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INVESTIGATION OF GLUTATHIONE METABOLISM IN FILAMENTOUS FUNGI

(A SHORT AUTO-REVIEW)

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Introduction

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine; GSH) is one the most abundant low molecular weight thiols found in biological systems. It has been detected in most eukaryotic cells including all the fungi tested thus far and also in many prokaryotes [1]. As a multifunctional primary metabolite, it plays a crucial role in numerous important biochemical processes. In fungi, GSH is involved in the transport of amino acids into the cells and the vacuoles (γ-glutamic cycle), the protection against oxidative stress, the detoxification of xenobiotics and heavy metals and it also takes part in the regulation of cell morphology and life cycle [1–3]. Not surprisingly, the GSH metabolism and related biochemical pathways are among the most frequently studied fields in fungal physiology. Here we summarize the results of some projects currently in progress in our laboratory where the investigation of the GSH metabolism of fungi is of primary interest.

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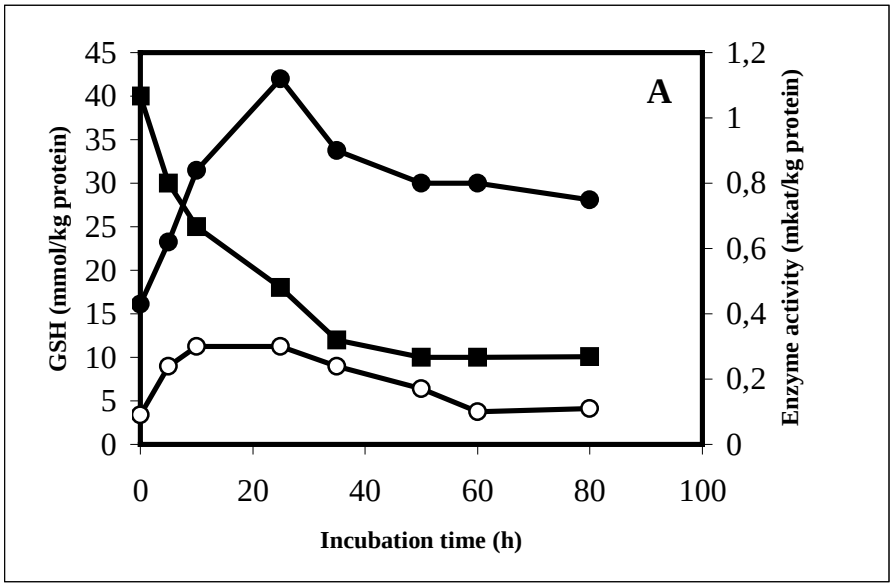
Toxicity of phenylacetic acid (PA) and phenoxyacetic acid (POA) in *Penicillium chrysogenum* and *Aspergillus nidulans*

The most frequently used side-chain precursors in industrial scale penicillin production are PA (for penicillin G synthesis) and POA (for penicillin V synthesis). Both organic acids are toxic for fungal cells and their toxicity is usually explained with their protonophoric character [4]. In our experiments, POA as well as PA disturbed significantly the GSH metabolism of *P. chrysogenum*. When these compounds were added at sublethal concentrations to the culture media they induced the GSH-dependent detoxification pathway of the cells, which was clearly indicated by the enhanced glutathione S-transferase (GST) and γ-glutamyltranspeptidase (γGT) activities and the decreased GSH levels. At higher concentrations, PA and POA even depleted the GSH pool, increased the intracellular peroxide and superoxide levels and finally killed the cells [5, 6] (Figure 1). In addition to *P. chrysogenum*, POA and PA had similar physiological effects on *A. nidulans* and *A. chrysogenum* (Table I). According to our recent observations, GST alone was unable to catalyze any direct conjugation reaction between GSH and either PA or POA. We therefore hypothesised that, similar to the degradation of other aromatic molecules including benzole, naphtol, anthracene, the epoxide intermediates of the hydroxylation reactions catalyzed by aromatic ring monooxygenases should be responsible for both the toxicity of the penicillin side-chain precursors and their inductive effect on GST [7, 8].

