Acta Microbiologica et Immunologica Hungarica, 48 (3–4), pp. 393–411 (2001)

THE GLUTATHIONE METABOLISM OF THE β-LACTAM PRODUCER FILAMENTOUS FUNGUS *PENICILLIUM CHRYSOGENUM**

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(Received: March 26, 2001; accepted: April 20, 2001)

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine; GSH) shares structural similarities with the β-lactam biosynthetic intermediate ACV-tripeptide {δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine}. Not surprisingly, GSH has been reported to inhibit the β-lactam biosynthetic machinery quite effectively and, hence, strategies to decrease the intracellular GSH concentrations without influencing negatively the physiological status of idiophasic mycelia would attract industrial interests. Here we present a detailed map of the GSH metabolic network of *P. chrysogenum* and show a promising way to keep the GSH pool selectively down under penicillin producing conditions. This procedure includes a well-controlled and transient lowering of pH at the beginning of the production phase, and it relies on the GSH-dependent detoxification of the protonophore penicillin side-chain precursors phenoxyacetic acid (POA) and phenylacetic acid (PA). Encouraging preliminary fed-batch fermentation experiments have been performed to test this technological proposal. Interestingly, the mechanism of the activation of POA and PA to the appropriate CoA derivatives has remained yet to be answered but the involvement of GSH seems to be rather unlikely in this case. Our data also challenge the hypothesis that the formation of different kinds of penicillins would be an alternative to GSH-dependent detoxification processes in *P. chrysogenum*.

Keywords: *Penicillium chrysogenum*, gluthathione metabolism

* This paper was written to commemorate to the fiftieth anniversary of the foundation of the Hungarian Society for Microbiology.

Introduction

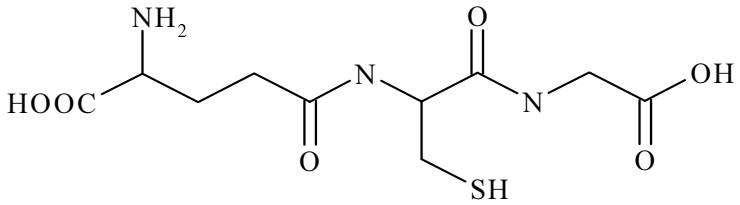
Glutathione (γ-L-glutamyl-L-cysteinyl-glycine; Fig. 1, GSH) is one of the most abundant low molecular weight thiols found in biological systems [1]. In fungi GSH was proved to play a crucial role in numerous physiological processes including (i) maintenance of a suitable reduced milieu for biosynthetic enzymes *via* stabilization of -SH groups [2], (ii) elimination of reactive oxygen species (ROS), e.g. peroxides [3–6], (iii) detoxification of harmful metabolites (e.g. formaldehyde and methylglyoxal) [7, 8], xenobiotics (*via* GSH S-conjugation) [9, 10] and heavy metal ions (e.g. Cd^{2+} and CrO_4^{2-}) [11, 12] as well as (iv) transportation of amino acids and peptides *via* the γ-glutamyl cycle [13]. In addition, GSH may also serve as a major sulphur storage compound in fungi [14].

In terms of cell morphology and cell physiology, GSH/GSSG redox imbalances of fungal cells are considered as early signal transduction events of dimorphic (yeast↔hypha) transitions [15–19], cell differentiation processes including sporulation and germination *via* transient hyperoxidant states (Dioxygen Avoidance Theory of Cell Differentiation) [20–22] and autolysis. It is notable that fungal autolysis more recently turned out to be energy-dependent and, at least in some aspects, reminiscent of the apoptosis of higher eukaryotes [23, 24].

