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Adaptive response to oxidative stress in the filamentous fungus *Aspergillus niger* B1-D

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Abstract

In the present study, we used a recombinant filamentous fungus strain, *Aspergillus niger* B1 D, as a model system, and investigated the antioxidant defences in this organism. Our findings indicate that pretreatment with low concentrations of H_2O_2 completely prevents killing by this oxidant at high concentrations. It shows that *A. niger* adapts to exposure to H_2O_2 by reducing growth and inducing a number of antioxidant enzyme activities, including superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, of which the induction of catalase is the most pronounced. Moreover the decline of these antioxidant enzymes activities after H_2O_2 detoxification, coincides with recommencement of growth. Results from monitoring the extracellular H_2O_2 concentration clearly indicate a very rapid detoxification rate for H_2O_2 in adapted *A. niger* cultures. A mathematical model predicts only very low concentrations of intracellular H_2O_2 accumulating in such cultures. Our results also show that glutathione plays a role in the oxidative defence against H_2O_2 in *A. niger*. On addition of H_2O_2, the intracellular pool of glutathione increases while the redox state of glutathione becomes more oxidized.

Introduction

H_2O_2 is an unavoidable by-product of all living organisms which rely on respiration for energy production. The mitochondrion is the main site of H_2O_2 production [1] where continuous production of superoxide caused by electron leakage takes place, and incomplete reduction of superoxide gives rise to H_2O_2 [2]. H_2O_2 production is also involved with a number of oxidase reactions, such as fatty acid catabolism β-oxidation. The cytotoxicity of H_2O_2 is generally thought to be due to its ability to damage macromolecules, including lipids, DNA, and proteins [3]. Compared with other reactive oxygen species (ROS) , H_2O_2 is less toxic, but is able to diffuse into different compartments from its original production sites before reaching its targets [4].

Abbreviations: CAT, Catalase; GPx, Glutathione peroxidase; GR, Glutathione reductase; GSH, Glutathione; GSSG, Oxidized glutathione; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; ROS, Reactive oxygen species; SOD, Superoxide dismutase; *t*-BHP, *tert*-butyl hydroperoxide.

Moreover, H_2O_2 can react with transition metal ions through the Fenton reaction [5] to produce the highly toxic hydroxyl radical, which greatly exacerbates the cytotoxicity of H_2O_2.

Yeasts have become a favourite model to study oxidative stress in microbial systems, and the research carried out in a range of yeast species, including mainly *Saccharomyces cerevisiae,* has contributed to our knowledge on how eukaryotes defend themselves from continuous damage caused by ROS. In *S. cerevisiae*, two catalases (CTT, CTA) [6] and some peroxidases, cytochrome *c* peroxidase (CCP) [7] and glutathione peroxidase (GPx) [8], have been shown to be involved in H_2O_2 catabolism. Mutants in which the genes for these catalases were deleted were significantly less able to acquire H_2O_2 tolerance compared with the wild type [9].

In filamentous fungi, more than one catalase gene has been shown to exist, and they are differentially regulated. For example, in *Aspergillus nidulans*, two differentially regulated genes, catA and catB, have been characterized [10–12]. CatA is preferentially expressed in spores, while catB is expressed only in mycelia, and both could be induced by oxidative stress. In

Aspergillus niger, intracellular catalase activities were shown to be induced by exogenous H_2O_2 [13,14]. Despite these earlier studies, the intrinsic role of catalase in filamentous fungi is still unclear, and to what extent catalase actually contributes to cellular oxidative stress defence via H_2O_2 detoxification is still unknown.

The tripeptide γ-L-glutamyl-L-cystinylglycine (glutathione—GSH) is one of the major antioxidant molecules in yeast cells, and is thought to play a vital role in buffering the cell against ROS [15]. Glutathione can react with oxidants nonenzymically, or the reaction can be catalysed by glutathione peroxidase, and the oxidized glutathiol (GSSG) could be recycled by glutathione reductase (GR) at the expense of NADPH. Mutants of *S. cerevisiae* deficient in glutathione synthesis [15–17] or glutathione recycling [18] showed increased sensitivity to H_2O_2, but were unaffected in their ability to adapt to H_2O_2. Generally, studies on the glutathione pathway involving filamentous fungi are rather scarce, and the findings are sometimes unclear and contradictory, compared with studies on yeasts. For example, GPx is thought to play only a minor role in the defence against oxidative stress in *A. niger* [19], In *Penicillium chrysogenum*, GSH concentration slightly increases in the presence of elevated concentrations of exogenous H_2O_2 while GSSG increased significantly, resulting in an imbalance of glutathione homeostasis. Meanwhile, GPx, GR, and glutathione cycling activities all were elevated by H_2O_2 addition [20].

Submerged culture is the system employed industrially for the production of a variety of metabolites of enormous economic importance produced by filamentous fungi [21]. The cultivation of these organisms in submerged culture involves vigorous agitation and aeration; thus it is not surprising that oxidative stress is frequently reported as occurring [22]. In the present work, we report on the physiological responses of a filamentous fungus, *A. niger* B1-D, to exposure to oxidative stress simulated by the addition of H_2O_2. We have monitored the activities of all major antioxidant enzymes reported from previous studies in filamentous fungi, which should allow a more coherent and complete understanding of the antioxidant defense adopted by this organism. We have examined the role of the glutathione pathway in this fungus in detail, to help clarify the antioxidant effects of GSH in the presence of elevated levels of H_2O_2.

