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Penicillium chrysogenum glucose oxidase – a study on its antifungal effects

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ABSTRACT

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Aims: Purification and characterization of the high molecular mass *Candida albicans*-killing protein secreted by *Penicillium chrysogenum*.

Methods and Results: The protein was purified by a combination of ultrafiltration, chromatofocusing and gel filtration. Enzymological characteristics [relative molecular mass (M_r) = 155 000, subunit structure α_2 with $M_{r,\alpha}$ = 76 000, isoelectric point (pI) = 5·4] were determined using SDS-PAGE and 2D-electrophoresis. N-terminal amino acid sequencing and homology search demonstrated that the antifungal protein was the glucose oxidase (GOX) of the fungus. The enzyme was cytotoxic for a series of bacteria, yeasts and filamentous fungi. Vitamin C (1·0 mg ml^{-1}) prevented oxidative cell injuries triggered by 0·004 U GOX in *Emericella nidulans* cultures but bovine liver catalase was ineffective even at a GOX : catalase activity ratio of 0·004 : 200 U. A secondary inhibition of growth in *E. nidulans* cultures by the oxygen-depleting GOX–catalase system was likely to replace the primary inhibition exerted by H_2O_2.

Conclusions: *Penicillium chrysogenum* GOX possesses similar enzymological features to those described earlier for other *Penicillium* GOXs. Its cytotoxicity was dependent on the inherent antioxidant potential of the test micro-organisms.

Significance and Impact of the Study: *Penicillium chrysogenum* GOX may find future applications in glucose biosensor production, the disinfection of medical implants or in the food industry as an antimicrobial and/or preservative agent.

Keywords: antifungal effect, *Candida albicans*, catalase, *Emericella nidulans*, glucose oxidase, oxidative stress, *Penicillium chrysogenum*, vitamin C.

INTRODUCTION

New approaches for treatment of invasive fungal infections are necessary because the incidence of such infections rose steadily from the 1970s (Ghannoum and Rice 1999). Moreover, new pathogenic species possessing intrinsic primary resistance and common pathogens rapidly developing secondary resistance to recently used antifungal agents are spreading widely and fast (White *et al.* 1998). The rise of AIDS-related mycoses and the spread of immunosuppressive therapies (Odds *et al.* 2003) also stimulate the need for novel antifungal agents. Present treatment options for systemic fungal infections are limited to only three classes of compounds, the polyenes, the azoles and the antifungal lipopeptides (Chen and Rodriguez 2003). Therefore, either the chemical modification of currently used drugs or the development of novel compounds together with the search for new molecular targets are of primary clinical interest

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(Rast *et al.* 2000; Haidaris 2003; Vincente *et al.* 2003). Novel discovery strategies including more effective screening of microbial natural products may lead to the development of new-type of antifungal drugs (Knight *et al.* 2003; Vincente *et al.* 2003). For example, screening of antifungal activities secreted by filamentous ascomycetes has led to the discovery of small molecular mass ($M_r \cong 6000$) cysteine-rich antifungal proteins, which are active against a broad range of filamentous fungi and occasionally against yeasts (Marx 2004). These proteins are considered as promising objects in future antifungal drug research and their mechanism of action is studied intensively (Kaiserer *et al.* 2003; Oberparleiter *et al.* 2003; Theis *et al.* 2003). In this study we demonstrate that a *Penicillium chrysogenum* strain, which is a good producer of both β-lactam and basic antifungal protein (PAF; Kaiserer *et al.* 2003), also secreted considerable *Candida*-killing activity in the exponential phase of growth. This activity was fully attributable to a high molecular mass protein, which was identified later as glucose oxidase (GOX). The antifungal effect of the *P. chrysogenum* GOX–bovine liver catalase system is also demonstrated and discussed in this paper.

MATERIALS AND METHODS

Growth conditions for protein production

Penicillium chrysogenum NCAIM 00237 (National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary) was maintained in Czapek-Dox medium on a rice surface and was grown in a defined medium consisting of 3 g l^{-1} $NaNO_3$, 0·5 g l^{-1} KCl, 0·5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0·05 g l^{-1} $FeSO_4 \cdot 7\ H_2O$ and 20 g l^{-1} sucrose dissolved in 25 mmol l^{-1} phosphate buffer (pH 5·8) (Marx *et al.* 1995; Pusztahelyi *et al.* 1997a,b). Culture flasks (500 ml) containing 100 ml of medium were inoculated with 10^5 spores and were incubated for 144 h and at 25°C with shaking (4·2 Hz). Samples were taken every 24 h between 1 and 6 days of incubation (Pusztahelyi *et al.* 1997a), and both dry cell weights (DCMs; Pusztahelyi *et al.* 1997a) and antifungal activity of the culture supernatant against 6 h exponential growth phase *Candida albicans* ATCC 1023 cells were determined.