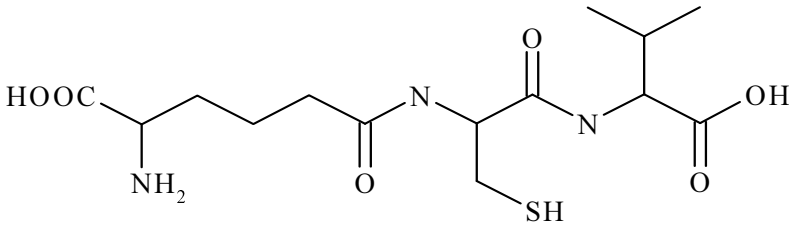
In β-lactam producer filamentous fungi (e.g. *Penicillium chrysogenum*, *Acremonium chrysogenum*) GSH also effects secondary metabolism because one of he most important intermediates of the β-lactam biosynthesis, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), is structurally analogous to GSH (Fig. 1). As a result, GSH suppresses β-lactam production by inhibiting ACV synthase and isopenicillin N synthase (IPNS) activities [25–29]. Paradoxically, the same tripeptide may contribute to the maintenance of the redox stability of the O_2-consuming IPNS [30, 31]. GSH may also take part in some additional steps of β-lactam biosynthesis including the intracellular transport of precursor amino acids [32, 33] and the activation of the side-chain precursors phenylacetic and phenoxyacetic acids (PA and POA, respectively) *via* the temporary formation of (*S*-acyl)glutathione intermediates [34]. Under β-lactam producing conditions, intracellular GSH reserves may serve as an endogenous sulphur source as well [35]. To sum up, GSH seems to be one of the most important, small molecular mass regulators of the β-lactam biosynthetic machinery.

The primary aim of mapping the GSH metabolism of *P. chrysogenum* was to develop a new strategy to decrease the intracellular GSH concentrations without influencing negatively the physiological status of idiophasic mycelia and to record the effect on penicillin productivity. Additional goals were to test the hypotheses that GSH is involved in the activation of penicillin side-precursors and the formation of

penicillins is an alternative to GSH-dependent detoxification of the side-chain precursors.



GSH



ACV

Fig. 1. Structural similarities between glutathione and ACV-tripeptide

Materials and methods

Culture conditions; minimal inhibitory concentration (MIC) determinations

The high β-lactam producing strain *P. chrysogenum* NCAIM 00237 was grown in flasks (500 ml) containing 100 ml of complex culture medium containing 2.0% glucose, 0.4% peptone, 0.4% yeast extract, 0.05% $MgSO_4$, 0.4% K_2HPO_4, 0.2% KH_2PO_4 [36–44]. The mycelia were separated by filtration on sintered glass, were washed and transferred immediately into a defined medium (pH 6.5–7.6) containing 0.4 or 0.93% K_2HPO_4, 0.2 or 0.55% KH_2PO_4, and also supplemented with 0-2.0% glucose, 2.0% 2-deoxy-D-glucose or 2.0% lactose as carbon sources, 0–60 mM sodium L-glutamate (Na-Glu), 0–40 mM $(NH_4)_2HPO_4$, 40 mM $NaNO_3$ or 10 mM $NaNO_2$ as

nitrogen sources and 20–60 mM cysteine (Cys), 20–60 mM methionine (Met) or 0–4.15 mM $MgSO_4$ as sulphur sources. Culture media normally contained 101 mM (2.0%) glucose, 60 mM (1.0%) Na-Glu and 4.15 mM (0.05%) $MgSO_4$ as carbon, nitrogen and sulphur sources, respectively, and 0.4% K_2HPO_4, 0.2% KH_2PO_4 (pH=7.6; normal buffer capacity culture medium) unless otherwise indicated. In some cases the buffer capacity of the culture media was increased by the addition of 0.93% K_2HPO_4 and 0.55% KH_2PO_4 (pH=6.5; increased buffer capacity culture medium). These media were also supplemented with 0-2.0% (w/v) PA or POA or their hydroxylated derivatives, 0.1% (w/v) 1-chloro-2,4-dinitrobenzene (CDNB), 0.15–0.25 % caffeine, 25 mM cAMP, 0.35–0.70 M H_2O_2, 0.5–2.0 mM *tert*-butyl hydroperoxide (*tert*-BOOH), 50–500 μM menadione (MQ), 0.5 mM diamide (a widely used compound to oxidise intracellular GSH to GSSG instantaneously and stoichiometrically) or 0–300 μM $FeCl_3$ as required. The starting mycelial dry weight was 5.0 mg/ml in each experiment and all the cultures were incubated with shaking at 25 °C and at 250 rpm for 5-10 h.

