Induction and catabolite repression of high-affinity glucoamylase in *Aspergillus terreus* strain 4

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The regulatory mechanism for glucoamylase synthesis in *Aspergillus terreus* strain 4 was studied. Synthesis of the enzyme was induced in the presence of maltose and other carbohydrates containing maltose units. Smaller amounts of enzyme were produced in the presence of glucose or other monosaccharides like sorbose and xylose. Enzyme production was maximal with 0.5% starch as the carbohydrate source. At higher concentrations of starch, production of the enzyme was less. In glucose medium, both extracellular and intracellular glucoamylase activities were very low. When starch medium was supplemented with glucose, the extracellular enzyme activity decreased with increasing concentration of added glucose. Intracellular amylase was also detected. There was no appreciable change in the level of this enzyme activity in the presence of added glucose. Further, when glucose was added to the growing culture after 2 d in starch medium there was a marked decrease in glucoamylase synthesis, which was also proportional to the amount of glucose added. Enzyme synthesis was resumed as soon as the glucose concentration in the culture medium fell below a critical level. All these observations strongly suggest that in *A. terreus* strain 4, extracellular glucoamylase synthesis is subject to catabolite repression.

Introduction

Although intensive investigations have been made to find microbial strains with strong glucoamylase activity, comparatively few studies have been carried out to elucidate the regulatory mechanism of this enzyme system. Further, the observations reported vary depending upon the organism studied. Thus glucoamylase synthesis has been found to be constitutive (Federici, 1984), partly constitutive (Attia & Ali, 1974; Takao *et al.*, 1986) or inducible (Barton *et al.*, 1972, 1975; Schellart *et al.*, 1976a). There are also reports of glucose and glutamic acid acting as catabolic repressors (Schellart *et al.*, 1976b).

In view of the commercial importance of glucoamylase, an intensive investigation was carried out in this laboratory. It yielded a good glucoamylase-producing strain, viz. *Aspergillus terreus* 4 (Ghosh *et al.*, 1990). The substrate constant for starch of this enzyme was 5.9 mg^{-1} . This value is exceptionally low as compared with the values reported for other mould glucoamylases (Middelhoven & Muylwijk, 1986). This paper reports the results of studies on the regulatory mechanism of glucoamylase synthesis in this strain.

Methods

Organism. The organism used in this study was *Aspergillus terreus* strain 4, isolated from spoiled potatoes and identified by the Commonwealth Mycological Institute (Kew, UK). Its glucoamylolytic activity was demonstrated by paper chromatography and by iodine-staining of the products of starch hydrolysis, catalysed by culture filtrates (Ghosh *et al.*, 1990).

Media and culture conditions. Stock cultures were maintained on 2% (w/v) malt extract agar and stored at 4 °C. For fermentation experiments, Czapek Dox (CD) medium was used as the basal medium. Starch-peptone (SP) medium containing 1% (w/v) starch and 0.5% peptone was also used in some experiments. The media were adjusted to pH 5.0 before autoclaving. Carbohydrates were sterilized separately and added aseptically to the sterilized media.

Erlenmeyer flasks (250 ml, each containing 40 ml medium) were inoculated with a spore suspension containing about $5-8 \times 10^6$ spores. All fermentations were run without shaking for 6 d at 28 °C.

Following incubation, the flask contents were filtered on dried and preweighed filter paper and washed thoroughly with distilled water. The filter paper with mycelia was then dried at 80 °C for 24 h (to a constant weight) and weighed.

Analytical methods. The culture filtrate was centrifuged at 10000 *g* for 10 min at 4 °C and the supernatant was used as the crude enzyme solution during initial studies of the time course of starch degradation (see Fig. 1). In all subsequent experiments the supernatant solution was dialysed against several changes of sodium citrate buffer (0.1 M, pH 5.0) for 24 h at 4 °C and this dialysed culture filtrate was used for enzyme

assays. To determine the intracellular enzyme activity, freshly harvested mycelium was thoroughly washed and homogenized in a tissue grinder with 0.1 M-sodium citrate buffer, pH 5.0.

