Induction and Catabolite Repression of Cellulase Synthesis in the Thermophilic Fungus *Sporotrichum thermophile*

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A strain of *Sporotrichum thermophile* (var. 2), grown in submerged culture at 44 °C, decayed crystalline cellulose by the secretion of cellulolytic enzymes into the culture medium. Dialysed culture filtrates, after growth on cellobiose, were also effective in degrading crystalline cellulose (Avicel). Cellulase, measured viscometrically with carboxymethylcellulose as substrate and therefore referred to as CM-cellulase, was induced by cellulose, glucomannan, cellobiose and, to a limited extent, by laminaribiose and cellobionic acid. Low CM-cellulase activities were also detectable when the organism was grown on other carbon sources. In culture media supplemented with readily metabolized non-inducing substrates, such as glucose, CM-cellulase activity did not increase after their exhaustion indicating that no derepression of constitutive CM-cellulase occurred. CM-cellulase synthesis induced during growth on cellobiose was strongly inhibited, although not completely suppressed, by glucose and other carbon sources. In cultures grown on glucose and cellobiose, glucose was utilized first and CM-cellulase synthesis was repressed; cellobiose utilization occurred only after glucose exhaustion and triggered CM-cellulase formation. When glucose was added to a culture growing on cellobiose, further utilization of the latter was prevented and CM-cellulase synthesis was inhibited. The use of glucose analogues gave some indications that cellobiose did not compete with glucose for the same transport carrier and that glucose catabolism was a prerequisite for the inhibition of CM-cellulase synthesis.

INTRODUCTION

Some thermophilic fungi have been reported to be strongly cellulolytic (Tansey, 1971), their role being of paramount importance in the colonization and degradation of cellulosic waste. Among these *Sporotrichum thermophile*, which has frequently been found in such composting cellulosic waste (von Klopotek, 1974), was reported to be a cellulose digester with an activity similar to that of mesophilic cellulase producers (Romanelli *et al.*, 1975; Coutts & Smith, 1976).

Cellulolytic micro-organisms produce a group of enzymes (the cellulase complex system) which act synergistically on cellulose. Besides endocellulases, termed C_x by Reese *et al.* (1950), exocellulases are also present; the latter split off cellobiose molecules from the non-reducing ends of cellulose chains. Some authors consider these exocellulases are the C_1 enzymes originally postulated (Reese *et al.*, 1950) as being responsible for conversion of crystalline cellulose into a form accessible to the C_x enzymes. However, Reese (1977) has suggested that C_1 is a special endocellulase acting on crystalline cellulose alone and not on carboxymethylcellulose.

In previous studies on the regulation of cellulase synthesis by cellulolytic micro-organisms, two different mechanisms have been proposed. (i) The cellulases are inducible by cellulose and its derivatives or by a few low molecular weight carbohydrates but their formation is

repressed by readily metabolized substrates like glucose (Reese, 1977); such a repression mechanism for enzyme synthesis is of widespread occurrence in micro-organisms and was first referred to as the 'glucose effect' (Epps & Gale, 1942) and later termed 'catabolite repression' (Magasanik, 1961). (ii) The cellulases are constitutive, but repressed by conditions favouring rapid balanced growth (Hulme & Stranks, 1971).

Irrespective of their constitutive or inducible nature, cellulases from mycelial organisms (Nisizawa *et al.*, 1972; Hulme & Stranks, 1970) as well as other fungal extracellular hydrolases (Cooper & Wood, 1973; Santos *et al.*, 1977) have been reported to be catabolite sensitive. Cooper & Wood (1973), working with the wilt organism *Verticillium albo-atrum*, were able to stimulate extracellular enzyme secretion by using a restricted supply of a carbon source. Their experiments were aimed at mimicking the situation encountered in natural environments where organisms living on the degradation products of polymeric substances, such as plant cell wall polysaccharides, have to deal with low concentrations of soluble nutrients. Growing the organism in batch cultures containing excess substrate did not result in induction, since all the enzymes tested were repressed.

In this paper we report that cellulase production by *Sporotrichum thermophile* is regulated by induction and catabolite repression and not by a derepression mechanism depending on a limited supply of carbon substrates.

METHODS

Organism, culture media and cultivation techniques. The organism used in this study was a strain of *Sporotrichum thermophile* (var. 2) isolated from decaying newspapers and identified by T. G. Barnes (for a detailed description of the organism, see Barnes, 1974). No clamp connections occur in the mycelium and since sporulation produces aleurospores, the organism can be considered identical with *Chrysosporium thermophile* as suggested by von Klopotek (1974). The strain was maintained on Difco Sabouraud maltose agar slants and stored at 2 °C.