Purification of *P. chrysogenum* antifungal protein

Mycelia from 800 ml of a 96 h culture were separated by filtration on sintered glass and the filtrate (protein content 110 mg) was concentrated on an Amicon Stirred Cell Model 8050 device supplied with Amicon PM30 ultrafiltration discs (Millipore Corporation, Bedford, MA, USA). The concentrated culture medium ($M_r > 30\,000$ protein fraction, $V = 40$ ml, protein content 31 mg) was dialysed against 25 mmol l^{-1} imidazole-HCl, pH 7·3 buffer and was loaded onto a PBE 94 chromatofocusing column (Pharmacia Biotech, Uppsala, Sweden; 1 × 18 cm, flow rate 30 ml h^{-1}, equilibrated with 25 mmol l^{-1} imidazole-HCl, pH 7·3 buffer). The pH gradient was developed by Polybuffer 74, pH 4·0, and the pH of the effluent was monitored as described elsewhere (Pócsi and Kiss 1988). Fractions (2·0 ml each) showing at least 40% growth inhibition on exponential phase *C. albicans* ATCC 1023 cultures were pooled (14 ml, protein content 4·3 mg) and concentrated on Amicon Ultrafree-CL Centrifugal Filter Units (Millipore Corporation).

The concentrated enzyme preparation from the chromatofocusing step ($V = 1·0$ ml) was applied to a Superdex 200 FPLC gel filtration column (Pharmacia Biotech; $V = 24$ ml, flow rate 60 ml h^{-1}), which was eluted with 0·2 mol l^{-1} NaCl solution prepared in 50 mmol l^{-1} sodium phosphate buffer, pH 7·0. One millilitre fractions with at least 40% inhibitory activity against *C. albicans* ATCC 1023 cultures were pooled and used in further enzymological and antimicrobial activity studies. The protein concentration of the final preparation ($V = 6·0$ ml) was 11 μg ml^{-1}.

Biochemical and enzymological characterization of the antifungal protein

Relative molecular mass (M_r) of *P. chrysogenum* antifungal protein was determined by SDS-PAGE on precast Novex 10% Tris–glycine gels using Mark12 M_r standards ($M_r = 2500$–$200\,000$) according to the instructions of the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA). Protein bands were visualized with Coomassie Brilliant Blue R (Pharmacia Biotech), and the glycoprotein character of *P. chrysogenum* antifungal protein was demonstrated by the periodic acid–Schiff method (Dewald *et al.* 1974).

Isoelectric point (pI) was determined by 2D electrophoresis, where the first run was carried out on precast Novex pH 3–7 IEF gels followed by SDS-PAGE on precast Novex 16% Tris–glycine gels as recommended by Invitrogen Life Technologies.

N-terminal protein sequencing by Edman degradation was performed from protein blotted onto polyvinylidene difluoride (PVDF) membrane as described earlier (Bogáti *et al.* 1996; Lindner *et al.* 1998). Homology search for short, nearly exact matches was carried out using NCBI protein–protein Blast (http://www.ncbi.nlm.nih.gov/BLAST), and multiple sequence alignment was generated by Florence Corpet's MultAlin software (http://prodes.toulouse.inra.fr/multalin/multalin.html) (Corpet 1988).

Penicillium chrysogenum GOX activities were measured by a modification of the D-glucose rate assay of Leary *et al.* (1992). The enzyme reactions were always performed in 0·1 mol l^{-1} Na_2HPO_4–KH_2PO_4, pH 6·6 buffer at 25°C. In standard GOX activity measurements, the D-glucose concentration was kept at constant value (50 mmol l^{-1}),

while it was varied in the range of 3·44–220 mmol l^{-1} (0·36–23·28 × K_m) in kinetic experiments. V_{max} and K_m parameters were calculated with the GraFit, Version 2·10 software (Erithacus Software Ltd, Horley, UK) (Emri *et al.* 2003). For D-xylose, a concentration range of 300–1000 mmol l^{-1} (0·44–1·45 × K_m) was selected. Michaelis–Menten type substrate saturation curves were fitted to means of initial reaction velocities calculated from four independent experiments.

