Repression and inactivation of α -amylase in *Thermomonospora* species during growth on cellobiose

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Thermophilic actinomycetes establish themselves as numerically dominant bacterial populations in selected high temperature environments by virtue of their exoenzymic ability to degrade the complex polysaccharides in thermogenic plant biomass. When *Thermomonospora curvata* and *Thermomonospora fusca* were grown on a mixture of cellulose and starch in mineral salts minimal medium, α -amylase was repressed via inhibition of maltose uptake by cellobiose. Addition of cellobiose to exponential phase cells growing on maltose or maltotriose triggered rapid degradation of extant amylase in the culture fluid of wild-type cells, but not in a protease-deficient mutant of *T. fusca*. A serine protease purified from *T. fusca* caused inactivation of the amylase in culture fluid of the mutant when added at a concentration approximating to that of the wild-type strain. The chelating agent, EDTA, accelerated inactivation by the protease, while the presence of calcium or amylase reaction products protected the amylase. Therefore, during growth in an environment containing multiple polysaccharides, these thermophiles control the levels of their extracellular depolymerizing enzymes via both inducer exclusion and proteolytic inactivation.

Keywords: actinomycete, amylase, cellobiose, protease, *Thermomonospora*

INTRODUCTION

Most microbial extracellular enzymes are controlled by catabolite repression and induction via a variety of mechanisms (reviewed by Priest, 1992; Saier *et al.*, 1996). Extracellular enzymes differ from those located in the cytoplasm because they are induced by their products rather than by substrate. Studies on inducer control have, to date, concentrated on the enterics (reviewed by Saier & Ramseier, 1996) or on Gram-positive bacteria having low (<55 mol %) G+C ratios (reviewed by Saier *et al.*, 1995, 1996). Here we describe some aspects of inducer control over the biosynthesis of an unusual α -amylase (Collins *et al.*, 1993) in *Thermomonospora* species (thermophilic actinomycetes which have a G+C ratio >65 mol %). We show that this control involves competition between amylase and cellulase inducers, and also proteolytic degradation of extant amylase after the introduction of cellobiose to cultures growing on starch or its degradation products.

METHODS

Micro-organisms and culture preservation. *Thermomonospora curvata* strain CUB993 was isolated from municipal solid waste compost (Stutzenberger, 1971). *Thermomonospora fusca* strain YX was isolated and described by Bellamy (1977). *T. fusca* ER1, a proteinase-deficient mutant of strain YX, was derived by Wilson (1992). *T. fusca* YX and ER1, as well as a highly purified preparation of the strain YX serine proteinase (in which strain ER1 is deficient), were generous gifts from David B. Wilson, Cornell University, USA. Cultures were maintained at -86°C in 33 % (v/v) glycerol and added as 1 % (v/v) inoculum; cells were subcultured at least once under test conditions to remove the possibility of interference by glycerol.

Medium and growth conditions. Cultures were grown in mineral salts minimal medium (Stutzenberger, 1972a) supplemented with 0.01 % yeast extract (Sigma). Carbon sources were 0.5 % soluble starch (Fisher Scientific), 0.5 % ground cotton fibres (surgical grade, Johnson & Johnson), or 10 mM maltose or maltotriose (Sigma). Carbon sources and vitamins were autoclaved as concentrated stocks and added aseptically to the salts medium after cooling. In experiments in which sugars were added to cultures during growth, the sugars were autoclaved dry in duplicate flasks into which the cultures were

poured aseptically to avoid volume changes. All cultures (100 ml initial volume) were shaken (140 r.p.m.) at 55 °C in baffled 250 ml Erlenmeyer flasks inclined at an angle of 30 ° from vertical for maximal aeration. Growth was measured spectrophotometrically (Milton Roy Spectronic 1201) in a 1 cm light path at 610 nm using culture samples diluted to an OD₆₁₀ of 0.1–1.0 (1.7 OD₆₁₀ units = 1 mg dry cell wt ml⁻¹) in fresh mineral salts medium. Culture fluids used in enzyme analysis or stability studies were clarified by centrifugation for 10 min at 13600 g at room temperature.