The following liquid media were used for submerged cultures: medium I had the same composition as that of Eggins & Pugh (1962) but without asparagine and with the addition of trace elements (10 ml l⁻¹ of the SL₆ solution of Pfennig & Lippert, 1966), CaCl₂·2H₂O (100 mg l⁻¹) and iron(III) ammonium citrate (10 mg l⁻¹); medium II had the same composition as medium I, but (NH₄)₂SO₄ was replaced by an equivalent amount of Difco Casamino acids. Organic substrates were sterilized separately and mixed aseptically with the medium to give a final concentration ranging from 0.01 to 1 % (w/v) (see individual experiments).

For inoculation, conidia (from a Difco Sabouraud maltose agar plate) were harvested in sterile 0.9 % (w/v) NaCl solution (50 ml) supplemented with Tween 80 (4 drops) and vigorously shaken on a gyratory shaker. The spore suspension was then filtered through a thin layer of cotton wool sandwiched between two layers of cotton gauze and washed by centrifugation with sterile saline. The number of conidia in the inoculum was estimated turbidimetrically using a calibration curve which gave a direct relationship between the absorbance (at 400 nm) of the diluted spore suspension and the spore number. Inoculations were carried out with different initial spore densities (see individual experiments).

Culture flasks (up to 1 l baffled Erlenmeyer flasks, or up to 6 l flat-bottomed round flasks) were incubated at 44 °C with shaking or with stirring. Under these conditions spore germination occurred about 7 h after inoculation.

The growth of the organism was measured by weighing a sample of previously washed mycelium dried on a filter paper.

Preparation of cell extracts. The mycelium in a sample of growing culture was harvested on filter paper by filtration under reduced pressure. After washing and resuspension in buffer (0.05 M-sodium acetate buffer at pH 5.0, or at pH 5.75 when necessary), the mycelium was homogenized in the cold by sonication (Branson, model S-110 set at maximum power).

Enzyme assays. CM-cellulase (endo-1,4-β-glucanase; EC 3.2.1.4) was determined viscometrically according to Husemann & Werner (1963). A 0.5 % (w/v) solution of carboxymethylcellulose (CMC 7HF, DS = 0.57 to 0.87; Hercules, New York, U.S.A.) in 0.05 M-sodium acetate buffer (6 ml, pH 5.0) was pipetted into an Ostwald viscometer placed in a water-bath at 40 °C. The enzyme reaction was started by adding 1 ml of the sample solution (culture filtrate, cell extract or a dilution thereof) followed by immediate mixing. Flow rates (usually four measurements) were determined at intervals during the first 10 min of enzyme reaction. By plotting the ratios $\eta_{\text{sp}t_0}/\eta_{\text{sp}t_x}$ ($\eta_{\text{sp}t_x}$, specific viscosity after different times of reaction) against reaction time, a straight line was obtained for cellulase solutions of sufficient dilution. The unit of cellulase activity was arbitrarily defined as the slope of that line multiplied by 1000.

Cellulolytic activity of concentrated culture filtrates (concentrated on a rotating evaporator under reduced pressure at 30 °C followed by dialysis against buffer at 2 °C) was demonstrated by analysing the reducing sugars released after reaction with Avicel (type SF, American Viscose Corp.; 6 %, w/v, final concentration) in 0.05 M-sodium acetate buffer, pH 5.0, at 40 °C.

Colorimetric determinations. Reducing sugars in culture fluids were determined colorimetrically by the procedure of Somogyi (1952) and Nelson (1944) using the corresponding sugars as standards. Glucose was specifically determined by the glucose oxidase method (Biochemica Test Combination, Boehringer).

Pentoses, alone or in the presence of cellobiose, were determined by the orcinol method (Herbert *et al.*, 1971) using individual sugars as standards. Cellobiose interference was negligible. The cellobiose concentration in media containing a mixture of cellobiose and sorbose or cellobiose and fructose was determined by the *o*-toluidine method (Dawes *et al.*, 1971).

Ammonia in the culture filtrates was assayed by the phenol-hypochlorite method described by Bernt & Bergmeyer (1970).

The protein content of cell extracts and concentrated culture filtrates was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Protein was precipitated with 4 % (w/v, final concentration) trichloroacetic acid in the cold.

Chemicals. All reagents used were of analytical grade. 2-Deoxyglucose, 6-deoxyglucose and 3-*O*-methylglucose were from Calbiochem. D-[U-¹⁴C]Glucose (198 mCi mmol⁻¹) was from The Radiochemical Centre, Amersham.

Cellobiitol was prepared by reduction of cellobiose with NaBH₄ by the method of Lewis *et al.* (1963). The product was crystallized in the cold in ethanol/water and its purity was checked by descending paper chromatography with the solvent system ethyl acetate/pyridine/water (8:2:1, by vol.) and with the alkaline silver nitrate spray reagent according to Trevelyan *et al.* (1950).

1,4-β-D-Xylopyranosyl-D-xylose (xylobiose) was prepared according to Sieber (1972) by partial acid hydrolysis of an acidic xylan obtained by alkaline extraction of beech holocellulose. The disaccharide was isolated from the mixture by chromatography on a charcoal/celite column (elution with a linear gradient of water/20 % ethanol) and purified by preparative paper chromatography.