Under selected experimental conditions, the idiophasic (between 50–80 h of incubation) changes in the GSH-metabolism were also studied, and penicillin productivity values were determined at about 80 h incubation time.

Specific intracellular GSH concentration and some specific enzyme activities were also determined in wild-type *P. chrysogenum* strain that was kindly provided by Prof. Dr. G. Winkelmann (Department of Microbiology and Biotechnology, University of Tübingen). The wild-type strain was cultured exactly under the same conditions as described above for the industrial strain.

Minimal inhibitory concentration (MIC) values for the penicillin side-chain precursors and their derivatives were determined by agar dilution method using solid media containing 2.0% lactose, 1.0% Na-glutamate, 0.93% K_2HPO_4, 0.55% KH_2PO_4, 0.05% $MgSO_4$, 2.0% agar (pH 6.5). Approximately 5.0×10^3 *P. chrysogenum* spores were spotted onto agar discs (diameter 3.0 cm, height 1.5 cm) and were incubated at 25 °C for 5 days [42–44].

Enzyme activity determinations and analytical procedures

Specific glutathione producing activity (GPA) [45], and specific glutathione reductase (GR) [46], glutathione peroxidase (GPx) [47], glutathione S-transferase (GST) [48], γ-glutamyltranspeptidase (γGT) [49], glucose-6-phosphate dehydrogenase (G6PD) [36], catalase [50] and superoxide dismutases (SODs; CuZnSOD and MnSOD) [51] activities were measured in cell-free extracts after disrupting *P. chrysogenum* mycelia by a Type X25 X-press (AB Biox, Göteborg, Sweden).

For monitoring changes in the intracellular concentrations of GSH and oxidized glutathione (GSSG), harvested mycelia were treated with 5% (w/v) 5-sulfosalicylic

acid and the extracts were neutralized with 0.5 M NaOH [40, 42]. The GSH and GSSG concentrations were measured according to Anderson [52].

The formation of β-lactams was quantified by the method of Bundgaard and Ilver [53] using penicillin V potassium salt as standard. Intracellular protein contents were measured according to Peterson [54].

The dissociation constants of PA and POA and their derivatives were determined by pH-titration in aqueous solution [43, 55].

Intracellular iron levels were monitored by atomic absorption spectrometry [44]. Cell-free extracts were also screened for (*S*-phenoxyacetyl)glutathione using a HPLC equipment and a standard prepared according to Ferrero et al. [34].

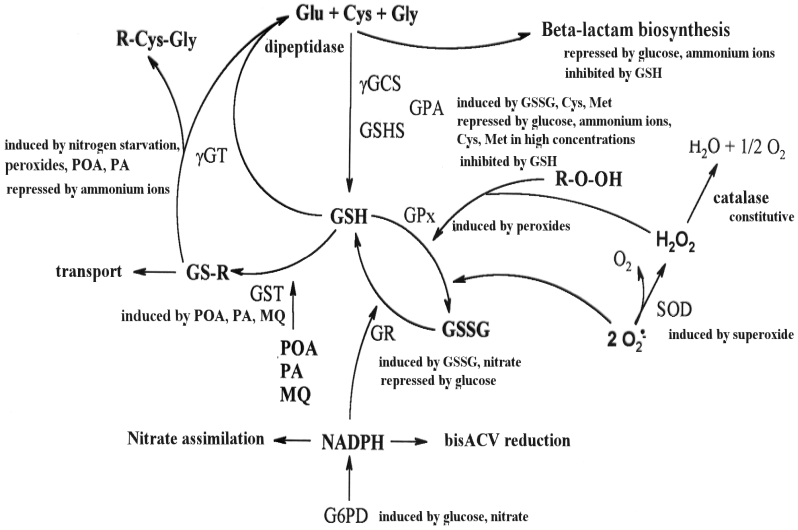


Fig. 2. Summary of the GSH metabolism of *Penicillium chrysogenum* NCAIM 0023

Results and Discussion

Mapping the GSH-metabolism of a high β-lactam producer industrial strain of P. chrysogenum