Table I

Effect of PA and POA on GSH metabolism in different fungi. Specific activity and production values are expressed as Mean ± S.D., calculated from 4 independent experiments
* P < 1%. P values were calculated using the Student's t-test

strain	treatment	GSH nmol/mg dry weight	GST μkat/kg protein	γGT μkat/kg protein
P. chrysogenum NCAIM00237	control	18 ± 0.2	400 ± 45	60 ± 8
	0.25% PA	6.1 ± 0.5*	650 ± 80*	250 ± 40*
	0.5% POA	7.5 ± 0.8*	700 ± 10*	30*
A. nidulans PW1	control	9.5 ± 1	70 ± 8	7 ± 1
	0.1% PA	3.3 ± 0.5*	160 ± 20*	30 ± 5*
	0.65% POA	3.5 ± 0.5*	120 ± 15*	18 ± 2*
A. chrysogenum ATCC46117	control	4 ± 0.2	60 ± 5	19 ± 1
	0.1% POA	1 ± 0.1*	80 ± 8*	24 ± 2*



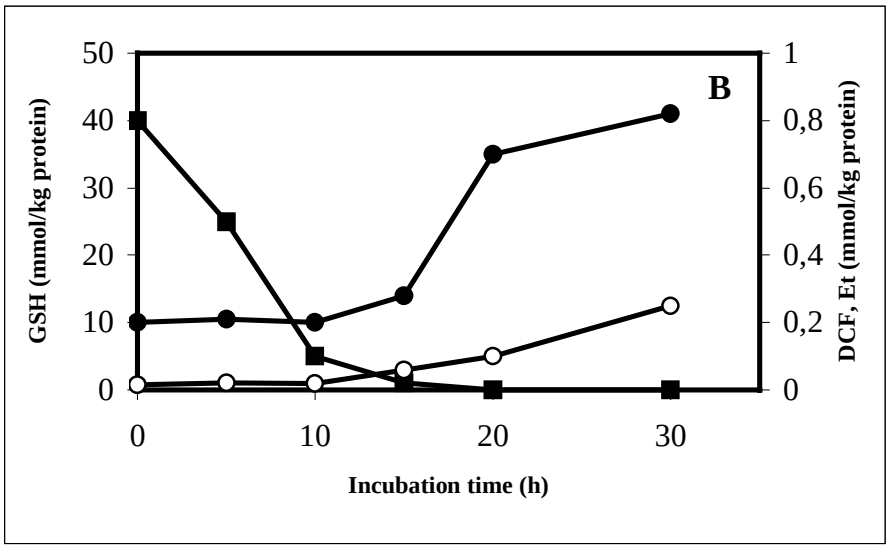


Figure 1. Effect of 0.5 % POA on the GSH level (■), specific GST (●) and γGT (○) activities in *P. chrysogenum* (A). Changes in the intracellular GSH (■), superoxide (Et; ●) and peroxide (DCF; ○) levels in the presence of 0.75 % POA (B)

The hydroxylation of PA and POA has already been described in several fungi, including the most favoured filamentous fungus model organism *A. nidulans*. In this micro-organism, the first hydroxylation of PA was catalyzed by phenylacetic acid 2-hydroxylase (PAH) [7,9]. This cytochrome P450 dependent monooxygenase was probably not the only enzyme that hydroxylates PA, since after disrupting the gene coding for PAH (phacA) the mutant *A. nidulans* cells still produced some 2-hydroxy-PA. Therefore the involvement of at least one more aspecific hydroxylase in the degradation of PA seemed to be probable [7, 9].

In a study, where the physico-chemical and toxicological properties of some hydroxylated derivatives of POA and PA were compared, we found that the hydroxylated compounds were less toxic for *P. chrysogenum* and they induced the GSH-dependent detoxification pathway only at considerably higher concentrations [10]. We therefore failed to demonstrate any correlation between the toxicity and the acidity of the tested compounds. For example, PA was more toxic (MIC_{PA}= 0.36 %) than 2-hydroxy-PA ($MIC_{2OH\text{-}PA}$= 0.74 %), while 4-hydroxy-PA was essentially non-toxic ($MIC_{4OH\text{-}PA}$ = 2 %) in spite of the fact that their first acidic dissociation constants were very similar (pK_1= 4.08, 4.19, 4.16, respectively) [10]. These results indicate that the protonophoric character alone cannot explain satisfactorily the toxicity of these side-chain precursors. Unfortunately, neither Rodríguez-Sáiz et al. [9] nor we could detect any increase in the toxicity of PA in loss-of-function *P. chrysogenum* and *A. nidulans* PAH mutants (Table II). As a consequence, PAH could not take part in the metabolic activation of PA prior to GSH S-conjugation, which may shed some light in the possible importance of aspecific hydroxylation in the generation of toxic PA and POA derivatives.