Glucomannan was extracted from dried tubers of *Orchis* sp. according to Buchala *et al.* (1974) and freeze-dried.

Laminaribiose was isolated from an enzymic hydrolysate of pachyman obtained by the action of endo-1,3-β-glucanase (EC 3.2.1.6) from *Rhizopus arrhizus* QM6789 (obtained by courtesy of Dr E. T. Reese).

Amorphous cellulose was prepared as follows. Avicel (5 g) was evenly dispersed in water (10 ml) and dissolved at room temperature by slowly adding concentrated phosphoric acid (85 %, 100 ml). The dissolved cellulose was immediately reprecipitated in cold water, washed with running tap water to neutrality and finally homogenized in distilled water with an Ultra-Turrax homogenizer.

Cellobionic acid was synthesized by chlorite oxidation of cellobiose (Westermarck & Eriksson, 1974).

Cellobiose uniformly ¹⁴C-labelled in the reducing glucose moiety was prepared enzymically using cellobiose phosphorylase (EC 2.4.1.20) derived from *Cellvibrio gilvus* according to the procedure of Swisher *et al.* (1964) with some modifications. *Cellvibrio gilvus* (ATCC 13127) was grown in a 2 l flat-bottomed round flask stirred magnetically at 30 °C. The medium used was that described by Hulcher & King (1958) except that vitamins were omitted and cellobiose was included at 0.2 % (w/v). After 30 h the cells were harvested by centrifugation, washed with cold 0.05 M-sodium citrate buffer at pH 6.2, and either used immediately or frozen in 10 % (w/v) glycerol for storage. The washed cell paste was resuspended in an equal volume of buffer and cell extracts were prepared by lysozyme digestion (160 mg lysozyme in 20 ml cell suspension) at 37 °C for 20 min. DNA was degraded by the addition of a few crystals of deoxyribonuclease I (from bovine pancreas, B-grade; Calbiochem) and the extraction was completed by a short ultrasonic treatment. Unbroken cells and cell debris were removed by centrifugation (13000 g) for 20 min at 2 °C and the supernatant was subsequently used as a crude source of cellobiose phosphorylase. The reaction mixture for the preparation of cellobiose contained D-[U-¹⁴C]glucose (0.1 mmol, containing 0.5 mCi), α-D-glucose 1-phosphate (di-Na; 0.25 mmol), MgCl₂·6H₂O (0.0225 mmol) and NaF (0.05 mmol), all in 2.5 ml sodium citrate buffer (0.05 M) at pH 6.2, and 3.5 ml cell extract. After incubation at 37 °C for 15 min, 12 ml absolute ethanol were added, the reaction mixture was boiled for 5 min and filtered through a Whatman GF/A glass-fibre filter. The filtrate was evaporated to dryness, the residue was redissolved in water and deionized by passage through an Amberlite MB2 (mixed bed) column (10 ml). Paper chromatography of a small sample in ethyl acetate/pyridine/water (2:1:2, by vol., upper phase) as solvent revealed that almost all the radioactivity present could be attributed to cellobiose and glucose. These two compounds and a few other minor radioactively labelled substances were separated on a charcoal/celite (1:1, w/w) column (1.5 × 25 cm). The sample, dissolved in 5 ml H₂O, was added to the top of the column which was then successively eluted with water (400 ml), a linear gradient of water/20 % ethanol (500 ml) and finally with 25 % ethanol (400 ml). Fractions of 5 ml

Table 1. *Cellulolytic activity of Sporotrichum thermophile grown on different types of cellulose (and on glucose and cellobiose as control)*

Medium II (3 l) was inoculated with conidia to an initial density of 3×10^6 ml⁻¹ and stirred magnetically at 44 °C for 7 h in order for germination to occur. The washed, young mycelium was used to inoculate medium II (300 ml in 500 ml baffled Erlenmeyer flasks; 0.225 mg mycelium ml⁻¹) supplemented with glucose (0.2 %, w/v) or cellobiose (0.2 %, w/v) or different kinds of cellulose: Avicel, 1 % (w/v); reprecipitated Avicel, about 1 % (w/v); CF 1, fibrous cellulose powder (Whatman), 1 % (w/v); CC 31, microgranular cellulose powder (Whatman), 1 % (w/v); CM 32, insoluble carboxymethylcellulose (Whatman), 1 % (w/v); CMC 7HF, soluble carboxymethylcellulose (Hercules), 0.2 % (w/v). The cultures were swirled in a water-bath at 250 rev. min⁻¹ at 44 °C. At intervals between 2 and 10.5 h after inoculation, samples were withdrawn and the culture filtrates were analysed for CM-cellulase activity.