Materials and methods

Strain and cultivation

A recombinant strain of *A. niger* B1-D [23] has been used throughout this study. Aspergillus complete medium was used for the batch cultivation [24]. The composition of the medium is 50 g glucose, 5 g NH_4Cl, 20 ml salt solution, 10 ml vitamin solution, and water to 1.0 L. Salt solution was KCl, 26 g; $MgSO_4 \cdot 7H_2O$, 26 g; KH_2PO_4, 76 g; trace-element solution, 50 ml; and water to 1 L. Trace-element solution was $Na_2B_4O_7 \cdot 10H_2O$, 40 mg; $CuSO_4 \cdot 5H_2O$, 400 mg; $FePO_4 \cdot 2H_2O$, 800 mg; $MnSO_4 \cdot 2H_2O$, 800 mg; $Na_2MoO_4 \cdot 2H_2O$, 800 mg; $ZnSO_4 \cdot 7H_2O$, 8 g; and water to 1 L. Vitamin solution: *p*-aminobenzoic acid, 20 mg; thiamine hydrocholoride, 50 mg; biotin, 10 µg;

nicotinic acid, 100 mg; calcium D-pantothenic acid, 200 mg; pyridoxine monohydrochloride, 50 mg; riboflavin, 100 mg; and water to 1 L.

Shake flask cultivation was carried out in 500-ml conical flasks containing 200 ml sterile medium. An inoculum of 1×10^5 spores per milliliter was used. The inoculated flasks were placed in a rotary shaker (New Brunswick Scientific, Edison, USA) at 200 rpm and $25 \pm 0.1°C$. After 70 h incubation, a stock solution of H_2O_2 was aseptically added to the culture to the desired final concentration by syringe filtration through a sterile 0.2-µm cellulose acetate membrane (NALGENE, Fisher, UK). In adaptation experiments, 100 µM H_2O_2 was added to batch cultures of *A. niger* at 70 h, followed by 10 or 20 mM H_2O_2 2 h later. For comparison, 10 or 20 mM H_2O_2 was added without pretreatment of 100 µM H_2O_2.

Biochemical assays

Biomass was estimated according to the method of Bai et al. [24]. Five milliliters of fungal culture was withdrawn and filtered through a 4.25-cm-diameter GF/C filter, which has a particle retention time size of approximately 1.2 µm (Whatman Ltd., UK). The filter cake was washed twice with 5 ml distilled water, dried for 20 min in a microwave oven (650 W) on medium–low power, and cooled in a desiccator before weighing. Glucose concentration of the filtrate was determined by an enzymatic assay kit (r-biopharm, Germany).

H_2O_2 concentration of the filtrate was assayed by the FOX method [25]. Fifty microliters of properly diluted sample was added to 950 µl FOX1 reagent (100 µM Xylenol orange, 250 µM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM H_2SO_4) in a cuvette (Fisher, UK), vortexed, and incubated at room temperature for 5 min to allow colour development. The absorbance was read at 560 nm. The signal was read against a H_2O_2 standard curve measured at the same time along with samples and was linear in the 0–100 µM concentration range. For the authenticity of H_2O_2, the same sample pretreated with 200 U/ml catalase was used as blank. For the assay of biomass, glucose, and H_2O_2, all samples were analyzed in triplicate.