The complex GSH metabolic network of *P. chrysogenum* NCAIM 0023 is presented in Tables I–III as well as in Fig. 2. Specific penicillin production values observed in normal and increased buffer capacity culture media are summarized in Table IV. Because a more detailed presentation and discussion of experimental data can be found in previous publications [36–44] here we turn our attention only to the most remarkable findings and their interpretation.

One of the most notable observations was that the intracellular GSH levels could not be decreased and, concomitantly, the penicillin yields could not be increased by changing solely the carbon, nitrogen and sulphur sources. The only way to keep the GSH pool selectively down during the penicillin production phase was to take advantage of the GSH-dependent detoxification of the penicillin side-chain precursors PA and POA through the subsequent action of GST and γGT (Tables I–IV) [43]. However, the inclusion of either Met or NH_4^+ ions into the culture media prevented the POA-induced idiophasic depletion of the GSH pool, and, simultaneously, decreased profoundly the penicillin yields (Table IV). Met added at low concentration stimulated the *de novo* GSH synthesis resulting in higher intracellular GSH levels (Tables I–III). Paradoxically, the specific GPA was significantly decreased by NH_4^+ ions. Nevertheless, the specific activity of γGT, which plays a pivotal role in both GSH-dependent detoxification (it degrades GSH S-conjugates) and amino acid uptake and transport (it splits GSH in the γ-glutamyl cycle) processes [1, 13, 17, 56], was halved in the presence of NH_4^+ in comparison to Na-Glu [39]. The repression of γGT by NH_4^+ is likely to be a key element in the maintenance of high intracellular GSH levels in *P. chrysogenum* mycelia [57] even in the presence of POA (Tables I and IV) [38] and, hence, may contribute to the observed ammonium ion repression of the penicillin biosynthesis itself (Table IV) [26]. Fortunately, POA increased the specific activities of the GSH-dependent detoxification pathway even in the presence of NH_4^+ ions (although to a lesser extent as with Na-Glu) [38], which may give us the opportunity to decrease the intracellular GSH pool under penicillin producing conditions by stimulating the influx of side-chain precursors into the cells (see below).

Table I

Specific activities and regulations of GSH-dependent and GSH-metabolism related enzymes in Penicillium chrysogenum[a] *[36–44]*

Enzymes	Specific activity[b] {mkat (kg protein)$^{-1}$}	Specific activity was increased by	Specific activity was decreased by	Specific activity was not affected by
GPA	0.060	any kinds of GSH/GSSG redox imbalances	60 mM Cys or Met	nitrogen starvation
		20 mM Cys or Met	**NH_4^+ nitrogen source**	NO_3^- or NO_2^- nitrogen sources
		carbon starvation		sulphur starvation
		lactose carbon source		2-deoxy-D-glucose
				caffeine
GR	3.1	any kinds of GSH/GSSG redox imbalances	carbon starvation	NH_4^+ nitrogen source
				nitrogen starvation
		NO_3^- or NO_2^- nitrogen sources with glucose carbon source but not with lactose	lactose carbon source	NO_3^- or NO_2^- nitrogen sources with lactose carbon source
				Cys, Met
				sulphur starvation
				2-deoxy-D-glucose
				caffeine
				intracellular iron levels
GPx	0.48	intracellular peroxide levels		increased superoxide levels
				GSH/GSSG redox imbalances
				nitrogen sources
GST	0.51	MQ		intracellular peroxide levels
		penicillin side-chain precursors		GSH/GSSG redox imbalances
				nitrogen sources
				CDNB

Table I (continued)