Table II

Toxicity of PA in different *A. nidulans* strains.
"M" is a loss-of-function PAH mutant of *A. nidulans* PW1 created by Mingot et al. [7] and "C" is the appropriate control strain.
Minimal inhibitory concentrations (MIC) were determined at pH 5.0.

Strains	MICPA (%)
FGSC26 (biA1 veA1)	0.4
PW1 (argB2 biA1 methG1 veA1)	0.2
C (argB2 biA1 methG1 veA1 p35::arg)	0.4
M (argB2 biA1 methG1 veA1 ΔphacA::arg)	0.4

We compared the GST isoenzyme patterns in *P. chrysogenum* cultures under penicillin producing and non-producing conditions. Surprisingly, POA induced GST1, one of the two major *P. chyrsogenum* GST isoenzymes, which located in the microsomal cell fraction. It is worth noting that aromatic ring monooxygenases including PAH have also been located in the microsomes [7]! Furthermore, GST1, in contrast to the cytosolic GST2 isoenzyme, was shown to possess a significant epoxidase activity, too.

To sum it up, both the formation of reactive epoxide intermediates and the concomitant shrinkage (even depletion) of the GSH pool are likely of paramount importance in the manifestation of the toxic effects of PA and POA in fungi. Of course, the protonophoric character of PA and POA might contribute to their toxicity, too but the significance of this property should not be overestimated. The toxic epoxide derivatives were formed most likely in the microsomes due to the action of aspecific hydroxylases and were detoxified immediately by microsomal GST. PAH, as the first enzyme in the homogentisic acid pathway of PA degradation, could be important in the elimination of the toxic penicillin side-chain precursors from the cell [9]. Alternatively, in penicillin producer species, the incorporation of PA or POA into β-lactam molecules seemed to be a very efficient way to avoid the modification of biomolecules by harmful aromatic epoxides [9].

Ageing and autolysis in *Penicillium chrysogenum* and *Aspergillus nidulans*

The potential importance of GSH metabolism and redox processes in the morphological changes of fungi has already been emphasized in different organisms by several authors [2, 3, 11]. In our laboratory, we studied the morphological and redox changes in carbon limited, autolysing cultures of *P. chrysogenum* and *A. nidulans*. Although both species showed very similar pellet morphology in the exponential and stationary phases of growth, the morphological features of autolysing and post-autolytic cultures were completely different.

In starving *P. chrysogenum* cultures the stationary phase was followed by the fast disintegration of the pellets to short filaments, which finally fragmented predominantly to two-celled round-ended “yeast-like” hyphal pieces [12, 13]. The autolysis resulted in a significant loss of biomass. It is worth noting that after addition of an extra dose of glucose to the culture media these “yeast-like” surviving morphological forms germinated simultaneously at both ends and, as a result, new thin hyphae appeared in the cultures [12, 13].

In *A. nidulans* cultures, after the stationary phase of growth the dry cell mass decreased only slightly, and the cultures preserved their pelleted cell morphology. The

autolysis of mycelia resulted in a continuous and slow reduction in the diameter of pellets instead of the fast disintegration process observable in *P. chrysogenum* cultures. In autolysing *A. nidulans* cultures some mycelia even developed conidiophors mainly in the centre of the pellets but formation of conidia was never observed. The autolysis of the outer looser parts of the pellets that consisted of short filaments gave rise to only a very few "yeast-like" fragments, which were abundant in *P. chrysogenum* cultures. After glucose supplementation the "yeast-like" morphological forms germinated and developed into pellets again. In old *A. nidulans* cultures the pellets disintegrated at last but the fragments always preserved their filamentous morphology. The "yeast-like" cells never became dominant in autolytic and post-autolytic phase *A. nidulans* cultures.

The morphological changes described above in autolytic *P. chrysogenum* and *A. nidulans* cultures coincided with some spectacular changes in the cell physiology. For example, both species started to utilize alternative energy and carbon sources, e.g. proteins and peptides, after the starting glucose had been metabolised. The extracellular hydrolase production (protease, chitinase) of both fungi was also considerable [12–14]. In the case of *A. nidulans*, γGT, an enzyme responsible for the hydrolysis and, hence, the mobilisation of GSH reserves [15], was induced and, as a consequence, the GSH pool shrank fast in this fungus. The enhanced ammonia efflux observed in both species also suggested intensive amino acid degradation during autolysis. As far as the NADPH production of *A. nidulans* is concerned, the importance of the oxidative pentose phosphate shunt decreased while that of the NADP-dependent isocitrate dehydrogenase increased as a function of culture time.