After fungal cells were harvested, filtered, and washed, the filter cake was resuspended in the same volume of sodium phosphate buffer (50 mM, pH 7.0). The fungal cells were disrupted by a high-pressure cell disrupter (Model 4000, Constant System Ltd., UK). Cell-free extracts were separated from cell debris by centrifugation at 4°C, 18,407 $\times g$ for 30 min. The clear supernatant was used to assay enzyme activities immediately. SOD activity was measured by its ability to inhibit the reduction of cytochrome *c* by the superoxide radical produced by the xanthine/xanthine oxidase system, which was describe by Crapo et al. [26]. Operating concentrations were as follows: 0.1 mM xanthine, 0.1 mM EDTA, 50 mM Na_2CO_3 (pH 10.2), 10 µM ferricytochrome *c*, and enough xanthine oxidase to cause $\Delta A_{550 \text{ nm}} = 0.025/\text{min}$. CAT activity was determined by a spectrophotometric method [27]. GPx activity was measured spectrophotometrically according to the disappearance rate of NADPH [28]. GR activity was determined by a spectrometric method described by Lee et al. [29]. The DTNB-GSSG reductase
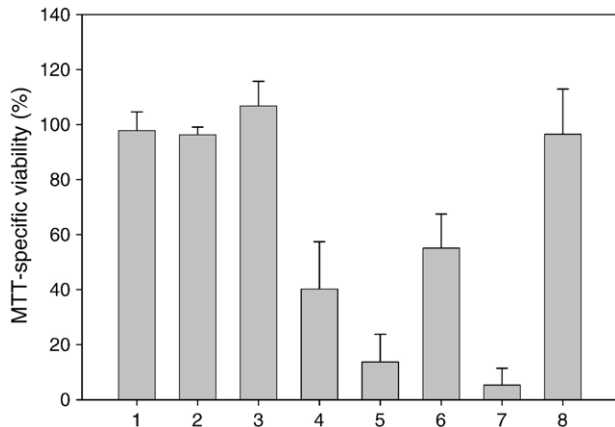
Fig. 1. Effect of high concentrations of H_2O_2 addition on the viability of *A. niger* B1-D. Seventy-two-hour-old batch cultures of *A. niger* were incubated with 10 or 20 mM H_2O_2 for 2 h and then sampled for the viability assay. Results were then standardized based on prestress values. Some cultures were pretreated with 100 μM H_2O_2 for 2 h before challenge with 10 or 20 mM H_2O_2. Others had 100 μg/ml cycloheximide added during the pretreatment. The effect of cycloheximide was elucidated by incubating with 72-h-old batch culture for 2 h. Results were obtained from three independent experiments and are expressed as mean ± SD. (1) Control; (2) 10 mM H_2O_2 (pre-treated with 100 μM H_2O_2 for 2 h; (3) 20 mM H_2O_2 (pre-treated with 100 μM H_2O_2 for 2 h). (4) 10 mM H_2O_2. (5) 20 mM H_2O_2; (6) 10 mM H_2O_2 (pretreated with 100 μM H_2O_2 and 100 μg/ml cycloheximide for 2 h); (7) 20 mM H_2O_2 (pretreated with 100 μM H_2O_2 and 100 μg/ml cycloheximide for 2 h); (8) 100 μg/ml cycloheximide.

recycling assay was used to measure total GSH and GSSG [30]. Protein concentrations in the cell-free extracts were assayed by the Bradford method [31]. For the enzymatic assays, triplicate samples were collected and disrupted, and the supernatants were used individually.

MTT-specific cell viability was measured by a method of Emri et al. [32] with modification. Two milliliters of broth was aseptically withdrawn, filtered, washed, and added to a vial into which 2 ml of fresh medium and 50 μl of 5 mg/ml solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were also added. The sample was incubated for 24 h at 30°C causing the cells to turn purple, after which time 1 ml of 10% (w/v) solution of sodium dodecyl sulfate (SDS) in 20 mM HCl was added. The sample was incubated for a further 24 h at 30°C, after which time it was centrifuged at 9279 ×*g* for 5 min and the MTT-formazan content of the supernatant was measured spectrophotometrically at 570 nm. A blank lacking cells was also carried out along with samples, the absorbance of which was subtracted from absorbances of samples. Results were expressed as $A_{570 \text{ nm}}$/g DCW. All samples were analyzed in triplicate.

Results

Fig. 1 shows the effects of H_2O_2 addition on the viability of *A. niger* B1-D. Direct addition of high concentrations of H_2O_2, either 10 or 20 mM, leads to reduced viability, indicating that these concentrations are lethal to this culture. However, pretreatment with a low, nonlethal concentration of H_2O_2 (100 μM) for 2 h prior to exposure to lethal concentrations completely re-

moved the deleterious effects by H_2O_2. These results proved that batch cultures of *A. niger* can adapt to H_2O_2 via preexposure to low levels of this stressor. The ability to adapt to H_2O_2 is compromised by 100 μg/ml cycloheximide, a conventional protein translation inhibitor, indicating that a *de novo* protein synthesis was required for this adaptive response. Cycloheximide itself has no noticeable effect on the viability of the culture for a 2-h treatment.

Fig. 2 shows the effects of H_2O_2 addition on the dry cell weight (DCW) and glucose concentration versus time in *A. niger* B1-D. From the figure, it can be seen that *A. niger* grew exponentially during the time between 70 and 96 h, and protein concentration remained stable in that period. However, a temporary reduction in growth was observed after exposure to 100 μM H_2O_2. Consequent exposure to 10 mM H_2O_2 led to further inhibition. Nevertheless, glucose was still utilized, indicating that the culture was still metabolically active. The culture recovered from the effects of the oxidative stress within 4 h after addition of the H_2O_2.

The response in terms of intracellular protein content was slightly different from that of cell mass: protein content decreased to 67% of its original value after additions of 100 μM H_2O_2, but took longer to recover to prestress levels. Overall, pretreatment with low concentration of H_2O_2 changed *A. niger*'s subsequent metabolism profoundly at the expense of growth.

