Cell Wall-associated 1,4- β -D-Xylanase in *Cryptococcus albidus* var. *aerius*: *in situ* Characterization of the Activity

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1,4- β -D-Xylanase (1,4- β -D-xylan xylanohydrolase; EC 3.2.1.8) has been detected in both cell-free extracts and culture fluids of the yeast *Cryptococcus albidus* var. *aerius* grown on glucose as the only carbon source. Mild acid treatment of whole cells proved that the enzyme was extracellularly located. The activity remained almost completely linked to the wall after cell breakage, only being liberated in the presence of salt at high concentration. After release, the enzyme became very unstable and so has been characterized *in situ* in 'permeabilized' cells. The maximum production took place at the beginning of the exponential growth phase. The optimum pH and temperature for activity were 5.0 and 40 °C, respectively. The enzyme degraded xylan and xylo-oligosides by an endo-splitting mechanism giving xylobiose, xylotriose and xylose as the main end-products. Activation energy and kinetic constants for xylan degradation were determined. Several metal ions such as Ag⁺ and Hg²⁺ inhibited the enzyme. The possible function of this endo-xylanase in *Cr. albidus* var. *aerius* is discussed.

INTRODUCTION

Cryptococci constitute a group of micro-organisms which differ from typical yeasts in a number of characteristics. Some of these concern the composition and structure of their cell envelopes. Bacon *et al.* (1968) described the occurrence of 1,3- α -glucan as a component of cryptococcal walls, and unusual sugars such as xylose, galactose and glucuronic acid have been isolated from extracellular polysaccharides (Golubev & Vdovina, 1974) and walls (Notario, 1977) of cryptococci. However, little attention has been paid so far to the lytic enzymes able to break down 1,3- α -glucans and, especially, xylose-containing polysaccharides in these yeasts. Only recently, Notario *et al.* (1976*a*) and Meyer & Phaff (1977) demonstrated the presence of β -xylosidases and 1,3- α -glucanases in several cryptococci.

Although Biely *et al.* (1978) described the growth of certain yeasts on xylan and hemicelluloses, the existence of endo-xylanases among yeasts has been described (and then only briefly) in very few papers (e.g. Stevens & Payne, 1977). The present paper describes the detection, location and partial characterization of endo-xylanase activity in the yeast *Cryptococcus albidus* var. *aerius*.

METHODS

Organism and culture conditions. *Cryptococcus albidus* var. *aerius* (CECT 1280), obtained from the Spanish Type Culture Collection (Salamanca, Spain), was grown as described by Notario *et al.* (1976*b*).

Substrates and chemicals. Commercial larchwood xylan (Sigma) was purified by the procedure of Aspinall & McKay (1958) as modified by Stevens & Payne (1977). A fraction containing about 98.5% xylose and

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1.5% glucose was used for the enzyme assays. Xylan was partially hydrolysed as described by Wolfrom & Franks (1965) and different xylo-oligosides were separated and characterized according to Whistler & Tu (1952). *p*-Nitrophenyl- and methyl- β -D-glucopyranosides were purchased from Koch-Light. Phenylmethylsulphonyl fluoride (PMSF) was from Sigma, and celluloses (Whatman CC-31, CM-52 and DE-52) were from BDH.

Enzyme preparation. Crude enzyme extracts were prepared from culture fluids and cell-free extracts of exponentially growing cells were obtained as described by Villa *et al.* (1976) and Notario *et al.* (1976b), respectively. In some experiments 0.02 M-PMSF was present throughout the enzyme preparation.

Enzyme assays. Standard assays contained 0.2 ml enzyme preparation plus 0.8 ml substrate (0.15%, w/v, solutions in 50 mM-sodium acetate buffer, pH 5.5) and were incubated at 37 °C for 5 min. Assays were stopped by adding 0.3 M-ZnSO₄ (1 ml) and 0.3 M-Ba(OH)₂ or by heating at 100 °C for 5 min followed by centrifuging. The reaction products were determined in the supernatant fractions.