Substrate	CM-cellulase activity (U ml ⁻¹)				
	2 h	4 h	6 h	8 h	10.5 h
Avicel	20	1350	4160	3200	5300
Reprecipitated Avicel	25	180	425	840	2365
CC 31	44	1900	8000	6600	14500
CF 1	35	2750	6800	7040	11600
CMC 7HF	300	820	2300	1100	900
CM 32	19	220	680	400	580
Cellobiose	250	4400	—	—	—
Glucose	15	50	—	—	—

were collected and those containing glucose (no. 61 to 107) and cellobiose (no. 159 to 188) were pooled. The total glucose yielded 143×10^6 c.p.m. and the cellobiose 281×10^6 c.p.m. as measured in Instagel (Packard) in a liquid scintillation spectrometer.

RESULTS

Sporotrichum thermophile is truly cellulolytic

Sporotrichum (Chrysosporium) thermophile is a thermophilic deuteromycete, having its temperature optimum for growth between 35 and 50 °C. Table 1 shows the cellulolytic activity of the culture filtrates when the mycelium was grown in submerged cultures in the presence of different cellulosic substrates. A few hours after inoculation, there was already a considerable amount of CM-cellulase activity present in the filtrates of all the cultures on crystalline cellulose and on cellobiose. In the culture on Avicel, the increase in CM-cellulase activity was paralleled by the degradation of the crystalline substrate, as deduced from the decrease (40 % after 10 h) in total dry substance (biomass produced plus residual cellulose). In the culture on amorphous cellulose (reprecipitated Avicel), despite the fact that CM-cellulase activity did not rise significantly during growth, there was an even more rapid degradation of the substrate, no cellulose being left after 6 h incubation.

When grown in a medium supplemented with cellobiose and in the absence of any cellulose, the organism also produced extracellular enzymes capable of degrading Avicel. A 10-fold concentrated and dialysed filtrate from a culture grown on cellobiose, whose CM-cellulase activity was 52 500 U ml⁻¹, released 0.97 µmol reducing sugar equivalents min⁻¹ (mg protein)⁻¹ when incubated with crystalline cellulose (Avicel) at 40 °C and at pH 5.0.

Conditions for CM-cellulase synthesis

Although CM-cellulase was produced in the presence of cellulose, other polysaccharides, with the exception of glucomannan, failed to induce CM-cellulase synthesis (Table 2). Culture filtrates from the cultures listed in Table 2 were also tested for their ability to degrade xylan, starch, pectin and glucomannan. No xylanase, amylase or endo-polygalacturonase activities were found in any of them, but the two filtrates showing high CM-cellulase activity

Table 2. *CM-cellulase synthesis in the presence of non-cellulosic polysaccharides as carbon sources (and with cellobiose as control)*

Erlenmeyer flasks (500 ml) containing 250 ml medium II and the organic substrate were inoculated with conidia (initial density $2 \times 10^6 \text{ ml}^{-1}$) and shaken on a rotary shaker ($200 \text{ rev. min}^{-1}$) in a water-bath at 45°C . The extracellular CM-cellulase activity was determined between 9 and 12 h after inoculation.

Organic substrate	Concn (%, w/v)	CM-cellulase* (U ml^{-1})
Cellobiose	0.2	6800
Orchis glucomannan (soluble)	0.05	5300
Beechwood xylan (only partly soluble)	approx. 1	500
Soluble starch	0.2	168

* Maximum value obtained during growth.

Table 3. *CM-cellulase production by Sporotrichum thermophile grown on different organic substrates as carbon source*

Erlenmeyer flasks (250 ml) containing 100 ml medium I and 0.5 % (w/v) organic substrate were inoculated with conidia to an initial density of $4 \times 10^6 \text{ ml}^{-1}$. The cultures were shaken (reciprocal shaker, 60 strokes min^{-1}) in a water-bath at 35°C for 12 h, in order for germination to occur, before raising the temperature to 48°C . The growth was checked visually and the cultures were harvested between 4 and 10 h after germination.

Organic substrate	CM-cellulase (U ml^{-1})	Dry weight (mg ml^{-1})	CM-cellulase specific activity [U (mg dry wt) $^{-1}$]
Glucose	15	0.59	25
Mannose	36	0.48	75
Fructose	68	0.31	219
Xylose	69	0.49	140
Glucosamine	26	0.54	48
Cellobiose	930	0.48	1937
Sucrose	56	0.50	112
Lactose	72	0.29	248
Glycerol	36	0.16	225
Mannitol	81	0.46	176
Sorbitol	18	0.37	48
Gluconate (Na)	29	0.24	120
Malate (Na)	40	0.18	222
Pyruvate (Na)	36	0.27	133
Lactate (Na)	14	0.18	77
Acetate (Na)	56	0.36	155
Succinate (Na)	32	0.19	168
Citrate (Na)	36	0.12	300
Glutamate (Na)	60	0.23	260
Asparagine	30	0.30	100
Salicin	50	0.23	217
Arbutin	52	0.24	216
Amygdalin	28	0.29	96

were also able to decrease the viscosity of a glucomannan solution, the highest glucomannanase activity being found in the filtrate of the culture grown on glucomannan.