Antioxidant enzymes have been thought of as the first line of defence in response to oxidative stress. In the present study we monitored four key antioxidant enzyme activities, SOD, CAT, GPx, and GR, previously reported as being of importance in oxidative stress defence in filamentous fungi [14,20,22], and the results are presented in Fig. 3. In the control, the activities of all four enzymes remained stable during the time between 70 and 96 h. However, CAT, GPx, and GR activities were all significantly induced by 100 μM H_2O_2, further enhanced by subsequent addition of 10 mM H_2O_2, and then began to decline 4 h after addition, coinciding with the recovery in growth. It is noticeable that the induction of CAT activity showed the greatest increase. Though not directly connected with H_2O_2 detoxification, intracellular SOD activity was also enhanced. Its maximum appeared 8 h after addition of 10 mM H_2O_2, and SOD activity declined more slowly compared with the other three intracellular antioxidant enzymes. This is consistent with other reports which proposed that SOD activity is mainly induced by superoxide, but could be increased moderately by H_2O_2 [14]. The intrinsic connection of these two stresses is still ambiguous, and we propose that addition of H_2O_2 to living cultures, especially in large quantities, may lead to an overabundance of molecular oxygen when H_2O_2 is dismutated to H_2O and O_2 by CAT. By contrast, addition of H_2O_2 at lethal doses failed to induce these activities. As shown in Table 1, activities of SOD, CAT, and GR fell significantly after addition of 10 mM H_2O_2 to unadapted cultures, while adding 20 mM H_2O_2 to such cultures reduced activities to almost unmeasurable levels. Therefore, the induction of antioxidant enzymes activities is essential for the cells to acquire the ability to adapt to H_2O_2.

Fig. 4 shows the changes in the concentrations of GSH and GSSG in response to H_2O_2 addition. Compared to the control, both
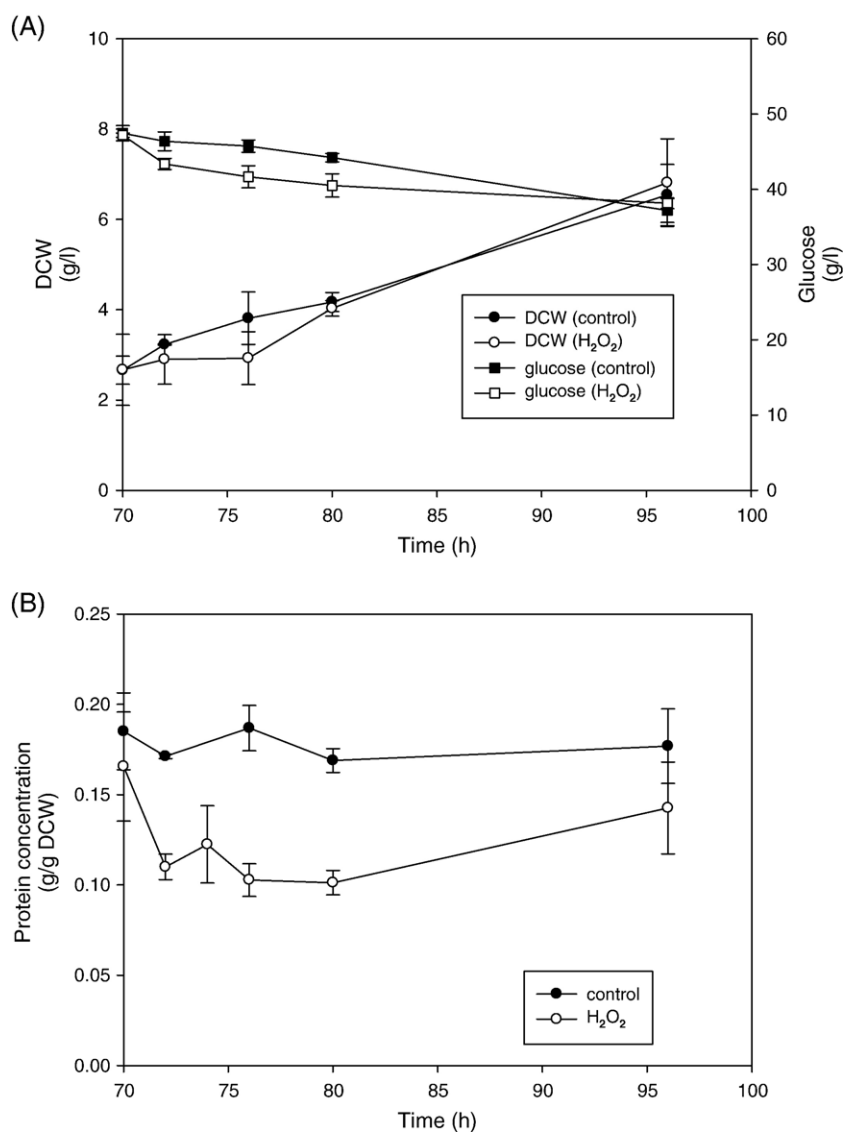
Fig. 2. Effect of H_2O_2 addition on the dry cell weight (DCW), glucose concentration, and intracellular protein content in batch cultures of *A. niger* B1-D. H_2O_2 was added to the culture at 70 and 72 h, to final concentrations of 100 μM and 10 mM, respectively. The results were obtained from three independents experiments and expressed as mean±SD.

GSH and GSSG concentrations increased significantly following addition of 100 μM H_2O_2, and then 10 mM H_2O_2, but the ratio of GSH/GSSG decreased. These results indicate that glutathione is implicated in oxidative defence against H_2O_2 in *A. niger*.

