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ORIGINAL PAPER

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Screening of chitin deacetylase from Mucoralean strains (Zygomycetes) and its relationship to cell growth rate

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Abstract Chitin deacetylase (CDA) is an enzyme that catalyzes the hydrolysis of acetamine groups of *N*-acetyl-D-glucosamine in chitin, converting it to chitosan in fungal cell walls. In the present study, the activity in batch culture of CDA from six Mucoralean strains, two of them wild type, isolated from dung of herbivores of Northeast Brazil, was screened. Among the strains tested, *Cunninghamella bertholletiae* IFM 46114 showed a high intracellular enzyme activity of 0.075 U/mg protein after 5 days of culture, and a wild-type strain of *Mucor circinelloides* showed a high intracellular enzyme activity of 0.060 U/mg protein, with only 2 days of culture, using *N*-acetylchitopentaose as substrate. This enzyme showed optimal activity at pH 4.5 in 25 mM glutamate-sodium buffer at 50°C, and was stable over 1 h preincubation at the same temperature. The kinetic parameters of CDA did not follow Michaelis-Menten kinetics, but rather Hill affinity distribution, showing probable allosteric behavior. The apparent K_{HILL} and V_{max} of CDA were 288 ± 34 nmol/l and 0.08 ± 0.01 U mg protein^{-1} min^{-1}, respectively, using *N*-acetylchitopentaose as substrate at pH 4.5 at 50°C.

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Introduction

Chitin deacetylase (CDA; E.C. 3.5.1.41) is an enzyme that catalyzes the hydrolysis of acetamine groups of *N*-acetylglucosamine in chitin, promoting the conversion to chitosan. This enzyme was first identified and partially purified from extracts of *Mucor rouxii* [5], and has been purified and characterized from other Zygomycetes strains, such as *Absidia coerulea* [11], and from *Aspergillus nidulans* and *Colletotrichum lindemuthianum* [1, 16]. Enzymes of this type have been reported to occur in some insect species [6]. These enzymes are glycoproteins secreted either into the periplasmic region or into the culture medium and exhibit remarkable thermal stability and specificity for water-soluble β-(1,4)-linked *N*-acetyl-D-glucosamine polymers. However, they vary considerably in their molecular weight and carbohydrate content 17].

In Zygomycetes, CDA has an important role in fungal growth, being involved in cell-wall chitosan biosynthesis in tandem action with chitin synthase [10], and it could also be involved in deacetylation of chitin oligosaccharides during autolysis after the action of endochitinase on cell walls. An alternative biological role involving the enzyme in plant-pathogen interactions has been suggested for CDA from *C. lindemuthianum*, a plant pathogen, and the enzyme is extracellular and active on chitin oligomers. These oligomers, but not their deacetylated form, elicit plant-defense mechanisms (e.g., callose formation) [13].

Chitosan is a natural polymer that has great potential in biotechnology and in the biomedical and pharmaceutical industries. It occurs in fungal cell walls in addition to chitin. Both compounds have been isolated from Zygomycetes [3, 4, 14]. Commercially, it is produced from chitin via a harsh thermochemical process that

shares most of the disadvantages of a multi-step chemical procedure. It is environmentally unsafe and not easily controlled, leading to a broad and heterogeneous range of products [9]. An alternative or complementary procedure exploiting the enzymatic deacetylation of chitin could potentially be employed, especially when a controlled and well-defined process is required. The aim of this work was to screen CDA activity from different Mucoralean species obtained from culture collections and natural habitats, and to study and characterize the kinetics of the enzyme.

Materials and methods

Microorganisms

Mucoralean strains: *Mucor circinelloides* and *Syncephalastrum racemosum* were isolated from dung of herbivores of Northeast Brazil. *Cunninghamella bertholletiae* (IFM 46114), *Cunninghamella echinulata* (URM 2136), *Cunninghamella ramosa* (URM 1918), and *Cunninghamella elegans* (IFM 30505) were obtained from culture collections: URM (Department of Mycology, UFPE, Brazil) and IFM (Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan).

