PURIFICATION, HETEROLOGOUS EXPRESSION AND MOLECULAR CHARACTERIZATION OF CHITIN DEACETYLASE FROM *RHIZOPUS CIRCINANS*

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Abstract

Chitin, a polymer of β-(1-4) linked N-Acetyl-Glucosamine, is one of the most abundant biopolymer found in nature. The deacetylated derivative of chitin, chitosan, is naturally found in the cell wall of some fungi, mainly in *Zygomycetes*, like *Rhizopus circinans*. Chitosan biosynthesis is catalyzed by chitin deacetylase (CDA) that hydrolyzes the N-acetamide groups on chitin chains. CDA activity has been described in several Bacteria and Fungi. A cDNA library was constructed from *R. circinans* and several cDNA homologous to CDA sequences from different fungal species were isolated. These cDNA were expressed in the *Pichia pastoris* expression system.

Introduction

Chitin, a polymer of β-(1-4) linked N-Acetyl-Glucosamine, is one of the most abundant biopolymers in nature. It is widely distributed in the exoskeleton of crustaceans and insects, in the cell walls of most fungi and some algae. Chitosan is naturally found in the cell wall of *Zygomycetes* and in the ascospore of *Saccharomyces cerevisiae* (1). It has been demonstrated that chitosan biosynthesis requires the coordinated action of chitin synthase (E.C. 2.4.1.16) and chitin deacetylase (E.C. 3.5.1.41).
Chitin deacetylase (CDA) catalyzes the conversion of chitin into chitosan by deacetylation of N-acetyl-glucosamine residues of nascent chitin chains. CDA activity has been described in several fungi: *Mucor rouxii* (2)*, Colletotrichum lindemuthianum* (4)*, Aspergillus nidulans* (5), *Gongronella butleri* (3). It was suggested that fungal CDA plays an important role in the fungal growth. The aim of this work was the isolation of a CDA cDNA from *Rhizopus circinans* and its heterologous expression in the *Pichia pastoris* system.

Results and discussion

Filamentous fungus *Rhizopus circinans* was grown in a liquid nutrient medium in a rotary shaker at 30 °C for 36 H. Total RNA was isolated and the poly(A)$^+$RNA was purified (Poly-Attract, Promega). The cDNA were prepared from this poly(A)$^+$RNA using the SMART cDNA library construction kit (Clontech). The cDNA were ligated into λTriplEx2 and then the ligation mix was packaged (Gigapack® III Gold Packaging Extract, Stratagene). The library was amplified and had a titer about 9.10^9 pfu/ml.
The library was screened by PCR with degenerate primers. One fragment (fragment RC) was amplified and cloned into pGEM®-T Easy (Promega). The sequence of the insert showed high

homology to reported fungal chitin deacetylases. Deduced amino sequence was compared to chitin deacetylase sequences in GenBank database. 67 % of identity was found with *Mucor rouxii* CDA.
A poly-his tag was added to the N- or C- terminus of the RC cDNA and the modified cDNA was cloned into pPICZ□A expression vector for *Pichia pastoris* (Invitrogen). The transformed cells were selected on the basis of their resistance to Zeocin. Ten transformants of each genetic construct were cultured in liquid medium during 120 h. The supernatant of each transformant was assayed for CDA activity (6). The majority of the transformants showed a high CDA activity (fig. 1). The presence of the tag did not impair the enzyme activity. Moreover CDA with a N-terminal tag displayed higher activity than CDA with C-Terminal tag or without tag.
Immunodetection on western blot with anti-CDA or anti-His antibodies was carried out on transformant supernatants. SDS-PAGE and immunoblotting with anti-CDA antibodies exhibited a signal at ~75 kDa in every analyzed clone. On the other hand, the reactivity against anti-His antibodies showed differences according to tag position. The signal was very low in CDA clones with C-terminal tag whereas it was strong in the CDA clones with N-terminal tag. This observation could be correlated with a partial hydrolysis of the protein C-terminal extremity by yeast proteases.
The recombinant enzyme with an N-terminal poly-his tag was purified by affinity chromatography (HiTrap, GE Healthcare). The enzyme was judged to be electrophoretically homogeneous as tested by SDS-PAGE. The enzyme band migrated at a position corresponding to a molecular mass ~ 75 kDa. The characterization of recombinant CDA is under development.

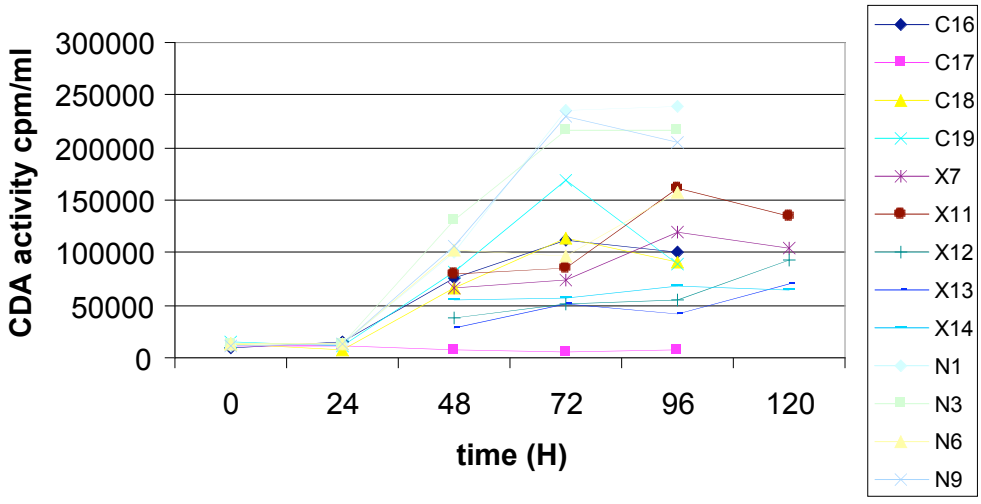

Figure 1 : Chitin deacetylase activity measured by radiometric assay (^{3}H-glycolchitin substrate) in the supernatant. C16-C19 : clones with a C-terminus tag; X7,X11-X13: clones without tag; N1, N3, N6 and N9 : clones with a N-terminus tag

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