Antimicrobial activity and mechanism of action studies

The antimicrobial activity of *P. chrysogenum* GOX was tested on the following fungal species: *C. albicans* ATCC 1023, *C. dubliniensis* NCPF 3949, *C. glabrata* DSM 6425, *C. krusei* DSM 6128, *C. parapsilosis* ATCC 22019, *Saccharomyces cerevisiae* AH 109 (Clontech, Palo Alto, CA, USA), *Aspergillus giganteus* AG090701 (Strain Collection of the Department of Molecular Biology, University of Innsbruck, Innsbruck, Austria), *A. niger* CBS 120.49, *A. terreus* 304 (Strain Collection of the Department of Molecular Biology, University of Innsbruck), *Emericella nidulans* FGSC26, *P. chrysogenum* Q176 ATCC 10002, *P. chrysogenum* NCAIM 00237. Bacteria including *Bacillus subtilis* ATCC 6633, *Escherichia coli* TG2 (Sambrook *et al.* 1989), *Serratia marcescens* ATCC 14756 and *Staphylococcus aureus* NCTC 6571 were also included in this study.

Test micro-organisms were cultivated in 100 μl YPD medium (20 g l^{-1} peptone, 10 g l^{-1} yeast extract and 20 g l^{-1} glucose) in 96-microtitre plates according to Lee *et al.* (1999). To each exponential growth phase microculture, 20 μl aliquots of *P. chrysogenum* GOX were added in a final concentration of 1·34 μg ml^{-1} (enzyme activity 0·09 U). After 5 h of incubation at optimal temperature, antimicrobial activity was determined by adding 10 μl aliquots of 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) solution (5·0 mg ml^{-1} MTT dissolved in phosphate-buffered saline, pH 7·4) to each well. The plates were incubated at 37°C for another 4-h period before the formazan crystals were solubilized by the addition of 30 μl of 20% (w/v) SDS solution prepared in 20 mmol l^{-1} HCl. After further incubation at 37°C for 16 h, the turbidity in each well was read at 570 nm on a BIO-TEK EL 340 Microplate Bio Kinetics Reader (Winooski, VT, USA).

Exponential growth phase 6 h *C. albicans* ATCC 1023 microcultures were always used to track antifungal activity in the purification steps of *P. chrysogenum* GOX. However, we used 14-h exponential growth phase *E. nidulans* FGSC26 microcultures to study the mechanism of action of *P. chrysogenum* GOX.

To characterize the mechanism of action, *P. chrysogenum* GOX and *A. niger* GOX (Sigma G-7141) were used within the range of 0–1·0 U and, in selected experiments, *E. nidulans* FGSC26 mycelia were treated with H_2O_2 (0–150 mmol l^{-1}). Microcultures were also supplemented with bovine liver catalase (0–200 U; Sigma C-40) or vitamin C (1 mg ml^{-1}) as required. When catalase was included in the reaction mixtures its activity was checked after 9 h of incubation according to Emri *et al.* (1999) before the MTT reaction was completed by the addition of SDS–HCl.

By definition, 1 U GOX activity converts 1 μmol β-D-glucose to D-glucono-δ-lactone and H_2O_2 in 1 min at 25°C and pH 6·6, and 1 U catalase activity decomposes 1 μmol H_2O_2 in 1 min at 25°C and pH 7·0.

Steady-state H_2O_2 concentrations in GOX–catalase systems

For enzyme catalysed consecutive coupled reactions eqn (1), the steady-state concentration of S_2 can be calculated under the premises $[S_1] \gg K_{m,1}$ and $[S_2] \ll K_{m,2}$ (Keleti 1985).

$$S_1 \xrightarrow{E_1} S_2 \xrightarrow{E_2} P \qquad (1)$$

Assuming that both enzymes obey Michaelis–Menten kinetics, the changes in $[S_2]$ are influenced solely by the formation and conversion reaction rates as shown in eqn (2), where $E_{t,1}$ and $E_{t,2}$ stand for the total concentrations of E_1 and E_2, $k_{cat,1}$ and $k_{cat,2}$ represent the first order reaction rate constants of the enzyme-catalysed reactions, and $K_{m,2}$ is the Michaelis constant of E_2 (Keleti 1985).

$$d[S_2]/dt = k_{cat,1} * E_{t,1} - \frac{k_{cat,2} * E_{t,2}}{K_{m,2}} * [S_2] \qquad (2)$$

Under steady-state conditions, $d[S_2]/dt = 0$ and, hence, $[S_2] = [S_2]_{SS}$, the steady-state concentration of S_2, as shown by eqn (3).

$$[S_2]_{SS} = \frac{k_{cat,1} * E_{t,1}}{k_{cat,2} * E_{t,2}} * K_{m,2} \qquad (3)$$

Determination of protein content and dry cell mass values

Protein was determined by the method of Bradford (1976) using BSA as standard. In chromatographic fractions measuring the UV absorption at 280 nm monitored the protein content. DCM values were estimated according to Pócsi *et al.* (1993).