Although the morphological changes observed during autolysis of *P. chrysogenum* and *A. nidulans* were quite different, the time-course of intracellular peroxide and superoxide levels were surprisingly similar [16]. The concentrations of these two reactive oxygen species (ROS) were lower in the stationary phase than those recorded in the exponential phase but increased steadily later on (Figure 2). In fact, there was a strict correlation between the age of the cultures and the amount of peroxides and superoxide accumulated inside the cells. These observations are in good accordance with the propositions of the Free-radical Theory of Ageing [17] and, therefore, the use of the term "ageing" to describe the physiological changes taking place in aged *P. chrysogenum* and *A. nidulans* cultures can be recommended [12–14, 16].

The specific activities of antioxidative enzymes changed similarly as a function of age in both fungal species. The superoxide dismutase (SOD) activities were always proportional to the intracellular superoxide levels and, therefore, high specific SOD activities were typical in aged cultures. Despite of the high peroxide levels observed in ageing fungal cells, glutathione peroxidase, glutathione reductase and catalase were

active mainly in the stationary and early autolytic phases of growth [16]. In aged cells, other peroxide eliminating enzymes (alternative oxidase, thioredoxin peroxidase) were likely to replace these enzymes.

The changes in the GSH/GSSG redox ratios during autolysis were significantly different in the two fungi studied (Figure 3). In *A. nidulans*, the GSH levels decreased steeply after the stationary phase and there was a small decrease in the GSSG concentration, too. Hence, the GSH/GSSG ratio decreased steadily during ageing. In the case of *P. chrysogenum*, the GSH content of the cells remained constant, while there was a drop in the GSSG level. Therefore, the GSH/GSSG ratio even increased, i.e. improved as a function of fermentation time concomitantly with autolysis and fragmentation [16]. Moreover, the re-addition of glucose to ageing *P. chrysogenum* cultures, which re-initiated the vegetative growth of the surviving fragments, resulted in a significant decrease in the intracellular GSH concentration [15]. These data suggest a possible correlation between cell morphology and the GSH/GSSG ratios.

In the opportunistic human pathogenic dimorphic fungus *Candida albicans*, the intracellular level of GSH also decreased during yeast → mycelial transition, probably due to a profound increase in the activity of γGT in the germ tubes [3, 18]. In the case of another dimorphic fungus, the industrially significant *Aureobasidium pullulans* we also found significantly higher GSH levels in yeast cells as compared to mycelia [19]. However, the GSH/GSSG ratios were similar in the two morphological forms since yeast cells contained higher GSSG concentrations as well. Unfortunately, the intracellular GSH and GSSG concentrations are influenced by numerous endogenous and environmental factors and even the cellular distribution of the tripeptide responds to different kinds of metabolic stresses. For example, nitrogen starvation provokes the accumulation and storage of GSH within the vacuoles [15]. This means that the calculated overall GSH/GSSG redox balances of the cells do not reflect properly the redox status of the cytosol and the endoplasmic reticulum, where the biosynthesis and assembly of cell wall constituents take place. The verification of any possible causal connection between the cell morphology and GSH metabolism of autolytic and post-autolytic fungal cells therefore needs further studies.

