Induction and repression of α -amylase production in batch and continuous cultures of *Aspergillus oryzae*

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The intra- and extracellular concentrations of α -amylase in *Aspergillus oryzae* have been measured during batch culture of a wild-type strain and two recombinant strains. The mean intracellular level for the two recombinant strains was about four to five times the level of the wild-type strain. The recombinant strains also had a higher α -amylase productivity, whereas the residence time of the intracellular α -amylase pool was approximately the same for the three strains. At high glucose concentrations there was a low constitutive synthesis of α -amylase, whereas at low glucose concentrations derepression resulted in an increased production rate. Shifts from a glucose- to a maltose-limited chemostat showed that maltose induces both the production and secretion of α -amylase. Finally, from immunoblots, both a glycosylated and an unglycosylated α -amylase have been detected.

Keywords: *Aspergillus oryzae*, batch culture, continuous culture, α -amylase, intracellular measurements

INTRODUCTION

A large number of industrial enzymes are derived from filamentous fungi belonging to the genus *Aspergillus*. It is a characteristic of these fungi that they are common and mostly harmless, and that they secrete a number of extracellular enzymes in relatively large quantities.

In this study α -amylase production by *Aspergillus oryzae* is considered. *A. oryzae* has been used in Asian food preparation for thousands of years. Recently, it has been shown that *A. oryzae* exhibits potential as a host for the production of many different heterologous proteins, both proteins from other fungi (Christensen *et al.*, 1988; Høj-Jensen *et al.*, 1989) and mammalian proteins (Ward *et al.*, 1992; Tsuchiya *et al.*, 1993). Today, *A. oryzae* is used industrially in the production of α -amylase, proteases and the heterologous lipase from *Humicola lanuginosa* (Barbesgaard *et al.*, 1992).

The mechanisms behind growth and product formation are critical in the optimization of a production process. Despite many years of experience with the culture of *A. oryzae*, these mechanisms are poorly understood and only a few attempts have been made to analyse the regulation

of the production of α -amylase in *A. oryzae*, Yabuki *et al.* (1977) and Erratt *et al.* (1984) showed that α -amylase production in *A. oryzae* is induced by maltose and starch, respectively, and other studies with the related fungus *A. awamori* have indicated glucose repression on the production of α -amylase (Bhella & Altosaar, 1987). Common to these experiments, however, is the fact that they have all been carried out in shake flasks where the culture conditions are poorly controlled.

In this study the intra- and extracellular concentrations of α -amylase were measured throughout cultivation in bioreactors, with the aim of investigating the influence of the substrates glucose and maltose on α -amylase production by *A. oryzae*. α -Amylase is a starch-degrading enzyme, which catalyses the hydrolysis of internal α (1–4)-glucosidic bonds in amylose, amylopectin and glycogen, but is not able to attack α (1–6)-glucosidic linkages in the branched amylopectin and glycogen. α -Amylase comprises a single polypeptide chain with 478 amino acid residues and one N-linked carbohydrate side-chain (McKelvy & Lee, 1969).

METHODS

Organism and culture conditions. The three strains of *A. oryzae* were obtained from Novo Nordisk. The wild-type strain (A1560) originated from wild-type strain IFO4177 from the Institute for Fermentation, Osaka, Japan. Strain CF1.1 is a

Abbreviations: AMG, amylglycosidase; FAU, α -amylase activity unit defined as the amount of α -amylase that hydrolyses 5.26 g starch at 37 °C in 1 h; FIA, Flow Injection Analysis; PNGase F, N-glycosidase F

transformant of A1560 containing additional copies of the α -amylase gene. The third strain, CF2.1, is a morphological mutant of CF1.1 that was isolated after treatment of CF1.1 spores with nitrosoguanidine (NTG).

Conidia from 6–8-d-old cultures (Carlsen, 1994) were inoculated into a defined batch medium containing (l^{-1}): 25.0 g glucose (monohydrate), 7.3 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4 , 1.0 g $MgSO_4 \cdot 7H_2O$, 1.0 g NaCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.5 ml trace metal solution (14.3 g $ZnSO_4 \cdot 7H_2O$, 2.5 g $CuSO_4 \cdot 5H_2O$, 0.5 g $NiCl_2 \cdot 6H_2O$ and 13.8 g $FeSO_4 \cdot 7H_2O$). The medium for continuous culture was identical to the batch medium except for the carbon and nitrogen content [8.0 g glucose monohydrate l^{-1} and 5.0 g $(NH_4)_2SO_4$ l^{-1}].