Enzymes	Specific activity[b] {mkat (kg protein)$^{-1}$}	Specific activity was Increased by	Specific activity was decreased by	Specific activity was not affected by
γGT	0.12	intracellular peroxide levels **penicillin side-chain precursors** nitrogen starvation	**NH_4^+ nitrogen source**	carbon sources sulphur sources intracellular superoxide levels MQ GSH/GSSG redox imbalances CDNB
G6PD	2.4	NO_3^- or NO_2^- nitrogen sources with glucose carbon source but not with lactose	lactose carbon source caffeine exogenous cAMP	intracellular peroxide levels intracellular superoxide levels GSH/GSSG redox imbalances
Catalase	5800			any tested culture media components or additives
CuZnSOD[c]	46 mU (kg protein)$^{-1}$	intracellular superoxide levels		intracellular peroxide levels GSH/GSSG redox imbalances
MnSOD[c]	18mU (kg protein)$^{-1}$	intracellular superoxide levels		intracellular peroxide levels GSH/GSSG redox imbalances

[a] - Specific enzyme activities determined at 5-10 h of incubation are summarized.
[b] - Culture media contained 101 mM glucose, 60 mM Na-Glu and 4.15 mM $MgSO_4$ as carbon, nitrogen and sulphur sources, respectively, in the normal buffer capacity culture medium unless otherwise indicated.
[c] - One unit of SOD activity was defined as the amount of enzyme that inhibited the Nitro Blue Tetrazolium oxidation rate of the control by 50 % [51]. In the MnSOD assay, reaction mixtures were also supplemented with 4.0 mM NaCN.

Table II

Specific concentrations of GSH and GSSG in Penicillium chrysogenum[a] *[36–44]*

Parameters	Specific concentrations[b] {mmol (kg protein)$^{-1}$}	Specific concentration was increased by	Specific concentration was decreased by	Specific concentration was not affected by
GSH	26	lactose carbon source carbon starvation Cys, Met diamide (paradoxically)	oxidative stress generated by *tert*-BOOH and MQ sulphur starvation **side-chain precursors depending on the pH control of the media but not with NH_4^+ ions** POA added at MIC, CDNB	nitrogen source nitrogen starvation caffeine 2-deoxy-D-glucose H_2O_2 up to 0.70 M intracellular iron levels
GSSG	2.0	oxidative stress generated by any kinds of stressors **side-chain precursors depending on the pH control of the media** **but not with NH_4^+ ions** POA added at MIC CDNB		carbon source nitrogen source sulphur source caffeine

[a] – Specific concentrations determined at 5–10 h of incubation are summarized.
[b] – Culture media contained 101 mM glucose, 60 mM Na-Glu and 4.15 mM $MgSO_4$ as carbon, nitrogen and sulphur sources, respectively, in the normal buffer capacity culture medium unless otherwise indicated.

In accordance with previous observations [58], the toxicity of the protonophore side-chain precursors, which are transported across the plasma membrane solely by free diffusion [59,60], was dependent on the pH of the culture medium (Tables I–IV) [38,42], and, most likely, was connected with the formation of toxic epoxide intermediates of hydroxylation reactions on the aromatic rings [42, 43]. The idea that other factors than the protonophoric character of these side-chain precursors were responsible for their cell toxicity was supported by the observation that the fungus

could not tolerate POA at 0.7% (equals to MIC for POA) or higher concentrations even in an increased buffer capacity culture medium (Table IV). On the other hand, *P. chrysogenum* was shown to produce penicillin V quite effectively in a normal buffer capacity culture medium containing 0.4% POA, which was characterized with low intracellular GSH concentrations and very severe GSH/GSSG redox imbalances [38].