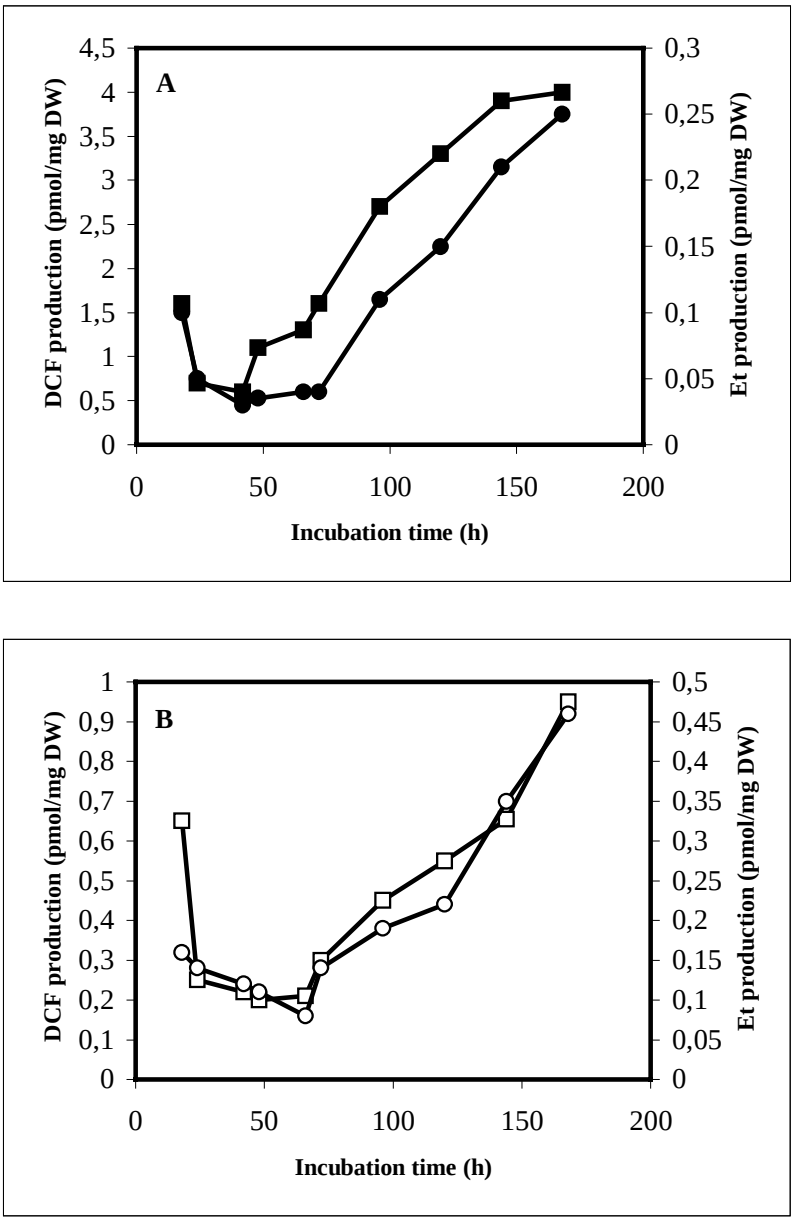


Figure 2 Changes in the intracellular peroxide (DCF; ■ and □) and superoxide (Et; ● and ○) levels in *A. nidulans* (A) and *P. chrysogenum* (B) cultures

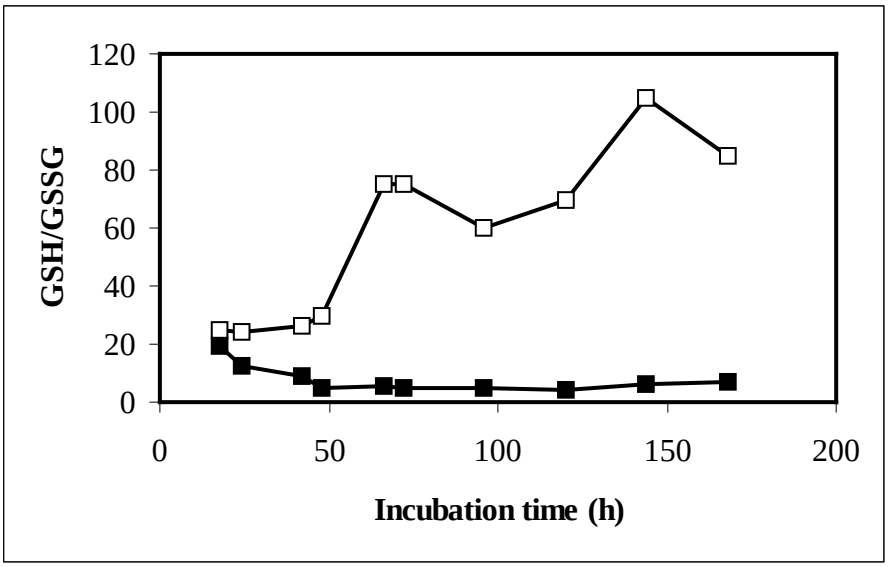


Figure 3 Changes in the GSH/GSSG ratios in *A. nidulans* (■) and *P. chrysogenum* (□) cultures

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