Culture conditions

Strains were maintained on PDA (potato dextrose agar) slants at 4°C. Cultures were subcultured (monosporic culture) on PDA plates, incubated at room temperature (28°C) for 5 days and used as a starter to prepare inoculum. Spore suspension (10^5 spores/ml final concentration) was used to inoculate a 500-ml Erlenmeyer flask containing 150 ml nutrient broth (YPD): 2% D-glucose, 0.3% yeast extract, 1% polypeptone, the pH was adjusted to 4.5 [7], and the culture grown at 28°C for 120 h on a orbital shaker at 150 rpm.

Enzyme extraction, partial purification and assay of CDA

CDA was isolated from mycelia harvested by filtration and disrupted by grinding with glass powder in a homogenizer at 4°C to give a crude extract [5], and was partially purified by ammonium sulfate treatment, initially by 60% followed by 85% saturation at 4°C, and allowed to settle overnight. The precipitate was recovered by centrifugation and the pellet formed was solubilized in 80 ml 25 mM sodium glutamate, pH 4.5, followed by dialysis against the same buffer. Enzyme activity was assayed by measurement of acetic acid released from *N*-acetylchitopentaose in 30 min at 50°C, using an F-KIT (Boehringer Mannheim, Mannheim, Germany). One unit of CDA activity was defined as the amount of enzyme required to produce 1.0 μmol acetic acid per minute when incubated with *N*-acetylchitopentaose under standard conditions.

Protein assay

Protein concentration was determined according to the method of Bradford [8] using bovine serum albumin as a standard.

Kinetic characterization

Optimal pH was evaluated by measuring CDA activity in 25 mM sodium glutamate buffer (pH 3.5–4.5) and 50 mM MES-NaOH buffer (pH 6.5–8.5). The optimal temperature for activity and thermostability was evaluated by incubation in a water bath at each temperature. Kinetic constant values were measured at optimal conditions of pH and temperature using *N*-acetylchitopentaose as substrate.

Results and discussion

Among all Mucoralean strains from culture collections and natural isolates, *C. bertholletiae* IFM 46114 showed the highest intracellular CDA activity (0.075 U/mg protein), at 5 days of cultivation. However, a higher enzyme activity among native strains (0.060 U/mg protein) was observed in *M. circinelloides* cultivation at 2 days of growth (Table 1).

The maximum growth rates of these fungi and their respective CDA activity, which varied widely among the

Table 1 Chitin deacetylase (CDA) activity from different Zygomycetes strains related to specific growth phase (μmax), determined by the release of acetic acid at 50°C in 30 min at pH 4.5 using 166 μmol *N*-acetylchitopentaose as substrate

Strains	Enzyme activity (U/mg protein), 2 days	Enzyme activity (U/mg protein), 5 days	μmax
Cunninghamella ramosa URM 1918	nd[b]	0.018	0.089
Cunninghamella bertholletiae IFM 46114	nd	0.075	0.137
Cunninghamella echinulata URM 2136	nd	0.044	0.165
Cunninghamella elegans IFM 40505	nd	0.042	0.113
Syncephalastrum racemosum (wild type)[a]	0.047	nd	0.056
Mucor circineloides (wild type)[a]	0.060	nd	0.025

[a]Obtained from herbivorous dung (Northeast Brazil)
[b]Not detectable

species evaluated, are also shown in Table 1, . We observed that *S. racemosum* and *M. circinelloides* wild strains showed early CDA activity and lower μmax, as compared with *Cunninghamella* species, which showed CDA activity only after 5 days in culture, with μmax ranging from 0.095 to 0.145 U mg protein^{-1} h^{-1}. Davis and Bartinicki-Garcia [10] showed that, in Zygomycetes, CDA has an important role in fungal growth, being involved in cell-wall chitosan biosynthesis in tandem action with chitin synthase.

