Purification and Some Properties of *Candida albicans* Exo-1,3- β -glucanase

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An exo-1,3- β -glucanase was purified from blastoconidia of *Candida albicans* 1001. The purified enzyme appeared as a single protein band by PAGE, and split into two subunits (M_r approximately 63 000 and 44 000) when analysed by SDS-PAGE. The pI of the enzyme was 4 and a K_m of 1.7 mg ml⁻¹ was estimated for laminarin as substrate. Despite its very reduced activity on the synthetic substrate *p*-nitrophenyl β -D-glucoside, *C. albicans* exo-1,3- β -glucanase hydrolysed 1,3- β -glucan by an exo-splitting mechanism and was inhibited by glucono- δ -lactone and by Hg²⁺ and Ag⁺ cations. The active exo-glucanase was mainly located in the periplasm, but it was also present inside the cytoplasmic membrane in small amounts and was secreted into the culture medium. The electrophoretic mobility of the enzyme from all three locations was the same.

INTRODUCTION

Candida albicans is a dimorphic yeast of medical importance. The basis of dimorphism in this species and its potential relationship to pathogenicity (Odds, 1985) are two major aspects of interest in research on *C. albicans*. Glucan, with either 1,3- β - or 1,6- β -linkages, represents the main structural component of the cell wall of *C. albicans* (Sullivan *et al.*, 1983; Gopal *et al.*, 1984) and this suggests an important role for glucanases as autolysins during growth and morphogenesis of this organism.

Although all fungal species that contain glucan in their cell walls apparently produce glucanases, there is a striking diversity in the different glucanase systems that have so far been characterized. Variations in the number of enzymes and the mode of action and properties of these proteins in different fungi make it difficult to present a unifying picture of the system. Therefore, a detailed characterization of the relevant enzymes is required to understand the biological role of glucanases as autolysins in each particular organism.

We have reported (Molina *et al.*, 1987) that the bulk of glucanase activity produced by *C. albicans* consists of an exo-1,3- β -glucanase that, unlike most exo-hydrolysing enzymes, has an almost negligible activity on the synthetic substrate *p*-nitrophenyl β -D-glucoside (pNPG). In this communication we present the subunit structure of this enzyme and its most relevant properties.

METHODS

Organism, media and growth conditions. *C. albicans* 1001 from the Spanish Type Culture Collection (recently deposited as ATCC 64385) was used throughout this study. Yeast cells were grown in YED medium (containing 15 g Difco yeast extract and 30 g glucose per litre), by shaking at 28 °C in an orbital incubator (Gallenkamp). Cells were usually harvested at the late exponential phase of growth, after approximately 15 h incubation.

Enzyme preparations. The preparation was obtained from 10 l of culture (containing approximately 2×10^8 cells ml⁻¹). The cells were suspended in 0.01 M-sodium succinate, pH 5.3, and mechanically broken to obtain 2469 ml of

Abbreviations: MUG, 4-methylumbelliferyl β -D-glucoside; pNPG, *p*-nitrophenyl β -D-glucoside.

cell-free extract; supernatant fluid (40000 g) from this crude extract was prepared essentially as described previously (Molina *et al.*, 1987), with the exception that dialysis was done before, and not after, centrifugation of the extracts. These preparations were stored frozen at -20°C . To precipitate the enzyme, solid ammonium sulphate (Merck) was added to 60% saturation, and the solution maintained for 1 h at 4°C . Precipitated proteins were pelleted by centrifugation (30000 g), dissolved in a small amount of 0.01 M-sodium succinate, pH 5.3, and dialysed overnight against the same buffer.

DEAE-Biogel A (Bio-Rad) chromatography was done in a column (3.2×45 cm) equilibrated with the same buffer; retained proteins were eluted with a linear NaCl gradient (0 to 0.3 M) in the same buffer at a flow rate of 12 ml h^{-1} , 2.5 ml fractions being collected.

