Acta Microbiologica et Immunologica Hungarica, 50 (1), pp. 67–76 (2003)

AUTOLYSIS AND AGEING OF *PENICILLIUM CHRYSOGENUM* UNDER CARBON STARVATION: RESPIRATION AND GLUCOSE OXIDASE PRODUCTION*

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(Received: 30 October 2002; accepted: 18 November 2002)

During the exponential growth phase of *Penicillium chrysogenum* NCAIM 00237 the effective conversion of glucose and O_2 to gluconate and H_2O_2 by glucose oxidase (GOX) was the most likely source of intracellular ROS measured. In glucose-supplemented autolysing cultures, the increased of intracellular ROS concentration was attributed to respiration in the absence of any significant GOX activity. The induction of GOX and catalase by glucose and H_2O_2 was clearly age-dependent in *P. chrysogenum*. In ageing cryptic growth phase cultures, superoxide dismutase and cyanide-resistant respiration were the major elements of antioxidative defence but these activities were insufficient to prevent the progressive accumulation of ROS and the concomitant decrease in cell vitality.

Keywords: ageing, autolysis, apoptosis, catalase, glucose oxidase, mitoptosis, *Penicillium chrysogenum*

Introduction

The growth of the high β-lactam producer *Penicillium chrysogenum* NCAIM 00237 was characterised by five phases including exponential (up to 34 h of incubation), deceleration (34–40 h), stationary (40–50 h), autolytic (50–148 h) and cryptic or post-autolytic (from 148 h) phases of growth [1–4]. During exponential growth, glucose was metabolised actively by the cells, and the exploitation of this carbon source fuelled the fast elongation and branching of hyphae, agglomerating ultimately pellets. In terms of cell physiology, this growth phase was characterised by surprisingly high intracellular peroxide, superoxide and glutathione disulphide (GSSG) levels and, consequently by rather low glutathione (GSH)/GSSG redox ratios [3]. Due to the accumulation of reactive oxygen species (ROS) and the decreased GSH/GSSG

* Previous parts of this series of papers were published by Pusztahelyi et al. [1, 2] and Sámi et al. [3, 4]. This paper was written to commemorate to the fiftieth anniversary of the foundation of Acta Microbiologica et Immunologica Hungarica.

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1217-8950/2003/$ 20.00

redox capacity of the cells, the specific activity of different antioxidative enzymes including catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) was very high as compared to the other growth phases. Intracellular ROS and GSSG levels started to decrease in the deceleration and stationary phases while the specific antioxidative enzyme activities declined only after the onset of autolysis [3].

Carbon-depleted cultures supplemented with an extra dose of glucose exhibited increased intracellular peroxide levels and decreased GSH concentrations, provoking elevation of the GPx and GR activities. Interestingly, the intracellular superoxide and GSSG levels remained low, and the catalase activity did not respond to carbon supplementation either [3]. These findings indicated that although there was some overlapping between the generation of ROS and the oxidative stress responses in exponentially growing and carbon-supplemented autolytic *P. chrysogenum* cultures. The molecular backgrounds of these physiological changes might be very different.

Here we present some experimental data on the two major ROS generating metabolic pathways in *P. chrysogenum*, namely the direct oxidation of glucose by glucose oxidase (GOX) and the regeneration of NAD^+ and FAD through the cytochrome-dependent mitochondrial respiratory chain. It was shown that the formation of ROS in these pathways depended highly on the growth phase of the culture. The contribution of the cyanide-resistant respiratory pathway to the antioxidative defence of the cells was also demonstrated.

Materials and methods

Culture conditions. *Penicillium chrysogenum* NCAIM 00237 was maintained and grown as described in previous publications [1–4]. The complex culture medium consisted of glucose (50 mM), casein peptone (0.4%; Merck, Darmstadt, Germany), yeast extract (0.4%; oxoid, Basingstoke, UK), corn steep liquor (0.24%; Ászár Starch-producing Company, Ászár, Hungary), 0.2% KH_2PO_4, 0.8% Na_2HPO_4 and 0.05% $MgSO_4.7H_2O$ (pH 7.4). Culture flasks (500 ml) containing 100 ml of medium were inoculated with 10^8 spores each and were incubated at 200 rev min^{-1} at 25 °C. The effect of glucose supplementation on the carbon-depleted cultures was studied by addition of a glucose solution (2.5 M) in the final concentration of 58 mM to selected flasks at the 88, 115 and 133 h of incubation.