Fig. 5 shows the changes in H_2O_2 concentrations in the culture fluid during the process of culture adaptation. The detoxification of 10 mM H_2O_2 in the adapted culture is significantly faster than in the unadapted cultures. This is probably due to the significant induction of antioxidant enzymes activities in the adapted culture, and the marked decrease in such activities in undapted cultures. Altogether, these results strongly support the view that the acquisition of H_2O_2 tolerance was due to increased capacity to remove the oxidant itself, thus avoiding prolonged exposure to H_2O_2 and associated deleterious intracellular events. Further, it has been proposed that the decomposition rate of exogenous H_2O_2 by

intact viable cells is a first-order reaction [33]: this implies that the decline in H_2O_2 concentration in the cultures should be exponential. In the present study, we found that the detoxification profile of 100 μM H_2O_2 in cultures of *A. niger* did indeed follow this trend, as supported by the high value of R^2, as did the detoxification profile of 10 mM H_2O_2 in adapted cultures, but not in unadapted cultures (Fig. 5B).

Discussion

Adaptation to H_2O_2

The intracellular concentration of H_2O_2 in aerobically growing *Escherichia coli* is estimated between 0.1 and 1 μM [34]. In the present work, we found that *A. niger* is able to cope with up to 100 μM exogenous H_2O_2, and adapt to up to 20 mM
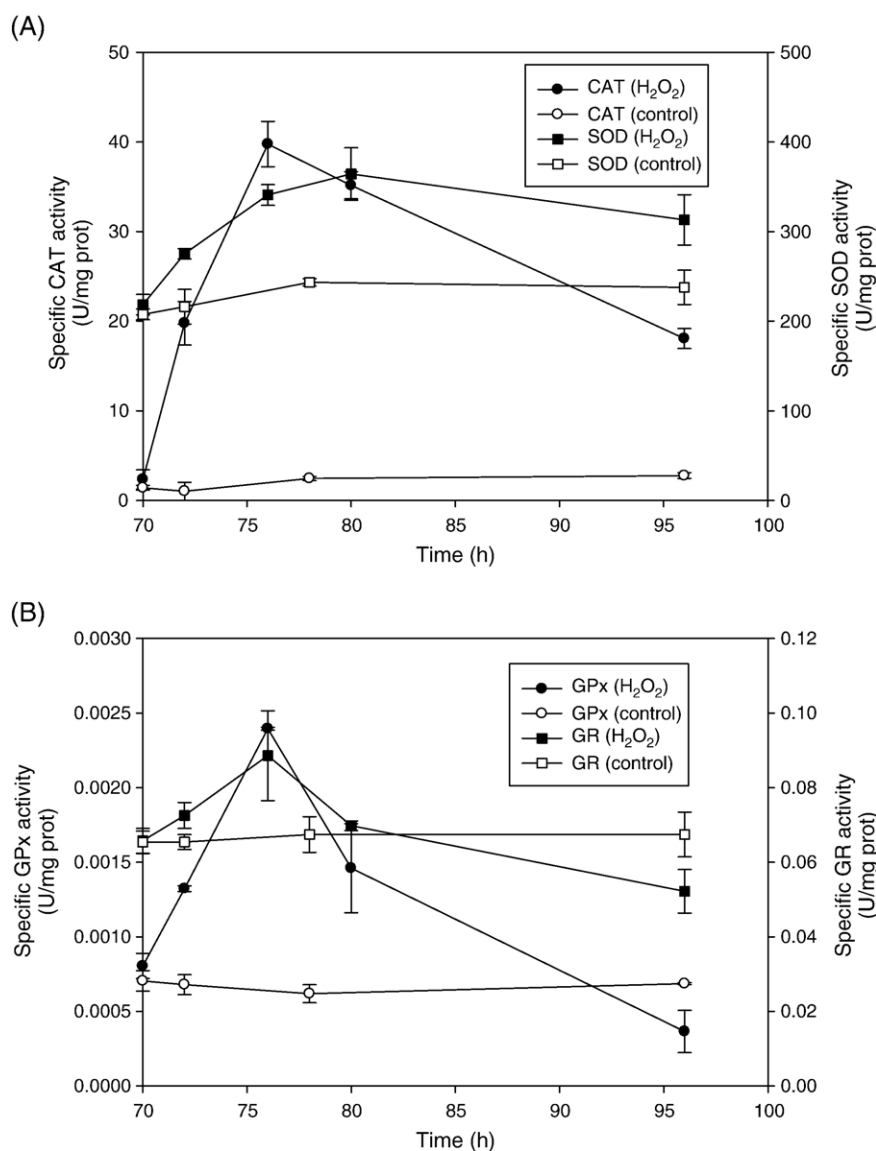
Fig. 3. Effect of H_2O_2 addition on the activities of antioxidant enzymes in batch cultures of *A. niger* B1-D. H_2O_2 was added to the culture at 70 and 72 h, to final concentrations of 100 μM and 10 mM, respectively. The results were obtained from three independent experiments, and expressed as mean±SD.