Statistical analysis

Presented experimental data are means of three to four independent experiments. The variations between experiments

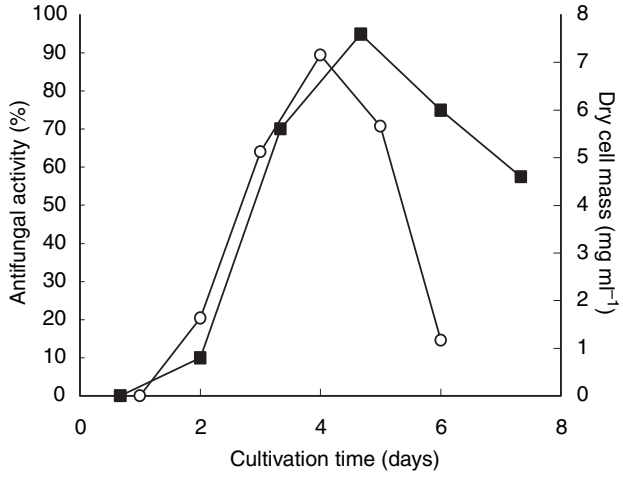

Fig. 1 Growth of *P. chrysogenum* (■) and *Candida*-killing effect of undiluted culture supernatants (○). Equal protein amounts (2·8 μg) were transferred to each well on a microtitre plate

were estimated by S.D. values for each procedure (Emri *et al.* 1997,1999,2003).

Chemicals

Unless otherwise indicated, all the chemicals were purchased from Sigma-Aldrich Ltd, Budapest, Hungary.

RESULTS

Exponential growth phase *P. chrysogenum* NCAIM 00237 mycelia produced considerable *Candida*-killing activity when 20 g l^{-1} sucrose and 3 g l^{-1} $NaNO_3$ were used as carbon and nitrogen sources, respectively (Fig. 1). This antifungal activity reached its maximum at 96 h cultivation time just before the onset of the stationary phase of growth. Later, activity decreased concomitantly with the progressing autolytic loss of biomass (Fig. 1).

A purification scheme including ultrafiltration, chromatofocusing and gel-filtration steps (elution profiles are not shown) provided a high-purity protein preparation (Fig. 2a). As low as 0·17 μg quantities of antifungal protein per well prevented the growth of *C. albicans* ATCC 1023 in microplate test cultures. Although a faint band of a contaminant protein appeared on SDS-PAGE at $M_r = 97\,400$ (Fig. 2a), its quantity never exceeded 3% of the total protein content, as calculated with QuantiScan version 1.2 software (Biosoft, Cambridge, UK).

The purified antifungal protein had a native relative molecular mass of $M_r = 155\,000$ as determined by gel filtration, and possessed an α_2 subunit structure with $M_{r,\alpha} = 76\,000$ (Fig. 2a). Both chromatofocusing and 2D electrophoresis (Fig. 2b) gave a pI value of 5·4. Periodic