Sephadex G-75 gel filtration was done on a column (2.5×87.5 cm) previously equilibrated with 0.025 M-sodium succinate, pH 5.3. Before gel filtration, the enzyme-containing fraction from ion exchange chromatography was concentrated by dialysis against polyethylene glycol 20000 (at 4°C) and then dialysed against 0.025 M-sodium succinate, pH 5.3, before applying it to the column. Elution was with the same buffer at a flow rate of 2 ml h^{-1} , 1 ml fractions being collected. In all cases, the protein contents of column eluates were estimated from absorbance measured with an ultraviolet monitoring system (ISCO UA5 model).

Gel electrophoresis. Analytical PAGE under non-dissociating conditions was done in 10% (w/v) polyacrylamide gels at pH 8.9 according to the method described by Ornstein & Davis (1964). Enzyme activities were detected on the gels either by overlaying them with the fluorogenic substrate 4-methylumbelliferyl β -D-glucoside (MUG) (0.2% in 0.01 M-sodium succinate, pH 5.3, with 1% agar) or by cutting the gel into 1 mm slices, and checking activity on laminarin or pNPG. Hydrolysis of MUG was observed, after 30 to 60 min at 37°C , by UV illumination.

Analytical PAGE was also done under dissociating conditions with the same type of gels but in the presence of SDS and β -mercaptoethanol as described by Laemmli (1970). In all cases, protein bands were visualized by staining either with Coomassie brilliant blue or with silver nitrate (Merrill *et al.*, 1982).

Preparative gel electrophoresis was done in an Elfe TM electrophoresis unit (Genofit). Samples were applied to a 10% (w/v) polyacrylamide column and run for 6 h at 120 V and 4°C , 0.3 ml fractions being collected.

Isoelectric focusing. Analytical electrofocusing of the purified enzyme was done in an LKB-focusing system. The gradient was formed in a 7.5% polyacrylamide gel of 0.2 mm thickness, using ampholine carrier ampholytes for two different pH ranges, a wider one (3.5 to 9.5) and a narrower one (2.5 to 5). Focusing was done for 70 min and 120 min respectively, at 1120 V. Protein bands were stained with silver nitrate and enzyme activity detected with MUG, as described above.

Other determinations. Assays of 1,3- β -glucanase activity against laminarin and of β -glucosidase based on the hydrolysis of pNPG were as described previously (Molina *et al.*, 1987); 1 unit of activity (U) was defined as the amount of enzyme which released 1 nmol of the measured reaction product min^{-1} under the conditions of the reaction. The products generated by laminarin hydrolysis were identified by TLC using D-glucose as a standard.

Chemicals. Laminarin, pNPG and MUG were purchased from Sigma, Sephadex G-75 from Pharmacia, DEAE-Biogel, acrylamide and bis-acrylamide from Bio-Rad and gluculase from Dupont. All other reagents were of analytical grade from commercial sources.

RESULTS

In order to analyse the biochemical characteristics of *C. albicans* exo-1,3- β -glucanase, the enzyme was purified from supernatants of cell-free extracts (Table 1). A highly purified preparation was obtained after the gel filtration step, so that a single protein band was observed when the preparation was analysed by non-dissociating PAGE and stained with Coomassie blue (data not shown). However, similar gels stained by using the more sensitive silver nitrate method revealed the presence of some impurities (Fig. 1a). The active exo-1,3- β -glucanase was identified as the most prominent band in this preparation by its ability to hydrolyse laminarin assayed after slicing the corresponding part of the gel (Fig. 1d). Although the action of this glucanase on synthetic glucosides is very limited (Molina *et al.*, 1987), the MUG hydrolysis assay was sensitive enough to confirm this protein band as the active glucanase (Fig. 1c).