following preexposure. A 100 μM H_2O_2 leads to a temporary growth arrest and a decrease in protein content. Previous work in *A. niger* showed that protein concentrations decreased under conditions of oxidative stress [35]. In *S. cerevisiae*, exposure to H_2O_2 resulted in a slowdown of protein biosynthetic processes, and a stimulation of protein degradation pathways [36]. However, despite a general decline in protein content, some antioxidant enzymes activities could be greatly enhanced. In the present study, we showed induction of CAT, SOD, GPx, and GR activities by exogenous H_2O_2 in *A. niger*. On the other hand, direct addition of high concentrations of H_2O_2 failed to induce these activities. Meanwhile, we showed that detoxification of H_2O_2 in the adapted culture is by far more rapid compared with the unadapted cultures. Therefore, we propose that the induction of these enzymes is the mechanism by which such cultures acquire tolerance to high (potentially lethal) concentrations of H_2O_2 in *A. niger*. More importantly, we have demonstrated

that this tolerance is simply due to a rapid detoxification of exogenous H_2O_2, avoiding prolonged exposure and associated intracellular damage.

A number of papers have reported the induction of antioxidant enzymes activities by H_2O_2 in microorganisms [29,37,38], but the time course effects of H_2O_2 are not discussed. However,

Table 1
Effects of direct addition of H_2O_2 (10 or 20 mM) on the activities of antioxidant enzymes in batch cultures of *A. niger* B1-D

	CAT u/mg prot	SOD u/mg prot	GR mu/mg prot
Control	2.61±0.22	246.3±10.0	88.9±5.0
10 mM H_2O_2	1.65±0.15	31.7±9.6	69.1±6.6
20 mM H_2O_2	N.D.	N.D.	9.0±1.4

H_2O_2 was added at 72 h at the indicated concentrations, and incubated for 4 h. Results were obtained from three independent experiments and expressed as mean±SD.
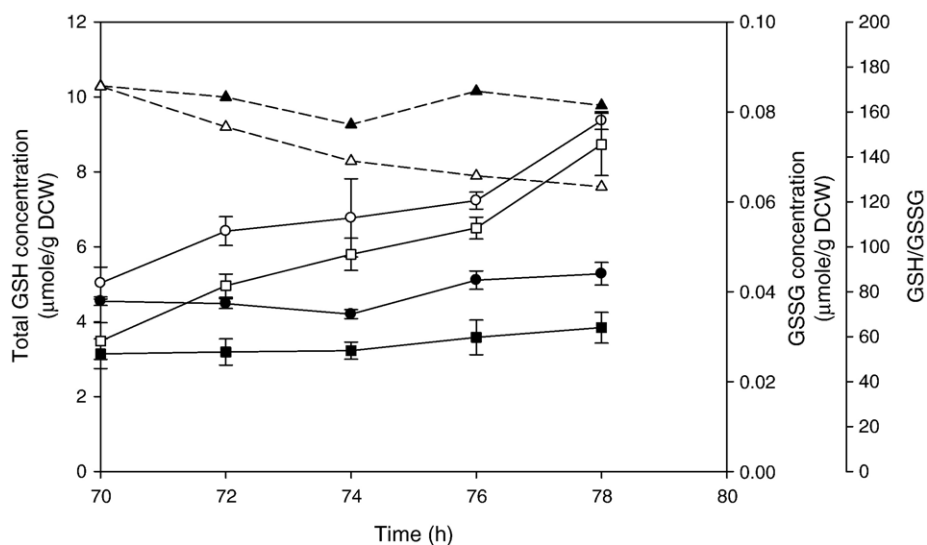
Fig. 4. Effect of H_2O_2 addition on the GSH and GSSG in batch cultures of *A. niger* B1-D. H_2O_2 was added to the culture at 70 and 72 h, to final concentrations of 100 μM and 10 mM, respectively. ● GSH (control); ○ GSH (H_2O_2); ■ GSSG (control); □ GSSG (H_2O_2); ▲ GSH/GSSG (control); △ GSH/GSSG (H_2O_2). The results were obtained from three independent experiments and expressed as mean±SD.

monitoring the changes in exogenous H_2O_2 levels with time is a key factor in attempting to understand the process of adaptation. Our results show that the antioxidant enzymes activities, those involved in the H_2O_2 detoxification, including CAT, GPx, and GR, peaked 4 h after addition of 10 mM H_2O_2 (Fig. 3). The decline in activity of these enzymes coincides with the recovery from growth arrest, meaning that cells are shifting from oxidative defence back to growth. This indicates that the cells may not keep the ability to tolerate severe H_2O_2 stress permanently. Overproduction of these antioxidant enzymes requires extra energy [24], so it makes sense that these enzyme activities are decreasing after exogenous H_2O_2 is completely detoxified, in order to avoid wasting energy. The mechanism through which this decline takes place is still obscure, but from our findings it is clear that activities of these antioxidant enzymes do not correlate well with endogenous H_2O_2 concentrations, since the complete removal of 10 mM H_2O_2 took places less than 1 h after addition in the adapted culture (Fig. 5), while the defensive enzyme activities remained high for much longer (Fig. 3).