All batch cultures were carried out in a 15 l MBR bioreactor and continuous cultures in a 7 l Chemap-bioreactor. The temperature was 30 °C and the pH was kept constant at 6.0 by adding 2 M H_2SO_4 or 4 M NaOH as necessary. Aeration was kept constant at 1 vol. air (vol. medium) $^{-1}$ min $^{-1}$. The dissolved oxygen tension was kept above 60 % by controlling the agitation rate. The biomass concentration was measured by filtering the sample on a dry and preweighed filter, washing with 0.9 % NaCl, followed by drying at 105 °C for 24 h.

Measurement of glucose. Glucose concentration was measured in cell-free samples by a Flow Injection Analysis (FIA) system based on the enzymic oxidation of β -D-glucose to D-glucolactone by immobilized glucose oxidase at pH 6.5. Hydrogen peroxide generated in this oxidation is detected in the FIA system by oxidation of luminol, whereby light with a maximum intensity at 430 nm is emitted (Benthin *et al.*, 1991; Carlsen *et al.*, 1995a).

Extracellular α -amylase measurements. A FIA system was used to measure the extracellular α -amylase concentration. The principle is the decolorization of a sample containing a blue iodine–starch complex by the α -amylase from the culture sample (Carlsen *et al.*, 1994). The α -amylase activity unit, FAU, is defined as the amount of α -amylase that hydrolyses 5.26 g starch at 37 °C in 1 h (Hansen, 1984). The FIA system measures the total amylolytic activity, but the exoacting glucoamylase activity does not give a substantial response by injection in the FIA system.

Preparation of intracellular α -amylase samples. The sample preparation for intracellular α -amylase measurements should be carried out as fast and as cold as possible to get a representative intracellular protein sample. The sample was therefore filtered rapidly in a cold Büchner funnel and washed with cold 0.9 % NaCl solution, whereafter the biomass was instantly frozen using liquid nitrogen. The frozen biomass was stored at –80 °C. Frozen biomass (100 mg) was resuspended in 1.5 ml 0.1 M sodium phosphate buffer, pH 5, and kept on ice. The buffer contained the inhibitors phenanthroline (10 mM), PMSF (1 mM), and pepstatin A (3 μ M) (all obtained from Sigma) to prevent unwanted proteolysis. Phenanthroline (and not the common EDTA) was used as metalloprotease inhibitor, since it has a much lower affinity for calcium which stabilizes α -amylase against protease degradation (Stein & Fischer, 1958).

The mycelium was disrupted in a Retsch bead-mill for 15 min with 0.5 ml glass beads (0.75–1.0 mm) at 4 °C. The samples were centrifuged (10 min, 15000 r.p.m., 4 °C) and the supernatant with the intracellular proteins was collected and stored at –20 °C.

Quantification of intracellular α -amylase. Intracellular samples were subjected to SDS-PAGE (10%, w/v, acrylamide containing 0.25 % bis-acrylamide, National Diagnostics) according to the method described by Laemmli (1970). Following electrophoresis, the separated proteins in the gel were electro-

phoretically transferred to a nitrocellulose membrane (pore size 0.45 μ m), which was then incubated with polyclonal rabbit antibodies (Novo Nordisk) raised against the *A. oryzae* α -amylase. The immuno-complex was detected with peroxidase-conjugated goat anti-rabbit antibodies (Dakopatts) and visualized by adding H_2O_2 and the hydrogen donor, 4-chloro-1-naphthal (Sigma). This resulted in a blue precipitate on the blot.

Quantitative values of the amount of protein were obtained by measuring the intensity of the blue bands with a laser scanner (LKB UltroScan XL Densitometer). Four α -amylase standards with a known amount of enzyme (1, 2, 4 and 10 mFAU) were added on each gel and used to generate a standard curve. Purified α -amylase produced by *A. oryzae* (obtained from Novo Nordisk) was used as a standard. In all cases a linear standard curve was obtained with a correlation coefficient better than 0.99 and a relative SD of the slope below 5 %. The samples were diluted to be within the linear range of the standard curve.

Deglycosylation of α -amylase. N-Glycosidase F (PNGase F, Sigma) which cleaves the N-glycan linkage of glycoproteins was used to deglycosylate α -amylase. Sodium phosphate buffer (20 μ l, 0.1 M, pH 8) and 100 μ g protein were added to 20 μ l sample and incubated for 20 h at 37 °C. SDS-PAGE and immunoblotting of the samples followed.

