Chitinase Activity from *Candida albicans* and its Inhibition by Allosamidin

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Candida albicans chitinase isolated using the Dyno-Mill disruption technique was characterized using an improved radiometric assay procedure. The enzyme had apparent temperature and pH optima of 45 °C and 6.5, respectively. The preparation yielded an apparent K_m of 3.9 mg chitin ml⁻¹ [17.6 mM-*N*-acetylglucosamine (GlcNAc) equivalents] and V of 2.3 nmol GlcNAc formed min⁻¹ (mg protein)⁻¹. The potential of the streptomycete antibiotic allosamidin as an antifungal agent is discussed in view of its dose-dependent inhibition of *C. albicans* chitinase activity (IC_{50} = 0.3 µM). Allosamidin was a potent competitive inhibitor of enzyme activity (K_i = 0.23 µM).

INTRODUCTION

Chitin, a (1→4)-β polymer of *N*-acetylglucosamine (GlcNAc) is a major structural component of most fungal cell walls (Gooday & Trinci, 1980). The enzyme chitin synthase (EC 2.4.1.16) catalyses the production of this macromolecule by polymerization of GlcNAc from the activated precursor UDP-*N*-acetylglucosamine. There is now compelling evidence that chitin synthase plays a fundamental role in morphogenesis (Cabib, 1987; Silverman *et al.*, 1988). A morphogenetic role has also been proposed for fungal chitinase (EC 3.2.1.14), which catalyses the hydrolysis of chitin to the disaccharide chitobiose (Bartnicki-Garcia, 1973). In this model, chitinase causes a limited degree of cell wall lysis so that the wall remains in a plastic and extensible condition. Significantly in this regard, chitinase activities have been detected in all actively growing chitinous fungi investigated (Gooday *et al.*, 1986). Thus, the regulation of growth and the initiation of budding, hyphal branches and septum formation may all be dependent upon a delicate balance between wall synthesis and wall lysis.

Chitin is not found in vertebrates and the potential of chitin synthase as a target for antifungal drugs which lack host toxicity was discussed at length by Gooday (1977). Disappointingly, polyoxins, which are competitive inhibitors of chitin synthase and effective agricultural antifungal agents, have so far proved unsuitable for treatment of experimental mycoses in mammals. In view of the major morphogenetic role proposed above for chitinase, it is conceivable that this lytic enzyme presents an alternative target for antifungal compounds directed against chitin deposition.

Recently, Sakuda *et al.* (1987) described the inhibition of ecdysis in the silkworm *Bombyx mori* and the common armyworm *Leucania separata* by the streptomycete antibiotic allosamidin. Chitinase plays an essential role during ecdysis in insects by catalysing the hydrolysis of integumental chitin. Thus, the insecticidal properties of allosamidin may reside in its potent competitive inhibition of insect chitinase activity (Koga *et al.*, 1987). *Candida albicans* is an important opportunistic fungal pathogen and chitinase activity was recently detected in this organism (Barrett-Bee & Hamilton, 1984). In the present study, we report a detailed characterization of chitinase in a cell-free preparation from *C. albicans* and the effect of allosamidin on enzyme activity.

METHODS

Chemicals. Allosamidin was a generous gift from Dr Akira Isogai of the University of Tokyo. All other chemicals were purchased from commercial sources and were of the highest grade available.

Organism and culture conditions. *C. albicans* (serotype A; NCPF 3153) was maintained on Sabouraud's dextrose agar (Oxoid) and grown in Sabouraud's dextrose broth (Oxoid) essentially as described by Hitchcock *et al.* (1986). Under these conditions the organisms grew exclusively in the yeast phase.

Mechanical disruption of cells. Cells were harvested by centrifugation, washed twice with distilled water and once with disruption buffer: 50 mM-Bis-Tris, 0.25 M-sucrose, 1 mM-EDTA.Na₂, pH 6.5. The organisms were resuspended in disruption buffer at a concentration of 4×10^9 cells ml⁻¹ and broken in either a Braun (FRG) or a Dyno-Mill Type KDL (Willy A. Bachofen, Basle, Switzerland) disintegrator. For Braun disintegration, cell suspensions (35 ml) were disrupted at a temperature of 4 °C using 1 mm diameter beads. Breakage was achieved using four 30 s disruption periods at 4000 r.p.m. interspersed with 30 s cooling periods. For Dyno-Mill disintegration, cell suspensions (200 ml) were disrupted at a temperature of 10 °C (maximum) in a 300 ml batch disintegration container, using 0.75–1 mm diameter beads. Breakage was achieved using four 1 min disruption periods at 3000 r.p.m. interspersed with 1 min cooling periods. The temperature was maintained using an LKB Multitemp II thermostatic circulator set at -13 °C throughout. Homogenates were recovered by aspiration. In the buffer mixture described above Braun and Dyno-Mill disintegration procedures resulted in approximately 40% and 70% cell breakage respectively, as judged by haemocytometer counts.