When the organism was grown in the presence of a variety of different low molecular weight organic substrates no appreciable amounts of CM-cellulase were formed except during growth on cellobiose (Table 3). Measurements of the biomass obtained after 4 to 10 h incubation indicated that the substrates utilized were not equivalent in supporting the growth of the organism. Even in the presence of substrates less readily utilized as carbon sources, no derepression of CM-cellulase synthesis was observed.

Other disaccharides and disaccharide derivatives having the same glycosidic bond as

Table 4. *CM-cellulase activity in the culture filtrate after growth in the presence of different disaccharides (and glucose) as carbon source*

In experiments (a), conidia (4×10^8 ml⁻¹) were pregerminated in medium I containing 1 % glucose. After germination, the washed mycelium was resuspended in fresh medium (20 ml) supplemented with different disaccharides and shaken (reciprocal shaker) in a water-bath at 48 °C. In experiments (b), conidia (2×10^6 ml⁻¹) were suspended in 50 ml medium II supplemented with cellobiose, laminaribiose or glucose and shaken in a water-bath at 44 °C. In both series of experiments, CM-cellulase activity was determined at intervals. The results indicate the maximum activities obtained, expressed as a percentage of the maximum activity after growth with cellobiose.

Substrate	Concn (%, w/v)	Glucosidic bond	CM-cellulase (%)
Cellobiose ^a	0.5	1,4-β	100
Gentiobiose ^a	0.5	1,6-β	8
Sophorose ^a	0.125	1,2-β	4
Maltose ^a	0.25	1,4-α	8
Trehalose ^a	0.5	1,1-α	4
Cellobiose ^b	0.2	1,4-β	100
Laminaribiose ^b	0.2	1,3-β	22
Glucose ^b	0.2	—	2

cellobiose were also tested for their possible stimulating effect on cellulase synthesis (experimental conditions as in Table 4). Such substances were: cellobiitol, cellobionic acid, cellobiose octaacetate, xylobiose (1,4-β-D-xylopyranosyl-D-xylose) and glucosylmannose (1,4-β-D-glucopyranosyl-D-mannose). Only glucosylmannose and cellobionic acid stimulated CM-cellulase synthesis: glucosylmannose was almost as effective (77 %) as cellobiose, whereas cellobionic acid had an inducing capacity of about one-third that of cellobiose. To establish if there was a relationship between the type of the glucosidic bond of different glucose dimers and CM-cellulase induction, the organism was cultivated on cellobiose, gentiobiose, sophorose, maltose, laminaribiose and trehalose (Table 4). Laminaribiose was the only compound found which stimulated CM-cellulase synthesis appreciably, although to a lesser extent than cellobiose. Sophorose, which is known to be a potent cellulase inducer in other organisms (Mandels *et al.*, 1962; Yamane *et al.*, 1970; Nisizawa *et al.*, 1971 *b*), had no effect.

CM-cellulase induction by cellobiose was studied in more detail in the experiment shown in Fig. 1. The biomass formed during the course of the culture was correlated with the utilization of cellobiose. The cellulase formed was immediately secreted into the culture medium, since the activities found in the cell extracts were very low throughout incubation.

CM-cellulase induction versus derepression

Working with several cellulolytic fungi, Hulme & Stranks (1971) came to the conclusion that cellulase is not formed as a response to any inducing substance, but rather is triggered by conditions reducing the rate of growth. Some experiments were performed to test such a possible mechanism of derepression of CM-cellulase synthesis in *S. thermophile*. The organism was first grown on glucose, but culture conditions were selected to obtain total glucose exhaustion before the mycelium entered the stationary growth phase. Even several hours after substrate depletion, cellulase did not significantly increase in the external medium (Fig. 2*a*). Similarly, resuspension of mycelium growing on glucose in fresh basal medium without glucose showed no derepression of CM-cellulase synthesis (Fig. 2*b*). The experiments shown in Fig. 3 were carried out to test the possible inhibitory effect of cellobiose itself, when supplied at different concentrations (up to 10 %, w/v). At very high cellobiose concentrations (5 and 10 %) CM-cellulase formation was only slightly inhibited (Fig. 3*a*) giving no indication of a catabolite repression of this enzyme by cellobiose. A limited supply of cellobiose (0.05 %) was, after 3 and 4 h incubation, accompanied by a correspondingly

Fig. 1. CM-cellulase synthesis by *Sporotrichum thermophile* during growth on cellobiose. Medium II (3 l) supplemented with cellobiose (0.25 % w/v) was inoculated with conidia to a density of 2×10^6 ml $^{-1}$ in a 6 l flat-bottomed round flask and stirred magnetically (400 rev. min $^{-1}$) in a water-bath at 44 °C. Samples for analysis were taken at intervals: ○, total CM-cellulase activity [U (ml culture filtrate) $^{-1}$]; ●, specific CM-cellulase activity [U (mg dry wt) $^{-1}$]; △, intracellular CM-cellulase activity [U in cell extract corresponding to 1 ml culture]; □, dry weight [mg (ml culture) $^{-1}$]; ■, residual cellobiose [μ mol (ml culture) $^{-1}$]. The arrow indicates the time of spore germination.