Gao et al. [11], working with CDA crude extract from *A. coerulea*, found an activity of 0.022 U/mg protein throughout 2 days cultivation, using a medium similar to that used in the present work. Similar results were obtained from *C. lindemuthianum* extracellular CDA: 0.0195 U/mg (crude extract) using glycol chitin as a substrate [15]. However, Tsigos and Bouriotis [16] working with the same fungus, but using *N*-acetylchitopentaose as substrate, found an activity of 0.002 U/mg.

Mycelial growth curves of different Zygomycetes strains are shown in Fig. 1. *C. echinulata* grew exponentially up to 24 h, followed by a stationary phase for 10 h and then it started to grow again, more slowly in than the previous exponential phase. This behavior suggests diauxic growth. An analogous behavior was observed with *C. bertholletiae*. However, with *C. ramosa* and *C. elegans*, the growth was slightly different. These cells did not appear to have a diauxic growth phase. We observed that the cells continued to grow for up to 4 days, followed by decline in cell growth.

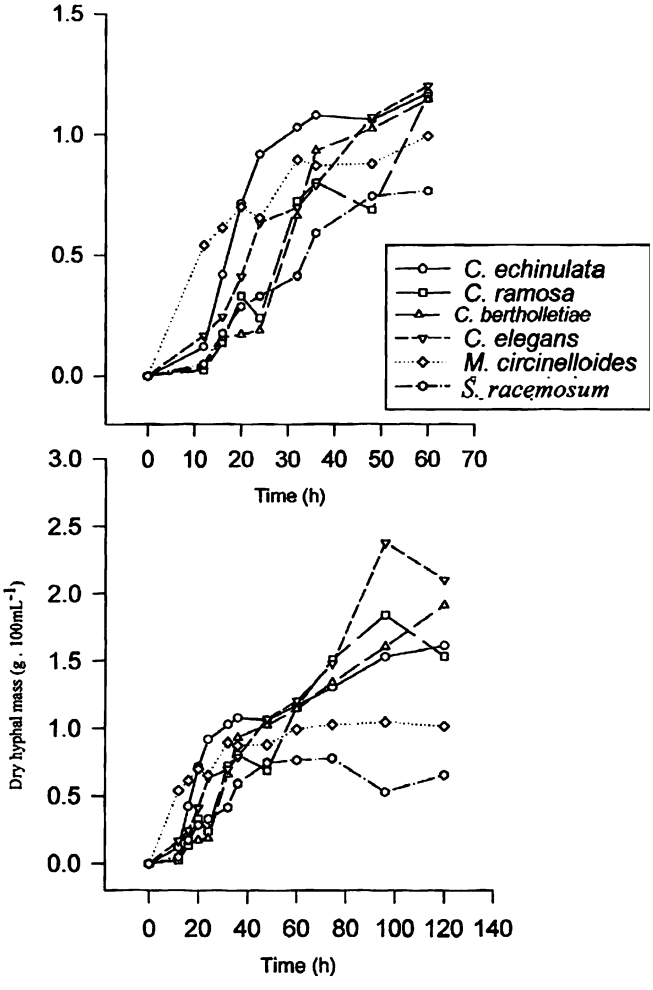

Fig. 1 Mucoralean strains grown in submerged culture at 28°C, 150 rpm using YPD medium [7]

Nevertheless, in cultures of *S. recemosum* and *M. circinelloides*, we observed cell growth with a fast exponential phase and a stationary period starting at 48 h; no diauxic behavior was observed. In both these latter cultures, we also observed CDA activity in the early phase of growth (2 days). *M. circinelloides* cells yielded lower biomass as compared to the biomass of other species (Fig. 1), suggesting that these cells are more selective than the others under the same growth conditions. These results illustrate that enzyme demand is greater at early stages of cell growth, due to cell wall creation. However, in all *Cunninghamella* species, CDA activity was detected only after 2 days of culture, and *C. bertholletiae* IFM 46114 showed the highest activity coinciding with the late exponential growth phase. The same was observed with *S. racemosum* and *M. circinelloides*, which had a late exponential growth phase after 2 days of culture. Amorim [2], Amorim et al. [3] and Tan et al. [14], studying chitosan production from different Zygomycetes strains, found that the late exponential growth phase generated the best yield of chitosan.