Enzymic disruption of cells. Cells were harvested by centrifugation and resuspended in 0.1 M-potassium phosphate, 0.1 M-2-mercaptoethanol, 0.1 M-EDTA.Na₂, pH 7.2, at 2×10^9 cells ml⁻¹ for 30 min at room temperature. Organisms were washed twice with distilled water and twice with 0.1 M-potassium phosphate, pH 7.2, and resuspended in 0.1 M-potassium phosphate, 0.9 M-sorbitol, 10 mM-EDTA.Na₂, pH 7.2, containing Zymolyase 20T (10 units ml⁻¹; Kirin Brewery, Japan) for 1.5 h at 37 °C with gentle agitation. Spheroplasts were harvested by centrifugation at 600 g (r_{av} , 10.7 cm) for 5 min, washed with 0.1 M-potassium phosphate, 0.9 M-sorbitol, 10 mM-EDTA.Na₂, pH 7.2, and resuspended in disruption buffer to a concentration of 1×10^{10} ml⁻¹. Breakage of spheroplasts was effected using 15 strokes of a hand-held Teflon-on-glass homogenizer at 4 °C. The procedure resulted in approximately 100% cell breakage.

Centrifugation of the cell lysates. The cell lysates from the three breakage procedures were centrifuged at 9000 g (r_{av} , 6.98 cm) for 20 min at 4 °C to remove unbroken cells and cellular debris, and the supernatants centrifuged at 145000 g (r_{av} , 8.1 cm) for 1 h at 4 °C. The high speed supernatant was stored at -20 °C as the soluble enzyme preparation.

Extraction of chitinase using digitonin. The procedure was essentially as described by Correa *et al.* (1982). Briefly, 1 g of cells was resuspended in 5 ml of 0.1% (w/v) digitonin, containing 25 mM-MES, pH 6.3, and 0.1% (v/v) mercaptoethanol. The suspension was incubated with shaking for 2 h at 37 °C, centrifuged for 10 min at 12000 g (r_{av} , 6.98 cm) and the supernatant retained.

Chitinase assay. Chitinase was assayed by the method of Molano *et al.* (1977) with the following modifications: chitosan (1 g; Sigma) was acetylated using 1.5 ml of acetic anhydride which contained 25 mCi (925 MBq) of radioactivity (Amersham: 500 mCi mmol⁻¹; 18.5 GBq mmol⁻¹); following homogenization in a Waring blender the chitin was filtered in a Buchner funnel using Whatman no.3 filter paper; after the chitin had been washed to neutrality it was resuspended in 300 ml 0.02% (w/v) sodium azide and 150 ml aliquots were ball-milled in 250 ml reagent bottles, containing thirty 5 mm diameter and thirty 10 mm diameter stainless steel balls (Bearing Service, Leeds, UK) at 120 r.p.m. for 48–72 h; the chitin was filtered as above, resuspended to approximately 3 mg ml⁻¹ in 0.02% sodium azide and stored at 4 °C.

Unless otherwise stated, reaction mixtures contained 60 µl [³H]chitin (2.6 mg ml⁻¹, 1.9 µCi mg chitin⁻¹, 70 kBq mg chitin⁻¹), 60 µl assay buffer (0.15 M-potassium phosphate, pH 6.5) and 60 µl diluted enzyme preparation. After incubation at 37 °C with vigorous shaking the reaction was stopped by the addition of 0.18 ml 10% (w/v) trichloroacetic acid. Chitin was separated from reaction products by filtration through glass wool tightly packed into a 200 µl micropipette tip. Samples (200 µl) of the filtrate were mixed with 5 ml of LKB Optiphase Safe scintillant and radioactivity was measured in an LKB Rackbeta 1217 liquid scintillation counter. All values shown are the means of at least triplicate determinations and variation between replicates was never greater than 10%.

Chitin deacetylase was assayed as described by Humphreys & Gooday (1984). The results indicated that this enzyme made a negligible contribution to the level of labelled reaction products.

Protein concentration was determined by the method of Bradford (1976) using a bovine serum albumin standard.