Fig. 2. Formation of extracellular CM-cellulase by *Sporotrichum thermophile* grown in the presence of glucose as carbon source. (a) Medium II (1 l) supplemented with 0.2 % (w/v) glucose was inoculated with conidia (2×10^6 ml $^{-1}$) in a 2 l flat-bottomed round flask and stirred magnetically in a water-bath at 44 °C. Samples for analysis were taken at intervals. (b) The same procedure was followed except that 2 h after germination, the mother culture (containing 0.3 % glucose) was filtered, and the mycelium was washed and resuspended in fresh culture medium without sugar carbon and reincubated at 44 °C. ○, CM-cellulase; ●, glucose; △, pH; ■, dry weight.

limited production of biomass and extracellular CM-cellulase, whereas the specific CM-cellulase activity [U (mg dry wt mycelium) $^{-1}$] was similar with all the different cellobiose concentrations used (Fig. 3b).

Inhibition of CM-cellulase synthesis by glucose and some other carbon substrates

Nisizawa *et al.* (1971a, b, 1972) and Whitaker (1971) reported that cellulase synthesis in *Trichoderma viride* is repressed by glucose. Glucose also exerts an inhibitory effect on CM-cellulase formation by *S. thermophile* (Fig. 4). Glucose was added to an exponentially growing batch culture of the organism with cellobiose as carbon source. The addition of

Fig. 3. Relationship between CM-cellulase formation and the cellobiose concentration in the culture medium. Six parallel cultures of 200 ml each, inoculated with conidia (2×10^6 ml $^{-1}$) and containing medium II but no sugar substrate, were shaken in 500 ml baffled Erlenmeyer flasks on a gyrotary water-bath (250 rev. min $^{-1}$) at 44 °C. At the time of germination, cellobiose was added to the cultures to give concentrations of 0.05, 0.5, 1, 5 and 10% (w/v), and CM-cellulase activity was measured at 1 h intervals. Activity in a control culture without cellobiose was measured at the time of inoculation and 3 h after inoculation. The histograms show (a) the absolute CM-cellulase activity [U (ml culture filtrate) $^{-1}$] and (b) the corresponding specific activity [U (mg dry wt) $^{-1}$].

glucose resulted in a marked decrease in CM-cellulase biosynthesis. This inhibition was found after only a short period and the effect was proportional to the amount of glucose added. CM-cellulase synthesis was resumed as soon as the glucose concentration in the culture medium fell below a critical level (between 1.5 and 2 μ mol ml $^{-1}$).

The experiment shown in Fig. 5 was carried out to establish whether or not cellobiose in dual substrate cultures, containing glucose and cellobiose, was actually taken up by the mycelium of *S. thermophile*. [14 C]Cellobiose concentration in the medium was measured throughout the growth of the culture. Glucose concentration was determined by the specific glucose oxidase method. The results show that glucose, although added when the organism was fully adapted to cellobiose, was immediately taken up by the mycelium and further cellobiose uptake and CM-cellulase formation were almost completely prevented.

Other substrates (Fig. 6) were tested for their possible inhibitory effect on CM-cellulase induction by cellobiose. Mannose (results not shown), xylose and, to a limited extent, gluconate, which were all almost equally good growth substrates, had similar inhibitory effects to that of glucose. Fructose and sorbose did not cause inhibition. Cultures with sorbose were included because this sugar has been reported to be transported by the same carrier as that used for glucose transport in baker's yeast (Cirillo, 1961) and in *Neurospora crassa* (Klingmüller, 1967; Scarborough, 1970). Growth with fructose or sorbose alone (results not shown) was very poor. Astonishingly, however, 50% of the sorbose was taken up by the mycelium in the culture grown on sorbose alone, but the biomass did not exceed that reached by the culture on fructose, a sugar which was not taken up by the mycelium. The organism grew on the Casamino acids and yeast extract present in the basic medium. In dual substrate cultures on fructose or sorbose with cellobiose, however, the growth was comparable to that of the control (cellobiose alone) and CM-cellulase formation did not seem to be significantly affected by the presence of these sugars. A different inhibitory effect on CM-cellulase synthesis to that caused by glucose and other substrates was observed in dual substrate cultures on cellobiose and acetate (Fig. 6f). The simultaneous presence of acetate resulted in a delay in cellobiose utilization which corresponded with a delay in CM-cellulase synthesis. Yield of mycelium (results not shown) was, however, only slightly affected. Attempts were made to mimic the inhibitory effect of glucose on CM-cellulase synthesis by using 3-O-methylglucose, 6-deoxyglucose and 2-deoxyglucose which are non-