CAT and GPx

In filamentous fungi, two peroxidases, CAT and GPx, have been reported to be associated with H_2O_2 detoxification. CAT catalyses the decomposition of H_2O_2 to water and oxygen, while GPx reduces peroxide at the expense of GSH and NADPH [13,14,20]. The relative extent to which these two enzymes contribute to H_2O_2 detoxification is still unclear. Our results show the prestressed level of GPx activity is far less than that of CAT. Similar results have been reported by a number of research groups using different organisms, such as *P. chrysogenum* [20], *Candida albicans* [38], and *S. cerevisiase* [8,9]. In addition, in the present study CAT activity increased 8.5-fold on addition of 100 μM H_2O_2, while GPx only rose by a factor of 1.6. Here, we propose that CAT plays more important role in H_2O_2
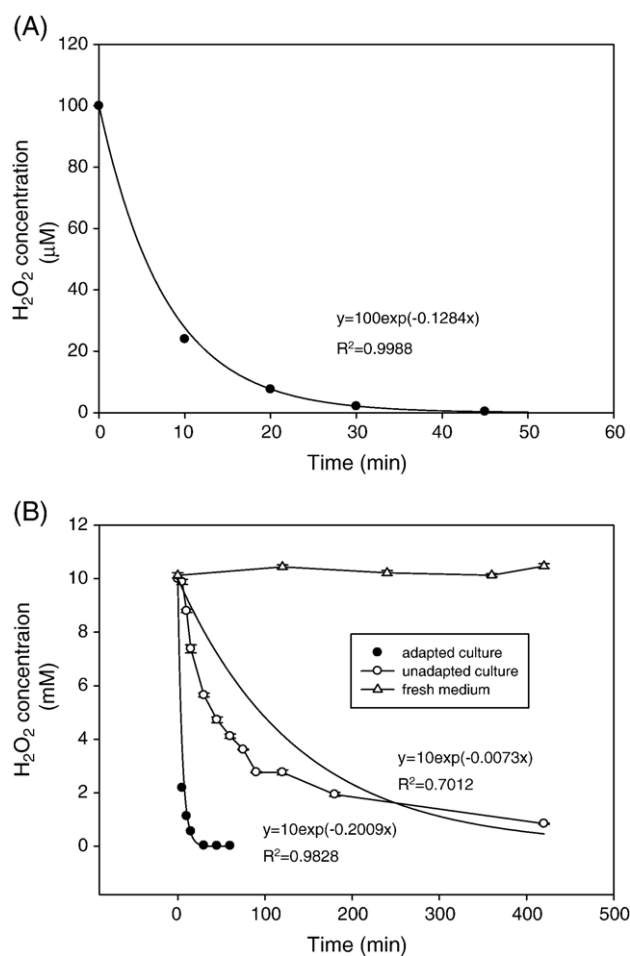

Fig. 5. H_2O_2 detoxification in batch cultures of *A. niger* B1-D. (A) H_2O_2 was added to batch culture at 70 h to final concentration of 100 μM. (B) H_2O_2 was added to batch culture, adapted (pretreatment with 100 μM H_2O_2 for 2 h) or not, at 72 h to final concentration of 10 mM. The results were obtained from three independent experiments and expressed as mean±SD.

detoxification in filamentous fungi. However, GPx has been shown to be indispensable in coping with stress induced by the addition of other peroxides, such as *tert*-butyl hydroperoxide (*t*-BHP), which cannot be detoxified by CAT [20]. In *S. cerevisiae*, a mutant with deleted CAT genes(*ctt1Δ/cta1Δ*) grew similarly to the wild type under nonoxidative stress conditions, and showed a similar susceptibility to H_2O_2, but its ability to adapt to H_2O_2 had been severely compromised [9]. On the other hand, a mutant carrying deletion of a GPx homologous gene was hypersensitive to both H_2O_2 and *t*-BHP [8]. In the wild type, the basal mRNA level of that gene was high, and the product of the gene was expressed constitutively [8]. From these results and our own findings, we hypothesize that, in fungi, CAT and GPx contribute to H_2O_2 detoxification at the same time under (normal) nonstress conditions, but CAT plays a more significant role in dealing with sudden bursts of H_2O_2, not only due to its overwhelmingly higher capacity to remove H_2O_2 but also the cells ability to rapidly boost the activity of this enzyme in response to H_2O_2. This adaptive capability has an intrinsic, central role in many areas of fungal life; for example, CAT has been shown to be implicated in the virulence of some fungi, in which the high capacity of the fungal cells to remove H_2O_2 is essential to counteract the burst of ROS generated by the host cells to kill the invading fungal pathogens [39].

Glutathione pathways (GPx, GR, GSH/GSSG)

The effect of H_2O_2 addition on GR follows the same trend as that of GPx. However, it is worth noting that the reductase activities are much higher than the peroxidase activities. Meanwhile, the redox state of glutathione, expressed as the ratio between the reduced from to the oxidized form, GSH/GSSG, is over 100. It has been reported that the redox state of glutathione in fungi is usually above 20 [40]. Here we show that this figure is higher in this *A. niger*, and the reduced state of glutathione corresponds to a high ratio of reductase activity to peroxidase activity.