Enzyme activity was followed by measuring the release of reducing sugars and xylose from the substrate. Reducing power was determined by Nelson's method (1944) or by that of Park & Johnson (1949). Xylose was measured as described by Dische & Borenfreund (1957). Results were always referred to xylose as standard. One unit of activity was defined as 1 nmol xylose or equivalent reducing power released from each substrate min⁻¹.

Xylanase assays *in situ*. The enzyme activity was determined in cells 'permeabilized' according to Serrano *et al.* (1973). Culture samples were centrifuged, washed twice with water and resuspended in water (final vol. 2 ml). The final cell concentration was always adjusted to 6×10^6 cells ml⁻¹. A mixture (0.2 ml) of toluene/ethanol (1:1, by vol.) was added and the suspension was vigorously shaken for 5 min. The cells were then repeatedly washed with water and 50 mM-sodium acetate buffer, pH 5.5, and resuspended in similar buffer (final vol. 1 ml). The resulting cell suspension was used as the enzyme preparation for standard assays. Controls without exogenous substrate were run in every case to avoid any possible interference due to autolytic reactions.

Enzyme characterization. Assays to determine the optimum pH were done using different buffers within the pH range from 2.5 to 10.5. Each assay contained 0.2 ml permeabilized cells, 0.4 ml xylan (0.15% w/v, in water) and 0.4 ml of the appropriate buffer. The influence of temperature was estimated between 5 and 80 °C. Enzyme inactivation by pH or temperature was determined from assays carried out under standard conditions but with enzyme preparations preincubated at the different pH values or temperatures for 10 min.

Inhibition experiments with metal ions were carried out by adding 0.2 ml of enzyme preparation to a mixture of 0.2 ml of freshly prepared inhibitor solution (up to 0.25 mM) and 0.6 ml of buffered substrate.

Control tests in which permeabilized cells were treated, in the absence of substrate, at different pH values or temperatures or incubated with metal ions were always performed.

Paper chromatography. Descending paper chromatography was used to check the purity of xylan and xylo-oligosides and to characterize the products of the enzymic reactions. Samples were chromatographed on Whatman no. 1 paper for 24 h with ethyl acetate/pyridine/water (8:2:1, by vol.) as solvent. Glucose, xylo-oligosides and xylose were used as references. Spots were developed with aniline phthalate (Partridge, 1949).

Miscellaneous determinations. Culture growth was determined by the increase in absorbance at 600 nm throughout incubation, or by determining the total cell number using a Thoma haemocytometer. Total protein was determined by Lowry's method and free glucose by that of Keston (1956). Enzyme location was studied as described by Arnold (1972). β -Glucanase and β -xylosidase were assayed as described by Notario *et al.* (1976a, b).

RESULTS AND DISCUSSION

Xylanase activity in crude enzyme preparations

Enzyme extracts were incubated in the presence of xylan for different times (up to 3 h). With longer incubations lower activities were found, and after 1 h only 5% and 2% (referred to 2.5 min tests) of the activity was recovered from cell-free extracts and culture fluids, respectively (Fig. 1a). Attempts to repeat the assays with the same preparations were completely unsuccessful because no xylanase activity could be detected. Extraction conditions were strictly controlled, but similar results were obtained whenever crude enzyme preparations were used. Attempts to stabilize the enzyme by addition of proteins (i.e. bovine serum albumin) were unsuccessful. The possible presence of proteases that could inactivate the enzyme was considered, but the presence of the protease inhibitor PMSF throughout the whole enzyme extraction and assay processes had no effect.

Fig. 1. Effect of incubation time (a) and cell concentration in the assays (b) on xylanase activity. Reactions were carried out under conditions described in the text with cell-free extracts (Δ), permeabilized cells ($\bullet$) and purified walls ($\circ$) as enzyme sources.