Enzyme analyses. All enzyme activities, with the exception of proteinase, were expressed as units = µmol product released min⁻¹. Amylase (EC 3.2.1.1) was estimated from reducing sugar liberation in 2 ml reaction mixtures containing 2% soluble starch, 0.1 M MES buffer, pH 6.0, and 0.1 ml cell-free culture fluid, incubated for 10 min at 65 °C. Cellulase (EC 3.2.1.4) was measured as endoglucanase activity based on reducing sugar generation in 2 ml reaction mixtures (2% Hercules type 7L CM-cellulose, 0.1 M MES, pH 6.2, and 0.1 ml cell-free culture fluid) incubated for 10 min at 65 °C. Reducing sugar concentrations were measured by the dinitrosalicylic acid method of Bernfeld (1955) and expressed as glucose equivalents ml⁻¹. Proteinase (chymotrypsin-like proteinase, EC 3.4.21.20) was measured in 0.3 ml reaction mixtures (final pH 8.5) containing equal volumes of 1.4% azoalbumin (Sigma) and cell-free culture fluid, incubated at 70 °C for 10 min. The reaction was stopped by the addition of 1.2 ml 10% trichloroacetic acid (TCA). Precipitated residual protein was removed by centrifugation (13600 g); 1 ml supernatant fluid was added to 1 ml 1 M NaOH and compared spectrophotometrically (1 cm light path) at 440 nm to corresponding blanks in which the TCA was added before culture fluid. Activity was expressed as units = µg azoalbumin hydrolysed min⁻¹.

Proteolytic inactivation of amylase. In preliminary experiments, the serine proteinase inhibitor PMSF (Sigma) was dissolved in 2-propanol and added (10⁻⁴–10⁻⁵ M final concentration) to exponential phase cultures to measure protection of amylase from proteolysis. Control flasks received an equal volume (0.1 ml) of 2-propanol without PMSF.

The rates of amylase inactivation by the purified proteinase were measured at 55 °C in *T. fusca* ER1 crude cell-free culture fluid, pH 8.0, to which was added proteinase (70 U ml⁻¹ final concentration) purified to electrophoretic homogeneity from *T. fusca* YX culture fluid (Gusek & Kinsella, 1987). Additives in some mixtures included 10 mM CaCl₂, 10 mM EDTA or a 40 mM (glucose equivalent) malto-oligosaccharide mixture produced from starch by *T. fusca* amylase at pH 6.0, 65 °C, then ultrafiltered through Amicon YM5 membranes to remove enzyme.

Measurement of soluble sugars. Uptake of maltose and other sugars by the actinomycetes was monitored as follows. Culture samples (2 ml) were removed aseptically at intervals during growth and centrifuged for 10 min at 13300 g at room temperature. The supernatant culture fluid was filtered through 0.45 µm pore size GHP13 Acrodiscs (Gelman Sciences) to remove residual particulates. Samples to be analysed for maltose in the presence of cellobiose were treated with purified β-glucosidase (type G4511, Sigma) to remove interference by cellobiose. Samples (20 µl) were pumped by a model 314 high pressure syringe pump (Isco) at a rate of 0.67 ml min⁻¹ through a RCM-Resex analytical column (7.8 × 300 mm, Phenomenex) at 85 °C in a regulated chamber (Bio-Rad). The retention times of malto-oligosaccharides were compared to those of known standards (Sigma) by refractive index changes at 35 °C internal cell temperature in a model

ERC-7510 detector (Erma Optical Works) linked to a CDS-402 data collection system (Varian Instrument Group).

The major malto-oligosaccharides liberated from starch by the extracellular amylases of the actinomycetes were also determined. Extracellular proteins from late exponential phase *T. curvata* and *T. fusca* cultures were concentrated about 10-fold and washed free of small molecular mass components on Amicon YM5 ultrafiltration membranes with 5 vols 0.05 M MES buffer, pH 6.0. These washed, concentrated proteins were incubated with 5% soluble starch at pH 6.0, 65 °C, for various intervals up to 8 h. Soluble malto-oligosaccharides were collected in Centricon-10 ultrafiltration cells (Amicon) and analysed in the HPLC system described above.

Other methods. Glucose concentrations estimated by HPLC were verified by the glucose oxidase colorimetric method using the Sigma manual assay kit 510-DA. Soluble protein was determined by the dye-binding method of Bradford (1976) according to the Bio-Rad technical bulletin 8L-0275.

Statistical analysis. Coefficients of variation were calculated (AT & T Statistical Quality Control Handbook, 1985) for assay methods by running six replicate determinations (four for HPLC methods) on representative samples. The values for amylase, growth, endoglucanase, reducing sugar, residual malto-oligosaccharides, proteolysis and soluble protein were ± 6.6, 14.5, 5.6, 1.3, 6.7, 2.1 and 3.7%, respectively. All data, except where otherwise noted, were the means of at least duplicate experiments.

RESULTS

Amylase production on starch as sole carbon source

During growth on starch as sole carbon source in mineral salts medium, the extracellular amylase levels in culture fluids of *T. curvata* and *T. fusca* reached maxima of about 17 and 7 U ml⁻¹, respectively, after 2–3 d growth at 55 °C. The soluble malto-oligosaccharides produced from starch by the extracellular amylases of either culture were maltose and maltotriose, with trace amounts of maltotetraose and maltohexose.