The fungi secreted CDA into either the periplasmic region or the culture medium, which would explain why CDA activity is detected in different phases due to differences in its distribution in the cell. In some Zygomycetes, such as *A. coerulea* and *M. rouxii* , CDA is found in the periplasmic region, with activities of 0.022 and 0.0305 U/mg, respectively, being obtained from these strains in 2 days culture using glycol chitin as substrate under the same culture conditions used here [12]. Nevertheless, CDA activity was detected in culture filtrates of *A. nidulans* and *C. lindemuthianum* [17]. Tokuyasu et al. [15] observed CDA activity in the culture medium of *C. lindemuthianum* only after the 8th day of growth, and the enzyme activity increased linearly until the 18th day when the culture stopped growing, which suggests that secretion of CDA begins after mycelial growth in the stationary phase has finished [15].

Regarding the effect of pH on CDA activity in C. *bertholletiae* (Fig. 2), optimal enzyme activity was observed at pH 4.5 in 25 mM sodium glutamate buffer, and lower CDA activity was observed at pH values below 3.5 and above 6.5. CDA from *C. bertholletiae* showed the same optimum pH value as that found in other Zygomycetes strains, such as *M. rouxii*. However, the optimum pH value varies considerably in some strains, being pH 11.5 for *C. lindemuthianum* and pH 7.0 for *A. nidulans* [17] and, in our study, pH 4.5 was optimal for *C. bertholletiae*, suggesting that the enzyme has

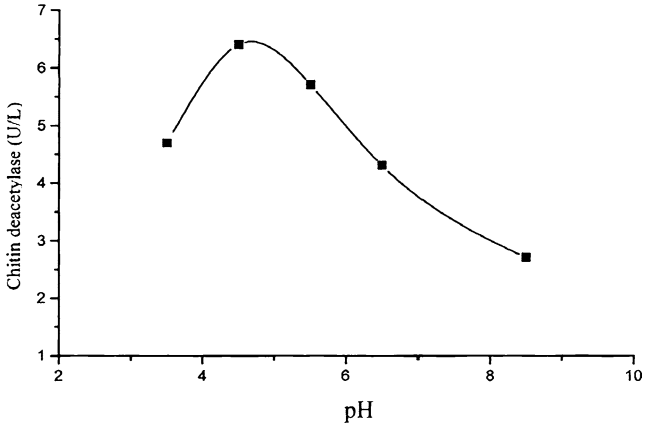

Fig. 2 Effect of pH on intracellular chitin deacetylase (CDA) activity from *Cunninghamella bertholletiae*. Standard deviations were too small to be represented

different kinetic characteristics from one strain to another.

The effects of temperature on the activity and stability of CDA from *C. bertholletiae* are shown in Figs. 3 and 4. The highest activity was detected when the reaction reached 50°C. The enzyme possessed a high thermal stability, maintained full activity even when incubated at 50°C for an hour. These results were similar to those found in *C. lindemuthianum* [15]. CDA from several fungi exhibits a remarkable thermal stability at their optimum temperature of 50°C [17].