Our results also clearly show that GSH does play a role in oxidative defence in *A. niger*. On the one hand, GSH reduces intracellular oxidants by reacting with them, enzymatically or nonenzymatically [40]. By contrast, GSH synthesis is boosted in attempting to rectify the imbalance of glutathione homeostasis. Emri et al. [20] showed that there were no significant changes in GSH levels in response to H_2O_2. The extremely high concentration of H_2O_2 (0.35 and 0.7 M) used in their work might account for this discrepancy; as we demonstrated here different concentrations of H_2O_2 impose distinct effects on microorganisms.

Estimation of intracellular concentration of H_2O_2

According to previous studies, H_2O_2 does not freely diffuse across biomembranes, and the fast removal of H_2O_2 by intracellular antioxidant enzymes is able to generate a concentration gradient between extracellular and intracellular milieu [4,33]. It is this gradient which provides the driving force for H_2O_2 entering the cells where it is decomposed. The intracellular concentration of H_2O_2 is usually much lower than the external one.

Under *in vivo* conditions, both CAT [41] and GPx [42] displayed first-order kinetics with their substrates, not exhibiting saturation effects. As such, the ratio between the concentration of intracellular and extracellular H_2O_2 is described by the [33]

$$[H_2O_2]_{in}/[H_2O_2]_{out} = R = k_{app}/(k_{GPx} + k_{CAT}), \quad (1)$$

where k_{app} refers to the first-order rate constant of the apparent decomposition rate by intact cells, and k_{GPx} and k_{CAT} refer to the first-order rate constants of GPx and CAT in the cell-free extract, respectively [33]. In the present study, we found that intracellular CAT activity is overwhelmingly higher than that of GPx. So the equation can be simplified to

$$[H_2O_2]_{in}/[H_2O_2]_{out} = R = k_{app}/k_{CAT}. \quad (2)$$

Further, k_{CAT} could be derived from intracellular CAT activity in the cell-free extract. In present study, one unit of CAT activity is defined as the decomposition of one micromole H_2O_2 in one minute in the presence of 10 mM H_2O_2 [27]. The value of the CAT activity (U_{CAT}, U/ml) measured in this assay is equal to that of the initial decomposition rate of H_2O_2 by the CAT (mM/min).

$$r_0 = k_{CAT} \ C_0$$

$$k_{CAT} = r_0/C_0 = U_{CAT}/C_0 = U_{CAT}/10, \quad (3)$$

where r_0 refers to the initial rate, when the initial substrate concentration is 10 mM (C_0). Finally, the gradient could be derived as

$$[H_2O_2]_{in}/[H_2O_2]_{out} = R = 10k_{app}/U_C. \quad (4)$$

The estimated gradient is a lower limit, because only CAT is considered for H_2O_2 detoxification in our model; if other enzymes consume H_2O_2, the gradient will be steeper. Nevertheless, our model gives us valuable insights into the profile of the intracellular concentration of H_2O_2 which is distinct from the extracellular one. k_{app} and k_{CAT} from adapted and unadapted *A. niger* are described in Table 2. There is a relatively small gradient between intracellular and extracellular H_2O_2 concentrations when 100 μM H_2O_2 is added. This suggests the rate at which exogenous H_2O_2 entering the cells is beyond their capacity to remove it, and accumulation of H_2O_2 inside the cells occurs quickly. By contrast, the estimated gradient in the

Table 2
First-order constants of apparent decomposition rate of H_2O_2 by intact cells, k_{app}; first order constants of decomposition rate of H_2O_2 by CAT in cell-free extract, k_{CAT}; H_2O_2 concentration gradient between intracellular and extracellular milieu

	k_{app}	k_{CAT}	$[H_2O_2]_{in}/[H_2O_2]_{out}$
100 μM	0.1284	0.154	0.834
10 mM	0.2009	0.758	0.265

100 μM H_2O_2 was added to batch culture of *A. niger* B1-D at 70 h (unadapted); 10 mM H_2O_2 was added to batch culture of *A. niger* B1-D pre-treated with 100 μM H_2O_2 for 2 h at 72 h.

adapted culture is much higher than in unadapted cultures. This shows that *A. niger* has a greatly enhanced capacity to remove H_2O_2 after adaptation, and the cells' ability to remove H_2O_2 becomes comparable to the rate of H_2O_2 entering the cells. Thus, accumulation of this oxidant inside the cells is avoided. By gaining new insights into the detoxification rate of exogenous H_2O_2 by intact adapted cells, and into the intracellular antioxidant enzyme activities, we have shown that enhanced degradation of H_2O_2, which alters the concentration gradient of the stressor and minimises intracellular accumulation, is the mechanism of the adaptive effect.

Conclusions

Pretreatment of *A. niger* with H_2O_2 at a nonlethal concentration confers greatly enhanced resistance to killing by H_2O_2 at lethal concentrations in the early exponential phase of growth. This adaptation involves the induction of a number of antioxidant enzymes, predominantly catalase. Enhancement of the activities of these antioxidant enzymes prevents accumulation of H_2O_2 inside the cells, as supported by the increased decomposition rate of external H_2O_2 by intact cells, and higher gradient of H_2O_2 concentration between intracellular and extracelluar milieu estimated from a mathematical model.

Acknowledgments

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