RESULTS AND DISCUSSION

Comparison of extraction procedures

A number of extraction procedures were compared with the aim of isolating sufficient quantities of chitinase for future purification studies and for the characterization of potential enzyme inhibitors. High specific activities were noted for chitinase preparations isolated by

both the Braun [0.54 ± 0.04 nmol GlcNAc formed min^{-1} (mg protein) $^{-1}$] and the Dyno-Mill [0.46 ± 0.03 nmol GlcNAc min^{-1} (mg protein) $^{-1}$] techniques when compared with the spheroplast breakage procedure [0.08 ± 0.01 nmol GlcNAc min^{-1} (mg protein) $^{-1}$] (all values are the means of at least three separate preparations $\pm$ SE). However, the total activity was greater for the Dyno-Mill procedure (80.3 ± 4.6 nmol GlcNAc formed min^{-1} (10^{11} cells) $^{-1}$) than for the Braun [52.1 ± 2.8 nmol GlcNAc min^{-1} (10^{11} cells) $^{-1}$] or spheroplast [17.8 ± 1.5 nmol GlcNAc min^{-1} (10^{11} cells) $^{-1}$] techniques, reflecting the higher efficiency of cell breakage. Furthermore, the Dyno-Mill provided an ideal method for the isolation of large quantities of soluble enzyme (approximately 130 ml per preparation) and was used for enzyme kinetic and inhibition studies.

During the conversion of *Saccharomyces cerevisiae* cells to protoplasts, approximately half of total yeast chitinase activity was liberated into the medium (Elango *et al.*, 1982). The authors concluded that this portion of enzyme activity was located in the periplasmic space. In the present study, the specific and total chitinase activities extracted by the spheroplast procedure were much lower than those extracted using the Dyno-Mill or Braun homogenizers. At least part of the explanation for this observation may be that as with *S. cerevisiae*, a proportion of *C. albicans* chitinase is located in the periplasmic space and is liberated into the medium during spheroplast formation. Similarly, the high specific activity of chitinase released from cells following incubation with digitonin [1.61 ± 0.04 nmol GlcNAc formed min^{-1} (mg protein) $^{-1}$] may be due to preferential extraction of the periplasmic enzyme by detergent treatment. Enzyme preparations isolated using the Braun and Dyno-Mill procedures had lower specific activities than digitonin-extracted chitinase. However, the mechanical disruption procedures yielded preparations with much higher levels of total chitinase activity than those isolated using the detergent [11.6 ± 1.1 nmol GlcNAc formed min^{-1} (mg protein) $^{-1}$]. One explanation is that the digitonin extraction procedure is inefficient. Alternatively, the proportion of total chitinase activity in the periplasmic space may be considerably lower in *C. albicans* than in *S. cerevisiae*. In agreement with the results of Elango *et al.* (1982) for *S. cerevisiae*, we were unable to detect chitinase activity in the growth medium following a 16 h incubation period. This suggests that export of chitinase to the periplasmic space was not followed by its release from the cell.

Properties of chitinase extracted by cell homogenization

The most sensitive assay for chitinase is a radiometric one (Cabib, 1987) and we used a modified version of the method described by Molano *et al.* (1977) for the studies described here. As discussed by Barrett-Bee & Hamilton (1984), the state of sub-division of chitin particles can influence reaction velocity. In addition, we have noted that the degree of reproducibility between replicate assays is much improved following milling of the chitin to a very fine suspension using stainless steel balls. This, combined with our introduction of a modified filtration method for the separation of reaction products from chitin, has resulted in a rapid, economical, sensitive and highly reproducible assay for chitinase activity.

A plot of product formed as a function of time did not yield a straight line (Fig. 1*a*), in keeping with the results of Molano *et al.* (1977). However, a plot of the rate of reaction against enzyme concentration was linear (Fig. 1*b*). Standard assay conditions were chosen where activity was on the linear portion of a progress and concentration curve. Chitin is insoluble in aqueous solutions so that it is difficult to interpret kinetic data for experiments where chitin is substrate. However, standard Michaelis-Menten kinetics were obtained and the preparation used for Fig. 1 yielded an apparent K_m of 3.9 mg chitin ml^{-1} and a maximum enzyme reaction velocity (V) of 2.3 nmol *N*-acetylglucosamine formed min^{-1} (mg protein) $^{-1}$. As noted by other workers (Barrett-Bee & Hamilton, 1984; Humphreys & Gooday, 1984), K_m and V values differed slightly for each preparation of radioactive chitin. There has been only one previous report of chitinase activity in *C. albicans* (Barrett-Bee & Hamilton, 1984). However, these workers did not quote K_m or V values for their enzyme preparation. Using a radiometric assay similar to that described in the present study, Humphreys & Gooday (1984) described K_m and V values of 16.7 mg chitin ml^{-1} and 2.33 nmol diacetylchitobiose formed min^{-1} (mg protein) $^{-1}$ respectively for their cytosolic enzyme preparation isolated from *Mucor mucedo*. Similarly, Correa *et al.* (1982) reported a K_m for chitinase from *S. cerevisiae* of 3.9 mM, as expressed in GlcNAc equivalents. When expressed