Analysis of amylolytic activity. Erratt *et al.* (1984) described a method to assay α -amylase activity and other starch-degrading activity in an SDS-gel. Following SDS-PAGE, the gel was washed for 10 min in distilled water. The staining procedure comprises three steps. Firstly, the gel was saturated with starch (1 %, w/v, starch solution); 2 $\times$ 1 h at 60 °C followed by 40 min at 4 °C. Secondly, the gel was washed with distilled water and incubated for 2.5 h in a 0.15 M sodium citrate/phosphate buffer, pH 4.8 (fresh buffer after 1.5 h). Finally, the buffer was removed and the gel stained with a KI/I $_2$ solution (10 g KI, 1 g I $_2$ in 100 ml distilled water). Clear spots caused by amylolytic activity appeared in the blue gel.

RESULTS

Identification of α -amylase

SDS-PAGE followed by immunoblotting was chosen to identify α -amylase, since it specifically detects the protein of interest. This method also enables detection of more than one form of α -amylase, e.g. preforms, unglycosylated and overglycosylated forms. By separating the proteins it is easy to distinguish them from each other and, furthermore, it is possible to estimate their molecular mass.

Fig. 1 shows an immunoblot where intra- and extracellular samples were loaded next to each other. The samples were from a glucose-grown batch culture of the wild-type strain A1560 (lanes 4 and 5) and the recombinant strain CF2.1 (lanes 2 and 3). Both intra- and extracellular samples from the two strains revealed two clear bands, a thick band at 54 ± 2 kDa and a thinner band at 66 ± 2 kDa (mean values $\pm$ SD for five replicates). The first value correlates very well with the reported molecular mass of purified α -amylase from *A. oryzae* (Erratt *et al.*, 1984; McKelvy & Lee, 1969). From the amino acid sequence, α -amylase has been calculated to be 52.6 kDa, and since it contains a 1.5 kDa carbohydrate chain, the molecular mass is 54.1 kDa.

In the samples taken from cultures of the genetically manipulated strain, a third band was detected (Fig. 1,

Fig. 1. Immunological identification of α -amylase. Lanes: 1, molecular mass markers; 2 and 3, intra- and extracellular samples, respectively, from batch culture with the recombinant strain CF2.1; 4 and 5, intra- and extracellular samples, respectively, from batch culture with the wild-type strain A1560. The molecular mass markers were visualized by silver staining.

Fig. 2. Glycosylation of samples. Lanes: 1, molecular mass markers; 2, untreated CF1.1 sample; 3, CF2.1 sample deglycosylated by PNGase F; 4, untreated α -amylase standard; 5, α -amylase standard deglycosylated by PNGase F. Note that the 66 kDa band can also be seen in the α -amylase standard, which indicates that the standard was not 100% pure. Proteins were visualized by the immuno technique described in Methods.

lanes 2 and 3). It is a weak band just below the 54 kDa band and it was suspected to be the unglycosylated form of α -amylase, which as mentioned above has a molecular mass of 52.6 kDa.

Deglycosylation

To test whether the 52 kDa band indeed represented an unglycosylated form of α -amylase, deglycosylation of a

Fig. 3. Analysis of amylolytic activity. The figure shows an *in situ* activity gel. Lanes: 1, α -amylase standard; 2 and 3, intra- and extracellular samples, respectively, from batch culture with the wild-type strain A1560; 4 and 5, intra- and extracellular samples, respectively, from batch culture with the recombinant strain CF2.1. The molecular mass of the activity bands was determined by comparing a silver-stained molecular mass marker (separated from the gel after SDS-PAGE), taking into account that the *in situ* activity gel was 1.12 times longer than the silver-stained gel, because of the different treatment of the gels.

few samples was carried out. An intracellular sample of CF2.1 and the α -amylase standard were deglycosylated with PNGase F, and the samples were loaded on a SDS-gel together with untreated samples. As shown in Fig. 2, the α -amylase band in the deglycosylated samples split up into two clear bands, which indicates a partial deglycosylation. This was further supported by intensity measurements of the bands, which showed that the sum of the intensity of the bands in lane 3 was the same as for the 54 kDa band in lane 2 (Fig. 2). The deglycosylated form had the same position as the weak band of 52 kDa in the untreated sample of CF2.1 and this band was therefore believed to represent the unglycosylated form of α -amylase.