Glucoamylase activity was determined by the increase in glucose released after digestion of starch in the reaction mixture (5 ml) containing 4 ml 1% (w/v) soluble starch in 0.1 M-sodium citrate buffer (pH 5.0) and 1 ml appropriately diluted enzyme solution. Following incubation at 60 °C for 10 min the reaction was stopped by immersing the reaction tube in boiling water bath for 5 min and then glucose was measured by the glucose oxidase method (Bergmeyer & Bernt, 1974), using glucose as the standard. A control was prepared using heat-inactivated enzyme. One unit of glucoamylase activity (U) was defined as the amount of enzyme liberating 1 μ mol glucose min^{-1} under the assay conditions. Specific activity of the enzyme was expressed as units $(\text{mg protein})^{-1}$. Residual starch was determined colorimetrically by the method of Robyt & Whelan (1968). Protein was determined by the Lowry method, using bovine serum albumin as the standard. Paper chromatograms were developed in *n*-butanol/pyridine/water/benzene (5:3:3:1, by vol.) using Whatman no. 1 paper, and the sugars were detected by spraying with *p*-anisidine hydrochloride (Schellart *et al.*, 1976a).

All experiments in this investigation were repeated at least once, and duplicate flasks were used in each experiment. Data represent the mean values.

Chemicals. Soluble starch, amylose, maltotriose and bovine serum albumin were obtained from Sigma. Amylopectin was obtained from Koch-light. All other chemicals used were commercial products of analytical grade.

Results and Discussion

Time course of starch metabolism

In order to study the course of starch degradation by *A. terreus* 4, the strain was grown for several days in SP medium and the crude enzyme solution was used for glucoamylase assay (Fig. 1). The concentration of glucose in the culture filtrate was maximal (1.5 mg ml^{-1}) on day 3 of the fermentation; it then decreased,

Fig. 1. Time course of starch degradation and secretion of glucoamylase by *A. terreus* 4 growing in SP medium (1% starch/ 0.5% peptone). ○, Glucoamylase; □, starch; △, mycelial dry weight; ■, glucose; ●, pH.

becoming almost negligible on day 6. Extracellular glucoamylase became detectable on day 3 of incubation and it went on increasing as glucose concentration decreased, until day 6. Visible mycelial growth occurred on day 3; it was maximal on day 6. During the early growth phase the pH of the medium increased to 6.2 when there was maximum accumulation of glucose. It then decreased gradually to 5.0 on day 5 and remained unchanged on further incubation.

Stimulation/induction of glucoamylase synthesis

In preliminary growth experiments with different carbon sources in CD medium, maximal enzyme activity was found if the carbon source was starch, maltotriose, maltose, amylose or amylopectin. Sorbitol supported appreciable mycelial growth (3.9 mg ml^{-1}) with very little yield (0.8 U ml^{-1}) of extracellular glucoamylase. Hence in order to study the effects of various carbohydrates on glucoamylase synthesis, 1% of the individual carbohydrate was added to 2-d-old cultures pregrown in CD medium containing sorbitol in place of glucose, and the glucoamylase activity of the dialysed culture filtrate was measured after 4 d of incubation (Table 1). Extracellular glucoamylase enzyme activity was detected only when the culture was grown in the presence of maltotriose, starch, amylose or amylopectin. Of the mono- and disaccharides tested, only maltose was favourable for enzyme production. Therefore, glucoamylase synthesis in this strain seems to be induced by the addition of maltose or other carbohydrates containing maltose units, but not by the addition of glucose and other sugars tested. The amount of enzyme production was not related to the amount of mycelial growth, as most of the non-inducing sugars supported good growth.

Table 1. Effect of various carbohydrates on extracellular glucoamylase production by *A. terreus* 4

Cultures were pregrown for 2 d in CD medium containing sorbitol in place of glucose, and then the test carbohydrate was added to 1% (w/v) and incubation continued for a further 4 d. Glucoamylase production was then assayed in dialysed culture filtrate.

Carbohydrate	Glucoamylase (U ml^{-1})	Glucoamylase spec. act. [U (mg protein) $^{-1}$]	Mycelial dry wt (mg ml^{-1})
Starch	21	7	3.2
Amylose	18	6	3.0
Amylopectin	16	5.3	2.8
Maltotriose	20	6.7	2.9
Maltose	19	6.3	3.1
Lactose	3	1.0	2.6
Sucrose	2	0.7	2.5
Glucose	4	1.3	2.8
Sorbitol	5	1.7	2.5
Xylose	6	2.0	1.8

Fig. 2. Effect of starch substrate concentration on (a) glucoamylase activity in dialysed culture filtrate and (b) residual sugar in the culture fluid of *A. terreus* 4.