Fig. 4

Fig. 5

Fig. 4. 'Glucose effect' on CM-cellulase synthesis induced by cellobiose. Medium II (1 l) supplemented with 0.25 % (w/v) cellobiose was inoculated with conidia ($2 \times 10^6 \text{ ml}^{-1}$) in a 2 l flat-bottomed round flask and stirred magnetically in a water-bath at 44 °C. At the time indicated by the arrow, the culture was quickly divided into four portions of 250 ml each and placed in 500 ml baffled Erlenmeyer flasks to which different amounts of glucose were added. These cultures were incubated at 44 °C on a gyrotary shaker, and CM-cellulase activities were measured at intervals. Glucose concentrations in the cultures were (% w/v): ▲, 0.2; ■, 0.1; ●, 0.05; ○, 0 (control, cellobiose alone).

Fig. 5. 'Glucose effect' on cellobiose utilization and CM-cellulase synthesis. Medium II (300 ml) supplemented with 0.2 % (w/v) cellobiose was inoculated with conidia ($2 \times 10^6 \text{ ml}^{-1}$) and incubated in a 500 ml Erlenmeyer flask in a gyrotary water-bath (250 rev. min^{-1}) at 44 °C. After 6 h incubation [^{14}C]cellobiose (1.5 μCi) was added and the utilization of cellobiose was subsequently determined by measuring at intervals the radioactivity in 2 ml samples of culture filtrates. At the time indicated by the arrow the culture was quickly divided into two portions, to one of which glucose (0.2 %, w/v) was added. ○, CM-cellulase in the control; ●, CM-cellulase in the culture medium after glucose addition; □, residual cellobiose in the control; ■, residual cellobiose in the dual substrate culture; ▲, residual glucose.

(or only partly-) metabolizable glucose analogues. 3-*O*-Methylglucose had no effect on the CM-cellulase synthesis induced by cellobiose (Fig. 7*a*); 6-deoxyglucose, which, like 3-*O*-methylglucose, is not a hexokinase substrate and therefore not metabolized by the cells, caused limited inhibition (Fig. 7*b*); whereas 2-deoxyglucose, which is probably metabolized to 2-deoxyglucose 6-phosphate, delayed CM-cellulase synthesis appreciably (Fig. 7*c*). The growth of the organism in the presence of 2-deoxyglucose (plus cellobiose) was at first inhibited, but later resumed together with the extracellular CM-cellulase synthesis. The length of this lag phase was directly dependent on the amount of 2-deoxyglucose added to the medium.

DISCUSSION

In many cellulolytic organisms the synthesis of cellulases is adaptive, i.e. the enzymes are induced only in the presence of cellulosic materials (Stewart & Leatherwood, 1976; Reese, 1977). Low levels of cellulase are probably always constitutively formed, supporting the idea that the true cellulase inducer is a soluble product of cellulose degradation which can be readily taken up by the cells (Mandels & Reese, 1960). This view has been challenged by some authors who claim that cellulase synthesis is constitutive, being regulated by catabolite

Fig. 6. Effect of xylose, fructose, sorbose, gluconate and acetate on CM-cellulase formation induced by cellobiose. Conidia (2×10^6 ml $^{-1}$) were allowed to germinate in medium II without any additional sugar. The young mycelium was then filtered, washed and resuspended in fresh medium. The suspension was divided into 300 ml portions, placed in 500 ml baffled Erlenmeyer flasks and incubated in a gyrotary water-bath (250 rev. min $^{-1}$) at 44 °C. At time 0 the substrates (0.2 %, w/v, final concentration) were added aseptically: (a) cellobiose alone (control); (b) cellobiose plus xylose; (c) cellobiose plus fructose; (d) cellobiose plus sorbose; (e) cellobiose plus sodium gluconate; (f) cellobiose alone and cellobiose plus sodium acetate. Control cultures on xylose, fructose, sorbose, gluconate and acetate alone, without cellobiose, were run in parallel (results not shown). ○, CM-cellulase; ●, residual cellobiose; □, dry weight; ■, residual xylose; △, CM-cellulase in the control culture; ▲, residual cellobiose in the control culture.

repression alone (Hulme & Stranks, 1970, 1971; Suzuki, 1975). The results presented here show that cellulase synthesis in *Sporotrichum thermophile* is an adaptive phenomenon, CM-cellulase being formed, in significant amounts, only when the organism is grown in the presence of cellulose or of a few other substances like glucomannan, glucosylmannose, cellobiose, laminaribiose and cellobionic acid, of which cellobiose is the most effective. Both, crystalline and 'amorphous' reprecipitated cellulose were degraded efficiently during growth of *S. thermophile* on these insoluble substrates. Extracellular enzymes formed in cultures on cellobiose were also capable of attacking crystalline cellulose (Avicel) indicating that all the enzymes forming the cellulase complex (exo- and endo-cellulases) were formed