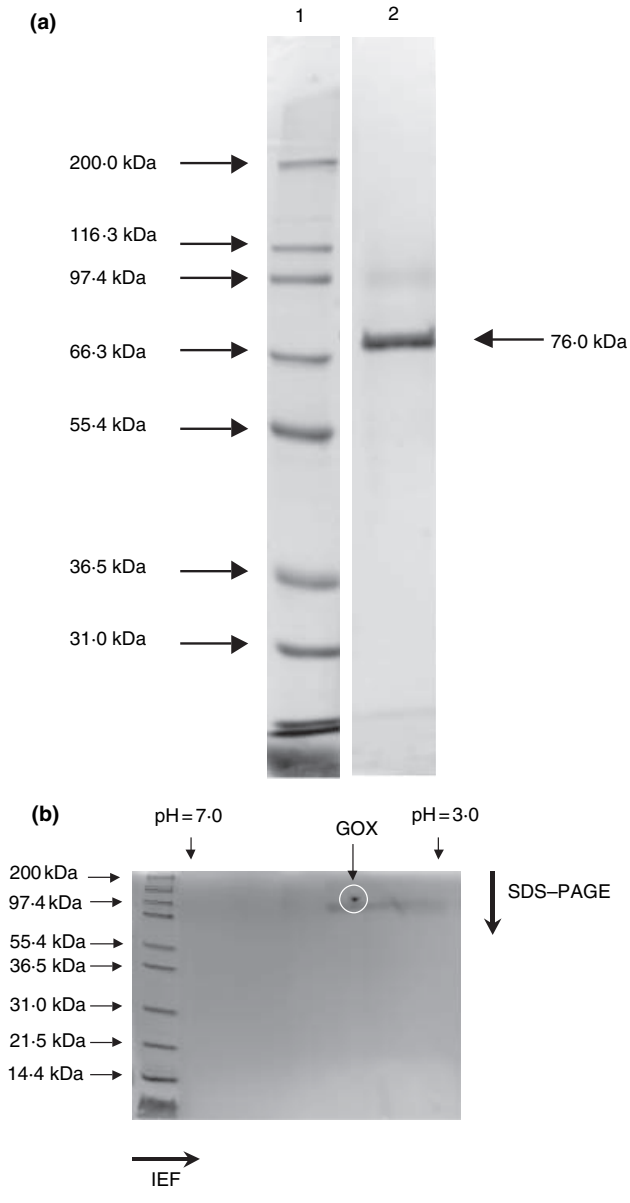

Fig. 2 Relative molecular mass (a and b) and isoelectric point (b) of *P. chrysogenum* GOX. (a) Lane 1 shows Coomassie-stained Mark 12 Standards; in lane 2, 5·0 μg purified GOX was loaded onto a precast Novex Tris–glycine gel (lane 2). (b) SDS-PAGE of 8·5 μg GOX on precast Novex 16% Tris–glycine gel. Note that GOX was prerun on precast Novex pH 3–7 IEF gel in this case

acid-Schiff staining of SDS-PAGE gels clearly demonstrated the glycoprotein character of the purified antifungal protein (data not shown).

Fourteen N-terminal amino acids were determined by Edman degradation, and a homology search using NCBI protein–protein Blast indicated close homology to *P. amagasakiense* and *Talaromyces flavus* GOXs (78·6%

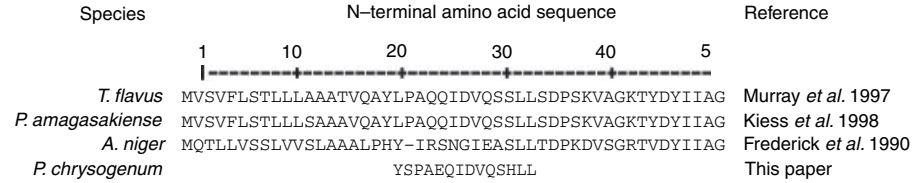

Fig. 3 Multiple sequence alignment of the N-terminal amino acids of *P. chrysogenum* GOX. The protein homology between *P. chrysogenum* GOX and that of the closely related *P. amagasakiense* was 78·6%

Table 1 Analysis of sensitivity of selected micro-organisms to *P. chrysogenum* GOX*

Micro-organisms	Sensitivity
Bacillus subtilis ATCC 6633	+++
Escherichia coli TG2	+++
Serratia marcescens ATCC 14756	+++
Staphylococcus aureus NCTC 6571	+++
Candida albicans ATCC 1023	+
Candida dubliniensis NCPF 3949	+
Candida glabrata DSM 6425	+++
Candida krusei DSM 6128	+++
Candida parapsilosis ATCC 22019	++
Saccharomyces cerevisiae AH 109	+++
Aspergillus giganteus AG090701	+
Aspergillus niger CBS 120·49	++
Aspergillus terreus 304	++
Emericella nidulans FGSC26	+++
Penicillium chrysogenum Q176 ATCC 10002	+++
Penicillium chrysogenum NCAIM 00237	+++

*The final concentration of GOX was 1·34 μg ml^{-1} (enzyme activity 0·090 U).
+++, highly sensitive, growth retardation >90%; ++, sensitive, growth retardation >50%; +, moderately sensitive, growth retardation >25%.