Cellular location of the xylanase activity

The pellet obtained after centrifuging for 40 min at 40000 g (containing some whole cells, wall debris, membranes etc.) had higher activity than the crude extract. When associated with cell structures the enzyme was apparently much more stable (only 18% loss of activity after 1 h incubation). Experiments were therefore carried out to establish its precise cellular location. As shown in Table 1, xylanase activity was completely lost after treatment of exponentially growing cells with 0.1 M-HCl, while neither cell viability nor α -glucosidase, a typical intracytoplasmic enzyme in *Cr. albidus* var. *aerius* (Notario *et al.*, 1976a), was affected. This finding suggested that xylanase was mainly located within the periplasmic space. Such a periplasmic location has been described for other yeast glycoside-hydrolases (Villa *et al.*, 1975).

Wall-associated xylanase activity

Walls were prepared and purified as described by Fleet & Phaff (1974) and checked for xylanase activity using suspension of purified walls as the source of enzyme. It was found that about 97% of the total activity was associated with the walls and in that condition appeared much more stable than in crude extracts (Fig. 1a). The linkage between xylanase and walls appeared rather weak since all the activity could be removed from the walls simply by washing with 2 M-NaCl. The solubilized enzyme was as unstable as that in crude extracts.

Xylanase activity in permeabilized cells

Wall isolation and purification is often time-consuming, and is especially so in *Cr. albidus* var. *aerius* (Notario *et al.*, 1976b). Moreover, preliminary evidence suggested that certain wall materials were always lost during purification. Permeabilized cells were thought to reflect more closely the situation *in vivo*, and no marked difference in enzyme stability, with respect to that associated with purified walls, was found after short incubation periods (2.5 to 5 min). No xylose or reducing sugars were released when permeabilized cells were incubated for similar periods at 37 °C in the absence of exogenous substrate. For these reasons, 5 min assays with permeabilized cells as the enzyme source were adopted for subsequent experiments.

Table 1. *Cellular location of endo-1,4-β-D-xylanase from Cryptococcus albidus var. aerius*

Xylanase activity is expressed as described in Methods. α-Glucosidase is expressed as μmol *p*-nitrophenol liberated from 0.25 % (w/v) *p*-nitrophenyl-α-D-glucoside min⁻¹ ml⁻¹ at 37 °C. Viable cells were counted under the microscope after 3 min suspension in 0.01 % (w/v) methylene blue prepared in 50 mM-sodium acetate buffer, pH 5.5.

	Treatment	
	None	0.1 M-HCl, 1 h
Percentage of viable cells	99	97.5
α-Glucosidase activity	2.49	2.45
Xylanase activity	12.85	trace

Production of xylanase in synchronous cultures

Unlike most fungal xylanases, which are only detected when micro-organisms are grown on xylan or its derivatives (King & Fuller, 1968), *Cr. albidus* var. *aerius* synthesized the enzyme when grown on glucose as carbon source. The enzyme appeared to be constitutive and higher activities were not obtained when xylan (1 %, w/v) or xylose (up to 10 %, w/v) were used as carbon sources. The maximum rise in xylanase activity took place at the beginning of the exponential phase of growth (12 h cultures), and another peak of activity was detected in the early stationary phase (36 h growth). This second peak coincided with those described for 1,3- and 1,6-β-D-glucanases in the same yeast (Notario *et al.*, 1976*b*). Only low levels of activity were found at other stages of growth (Fig. 2).

Enzyme characterization

Effect of incubation time. As previously indicated, enzyme activity declined progressively when assays were developed for periods up to 3 h. When permeabilized cells were used as the source of enzyme, only 14.5 % of the activity was recovered after 1 h incubation under standard conditions (Fig. 1*a*).

Effect of cell concentration. Activity increased linearly when the concentration of permeabilized cells in the assays was increased up to 2.5×10^6 cells ml⁻¹ (Fig. 1*b*). Further increases in the amount of cells did not give significantly higher levels of activity (between 3×10^6 and 7×10^6 cells ml⁻¹) and even had an inhibitory effect (above 7.5×10^6 cells ml⁻¹).

Effect of pH. The highest enzyme activity was obtained at pH 5.0. This was also considered to be the optimum pH since samples of permeabilized cells that had been maintained at pH 5.0 for 10 min before being assayed showed almost full activity. This optimum pH value is in the range reported for other lytic enzymes (Villa *et al.*, 1975). However, this enzyme had a narrower pH-dependence since slight deviations from the optimum value caused significant loss of activity (about 25 % loss at pH 4.5 or 5.5).