Fig. 7. Effect of (a) 3-*O*-methylglucose, (b) 6-deoxyglucose and (c) 2-deoxyglucose on CM-cellulase synthesis induced by cellobiose. Cultures (100 ml in 250 ml baffled Erlenmeyer flasks) were inoculated with conidia (2×10^6 ml $^{-1}$) and incubated in a gyrotary water-bath (250 rev. min $^{-1}$) at 44 °C. The basic medium II contained the following additional substrates. (a) ○, cellobiose alone (0.2 %, w/v); ●, cellobiose (0.25 %, w/v) plus 3-*O*-methylglucose (0.2 %, w/v). (b) ○, cellobiose alone (0.25 %, w/v); ●, cellobiose (0.25 %, w/v) plus 6-deoxyglucose (0.2 %, w/v). (c) ○, cellobiose alone (0.25 %, w/v); ●, ■, ▲, cellobiose (0.25 %, w/v) plus 0.025, 0.05 and 0.1 % (w/v) 2-deoxyglucose, respectively.

even in the absence of cellulose. However, the cellulase activity was estimated viscometrically and therefore only CM-cellulase was assayed. No attempt has been made during this work to distinguish between the induction of the different enzymes comprising the cellulase complex. The level of CM-cellulase induced by various insoluble cellulosic substrates varies appreciably. The highest activities were obtained by incubating *S. thermophile* with highly crystalline celluloses like Avicel, CC 31 or CF 1 (Table 1). Incubation on amorphous cellulose or on soluble or insoluble carboxymethylcellulose resulted in much lower activities. This does not necessarily imply higher enzyme production with crystalline substrates, since the results obtained could be due to less enzyme adsorption on these substrates. Cellobiose seems to be the most likely natural inducer. Other disaccharides (with the exception of laminaribiose and cellobionic acid) with a similar chemical structure had no stimulating effect. However, no evidence is given in this report that cellobiose was taken up by the organism as such and so acted as the true intracellular inducer of cellulase synthesis. Alteration of this disaccharide by extracellular or mural (cell wall-bound) enzymes, prior to its entry into the cell, cannot be excluded.

A difference between *S. thermophile* and other cellulolytic fungi is that, in the former organism, CM-cellulase is synthesized during active growth on cellobiose, whereas in other fungi, cellulase synthesis is triggered by a very low cellobiose concentration in the culture medium (Mandels & Weber, 1969; Cooper & Wood, 1973). In *Trichoderma viride*, concentrations exceeding 0.2 % sophorose (which is a potent cellulase inducer in this fungus) cause catabolite repression of cellulase formation (Nisizawa *et al.*, 1971*a*). This finding has recently been corroborated by Loewenberg & Chapman (1977), who investigated the metabolic fate of sophorose and found that several low, repeated feedings of this sugar induced the formation of much more cellulase than an equivalent single dose.

The strong inhibition of CM-cellulase synthesis in fully induced cultures of *S. thermophile* caused by glucose and other readily metabolized substrates can be interpreted in terms of catabolite repression. In dual substrate cultures with glucose and cellobiose the glucose is preferentially utilized and apparently without any lag phase. In consequence, prevention of cellobiose uptake is paralleled with inhibition of CM-cellulase formation. The delay in cellobiose utilization and CM-cellulase formation caused by the presence of acetate also shows that CM-cellulase synthesis in this fungus is directly dependent on cellobiose utilization. In the present work a few attempts have been made to see if this phenomenon could be interpreted as a competition between glucose and cellobiose for the same transport carrier. With the use of 2-deoxyglucose, which competitively inhibits glucose transport in other eukaryotic micro-organisms (Neville *et al.*, 1971; Romano, 1973), the 'glucose effect' in dual substrate cultures was only partially mimicked (Fig. 7c); growth and CM-cellulase synthesis were temporarily inhibited but both resumed after a lag period, the duration of which was directly correlated with the concentration of the inhibitor added to the medium, and was much shorter than with the same glucose concentrations. 3-*O*-Methylglucose and 6-deoxyglucose (not substrates for hexokinase), which are common glucose analogues used in transport studies for their lack of interference with the subsequent sugar metabolism, had almost no effect on CM-cellulase synthesis induced by cellobiose. These findings support the idea that glucose and cellobiose are taken up by separate carriers into this organism, the glucose transport system probably being constitutive. The very short time elapsing between the addition of glucose to an induced culture and the observed repression of CM-cellulase synthesis suggests that this repression may occur at the translational or post-translational level of enzyme synthesis. A similar conclusion was drawn by Nisizawa *et al.* (1972) in their study on cellulase catabolite repression in *Trichoderma viride*.

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