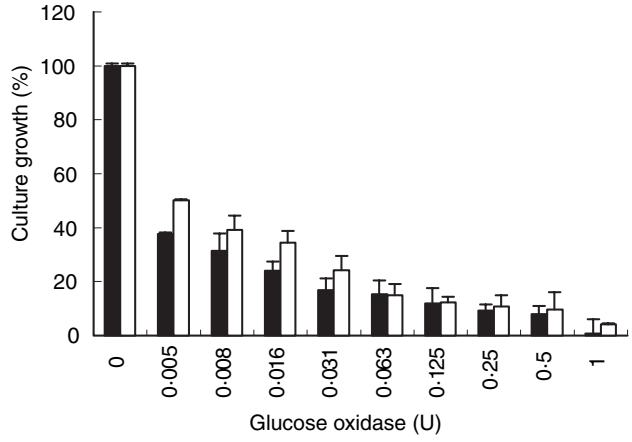

Fig. 4 Comparison of the antifungal effects of GOXs produced by *A. niger* (black columns) and *P. chrysogenum* (white columns) on 14 h exponential growth phase *E. nidulans* cultures. *E. nidulans* hyphae were treated with different GOX activities within the range of 0–1 U. Columns represent mean values calculated from three independent experiments; bars indicate S.D. values

sequence identity; Fig. 3). The purified *P. chrysogenum* antifungal protein indeed possessed high GOX activity.

Penicillium chrysogenum GOX oxidized D-glucose very effectively with kinetic parameters $K_{m,glucose} = 9{\cdot}5$ mmol l^{-1} and $V_{max,glucose} = 10{\cdot}38$ kat kg^{-1}. The enzyme also catalysed the reaction with D-xylose but at a much lower rate ($K_{m,xylose} = 690$ mmol l^{-1}, $V_{max,xylose} = 0{\cdot}777$ kat kg^{-1}). *Penicillium chrysogenum* GOX did not show any activities against D-fructose, D-galactose and D-arabinose. In the presence of D-glucose, all tested bacteria, yeasts and filamentous fungi were sensitive to *P. chrysogenum* GOX (Table 1). Surprisingly, the sensitivity varied in a broad range including highly sensitive bacteria and yeasts like *C. glabrata* and *C. krusei* and moderately sensitive fungi, e.g *C. albicans*, *C. dubliniensis* and *A. giganteus* (Table 1). It is worth noting that *P. chrysogenum* NCAIM 00237 itself was very sensitive to its own GOX.

In studies addressing the mechanism of action the highly GOX sensitive *E. nidulans* FGSC26 was used as test organism. As shown in Fig. 4, the antifungal effects of both *P. chrysogenum* and *A. niger* GOXs were dose-dependent. As little as 0·005 U GOX activity inhibited the growth of *E. nidulans* effectively, and 1 U GOX activity completely blocked the fungal growth.

Unexpectedly, bovine liver catalase was not able to neutralize the deleterious growth inhibitory effect of either *P. chrysogenum* or *A. niger* GOXs on *E. nidulans* cultures even at a GOX : catalase activity ratio of 0·004 : 200 U (Fig. 5). Importantly, catalase activity declined very slowly in the reaction mixtures tested: 50–60% of the starting activity was still detectable after 9 h of incubation together with GOXs. In contrast, oxidative cell injuries triggered by 0·004 U GOX activity could be prevented by 1·0 mg ml^{-1} vitamin C (Fig. 5). Similar to catalase, vitamin C was ineffective at high GOX activity (1·0 U).

The cytotoxicity of H_2O_2 was tested in a separate experiment (Fig. 6). The growth retardation caused by H_2O_2 treatment was dose-dependent and reached 100% in the presence of 50 mmol l^{-1} H_2O_2. Unlike with GOXs, bovine liver catalase was highly protective against H_2O_2 induced cell damages even at low activities, such as 4 U (Fig. 6).

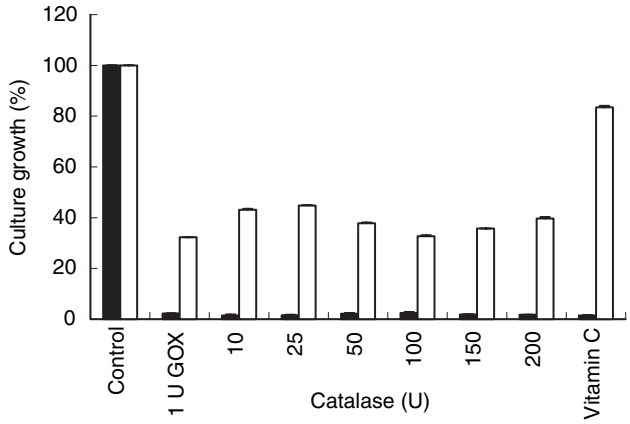

Fig. 5 Protective effect of bovine liver catalase (0–200 U) or vitamin C (1·0 mg ml^{-1}) on *E. nidulans* cultures, which were treated with either 1 U (black columns) or 0·004 U (white columns) *P. chrysogenum* GOX. Fungal cultures neither treated with GOX nor with catalase were used as controls. Columns represent means calculated from three independent experiments; bars indicate S.D. values. The same growth inhibition and cytoprotection patterns were observed with 1 and 0·004 U *A. niger* GOX and bovine liver catalase or vitamin C (data not shown)