The enzyme was further purified by preparative gel electrophoresis to yield a preparation showing a single protein band in non-dissociating gels stained with silver nitrate (Fig. 1b). The pure enzyme split into two protein bands when analysed by SDS-PAGE (Fig. 2) indicating that the active protein consists of two subunits. The M_r of the subunits was estimated in SDS-PAGE, by comparison with appropriate standards, to be 63000 and 44000 respectively, and given the apparent 1:1 stoichiometry of the two subunits the M_r of the intact enzyme must be 107000 daltons or a multiple of it. It is not possible to estimate the M_r of the native enzyme from gel

Fig. 1

Fig. 2

Fig. 3

Fig. 1. PAGE of purified *exo*-1,3- β -glucanase (after the gel filtration step: see Table 1) (a) stained with silver nitrate, (c) assayed for activity against MUG and (d) against laminarin after slicing the gel. (b) PAGE of the pure preparation (after preparative gel electrophoresis) stained with silver nitrate. The amounts of protein loaded were 4 μ g in a, b and d and 1 μ g in c.

Fig. 2. SDS-PAGE of purified *exo*-1,3- β -glucanase (after preparative gel electrophoresis), stained with silver nitrate. Values on the right indicate mobilities of the M_r standards.

Fig. 3. Analytical electrofocusing of the purified *exo*-1,3- β -glucanase (1 μ g) stained with silver nitrate. Values on the right represent pI standards.

Table 1. Purification of the *exo*-1,3- β -glucanase from cell-free extracts of *C. albicans*

Purification step	Total units*	Volume (ml)	Total protein (mg)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)
Cell-free extract (48000 g supernatant fluid)	62838 (100)	2469	7130	8.8	1.0
Freezing and thawing	69213 (110)	2385	3649	19	2.1
Ammonium sulphate precipitation	45855 (72.9)	445.5	461.9	99.3	11.3
DEAE-Biogel A	39151 (62.3)	98	24.5	1598	181.4
PEG concentration	27081 (39.1)	9.4	10.8	2507	284.6
Sephadex G-75 gel filtration	24486 (35.4)	108	1.9	12622	1432.7

* Figures for percentage yield are shown in parentheses.

filtration chromatography because activity is significantly retarded and elutes with an apparent M_r much less than 100000 (Molina *et al.*, 1987), probably due to an affinity for the Sephadex matrix that is also exhibited by other glucanases (Sanchez *et al.*, 1982a). However, the electrophoretic mobility of *C. albicans* *exo*-glucanase in non-dissociating 10% polyacrylamide gels, when compared to that of *Saccharomyces cerevisiae* *exo*-glucanase of similar isoelectric point, indicated that the M_r could be close to 100000 (data not shown). This would favour the idea of the enzyme consisting of two subunits with the indicated M_r values.

Fig. 4. PAGE analysis and MUG-hydrolysing activity of exo-1,3-glucanase in different cellular fractions. (a) Purified preparation (after gel filtration: see Table 1); (b) protoplast lysates; (c) cell-free extracts; (d) culture fluids; (e) partially purified *C. albicans* β -glucosidase.

The properties and mode of action of the enzyme were studied with the highly purified preparation obtained after the gel filtration step (Table 1). Optimum pH for activity was in the range of 5.5 to 6.0, at least a 70% reduction in activity being observed at pH values lower than 5.0 or higher than 7.0. For short incubation periods (15 min) the apparent optimum temperature was 50 °C, but preincubation of the enzyme (for 30 min) at this temperature reduced its activity by 50% when it was assayed subsequently. The purified β -glucanase preparation was active against laminarin (1,3- β -glucan) but inactive on pustulan (1,6- β -glucan). TLC of the reaction products released from laminarin showed that glucose was the only sugar produced. Consistent with this, periodate-oxidized laminarin (i.e. substrate with modified chain ends) (Goldstein *et al.*, 1965) was not hydrolysed. This observation clearly indicated an exohydrolytic mode of action for this β -glucanase; however, in contrast to what is usually observed with exo-1,3- β -glucanases, its activity against the synthetic substrate pNPG was very low (approximately 4% of the activity against laminarin).