The relationship between substrate structure and CDA activity of *M. rouxii* on chitinoligosaccharides [degree of polymerization (DP) 2–7] has been reported [17]. This latter study observed that the length of the oligomer is important for enzyme action, which could not effectively deacetylate chitin with a DP lower than three. Tetra-*N*-acetylchitotetraose and penta-*N*-acetylchitopentaose were fully deacetylated by the enzyme. Chitin oligosaccharides (DP 2–4) have also been used for

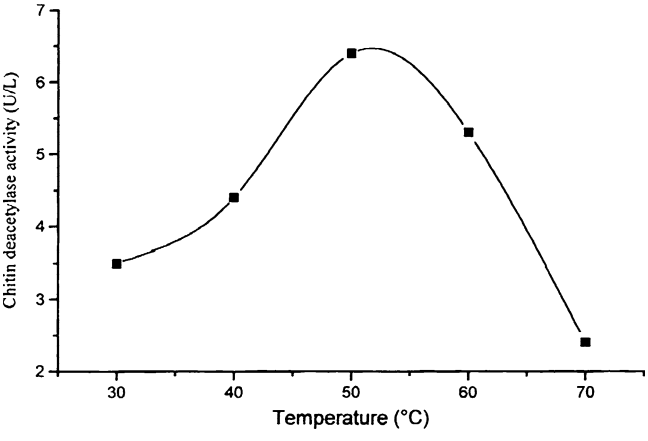

Fig. 3 Effect of temperature on intracellular CDA activity from *C. bertholletiae*. Standard deviations were too small to be represented

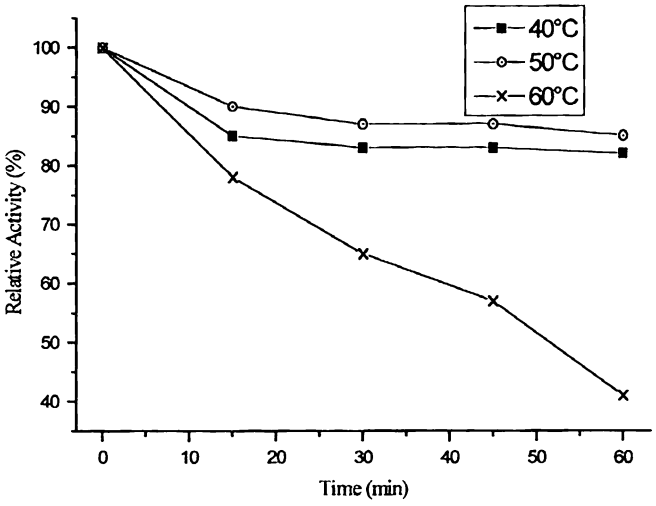

Fig. 4 Thermal stability of CDA activity from *C. bertholletiae* after pre-incubation at 40, 50 and 60°C for different times. Standard derivations were too small to be represented

model substrates to study the mode of action of CDA from *C. lindemuthianum* [17].

Experiments have also been performed elsewhere using CDA from *M. rouxii* and partially deacetylated water-soluble chitosans as substrates, showing that the enzyme was effective in deacetylating polymers, with up to 97% deacetylation [17].

Kinetic parameters of CDA from *C. bertholletiae* IFM 46114 were determined at pH and temperature conditions of maximal activity, obtained from Enz-Fitter plot analyses. They did not follow the Michaelis-Menten kinetics but rather Hill affinity distribution, which corresponds to a sigmoid curve, showing probable allosteric behavior. The values calculated for apparent K_{HILL} and $V_{\max}$ of CDA were 288 ± 34 nmol/l ($P = 1.66\times10^{-3}$) and 0.08 ± 0.01 U mg protein^{-1} min^{-1}, ($P = 3.19\times10^{-3}$), respectively, using *N*-acetylchitopentaose as substrate at pH 4.5 and at 50°C.

The CDA kinetic constants found in the present study have different behavior as compared to CDA activity from *A. nidulans*, which has an apparent K_{m} of 2.5 mM and $V_{\max}$ of 33.3 nmol/min, using *N*-acetylchitopentaose as substrate [1]. Extracellular chitin deacetylase activity from *C. lindemuthianum* has a K_{m} of 0.414 mM and $V_{\max}$ of 158 μmol min^{-1} mg protein^{-1} using the same substrate [15].

The development of a controllable process of enzymatic deacetylation, correlated with a growth-rate-related increase in cellular chitosan content, represents an attractive alternative for application in glycotechnology.

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