Effect of temperature. At the optimum pH the maximum xylanase activity was obtained at 50 °C. However, 5 % loss of activity was detected after 10 min preincubation under these conditions, whereas practically no inactivation occurred at 40 °C. The activation energy for the enzymic hydrolysis of xylan was estimated to be 15925 ± 1480 J mol⁻¹.

Effect of substrate concentration. The enzyme activity was determined at pH 5.0 and 37 °C for a series of increasing concentrations of xylan. From reciprocal plots, the apparent Michaelis-Menten constant (K_m) was estimated to be 0.121 mM. This K_m value is expressed on a molar basis assuming a molecular weight of 16200 for the purified xylan, corresponding to a DP value of 123 as determined by the enzymic procedure described by Sturgeon (1973).

Inhibition by metal ions. Zn²⁺, Mn²⁺, Pb²⁺, Hg²⁺ and Ag⁺ (as chlorides) were tested for any inhibitory effect on xylanase activity. A continuous decrease in activity was caused by

Fig. 2. Production of endo-1,4- β -D-xylanase (●) by *Cryptococcus albidus* var. *aerius* growing in asynchronous cultures. Culture growth (○); free glucose in the medium (□).

Fig. 3. Inhibitory effect promoted by metal ions on xylan degradation by endo-1,4- β -D-xylanase from *Cryptococcus albidus* var. *aerius*: Ag^+ (●), Hg^{2+} (○), Pb^{2+} (■), Mn^{2+} (□), Zn^{2+} (△).

increasing the concentration of Ag^+ , total inhibition being observed at concentrations higher than 0.15 mM. At very low concentrations (0.01 mM) all the divalent cations induced a rapid decrease of activity to about 40 to 60% of that in control experiments. However, less inhibition occurred at higher ionic concentrations, resulting in rather peculiar inhibition curves (Fig. 3). Inverse plots were non-linear and evaluation of K_i values by extrapolation was not possible. There is no clear explanation for such a phenomenon. It is possible that these ions at low ionic concentrations were able to promote direct inhibition of the enzyme activity, whereas at higher concentrations, despite some inhibition, they could provide more

Table 2. *Total reducing sugars released by the action of xylanase alone or with other enzymes on whole cells, purified walls and wall fractions isolated from Cryptococcus albidus var. aerius*

Walls purified from cells grown for 36 h were used both as enzyme source and as substrate. Those to be used as substrate were heated for 10 min at 100 °C to inactivate any endogenous lytic enzyme. Those to be used as the enzyme source were treated with toluene (as whole cells, see Methods). Toluene-treated walls contained similar amounts of xylanase to untreated walls but did not show detectable levels of β -xylosidase or glucanases unless incubations were maintained for very long periods (30 h at least). Whole cells and two xylose-containing fractions (wall fractions 1 and 2, containing 4 and 5% xylose, respectively), prepared from *Cr. albidus* var. *aerius* walls by a modification of Adam's (1965) procedure, were also used as substrate. Endo- and exo-1,3- β -D-glucanases and β -xylosidase, purified from cell-free extracts of *Cr. albidus* var. *aerius* (Notario, 1977), were also used. The final concentration of these enzymes in the assay reactions was adjusted to 125 units (defined by their activity on laminarin or *p*-nitrophenyl- β -D-xylopyranoside, as in Methods). Assay mixtures contained 0.5 ml enzyme (2.5 mg dry wt permeabilized walls, or purified lytic enzymes, in buffer) plus 0.5 ml of buffered substrate and were incubated under standard conditions for 2 or 5 h. Reactions were stopped by centrifuging and reducing sugars were determined in the supernatants. When enzyme mixtures were used the procedure included a 30 min pretreatment with xylanase alone before addition of the other enzymes.