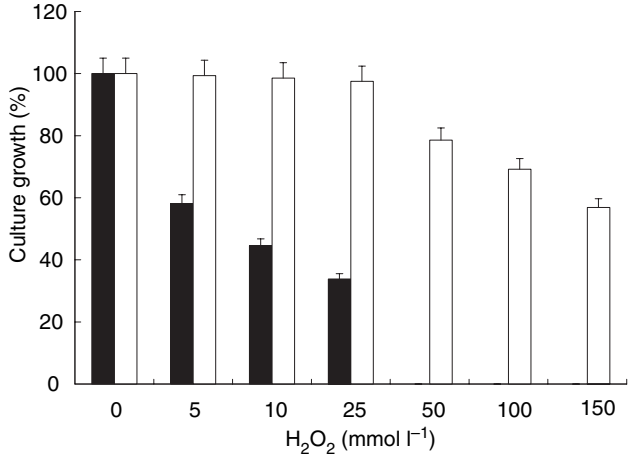

Fig. 6 The antifungal effect of 0–150 mmol l^{-1} H_2O_2 on *E. nidulans* hyphae (black columns). The white columns demonstrate the protective effect of 4 U bovine liver catalase against H_2O_2. Columns represent means calculated from three independent experiments; bars indicate S.D. values

Theoretical calculations (eqn 3) showed that the steady-state H_2O_2 concentration was low, approximately 1·48 μmol l^{-1} even in the presence of 1 U *P. chrysogenum* GOX and 10 U bovine liver catalase activities ($k_{cat,GOX}$ = 1609 s^{-1}, $k_{cat,catalase} = 8{\cdot}0 \times 10^5$ s^{-1}, $E_{t,GOX} = 9{\cdot}9 \times 10^{-9}$ mol l^{-1}, $E_{t,catalase} = 3{\cdot}36 \times 10^{-7}$ mol l^{-1}, $K_{m,catalase}$ = 25 mmol l^{-1}). $k_{cat,GOX}$ was calculated using V_{max} and M_r values shown above (Keleti 1985) while the kinetic parameters for catalase were taken from Abe *et al.* (1979). At lower GOX and higher catalase concentrations, $[H_2O_2]_{ss}$ (steady-state H_2O_2 concentration) decreased steeply (eqn 3).

DISCUSSION

The high β-lactam producer *P. chrysogenum* NCAIM 00237 was cultured in a medium containing sucrose and sodium nitrate as appropriate carbon and nitrogen sources. A high molecular mass (M_r = 155 000) protein with considerable *C. albicans*-killing activity was produced that also prevented the growth of bacteria, *Candida* and non-*Candida* yeasts as well as filamentous fungi. Under the same culture conditions, the small antifungal protein PAF (M_r = 6500) that has been characterized first from *P. chrysogenum* Q176 (Marx *et al.* 1995) was also secreted but this protein was reported to be effective only against filamentous ascomycetes (Kaiserer *et al.* 2003). N-terminal sequencing of the purified protein, homology search (protein–protein Blast) and subsequent enzymological studies clearly demonstrated that *P. chrysogenum* GOX was solely responsible for the observed antimicrobial activity. In accordance with previous observations, sucrose-nitrate culture media with slightly acidic pH (5·5–6·5) were advantageous for GOX production (Mischak *et al.* 1985; Rando *et al.* 1997).

Penicillium chrysogenum GOX was first purified by Eriksson *et al.* (1987), but the published enzymological characteristics (M_r = 175 000, $M_{r,\alpha}$ = 72 000, pI = 4·2) were quite different from those shown in this paper (M_r = 155 000, $M_{r,\alpha}$ = 76 000, pI = 5·4), which might be attributed to methodological differences. The enzyme kinetic properties of *P. chrysogenum* GOX presented in this work (K_m, V_{max}, k_{cat}) were highly comparable with those found in other *Penicillium* species (Garzillo *et al.* 1995; Witt *et al.* 1998).

Although the wide-ranging antibacterial activity of GOX produced by *P. notatum* (penatin or notatin) was reported more than 60 years ago (Coulthard *et al.* 1942; Kocholaty 1942,1943) no detailed study on the antifungal activity of *Penicillium* GOX has been performed thus far, to the best of our knowledge.