Fig. 1. Plots of product formed against time (a) and enzyme activity against enzyme concentration (b) for *C. albicans* chitinase.

as GlcNAc equivalents, the K_m for *C. albicans* chitinase (3.9 mg chitin ml⁻¹) has a value of 17.6 mM. The K_m value for *M. mucedo* chitinase (16.7 mg chitin ml⁻¹; Humphreys & Gooday, 1984) has a value of 72.8 mM-GlcNAc equivalents. These results indicate a marked variation in substrate affinity for the fungal chitinases studied to date.

The apparent temperature optimum for chitinase was 45 °C. However, in keeping with the studies of Barrett-Bee & Hamilton (1984) chitinase was assayed routinely at 37 °C, the temperature of the pathogen's human host. The enzyme showed a broad pH dependence with an optimum around 6.5. No detectable loss of enzyme activity occurred during storage periods of three months duration at -20 °C or 4 °C. Furthermore, the level of chitinase activity remained constant following fifteen freeze/thaw cycles over a period of two months. This extraordinary level of stability appears to be a common feature of many fungal lytic enzymes and may reflect a high level of protein glycosylation. For example, chitinase purified from *S. cerevisiae* is a glycoprotein composed of approximately 18% carbohydrate (Correa *et al.*, 1982).

Effect of allosamidin on enzyme activity

Koga *et al.* (1987) reported that allosamidin inhibited *Bombyx mori* chitinases competitively following Lineweaver-Burk analysis of a reaction where glycol chitin was substrate. K_i values were 0.07 μM and 0.11 μM for chitinases isolated from the alimentary canal and larval integument of the organism respectively. These results are in close agreement with those for *C. albicans* chitinase. The inhibition of chitinase by allosamidin was dose-dependent with an IC₅₀ value of 0.3 μM (Fig. 2). Kinetic analysis of this inhibition using a variety of inhibitor concentrations is shown in Fig. 3. The data suggest that allosamidin is a potent competitive inhibitor of *C. albicans* chitinase with a K_i of 0.23 μM. There has been only one other report of the inhibition of a fungal chitinase by allosamidin. Gooday *et al.* (1988) reported an IC₅₀ value of 3.2 μM (using 3,4-dinitrophenyl tetra-*N*-acetylchitotetraoside as chitinase substrate) for an enzyme preparation isolated from *Neurospora crassa*.

Thus, our results together with those of Gooday *et al.* (1988) suggest that fungal chitinase is highly susceptible to inhibition by allosamidin and that the antibiotic inhibits enzyme activity in a manner similar to that described previously for insect chitinase (Koga *et al.*, 1987). The potent inhibition of chitinase activity of the opportunistic pathogen *C. albicans*, coupled with the major role proposed for the enzyme in fungal growth and morphogenesis, implies that chitinase inhibition by allosamidin might be exploited to treat clinically important fungal infections. However, our preliminary studies indicate that the presence of allosamidin in the culture medium at a concentration of 30 μM has no effect on cell number in cultures sampled in the late exponential phase of growth. This result would suggest either that allosamidin cannot gain access to chitinase in *C. albicans* or that the enzyme is not essential for growth of this organism. Humphreys & Gooday (1984) have proposed a role for membrane-bound chitinase during

Fig. 2

Fig. 3

Fig. 2. Effect of allosamidin on chitinase activity [100% activity represents 0.44 nmol GlcNAc formed min^{-1} (mg protein^{-1})].

Fig. 3. Kinetic analysis of chitinase activity in the presence of allosamidin. Allosamidin concentrations were: (■) 0 μM ; (○) 0.1 μM ; (●) 0.2 μM ; (□) 0.5 μM ; (▲) 1.0 μM .

growth and morphogenesis in *M. mucedo*. If a similar role is performed by a membrane-bound chitinase in *C. albicans* then allosamidin apparently cannot gain access to the active site of this enzyme in intact organisms. Interestingly in this regard, we have recently confirmed the presence of a microsomal chitinase in *C. albicans* and work is under way to purify and characterize this enzyme.

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