Amylase inactivation during growth on cellulose

During the growth of the actinomycetes on a mixture of cellulose and starch, extracellular α-amylase levels rose initially, but fell rapidly, compared to cultures grown on starch as sole carbon source (Fig. 1a). This result prompted a study to determine the mechanism by which the presence of cellulose caused the inactivation of amylase in culture fluid. Since cellobiose is the predominant soluble sugar which accumulates from the action of the *Thermomonospora* extracellular cellulases (Stutzenberger, 1972b; Aguirre *et al.*, 1986), the disaccharide was tested for its ability to block amylase biosynthesis or to initiate inactivation of the enzyme in culture fluid. When *T. curvata* was grown on a mixture of maltose and cellobiose, the amylase levels in culture fluid were depressed nearly to the level of that observed during growth on glucose as sole carbon source (Fig. 1b). This depression of amylase levels in culture fluid of cells grown in a maltose/cellobiose mixture was found to be due to the ability of cellobiose to inhibit the uptake

Fig. 1. Extracellular α -amylase production by *T. curvata* during growth on various carbon sources in mineral salts minimal medium. (a) Amylase levels in culture fluid of cells grown on starch as sole carbon source (○) or on a mixture of starch and cellulose (△). (b) Amylase in cultures grown on 10 mM maltose (○), 10 mM glucose (△) or a mixture of 10 mM maltose and 10 mM cellobiose (□).

of maltose from the medium; when 1 mM cellobiose was added to a culture growing on maltose (10 mM initial concentration), a transient cessation of maltose uptake persisted for several hours (Fig. 2). During this time, cellobiose was taken from the medium in preference over maltose until the cellobiose concentration fell below the level of detection (detailed data not shown).

Cellobiose-induced inactivation of amylase

The ability of cellobiose to depress amylase biosynthesis in *T. curvata* could be explained by the inhibition of maltose uptake. However, it could not account for the

Fig. 2. Effect of 1 mM cellobiose (time of addition shown by arrow) on maltose uptake (△) by *T. curvata* during growth (○) in minimal medium.

rapid decrease of amylase activity in the fluid of cultures grown in the presence of cellulose. The cellulose did not bind amylase, nor did addition of cellobiose to cell-free culture fluid cause inactivation of extant amylase. Cellobiose is a cellulase inducer in *Thermomonospora* spp. (reviewed by Wilson, 1992), and therefore the inactivation of amylase in the presence of cellobiose was suspected to be related in some manner to cellulase induction. In *T. fusca*, the production of an extracellular serine proteinase has been temporally linked to cellulase biosynthesis (Gusek *et al.*, 1988). This relationship of proteolytic activity to cellulase induction in *T. fusca*, and in other species (Lovrien *et al.*, 1985), prompted studies to determine whether the cellobiose effect on extracellular amylase was due in part to proteinase activity. Our use of PMSF as a serine proteinase inhibitor in exponential phase cultures to determine its amylase-protective effect was thwarted by its ability to inhibit growth of the actinomycetes; moreover, results were difficult to interpret because of the instability of PMSF under alkaline, high temperature culture conditions (James, 1978). Therefore, we employed a comparison of *T. fusca* YX and its proteinase-deficient derivative strain ER1 (Wilson, 1992; Lao & Wilson, 1996), to evaluate the role of proteolysis in amylase inactivation.

Cellobiose introduced into a *T. fusca* culture growing on maltotriose did not cause immediate inducer exclusion of the malto-oligosaccharide as it did in the case of maltose (Fig. 3a). However, rapid induction of cellulase occurred, closely followed by peaking and decrease of amylase activity in culture fluid (Fig. 3b). Following cellobiose addition, proteinase activity of culture fluid increased about eightfold. The total protein concentration in culture fluid rose roughly in parallel with proteinase activity, so it was not apparent that the proteinase was functioning to recycle extracellular

Fig. 3. Effect of 1 mM cellobiose on *T. fusca*. (a) Growth (○) and maltotriose depletion (△) from fluid of culture receiving 1 mM cellobiose (time of addition shown by arrow). (b) Amylase production and degradation (□) after induction of cellulase (measured as endoglucanase, ●) by cellobiose addition as shown in (a). (c) Total extracellular protein (▲) and proteinase activity (■) during cellobiose addition as shown in (a).

protein components back into the cells during the latter stages of growth (Fig. 3c).

Amylase stability in proteinase-deficient culture

The role of proteolytic activity was more clearly defined by the response of the amylase accumulation pattern to cellobiose addition in the proteinase-deficient *T. fusca* ER1. The maximal amount of amylase activity was about the same as that in *T. fusca* YX. However, no

Fig. 4. Growth (○) and amylase activity (△) in a *T. fusca* ER1 culture during addition of cellobiose (times of addition and final mM concentrations contributed by each addition indicated by arrows and numbers, respectively).

amylase inactivation response was observed even after repeated addition of cellobiose (Fig. 4). The uptake of the cellobiose was reflected by cellulase induction within an hour after addition of the inducer (data not shown).