Analysis of amylolytic activity

Activity gels were carried out to determine the number of proteins with starch-degrading activity (Fig. 3). The gels showed two bands with starch-degrading activity at 54 kDa and 66 kDa for each analysed sample (both intra- and extracellular samples from cultures of A1560 and CF2.1). The α -amylase standard also showed the 66 kDa band, but it is very weak and cannot be seen on the photograph. Only one other starch-degrading enzyme has been reported to be produced by *A. oryzae*; an exoacting amyloglycosidase (AMG) (Fogarty, 1983), and it was therefore believed that the 66 kDa band represented this exoenzyme.

Growth and α -amylase activity in batch culture

Three batch cultures were carried out on a glucose medium, one with each of the three strains. Glucose, biomass, and intra- and extracellular α -amylase concentrations were measured during all three batch cultures,

Fig. 4. (a) Batch culture with A1560. Biomass concentration (▲) and extracellular α -amylase activity (■) plotted in a linear plot against the batch culture time with A1560. (b) Glucose concentration measured on-line (—) and intracellular α -amylase concentration (□) plotted against batch culture time with A1560. The absolute SD for the α -amylase concentration is given as vertical error bars.

and the results for the wild-type strain A1560 are shown in Fig. 4.

Exponential growth occurred during the first 10 h, followed by a decline in the specific growth rate. The α -amylase concentration also increased exponentially at the same rate in this period. This shows that α -amylase production is growth-related and the constant, specific rate of α -amylase production is given in Table 1. After

15 h, the glucose was exhausted (on-line glucose measurement in Fig. 4b), but α -amylase secretion continued to increase by a factor of 2 during the following 4 h (Fig. 4a).

The intracellular α -amylase concentration (Fig. 4b) was approximately constant at 3.5 FAU (g dry wt)⁻¹ during the first 15.0 h, but a drastic increase in the intracellular α -amylase occurred after the glucose was exhausted.

A similar trend was observed in the batch cultures of the other strains, but the final extracellular α -amylase concentration was four to five times higher for the recombinant strains compared with the wild-type strain (Table 1). The residence time for the intracellular pool of α -amylase was calculated from the mean intracellular concentration in the exponential growth phase and the specific α -amylase productivity (Table 1). In the calculation of the residence time, degradation of α -amylase was not considered since it has been shown to be very stable at pH 6.0 (Carlsen *et al.*, 1995b).

Continuous culture

A continuous culture with the wild-type strain A1560 was carried out. The culture was started as a glucose-limited chemostat, and after 118.75 h the feed was shifted to a maltose medium. The shift in the carbon source did not give rise to any changes either in the CO₂ and O₂ measurements in the exhaust gas, or in the dry weight measurements.

The extra- and intracellular α -amylase concentrations during the culture are shown in Fig. 5(a) and (b), respectively. After 100 h, a steady-state level in both the intra- and the extracellular concentrations was obtained. The steady-state level of the intracellular concentration was about 17 FAU (g dry wt)⁻¹ and since the specific α -amylase production rate was about 15 FAU (g dry wt)⁻¹ h⁻¹, it was found that the turnover time of the intracellular α -amylase pool was 1.1 h. When the glucose medium was changed to the maltose medium, the intracellular level of α -amylase decreased rapidly, followed by an increase to approximately the same level. At the same time, the extracellular concentration increased until it reached a new steady state at 1.2 FAU ml⁻¹. The residence time for the intracellular α -amylase pool at the steady-state level was only about 42 min. When the medium was changed back to glucose, the α -amylase production instantly decreased from 25 FAU (g dry wt)⁻¹ h⁻¹ to 16.4 FAU (g dry wt)⁻¹ h⁻¹. This was seen in the change in the extracellular α -amylase concentration, which can be de-

Table 1. Physiological variables for the three *A. oryzae* strains

Strain	Mean intracellular concn [FAU (g dry wt) ⁻¹ ± SD]	Specific productivity [FAU (g dry wt) ⁻¹ h ⁻¹]	Residence time (min ± SD)	Maximum extracellular α -amylase concn (FAU ml ⁻¹)
A1560	3.5 ± 1	7.5	28 ± 8	0.6
CF1.1	15 ± 1	24	38 ± 3	2.2
CF2.1	17 ± 1	30	34 ± 3	3.1

Fig. 5. (a) Chemostat experiment with A1560. After 118.75 h, the medium was changed from a glucose medium to a maltose medium and after 245.25 h, it was changed back to glucose, as shown. The dilution rate was 0.075 h^{-1} . The extracellular α -amylase concentration profile during continuous culture is shown plotted against time. The line after 245.25 h represents the simulated α -amylase concentration during a specific productivity of $16.4 \text{ FAU (g dry wt)}^{-1} \text{ h}^{-1}$. (b) The intracellular α -amylase level during the medium change from glucose to maltose (at 118.75 h) in continuous culture. The absolute SD is given as vertical error bars.

scribed as a wash-out profile using this specific production rate.