Determination of extracellular glucose and gluconate concentrations and of glucose oxidase (GOX) activities. Samples were filtered through sintered glass and the filtrates were used for glucose and gluconate measurements. Glucose concentrations were measured spectrophotometrically using *Aspergillus niger* GOX and horseradish

peroxidase (HRP) as auxiliary enzymes [5]. The reaction mixtures always contained 900 µl reagent {phenol (11 mM), 4-amino-antipyrine (0.76 mM), GOX (4 kU. l^{-1}) and HRP (1 kU. l^{-1}) prepared in 0.1 M NaH_2PO_4-K_2HPO_4 buffer (pH 6.6)} and 100 µl filtered culture medium. The reaction was followed at 500 nm for 3 min and the glucose concentrations were calculated using calibration curves (2.5–50 mM glucose).

In the gluconate rate assay, *Pseudomonas* sp. D-gluconate dehydrogenase (D-gluconate oxidoreductase, EC 1.1.99.3; GDH; Sigma G7275) was used as an auxiliary enzyme [6]. The reaction mixtures contained 900 µl phenazine methosulfate (100 µM) - 2,6-dichlorophenol-indolphenol (150 µM) reagent prepared in 33 mM KH_2PO_4-K_2HPO_4 buffer (pH 6.0), 20 µl (0.5 U) GDH enzyme preparation and 100 µl culture medium filtrate. The decrease in the absorbance was monitored at 600 nm for 1 min, and the gluconate concentrations were calculated using calibration curves that covered the concentration range of 0–25 mM.

For glucose oxidase measurements, mycelia from 8 ml aliquots of culture media were separated by filtration on sintered glass, washed and transferred immediately into 0.1 M KH_2PO_4-K_2HPO_4 buffer (pH 7.5; 8 ml). The samples were frozen at –30 °C, and cell-free extracts were prepared by X-pressing and centrifugation [7–9]. In addition to extracellular and intracellular soluble (soluble cell-bound) GOX activities, total cell-bound GOX activity was also measured as described before for *P. chrysogenum* γ-glutamyltranspeptidase [8] and *Acremonium chrysogenum* chitinase [10]. In this case, X-pressed mycelia were not centrifuged, and suspensions containing disrupted cells were used directly in GOX activity determinations [8–9]. The difference between soluble cell-bound and total cell-bound enzyme activities was attributed to cell-wall associated GOX.

GOX activities were measured by a modification of the glucose rate assay by Leary et al. [5]. The glucose concentration was kept at constant value (50 mM), and *P. chrysogenum* enzyme samples were added into the reaction mixtures instead of *A. niger* GOX. Calibration curves (0.39–5.00 U. l^{-1} *A. niger* GOX; Sigma G7141; 1 U oxidises 1.0 µmol D-glucose to D-gluconolactone and H_2O_2 per min) were used to calculate GOX activities.

Determination of total and cyanide-resistant respiration rates and dry cell weights (DCWs). Respiration was measured in a built-in-house oxygraphic cell (V=10 ml) at 25 °C, using a Clark-type polarographic oxygen electrode [11–12]. The cytochrome-dependent pathway was inhibited with 1 mM KCN, and the remaining respiration was regarded as cyanide-resistant [11–12].

DCWs were determined from 5 ml aliquots of the cultures as described by Pusztahelyi et al. [1–2].

Chemicals. Unless otherwise indicated, all the chemicals were purchased from Sigma-Aldrich (Budapest).

Reproducibility. All the experimental data presented here are mean of 3 independent measurements. The variations between experiments were estimated by standard deviations (S.D.), and the statistical significance of changes in physiological parameters were estimated by the Student's t-test. Only the probability levels of $P \leq 5\%$ were regarded as statistically significant.

Results

In the early exponential phase of growth (up to 20 h of incubation), *P. chrysogenum* cells metabolised 30.8% of the starting concentration of glucose (52→36 mM) without any significant conversion to gluconate (Figures 1D and 1E). At 20 h of incubation, only a small increase (about 1.16 mg. ml^{-1}) of DCW was recorded (Figure 1A). Later, between 20 and 26 h of incubation, exactly the half of the remaining glucose (36 mM) was converted to gluconate (18 mM, which is equal to 34.6% of the initial concentration of glucose) and the other half was metabolized by the fungus (Figures 1D and 1E). The DCW reached 3.4 mg. ml^{-1} by the 28 h when the utilisation of extracellular gluconate started (Figures 1A and 1E). The total of gluconate was consumed by the 36 h of incubation (Figure 1E; deceleration phase [1, 3–4]), resulting in a further 85% increase of the biomass (3.4→6.3 mg. ml^{-1}; Figure 1A).

