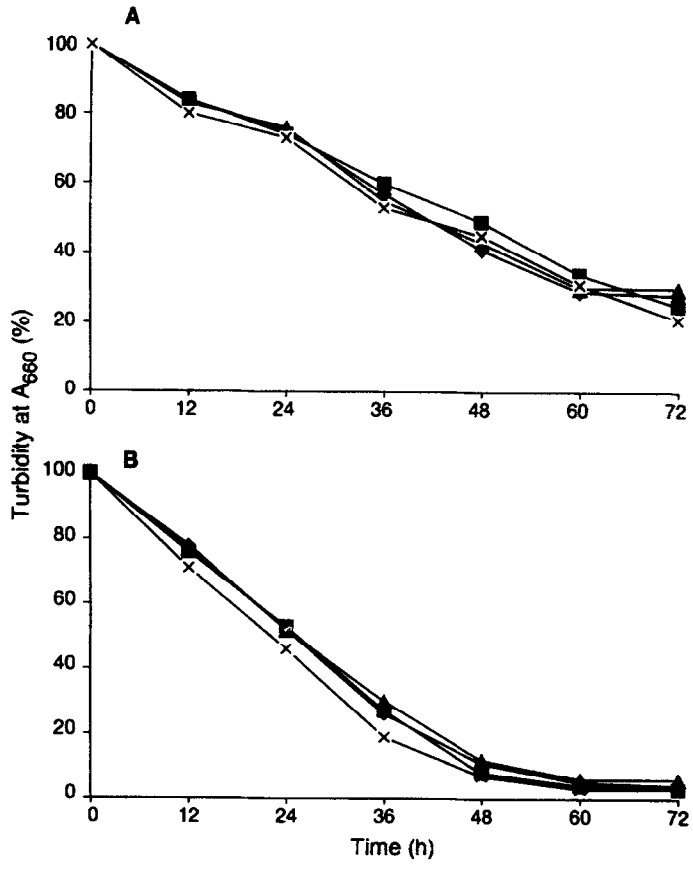

Fig. 2. Solubilization of Avicel by the crude cellulase (equivalent to 0.5 IU CMCase) from *C. thermocellum* strain YS produced on cellobiose (A) and Avicel (B) in the presence of varying concentrations of D-glucono-1,4-lactone. Symbols ■, ▲, ◆ and × represent the cellulase system produced in the presence of 0, 2.6, 5.8 and 8.4 mM of D-glucono-1,4-lactone, respectively. Each reading is the mean of two determinations from two different cultures.

Since cellobiose induces the production of cellulase in fungi only at concentrations as high as 1% or more, it is generally accepted that a transglycosylated product of cellobiose formed by the β-D-glucosidase could be the true inducer of cellulase (Beguin and Aubert, 1993). Whether cellobiose or the transglycosylated product of cellobiose becomes the true inducer of cellulase system in *T. reesei* was tested using either a β-D-glucosidase defective mutant or a specific inhibitor of β-D-glucosidase by Fritscher *et al.* (1990). These authors reported that cellobiose induced the production of cellulase in a β-D-glucosidase defective mutant of *T.reesei* and by *T.reesei* in the presence of β-D-glucosidase inhibitor. This showed that cellobiose could be the inducer of cellulase in *T. reesei.* The present study demonstrated that the production of CMCase, pNPCase and true cellulase by *C. thermocellum* in the presence of cellobiose and Avicel was not influenced by D-glucono-1,4-lactone, a potent inhibitor of β-D- glucosidase. The concentration of D-glucono-1,4-lactone used in the present study was at least 10^3 times higher than that shown to inhibit fungal β-D-glucosidases completely (Christakopoulos *et al.*, 1994a). It is therefore unlikely that the β-D-glucosidase of *C. thermocellum*, if present, will be able to synthesise any transglycosylated product in the presence of such high concentrations of D-glucono-1,4-lactone. Thus, for the first time, we have demonstrated that cellobiose is the inducer of cellulase in *C. thermocellum.*

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