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Purification and characterization of chitin deacetylase from *Scopulariopsis brevicaulis*

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Abstract

An extracellular chitin deacetylase from *Scopulariopsis brevicaulis* has been produced using chitin as sole carbon resource of culture medium. The enzyme activity was 10–11 units ml^{-1} culture supernatant after the strain was shaken at 200 rpm and 29 °C for 96 h. The enzyme was purified 74-fold at 38% yield through ammonium sulfate precipitation, and Sephadex G-25, and G-100 column chromatography. The apparent molecular weight of 55 kDa, as determined by SDS-PAGE and gel filtration chromatography, suggested that the enzyme exists as a single component. The enzyme was active on chitooligosaccharides with at least two *N*-acetyl-glucosamine residues, but the activity increased with the number of *N*-acetyl-glucosamine residues. When hexa-*N*-acetylchitohexaose was used as substrate, the optimum pH for enzyme activity was determined to be 7.5, and the optimum temperature was 55 °C. Under these conditions, the activity of enzyme was studied on water-soluble chitosan, chitin from *Aspergillus niger* and shrimp crystalline chitin. The structures of products were characterized by FT-IR, XRD and potentiometric titration. The results indicated that degree of substrate crystallinity had an important effect on enzyme activity. The enzyme had high deacetylating activity on amorphous chitin from *A. niger* mycelium (37% deacylation) and water-soluble chitosan (33%) but low. activity on shrimp crystalline chitin (3.7%).

Keywords: *Scopulariopsis brevicaulis*; Chitin deacetylase; Purification; Characterization

1. Introduction

Chitin, a linear polysaccharide composed of 1,4-linked 2-acetamido-2-deoxy-β-D-glucose units, is one of the most abundant, easily obtained, and renewable natural polymers. It is widely distributed in crustaceans, insects, and in certain fungi (Bartnicki-Garcia, 1968). Up to now, no important industrial applications have been found due to its insolubility in aqueous and organic solvents. Chitosan, the *N*-deacetylated derivative of chitin, is soluble in acid solutions, and has a wide range of uses; such as a natural insecticide (Thome & Van Daele, 1986), an antimicrobial agent (Liu, Du, Wang, Hu, & Kennedy, 2004; Liu, Du, Yang, & Zhu, 2004), a biopolymer for binding metals (Wan, Petrisor, Lai, Kim, & Yen, 2004), or a base for cosmetics (Hirano, 1989). At present, deacetylation of chitin to chitosan is usually achieved by thermo chemical treatment of chitin to remove the acetyl groups. This process uses strong alkali at high temperatures for extended periods of time, so it is environmentally unsuitable and not easily controlled, leading to a broad and heterogeneous range of products (Chang, Tsai, Lee, & Fu, 1997).

Deacetylation can also be achieved enzymically using chitin deacetylase (EC3.5.1.41) under mild conditions (Trudel & Asselain, 1990) which overcome most of disadvantages in the alkali treatment method. Chitin deacetylase had been isolated partially from extracts of many fungi (Araki & Ito, 1975; Kauss, Jeblick, & Yong, 1983; Siegrist & Kauss, 1990) and occurs in some insect species (Arachami, Gowri, & Sundara-Rajulu, 1986). The purification of chitin deacetylase from *Mucor rouxii* (Kafetzopoulos, Martrinou, & Bouriotis, 1993), *Absidia coerulea* (Gao, Katsumoto, & Onodera, 1995) and *Aspergillus nidulans* (Alfonso, Nuero, Santamaria, & Reyes, 1995) have been reported.