The total of GOX activity (cell-bound + extracellular) was constant during the exponential and deceleration phases of growth (24–36 h; Figure 2) while the specific activity decreased owing to the gain in the biomass (Figures 1A and 1F). The enzyme was secreted actively into the culture medium as shown by the decreasing soluble cell-bound (1.18→0.77 U. ml^{-1}; 24–36 h) and increasing extracellular (0.10→0.62 U. ml^{-1}; 20–36 h) specific activities (Figure 2). The cell-wall associated enzyme activity exhibited a constant value of about 0.22–0.28 U. ml^{-1} (Figure 2).

As far as the respiration is concerned, both the total and the cyanide-resistant respiration rates increased during exponential growth, reaching their maxima at 28 h incubation time (45.5 and 20.1 μM O_2 min^{-1} g^{-1} DCW, respectively) and declined in the deceleration phase (36 h; Figures 1B and 1C).

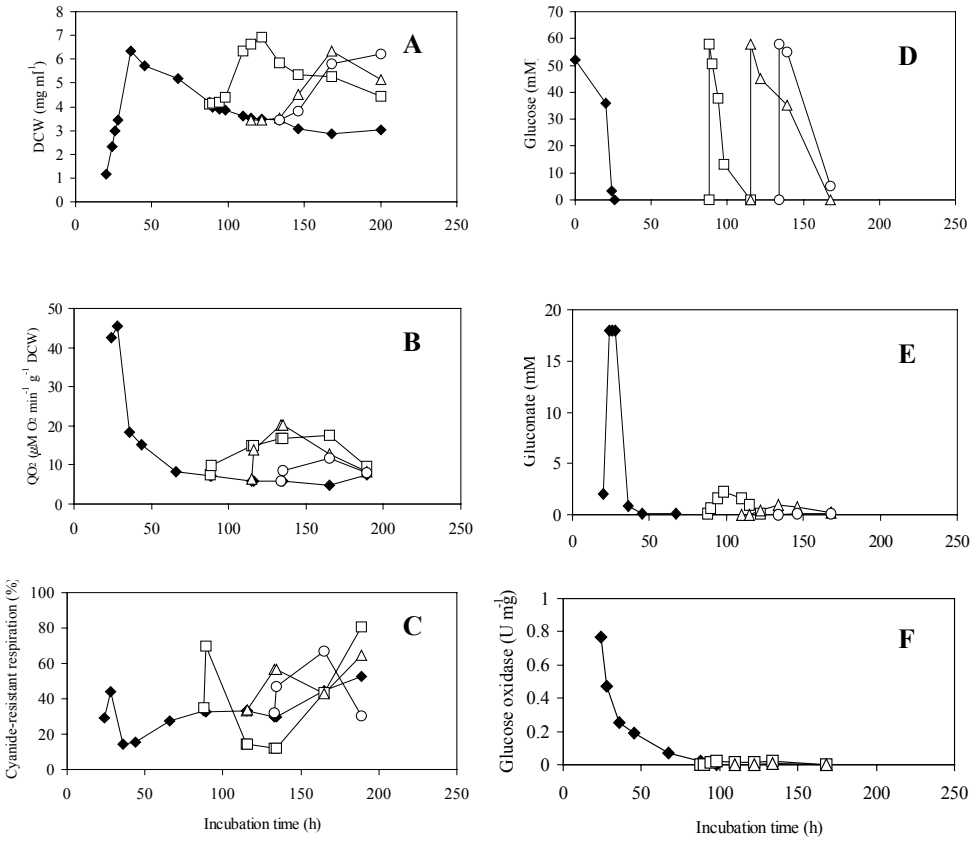

Fig. 1. Changes in DCWs (A), total (B) and cyanide-resistant (C) respiration rates, glucose (D) and gluconate (E) concentrations and in the specific total glucose oxidase activity (extracellular + total cell-bound) (F). Symbols ♦, □, Δ, and ○ stand for control and glucose supplemented (88, 115 and 134 h) cultures, respectively. One typical set of curves is presented

In the stationary and autolytic phases of growth, the glucose oxidase activity decreased steeply in all cell fractions studied and was eliminated completely by the 98th of incubation (Figures 1F and 2). [1–5] the total respiration reached a low constant value of about 5–7 μM O_2 min^{-1} g^{-1} DCW After 88 h incubation time while the ratio of cyanide-resistant respiration increased continuously throughout the stationary, autolytic and cryptic growth phases up to 52.4% (189 h; Figures 1B and 1C).