Amylase inactivation by purified protease

The role of a specific serine proteinase in the degradation of amylase was confirmed by incubating the crude amylase in *T. fusca* ER1 cell-free culture fluid with proteinase purified from *T. fusca* YX extracellular fluid. In the absence of the proteinase, the crude amylase underwent a small amount of heat activation similar to that observed earlier for amylase in *T. curvata* culture fluid (Glymph & Stutzenberger, 1977). This heat activation of the crude amylase was completely reversed by overnight incubation of the culture fluid containing the activated amylase at 5 °C. Conversely, the amylase in *T. fusca* ER1 culture fluid mixed with the purified proteinase lost about 80% of its activity after incubation for 24 h at 55 °C (Fig. 5). The inactivation of the amylase was increased by EDTA (80% of activity lost within 5 h), but was diminished by the presence of calcium (20% of activity lost after 24 h). In the presence of its malto-oligosaccharide reaction products, the amylase underwent a transient thermal activation (an increase in activity of about 60% relative to the control) and retained its activity over that of the zero time sample despite the presence of the proteinase.

DISCUSSION

Thermomonospora species produce a wide range of extracellular depolymerizing enzymes including amylase (Glymph & Stutzenberger, 1977), cellulase (Calza *et al.*,

Fig. 5. Inactivation of amylase in *T. fusca* ER1 culture fluid by the serine proteinase purified from *T. fusca* YX. ○, Amylase control (no proteinase added); △, amylase and proteinase; □, amylase with proteinase in the presence of malto-oligosaccharides from amylase hydrolysis of starch; ●, amylase with proteinase in the presence of 10 mM EDTA; ▲, amylase with proteinase in the presence of 10 mM Ca²⁺.

1985; Lin & Stutzenberger, 1995), pectinase (Stutzenberger, 1987), proteinase (Gusek & Kinsella, 1987) and xylanase (Ristroph & Humphrey, 1985; Stutzenberger & Bodine, 1992). Although these enzymes have all been studied as to their chemical and physical characteristics, only cellulase has received detailed attention as to the regulatory mechanisms which control its biosynthesis (reviewed by Wilson, 1992). Extracellular enzyme biosynthesis in *Thermomonospora* has both ecological and biotechnological implications (McCarthy *et al.*, 1988; Trigo & Ball, 1994), and therefore the factors which regulate their biosynthesis in these actinomycetes should be defined. This aspect would be particularly relevant during growth on a complex lignocellulosic biomass (McCarthy, 1987); the interaction and competition between inducers released concurrently from a variety of available biopolymers determine the sequence of appearance and the maximal levels attained by these enzymes (Stutzenberger & Jenkins, 1991).

In this study, we have shown that the presence of cellulose or cellobiose blocks induction of α -amylase via inducer exclusion and initiates inactivation of the amylase via proteolysis. The rapid decrease of α -amylase activity when the actinomycetes were grown in the presence of cellulose does not appear to be attributable to thermal instability of the enzyme, since amylase levels were relatively constant for several days under culture conditions in the absence of cellulose, and the enzyme retains its activity at temperatures up to 65 °C under

slightly alkaline cultural conditions (Glymph & Stutzenberger, 1977).

The cessation of amylase biosynthesis after cellulase induction would be advantageous to the actinomycetes on the basis of energy conservation, particularly since starch (which constitutes 2–6% of total polysaccharide in lignocellulosic plant material) is eliminated more rapidly than cellulose from degrading biomass (Lossin, 1970). However, the advantage of inactivation of extant amylase by proteinase is not clear. This serine proteinase, which is maximally produced during growth on cellulose (Gusek *et al.*, 1988), alters the cellulase isozyme pattern and is capable of extensively hydrolysing the actinomycete crude extracellular proteins (Wilson, 1992). Nevertheless, *Thermomonospora* is not adapted for growth at the expense of proteinaceous substrates, either intact or hydrolysed (Crawford, 1975), and therefore the amylase, even after extensive proteolytic degradation, could probably not be recycled for the synthesis of new enzymes or as carbon and energy sources. Whatever functions this proteinase may serve, it does not inactivate the amylase if it is protected by its malto-oligosaccharide products and therefore would not eliminate amylase activity from the environment before the supply of starch was exhausted. This feature is consistent with the observation (Wheatley, 1987) that the predictability of the selection of enzyme proteins for degradation is greatly influenced by the presence of their substrates and co-factors.

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