DISCUSSION

Immunoblotting, silver-staining and *in situ* α -amylase activity assays of polyacrylamide gels strongly indicated that the band at 54 kDa was α -amylase. The intensity of this band on immunoblots was therefore used to get a quantitative value of the intracellular α -amylase concentration. Two other bands were visualized on the immunoblot, a band at 66 kDa and a weak band at 52 kDa. *In situ* activity gels showed that the 66 kDa band has starch-degrading activity. Deglycosylation experiments indicate that the deglycosylated form of α -amylase was the 52 kDa band. The unglycosylated form of α -amylase was not detected in samples from wild-type strain

culture, whereas the recombinant strains, especially CF2.1, produced this unglycosylated form. This indicates that the post-translational step, glycosylation, cannot catch up with the production of the unglycosylated form at the higher production rate of the recombinant strains.

The unglycosylated form was detected both intra- and extracellularly which indicates that this form can also be transported over the cell membrane and wall. The ratio between the unglycosylated and the glycosylated form in the batch culture with the recombinant strain CF2.1 was $12 \pm 2\%$ for the intracellular samples and $11 \pm 2\%$ for the extracellular samples. If there is no secretion of the unglycosylated form, this would only be present extracellularly due to fragmentation and the intracellular ratio would therefore be larger than the extracellular ratio. Since the ratios were equal it seems that the glycosylation of α -amylase is not crucial for the secretion process.

Only two types of starch-degrading enzymes have been reported in *A. oryzae*, α -amylase and AMG, and the 66 kDa protein is therefore strongly believed to be AMG. Recognition of AMG by antibodies could be explained by the presence of AMG in small amounts in the α -amylase standard, which was used to immunize the rabbit.

The intracellular α -amylase concentrations for the three strains of *A. oryzae* during batch fermentations all follow the same trend. In the exponential growth phase where the glucose concentration was high, the intracellular α -amylase concentration was almost constant. The intracellular concentration for the recombinant strains was about four to five times higher than for the wild-type strain.

The specific productivity and the residence time for the intracellular pool were calculated for the exponential growth phase. The specific productivity for the recombinant strains was about three times higher than for the wild-type strain, which indicates that the higher intracellular concentration is not a consequence of limitations in the secretion system, but rather is caused by the higher production rate due to the additional genes encoding α -amylase. The residence times for the intracellular α -amylase pool were in the same range for the three strains, which supports the hypothesis that the secretion system can handle the higher productivity.

Hubbard & Ivatt (1981) give a rough approximation of the time course of processing a glycoprotein in a eukaryotic cell to be between 30 and 60 min, which correlates quite well with the residence time calculated for the three strains.

A drastic increase in the intracellular concentration occurred at the end of the batch cultures when the glucose was depleted, whereas the extracellular α -amylase production continued at the same rate. The increase in the intracellular α -amylase level is therefore not due to a reduction in the secretion process, but rather a derepression of production when glucose is depleted, resulting in increased α -amylase production. This is supported by the glucose-limited chemostat experiments where the specific α -amylase productivity was about two

times higher than during the exponential growth phase where the glucose concentration was high. In the glucose-limited chemostat, the residual glucose concentration was about 2 mg l⁻¹ and from other chemostat experiments it has been found that glucose concentrations below 5 mg l⁻¹ do not repress α -amylase production (Carlsen, 1994). Since there was no repression of glucose in the chemostat experiment, it is obvious that there was induction by maltose since the specific productivity increased more than 60% upon changing to a maltose medium in the chemostat. Thus, production of α -amylase by *A. oryzae* is induced by maltose and is partly repressed by glucose, i.e. there is a constitutive production at high glucose concentrations but with a twofold higher production rate at low glucose concentrations.

The rapid decrease in the intracellular concentration compared with the increase in the extracellular α -amylase concentration strongly indicates that there was a rapid increase in the secretion of α -amylase caused by maltose induction. The following increase in the intracellular concentration can be explained by increased α -amylase synthesis, which was also induced by maltose. Thus, the secretion was regulated faster than the synthesis of α -amylase. After 150 h, the intra- and extracellular concentration reached a steady-state level, where the production and the secretion were in balance. The significantly lower residence time for the intracellular α -amylase pool for the maltose-limited culture, also indicates that maltose induces secretion of α -amylase.

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