After glucose supplementation at any incubation time tested, both the total and the cyanide-resistant respiration rates increased considerably (Figure 1B and 1C). The

total respiration reached 26–44% (12–20 μM O_2 min^{-1} g^{-1} DCW) of that observed during the exponential growth with an 57–70% ratio of cyanide-resistant respiration. Similar to exponential growth, the consumption of the extra glucose resulted in decreasing total and cyanide-resistant respiration rates but the latter started to raise again when the cultures reached a new autolytic phase as indicated by the loss of biomass (Figures 1A, 1B and 1C).

No significant gluconate formation was observed in glucose supplemented autolytic cultures, and the GOX activities were also negligible (Figures 1E and 1F).

Discussion

The autolysis of filamentous fungi is a dynamic, energy-consuming process, which shares some common features with the apoptosis of higher eucaryotes [13]. Fungal autolysis is initiated and influenced by numerous intrinsic and extrinsic factors, including nutrient limitation [1, 14–15]. Although the molecular background of autolysis is poorly understood [15] transient accumulation of ROS and GSH/GSSG redox imbalances may be important signal transduction events of fungal cell death [16]. In fact, both catalase and superoxide dismutase (SOD) activities increased temporarily in late-stationary phase *Aspergillus niger* B1-D cultures [17], which might be indicative of a transient hyperoxidant state prior to autolysis [16]. On the contrary, the intracellular ROS and GSSG levels as well as almost all the antioxidative enzyme activities tested were highest in exponential and deceleration phase *P. chrysogenum* cultures, and autolysis was not preceded by any hyperoxidant state in this case [3].

As shown in Figures 1F and 2, exponentially growing *P. chrysogenum* produced and secreted high GOX activities and, as a consequence, converted the starting glucose to gluconate quite effectively (Figures 1D and 1E). Although *P. chrysogenum* has been known as a potent GOX producer [18] and as high as 60% glucose→gluconate conversion rates have been demonstrated in a defined medium [19–20] this is the first report on significant gluconate formation in a complex medium containing corn steep liquor. While GOX is located mainly in the cell-wall in the major industrial GOX producer *A. niger* [21], in *P. chrysogenum* GOX is predominantly a soluble enzyme (either extracellular or cell-bound), and only a minute amount of enzyme is trapped within the cell-wall (Figure 2). The precise location of the soluble cell-bound enzyme fraction has remained yet to be elucidated a periplasmic location seems, however to be probable [20].

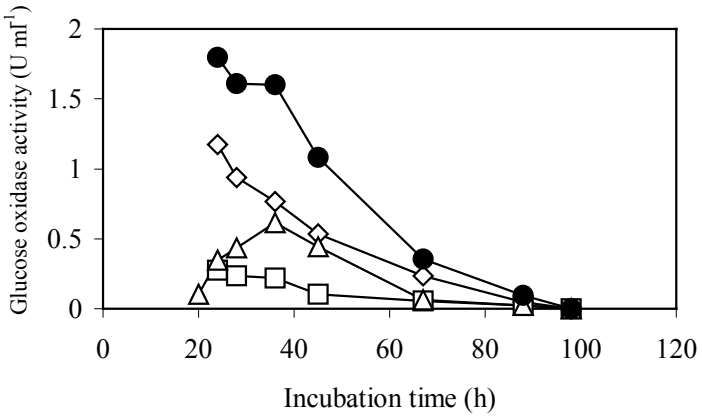

Fig. 2. Cellular distribution of glucose oxidase as a function of incubation time. Symbols ●, ◊, □ and Δ represent total, soluble cell-bound, cell-wall-associated and extracellular glucose oxidase activities, respectively. One typical set of curves is presented