Table III

GSH/GSSG redox balances in Penicillium chrysogenum[a] *[36–44]*

Parameter	Concentration ratio[b]	Concentration ratio was increased by	Concentration ratio was decreased by	Concentration ratio was not affected by
GSH / GSSG ratio	13	carbon starvation lactose carbon source Cys, Met	oxidative stress generated by any kinds of stressors **side-chain precursors depending on the pH control of the media but not with NH_4^+ ions** POA added at MIC sulphur starvation CDNB	nitrogen source caffeine

[a]- Concentration ratios determined at 5–10 h of incubation are summarized.
[b] – Culture media contained 101 mM glucose, 60 mM Na-Glu and 4.15 mM $MgSO_4$ as carbon, nitrogen and sulphur sources, respectively, in the normal buffer capacity culture medium unless otherwise indicated.

Importantly, *P. chrysogenum* tolerated any kinds of oxidative stress exceptionally well owing to the very high constitutive catalase and inducible GPx activities (Table I). The intracellular GSH concentrations and the redox capacity of the GSH/GSSG system observed in the trophophase even seemed to overweigh the needs raised later by the β-lactam production [43].

In addition, thioredoxin-dependent redox enzymes, e.g. peroxidases and broad-specificity disulphide reductases, were likely to play a crucial role in both the antioxidant defence of the cells and the stabilization of the ACV-tripeptide in its reduced form especially in the case of GSH/GSSG redox imbalances [61–63].

To sum up, the only technological possibility to keep the intracellular GSH concentrations low in idiophasic *P. chrysogenum* cultures seems to be a well-controlled and transient lowering of pH at the beginning of the production phase. The GSH-dependent elimination of protonophore side-chain precursor molecules flooded into the cells is expected to diminish intracellular GSH concentrations [43].

Table IV

Penicillin yields under different culture conditions [38,42,43]

Culture conditions	Idiophasic GSH levels[a] {mmol (kg protein)$^{-1}$}	Idiophasic GSSG levels[a] {mmol (kg protein)$^{-1}$}	Penicillin yields[b] {mol (kg protein)$^{-1}$}
Increased buffer capacity culture media			
2.0% lactose, **1.0% Na-Glu**, 0.93% K_2HPO_4, 0.55% KH_2PO_4, 0.05% $MgSO_4$, pH=6.5	60–65	1.1–1.4	0.1
2.0% lactose, **1.0% Na-Glu**, 0.93% K_2HPO_4, 0.55% KH_2PO_4, 0.05 %$MgSO_4$, pH=6.5, **0.5% POA**	12	4.0	3.5
2.0% lactose, **1.0% Na-Glu**, 0.93% K_2HPO_4, 0.55% KH_2PO_4, 0.05% $MgSO_4$, pH=6.5 **0.5% POA**, +**40 mM Met** at 35 h of incubation	20–25	not determined	1.5
2.0% lactose, **1.0% Na-Glu**, 0.93% K_2HPO_4, 0.55% KH_2PO_4, 0.05% $MgSO_4$, pH=6.5, **0.7% POA** (MIC)	not detectable starting from 15 h of incubation	44 at 25 h of incubation because of serious oxidative cell injuries	not determined
Normal buffer capacity culture media			
2.0% lactose, **0.8% Na-Glu**, 0.40% K_2HPO_4, 0.20% KH_2PO_4, 0.05% $MgSO_4$, pH=7.6,	85 at 15 h of incubation	2.3 at 15 h of incubation	0.1

Table IV (continued)

Culture conditions	Idiophasic GSH levels[a] {mmol (kg protein)$^{-1}$}	Idiophasic GSSG levels[a] {mmol (kg protein)$^{-1}$}	Penicillin yields[b] {mol (kg protein)$^{-1}$}