Effects of starch on glucoamylase production

Fermentations were carried out in CD medium containing varying concentrations of starch as the carbon source in place of glucose and observations were made every 24 h using dialysed culture filtrate as the enzyme solution (Fig. 2a, b). In early stages of the fermentation (up to 3 d), high levels of glucose (1.2–3.2 mg ml⁻¹) and some extracellular amylase activity (4–13.5 U ml⁻¹) were observed in all the cultures regardless of the starch concentration (0.3–3.0%) used. But the enzyme activity was maximal (24.5 U ml⁻¹) on day 6 of the fermentation with 0.5% starch when the residual glucose in the culture filtrate was very low (0.6 mg ml⁻¹). In contrast, a high level of glucose (2.8 mg ml⁻¹) and very poor enzyme activity (11 U ml⁻¹) were observed in an otherwise identical culture growing with 3.0% starch.

It seemed possible that the lower activity of the enzyme observed in the culture filtrate of high glucose

Fig. 3. Effects of carbon sources and glucose concentration on glucoamylase production by *A. terreus* 4. In (a) the carbon source was glucose at the concentration indicated; in (b) it was 0.5% starch plus glucose at the concentration indicated. ○, Extracellular glucoamylase; △, intracellular glucoamylase; ●, mycelial dry weight.

content might be due to product inhibition of glucoamylase by glucose. To check this possibility, varying amounts of glucose were added to different sets of reaction mixture containing starch and the enzyme sample. During glucoamylase assay under standard condition no decrease in activity was observed in any case as compared to the control, without added glucose. This indicates that glucose in the enzyme solution did not inhibit the activity of glucoamylase.

Effects of starch and glucose on glucoamylase formation

Two sets of fermentations were carried out for 6 d in basal CD medium containing as carbon source(s) (a) different concentrations of glucose, or (b) a combination of 0.5% starch and varying concentrations of glucose. During growth at the expense of glucose as the sole carbon source, extracellular glucoamylase was negligible irrespective of the glucose concentration used. The intracellular level was also very low in all cases (Fig. 3a). On the other hand, appreciable intracellular amylase

Fig. 4. Effect of glucose concentration on glucoamylase synthesis by *A. terreus* 4. Cultures were pregrown on basal CD medium (containing 0.5% soluble starch as the C-source) for 2 d. Glucose was then added (arrow) to the concentration indicated. At intervals thereafter, glucoamylase activity was assayed in dialysed culture filtrates (a), and residual glucose in the culture filtrate was determined (b).

activity was observed in cultures growing in the presence of starch and glucose (Fig. 3b). The glucoamylase activity of intracellular amylase was confirmed by chromatographic analysis of starch hydrolysate carried out with this enzyme, when only a glucose spot was observed. Further, in all cases starch was completely degraded in the course of fermentation as confirmed by staining the samples of 6-d-old culture filtrate with iodine solution. Again, increasing glucose concentrations greatly reduced the production of extracellular glucoamylase (Fig. 3b). This suggests that glucose represses synthesis of extracellular glucoamylase, but not that of intracellular amylase.

To further verify this observation, different batches of fermentations were set up for 2 d with basal CD medium

containing 0.5% soluble starch. Varying concentrations (0.05–0.2%) of glucose were then added to these 2-d-old cultures, and during a further 6 d of incubation, dialysed culture filtrate samples were analysed at intervals for glucoamylase activity (Fig. 4a). The amount of glucose present in the filtrate was also determined (Fig. 4b). Addition of glucose resulted in a marked decrease in glucoamylase synthesis. This inhibitory effect was proportional to the amount of glucose added. Enzyme synthesis was resumed as soon as the glucose concentration in the culture medium fell below a critical level (2.0–3.2 mg ml⁻¹). This strong inhibition of glucoamylase synthesis in fully induced cultures of *A. terreus* 4 caused by glucose can be attributed to catabolite repression.

Concluding remarks

From the above results it can be concluded that glucoamylase synthesis in *A. terreus* 4 is subject to both induction and catabolite repression, an observation not previously reported except for *Trichoderma viride* (Schellart *et al.*, 1976b). There are contradictory observations of some carbohydrates acting as inducers or repressors of glucoamylase synthesis. Thus maltose is reported to induce glucoamylase synthesis in *Aspergillus niger* (Barton *et al.*, 1972). In *T. viride*, however, maltose is not an inducer: glucoamylase synthesis in this mould is induced by starch, dextrins of five or more glucose units and, to a lesser extent, by maltotriose (Schellart *et al.*, 1976b). Contradictory reports have also been made for glucose, acting as inducer in *A. niger* (Barton *et al.*, 1969) but as a repressor in *T. viride* (Schellart *et al.*, 1976b), *Polyporus ostreiformis* (Pal *et al.*, 1980) and *Candida antarctica* (De Mot & Verachtert, 1987). Such confusing observations by different workers on inducing/repressing effects of the same carbohydrates on glucoamylase synthesis reflect different regulatory mechanisms operating in the various moulds studied.

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