Enzyme	Substrate ...	Reducing sugars released [$\mu\text{g (mg dry wt substrate)}^{-1}$]							
		Whole cells		Purified walls		Wall fraction 1		Wall fraction 2	
		2 h	5 h	2 h	5 h	2 h	5 h	2 h	5 h
Endo-1,3- β -D-glucanase		26	165	32	205	22	155	12	120
Exo-1,3- β -D-glucanase		68	350	83	407	75	407	15	237
β -Xylosidase		6	105	28	140	34	160	56	215
Xylanase		4	60	16	88	18	95	23	110
Xylanase + endo-1,3- β -D-glucanase		35	208	55	296	38	265	40	307
Xylanase + exo-1,3- β -D-glucanase		206	604	307	892	289	835	104	398
Xylanase + β -xylosidase		25	256	78	461	176	606	86	419

favourable conditions for the stability of the enzyme-substrate complexes or allow greater accessibility of the enzyme to the substrate. This possibility would be in agreement with that proposed by Villa *et al.* (1978) to explain the effect of NaCl on certain β -glucanases.

When applied to Hg²⁺, this explanation would be consistent with the proposed role of -SH groups in controlling the activity of yeast wall lytic enzymes (Notario *et al.*, 1976c). However, the fact that Hg²⁺ did not inhibit the activity completely, as it does with other lytic enzymes (Notario *et al.*, 1976b, c; Villa *et al.*, 1978), suggests either that -SH groups are not essential for the catalytic activity of the enzyme, or that *in situ* other -SH groups possibly involved in maintaining the enzyme structure might be less susceptible than in solubilized enzymes.

Substrate specificity and pattern of hydrolysis. Only xylan, xylo-oligosides and cellulose were used for these experiments. Cells grown for 36 h (showing low β -xylosidase but high xylanase activities) were chosen as the source of enzyme to avoid, as far as possible, the interference by other lytic enzymes present in the permeabilized cells. Toda *et al.* (1970) showed that certain xylanases were in fact cellulases. No activity against crystalline or pre-swollen celluloses was found, suggesting that the enzyme was a true xylanase. The enzyme was active on xylan and xylo-oligosides, but the degree of activity decreased in parallel with the chain length of the substrate and no xylobiase activity could be attributed to the xylanase.

Chromatographic analysis of supernatants of reaction mixtures showed that xylotetraose, xylotriose and xylobiose (after 30 min assays), xylotriose, xylobiose and traces of xylotetraose (after 2 h) and xylotriose, xylobiose and xylose (after 5 h) were the main products of the enzymic reactions. Traces of xylose were detected early in the tests but they appeared to be due to β -xylosidase activity. Similar results were obtained when purified walls (almost

free of β -xylosidase and glucanases) were used as the source of enzyme. These results suggested that the xylanase was an endo-splitting enzyme with a hydrolytic pattern similar to that described by Gorbacheva & Rodionova (1977) for an enzyme from *Aspergillus niger* able to break down xylans at random giving xylobiose > xylotriose > xylose as end-products.

Xylanase functions: action on whole cells and isolated walls

Xylanase alone or in combination with other lytic enzymes promoted significant release of reducing sugars from whole cells, purified walls and two xylose-containing wall fractions (Table 2). As expected from their respective patterns of hydrolysis, xylanase pretreatment caused a significant increase in β -xylosidase activity. In the case of β -glucanases a synergistic action was restricted to exo-enzymes. Notario (1977) suggested that the wall structure in this yeast would be strengthened by xylo-oligosidic bridges between glucan and mannan, and that their degradation might be crucial in morphogenetic events involving localized partial hydrolysis of the wall. The high instability of the enzyme studied, its occasional production at the beginning of active growth, and its co-operativity with other enzymes of the same yeast seem to support this hypothesis. However, the fact that neither complete wall dissolution nor protoplast formation occurred (even when all the enzymes were acting together) would indicate that other plasticizing factor(s) are also involved in wall dynamics in *Cr. albidus* var. *aerius*. On other hand, the production of xylanase by stationary phase cells could be related to a metabolic function such as the modification of wall materials or the digestion of external glycoproteins secreted into the culture medium by the cells.

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