All bacterial and fungal strains tested in this study were sensitive to the action of *P. chrysogenum* GOX including *P. chrysogenum* NCAIM 00237, the GOX producer strain itself (Table 1). In glucose-fed exponential growth phase *P. chrysogenum* cultures, high intracellular reactive oxygen species (ROS), e.g. peroxide and superoxide, and increased antioxidative enzyme activities were recorded (Sámi *et al.* 2001), which was likely the consequence of the oxidative stress generated by the H_2O_2 formation by GOX (Sámi *et al.* 2003). The exceptionally high oxidative stress

tolerance of glucose-grown exponential growth phase *P. chrysogenum* mycelia (Emri *et al.* 1997,1999) can be explained by a permanent H_2O_2 stress and a consequent and continuous induction of the anti-oxidative defence system (Sámi *et al.* 2003). Nevertheless, the addition of surplus endogenous GOX may disturb the sensitive balance between ROS generation and elimination.

The difference between the GOX tolerance of the fungus species tested, e.g. within the genus *Candida*, reflects inherent differences between the oxidative stress tolerance of these fungi. Resistance against myeloperoxidase-derived oxidants has been considered as a possible virulence factor associated with this opportunistic human pathogen (Maródi *et al.* 1991). The variation in GOX sensitivity of fungi, which have been observed by other authors as well (Popper and Knorr 1997; Cailliez-Grimal *et al.* 2002), might play an important role in fungus–fungus interactions and might therefore contribute to an ecological advantage for selected organisms in the competition for nutrients. In this respect, the antagonism of the GOX and chitinase-producing organism *T. flavus* against the GOX-sensitive mould *Verticillium dahliae* and the chitinase-sensitive fungus *Sclerotium rolfsii* has been reported (Madi *et al.* 1997).

The antimicrobial effects of GOXs are primarily based on the liberation of H_2O_2, which is a harmful oxidative stress-generating agent. In fact, the supplementation of test *E. nidulans* microcultures with 1·0 mg ml^{-1} vitamin C satisfactorily prevented oxidative cell injuries in the presence of 0·004 U of either *P. chrysogenum* or *A. niger* GOXs. Interestingly, bovine liver catalase was ineffective to eliminate the deleterious effects of GOXs under the same experimental conditions, although the protective effect of catalase against fungal GOXs has been reported in several cases (Kim *et al.* 1988; Geisen 1999). In fact, catalase was clearly protective against endogenous H_2O_2, and the steady-state H_2O_2 concentrations calculated for the used GOX–catalase coupled systems were almost negligible within the μmol l^{-1}–nmol l^{-1} range. Most likely, the low oxygen tensions controlled by the oxygen-consuming GOX–catalase systems resulted in a secondary inhibition of growth in the *E. nidulans* microcultures replacing the primary inhibition exerted by H_2O_2. In accordance with this observation, *Aspergillus* species were capable to grow at low oxygen tensions but no growth was recorded below 0·025% oxygen concentration (Hall and Denning 1994). Moreover, GOX–catalase systems used in the food industry have been reported to affect aerobic micro-organisms by depleting available oxygen (Dondero *et al.* 1993; Fuglsang *et al.* 1995).

Despite the abundant availability of commercial GOX, search for novel enzymes with more advantageous enzymological properties is still going on (Rando *et al.* 1997). As a result, new GOXs have been found and characterized in both the Kingdoms of Fungi (Hatzinikolaou *et al.* 1996) and Animals (Eichenseer *et al.* 1999; Ohashi *et al.* 1999). Although its medical application for the treatment of human mycoses is unlikely because of its ROS-generating properties, *P. chrysogenum* GOX may be used in the medicine as a component of electrochemical biosensors developed for the quantitative determination of glucose in the body fluids (Jaffari and Turner 1995; Liang *et al.* 2000). The antimicrobial activity of the enzyme may be exploited in the enzymatic removal and disinfection of bacterial and fungal biofilms from medical indwelling devices (Johansen *et al.* 1997). The sanitizing potential of GOXs could be utilized most effectively in two-component enzyme systems with lactoperoxidases (Popper and Knorr 1997) or fungal haloperoxidases (Hansen *et al.* 2003). Moreover, *P. chrysogenum* GOX alone or in combination with catalases may also gain a profitable application in the food industry as an antimicrobial and/or preservative agent (Tiina and Sandholm 1989; Dobbenie *et al.* 1995; Fuglsang *et al.* 1995).

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