The same highly purified exo-1,3- β -glucanase preparation was used for kinetic and inhibition studies. A K_m of 1.7 mg ml⁻¹ was estimated, using laminarin as substrate. As with other glucanases (Bull, 1967), the enzyme was efficiently inhibited by glucono- δ -lactone: a concentration of 10 mM caused 86% inhibition. Concentrations of 0.5 mM-Hg²⁺ and 1 mM-Ag⁺ caused 100% and 50% inhibition respectively. Finally, a pI of approximately 4 was measured for the active glucanase band in the purified preparation (Fig. 3).

The bulk of the cell-bound exo-glucanase of *C. albicans* is located in the periplasm (Molina *et al.*, 1987). Electrophoresis in non-dissociating gels, followed by detection of the active glucanase by hydrolysis of MUG was used to compare exo-glucanase in different cell fractions. The purified enzyme showed a single active band (Fig. 4a); a band of the same mobility was detected in cell-free extracts (Fig. 4c), concentrated protoplast lysates (Fig. 4b) (in this case of a very low

intensity) and culture medium (Fig. 4*d*). The first two preparations displayed another much more intense band of MUG-hydrolysing activity which corresponded to a distinct β -glucosidase enzyme (Fig. 4*e*) that was not detected either in culture medium samples (Fig. 4*d*) or as a protoplast secretion product (data not shown), confirming the cytoplasmic location of *C. albicans* β -glucosidase (Polacheck *et al.*, 1987).

DISCUSSION

Detailed characterization of β -glucanases in several fungal species indicates that these enzyme systems are complex with regard to the number of enzymes involved, and diverse from the point of view of differences between species. This complexity is most clearly illustrated by the studies done on *S. cerevisiae*. At least two 1,3- β -glucan degrading enzymes, which can be detected in protoplast lysates and among protoplast secretion products, can be distinguished from the two glucanases present in cell-free extracts and in the culture medium (Cenamor *et al.*, 1987). The complexity and diversity obscure the essential role(s) of the glucanase system in species bearing glucan as their major wall component. The necessity for such a complex system and the evolutionary divergence that it shows remain to be explained. Moreover, the difficulty of using the physiological substrates of these enzymes is a limitation on investigation of the system.

The 1,3- β -glucanase system of *C. albicans* is no exception to this general rule. The production of more than one β -glucanase has been documented for some strains and strain differences are the likely explanation for several discrepancies in the experimental results from different laboratories (Notario, 1982; Ram *et al.*, 1984). The most abundant 1,3- β -glucanase of *C. albicans* 1001 is clearly the *exo*-hydrolysing enzyme that we reported previously (Molina *et al.*, 1987) and have now further characterized. From the point of view of its kinetic properties (with laminarin as substrate), optimum pH and inhibition by glucono- δ -lactone, it does not differ very much from other *exo*-1,3- β -glucanases that have been analysed in detail. Examples of these are one of the *exo*-glucanases of *S. cerevisiae* (Sanchez *et al.*, 1982*b*) and *exo*-glucanases of *Pichia polymorpha* (Villa *et al.*, 1975), *Candida utilis* (Villa *et al.*, 1976) and *Kluyveromyces phaseolusporus* (Villa *et al.*, 1978). However, its greatly reduced activity on synthetic glucosides (4% of the activity on laminarin) and its subunit structure are properties not shared by most of the other known major *exo*-glucanases produced by fungal species, confirming the diversity of these enzyme systems among different species. Moreover, the capacity of the enzyme to hydrolyse synthetic glucosides such as pNPG also seems to vary between strains of *C. albicans* (Olivero & Larriba, personal communication).

The active *exo*-1,3- β -glucanase could be detected in small amounts in protoplast lysates, and was secreted into the periplasmic space and culture medium without any apparent modification affecting its electrophoretic mobility. This is consistent with the notion that the active enzyme is produced inside the membrane and exported to the outside medium. The previously suggested possibility of the enzyme being activated from an inactive precursor (Molina *et al.*, 1987) is also compatible with these findings and is currently being considered in this laboratory, as one of the properties to be studied by a more extensive characterization that includes cloning the gene.

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