In good agreement with the results of Nielsen et al. [19], we also found that gluconate was not utilised prior to the full consumption of all the starting glucose from the culture medium (Figures 1D and 1F). As a result of the action of GOX, the maximum gluconate concentration was 18 mM in the cultures (conversion yield 34.6%), suggesting that the highest possible extracellular H_2O_2 concentration was also about 18 mM. Although late-exponential phase *P. chrysogenum* cultures are known to tolerate H_2O_2 exceptionally well up to 700 mM [7] no information is available on the oxidative stress tolerance of lag phase or early exponential phase mycelia because it is quite difficult to obtain reproducible physiological data below 24 h of incubation. Nevertheless, the high intracellular ROS levels (peroxide, superoxide) and the high or increasing antioxidative enzyme activities (catalase, GR, GPx) recorded later in the exponential phase of growth (24–28 h; [3]) were likely the consequences of the oxidative stress generated by the earlier H_2O_2-formation by GOX. It is noteworthy that H_2O_2 has been shown to play a crucial role in the induction of *A. niger* glucose oxidizing system (GOX, lactonase and catalases [22]) and to stimulate fungal cyanide-resistant respiration (Figure 1C [12]). The latter element of the antioxidative defence may also contribute to the prevention of generation of ROS in metabolically active mitochondria [12, 23] especially when the total respiration rate is high (Figure 1B). The induction of antioxidative enzymes in the early exponential phase of growth explains satisfactorily the remarkable oxidative stress tolerance of late exponential phase *P. chrysogenum* mycelia [7–8].

Unexpectedly, the generation of free radicals after glucose supplementation in autolysing cultures [3] could not be attributed to GOX as indicated by the low specific enzyme activities (Figure 1F) and the very low glucose→gluconate conversion rates (Figure 1E). Under these circumstances, disintegrating mitochondria with partly disorganised cristae [24] are the most likely sources of ROS. The loss of mitochondrial function was clearly demonstrated by significantly reduced total respiration rates after glucose re-additions (Figure 1B). Again, increased cyanide-resistant respiration (Figure 1C) together with increased GPx and GR activities [3] were the most important elements of the primary defence against oxidative stress.

It is very important to emphasize the fact that although the intracellular peroxide level was high [3] and glucose was present in the culture medium neither GOX nor catalase induction was observed after glucose re-addition (Figure 1F [3]). It is the concerted transcriptional regulation of these enzymes, including goxB (mediator of the H_2O_2 effect) and goxE (mediator of the carbon effect) gene products in *A. niger* [22], which did not seem to work in glucose-supplemented autolysing *P. chrysogenum* cultures. We can therefore conclude that the synchronous upregulation of the glucose oxidizing system in the presence of glucose and H_2O_2 is age-dependent in *P. chrysogenum*.

We also considered the possibility that decreases in pH after glucose supplementation [1] might inactivate GOX as recorded in *A. niger* cultures at pH values below 3.5 [25–26]. In *P. chrysogenum*, glucose pulsing always resulted in pH drop down to 4.2–4.5 [1] while the stability of GOX was not affected by pH values even as low as 3.5 [18], which makes the pH inactivation of GOX rather unlikely in this case.

Dry cell weight, extracellular pH, NH_3 and amino nitrogen concentrations, extracellular chitinase and N-acetyl-β-D-hexosaminidase activities as well as intracellular GPx, GR and catalase activities, total respiration rate also reached a constant value prior to the onset of the cryptic growth phase after 148 h of incubation (Figures 1A and 1B [1–4]). This dynamic post-autolytic equilibrium between cell death and cryptic growth were characterised by the intracellular accumulation of ROS [3], increasing SOD activity [3], decreasing cell vitality [4] and increasing cyanide-resistant respiration (Figure 1C). The physiological changes taking place in surviving fragments were very similar to those described in ageing stationary cultures of *Saccharomyces cerevisiae* [27] and in yeast mother cell-specific ageing leading to apoptosis [28–29].

Like the programmed death phenomena of higher eucaryotes [30], mitochondrial disorganisation [24] may trigger cell death processes in ageing cultures of filamentous fungi, too. When the ROS concentrations in the cells increase in spite of the increasing SOD activity [3] and cyanide-resistant respiration (Figure 1C) the only

further measure that can be applied is the elimination of mitochondria (mitoptosis) and, as a consequence, of the respiration itself ([30]; Figure 1B). Further studies are needed to demonstrate if mitoptotic processes of fungi give rise to signal proteins activating cell death programmes [30].

Acknowledgements. The Hungarian Ministry of Education awarded a Széchenyi Scholarship for Professors to I.P., which is greatly acknowledged. This project was supported financially by the OTKA (grant reference numbers T025174 and T034315).

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