Normal buffer capacity culture media			
2.0% lactose, **0.8% Na-Glu**, 0.40% K_2HPO_4, 0.20% KH_2PO_4, 0.05% $MgSO_4$, pH=7.6, **0.4% POA**	not detectable starting from 10 h of incubation	38 at 15 h of incubation	2.0
2.0% lactose, **0.8% Na-Glu**, 0.40% K_2HPO_4, 0.20% KH_2PO_4, 0.05% $MgSO_4$, pH=7.6, **0.1% CDNB**	not detectable starting from 10 h of incubation	41 at 15 h of incubation	not detectable
2.0% lactose, **1.5% $(NH_4)HPO_4$** 0.40% K_2HPO_4, 0.20% KH_2PO_4, 0.05% $MgSO_4$, pH=7.6,	74 at 15 h of incubation	2.1 at 15 h of incubation	not detectable
2.0% lactose, **1.5% $(NH_4)HPO_4$**, 0.40% K_2HPO_4, 0.20% KH_2PO_4, 0.05% $MgSO_4$, pH=7.6, **0.4% POA**	81 at 15 h of incubation	2.5 at 15 h of incubation	not detectable

[a] – Unless otherwise indicated, specific GSH and GSSG concentrations determined between 50 and 80 h of incubation are shown.
[b] – Highest penicillin productivity values observed at about 80 h of incubation are presented.

This proposal has been tested preliminarily in a 5 l fed-batch penicillin V fermentation system [64, 65] using $(NH_4)_2SO_4$ as a nitrogen source and introducing different degrees of acidification for 5 h at the beginning of the production phase. When the pH was decreased from 6.4 to 5.4 a decrease in the intracellular GSH levels and some increase in the penicillin V yields were recorded (Sámi, L., personal communication). Further fermentation experiments are in progress to optimize the degree of acidification and to minimize its negative physiological effects (e.g. decreasing biomass [42]) under penicillin V producing conditions.

Obviously, the increased influx of toxic side-chain precursor molecules into idiophasic cells gives a certain risk to the fermentation process, which is rather difficult to estimate and control. Therefore, the construction of low GSH producer *P. chrysogenum* strains preferably also defective in the degradation of penicillin side-chain precursors can be highly recommended as a new strategy for further *P. chrysogenum* strain improvement [66, 67].

Table V

Specific glucose-6-phosphate dehydrogenase and catalase activities and specific GSH productions in a high β-lactam producer and in a wild-type strain of Penicillium chrysogenum[a]

Strain	G6PD[b] mkat (kg protein)$^{-1}$	catalase[b] kat (kg protein)$^{-1}$	GSH[b] mmol (kg protein)$^{-1}$
NCAIM 00237	2.4±0.2[c]	5.4±0.7[c]	25±2
Wild-type	2.3±0.2[c]	4.2±0.6[c]	23±2

[a] – Culture media contained 101 mM glucose, 60 mM Na-Glu and 4.15 mM $MgSO_4$ as carbon, nitrogen and sulphur sources, respectively, in the normal buffer capacity culture medium unless otherwise indicated.
[b] – Specific enzyme activity and specific GSH production values are expressed as Mean±S.D., calculated from 4 independent experiments.
[c] – Specific G6PD and catalase activities were not inducible by 0.35 M H_2O_2 [37].

Table VI

Ranking of acidity, toxicity, GSH-dependent detoxification and penicillin productivity of PA, POA and their hydroxylated derivatives [43]

Acidity (pK_1):
2-hydroxy-POA>POA≅4-hydroxy-POA>>PA≅3-hydroxy-PA≅2,5-dihydroxy-PA>4-hydroxy-PA≅2-hydroxy-PA>3,4-dihydroxy-benzoic acid
Toxicity (MIC):
PA>POA≅2-hydroxy-PA>2-hydroxy-POA>3-hydroxy-PA≅4-hydroxy-PA>4-hydroxy-POA≅2,5-dihydroxy-PA≅3,4-dihydroxy-benzoic acid
GSH-dependent detoxification (induction of GST and γGT):
PA>POA>2-hydroxy-PA≅2-hydroxy-POA>3-hydroxy-PA>4-hydroxy-PA≅4-hydroxy-POA≅2,5-dihydroxy-PA≅3,4-dihydroxy-benzoic acid
β-Lactam production:
PA>POA>>4-hydroxy-PA≅4-hydroxy-POA>3-hydroxy-PA>2-hydroxy-PA≅2-hydroxy-POA≅2,5-dihydroxy-PA≅3,4-dihydroxy-benzoic acid

Comparison of catalase and glucose-6-phosphate dehydrogenase activities in a high β-lactam producer and in a wild-type P. chrysogenum strain

As shown previously (Table I), high specific catalase and G6PD activities were found in *P. chrysogenum* NCAIM 0023 mycelia that were insensitive to either intracellular ROS concentrations or GSH/GSSG redox imbalances. The lack of regulation of these enzyme activities by oxidative stress is very unusual among fungi [3, 68]. G6PD is a key enzyme of the pentose phosphate pathway that is one of the major NADPH suppliers of *P. chrysogenum* cells. It is wellknown that the biosynthesis of the penicillin precursor amino acids L-α-aminoadipic acid, L-cysteine and L-valine requires at least 8 NADPH [69, 70], and the stabilization of the ACV-tripeptide in its reduced form is also likely to need significant quantities of NADPH [61, 62]. Therefore, the presence or absence of any regulatory element of the pentose phosphate pathway in *P. chrysogenum* may have an effect on the β-lactam production as well. Similarly, catalase may be of crucial importance in the elimination of peroxide generated by glucose oxidase [71]. According to Nielsen [70], when *P. chrysogenum* was grown in a defined medium, the yield of gluconate was as high as 0.60 moles gluconate (and, of course, 0.60 moles H_2O_2 also formed) per mole glucose. Obviously, an effective antioxidative enzyme system is needed to copy with the burst of ROS under these conditions.

As indicated in Table V, essentially the same specific G6PD and catalase activities were detected both in the high β-lactam producer *P. chrysogenum* NCAIM 00237 strain and in a wild-type strain. This means that the high G6PD and catalase activities not inducible by oxidative stress are intrinsic features of *P. chrysogenum* and are not the consequences of strain development. Obviously, these metabolic characteristics contributed to the success of *P. chrysogenum* as an industrial micro-organism.

As far as other antioxidative enzymes are concerned, more recently Diez et al. [72] did not find any correlation between MnSOD gene expression levels and penicillin G productivity in different *P. chrysogenum* strains.

It is worth noting that the strain improvement procedures did not influence the specific intracellular GSH concentrations either in *P. chrysogenum* NCAIM 00237 (Table V).

The activation of penicillin side-chain precursors

Interestingly, there are still some uncertainties in connection with the formation of CoA-activated PA and POA derivatives in idiophasic *P. chrysogenum* mycelia [29]. There are three options to form CoA-PA and CoA-POA in *P. chrysogenum* cells

including cytosolic acetyl-CoA synthase [73], microsomal phenylacetyl-CoA ligase [29, 74] or GST through the temporary formation of GSH S-conjugates of PA and POA [34]. Although S-conjugated PA was reported to be a good substrate for the microbody-located acyl-CoA:isopenicillin N acyltransferase [34] we failed to detect any (*S*-phenoxyacetyl)glutathione intermediate in idiophasic *P. chrysogenum* cells under penicillin V production conditions [42], which makes this route rather unlikely to function *in vivo*. Further research is needed to decide if either one of or both acetyl-CoA synthase and phenylacetyl-CoA ligase are involved in penicillin biosynthesis *in vivo* [29].

Is β-lactam production a detoxification process in P. chrysogenum*? Is it an alternative to GSH-dependent detoxification?*

Unfortunately, our data presented in Table VI do not support this exiting hypothesis. The toxicity of penicillin side-chain precursors and their hydroxylated derivatives correlated well with GSH-dependent detoxification but there was no correlation with antibiotic production. As it is certainly the case for many fungal antibiotics [75], β-lactams may play an important role in the defence of territory and nutrients of some *Penicillium* species [76].

Acknowledgements: The Hungarian Ministry of Education granted a Széchenyi Scholarship for Professors to I.P., which is greatly acknowledged. These projects are supported financially by the OTKA (grant reference numbers T025174, T034315 and F019929).

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