A cDNA of the *M. rouxii* encoding chitin deacetylase has been isolated, sequenced and further characterized (Kafezopoulos, Thireos, Vournakis, & Bouriotis, 1995). The chitin

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deacetylase from the *M. rouxii* and *A. coerulea* exhibited similar molecular weights (75 kDa), amino-terminal sequences, optima pH's (4.5) and optima temperatures (50 °C), and substrate specificities (four to six sequential *N*-acetylglucosamine residues). The CAD from *A. nidulans* exhibited different molecular weight (27 kDa), optimum pH (7.0), and substrate specificity (glycol chitin and oligomers of *N*-acetylglucosamine). Recently Martinou, Koutsiouli, and Bouriotis (2003) succeeded in cloning the chitin deacetylase gene from *Saccharomyces cerevisiae* and expressing it in *Escherichia coli.* The resultant chitin deacetylase apparent molecular weight, optimum temperature and pH were 43 kDa 50 °C and 8.0, respectively (Martinou, Koutsioulis, & Bouriotis, 2002). However, until now, name of these enzymes can be applied to the production of chitosan from chitin in the industrial field yet, due to very limited extent of deacetylation on such macromolecular substrates. Thus, finding a chitin deacetylase with high activity against the polysaccharide still is a very interesting quest.

In this paper, an extracellular chitin deacetylase from *Scopulariopsis brevicaulis* was produced using chitin as sole carbon resource in the culture medium, and isolated and purified by a very simple method. Its properties such as optimum temperature, thermal stability, pH, molecular weight and substrates were investigated, and its effect of deacetylation was evaluated with shrimp crystalline chitin, chitin from *Aspergillus niger* and water-soluble chitosan.

2. Materials and methods

2.1. Materials

S. brevicaulis was isolated from the soil around the chitin factory in Jinan of china. The soil around the chitin factory has plenty of chitin, and so those microorganisms producing chitin deacetylase have greater scope for growth in this soils. *N*-acetylchitooligosaccharides (dimer, trimer, tetramer, pentamer and hexamer) and 3-methyl-2-benzothiazolinonehydrazone were purchased from Sigma (St Louis, USA). Sephadex G-25, G-75 and Sephadex G-100, were purchased from Pharmacia (Uppsala, Sweden). *N*-acetyl-D-glucosamine and shrimp crystalline chitin were purchased from Liao Nin Xin Die Biotechnology Co. Ltd (Dalian, China). D-Glucosamine·HCl was purchased from Zhejiang Aoxing Biotechnology Co. Ltd (Taizhou, China). Molecular weight markers were purchased from Bio-Rad (Hercules, USA). Water-soluble chitosan (54% degree of deacetylation with an average molecular weight of 280,000) and chitin from *A. niger* (21% degree of deacetylation with an average molecular weight of 120,000) were prepared in our laboratory based on the method in the literature (Synowiecki & Al-Khateeb, 1997). All other chemicals used were commercial products of analytical grade.

2.2. Organism and culture conditions

S. brevicaulis was cultivated on agar slants [sucrose (3%), $NaNO_3$ (0.2%), K_2HPO_4 (0.1%), KCl (0.05%), $MgSO_4$, (0.05%)] for 72 h at 28 °C. The cells were harvested into sterile water. This suspension (6 ml, containing 5.0×10^8 cells ml^{-1}), was inoculated into a 500 ml Erlenmeyer flask containing liquid medium (80 ml, $NaNO_3$, 0.16 g; K_2HPO_4, 0.08 g; KCl, 0.04 g; $MgSO_4$, 0.04 g; peptone, 0.08 g; chitin, particle size less than 100 mesh, 0.4 g). The flask was shaken at 200 rpm and 29 °C for 96 h.

2.3. Preparation of chitin deacetylase

After incubation, and working throughout at 4 °C, the culture (120 ml) was centrifuged at $10{,}000\times g$ for 30 min. The supernatant was collected and mycelium (4 g) was homogenized in a cold mortar with sterilized silica powder (60 mesh, 6 g) to break the cells; the supernatant of the culture was added to the homogenate to form a suspension. The suspension was centrifuged at $10{,}000\times g$ for 30 min, and the supernatant was used as the crude extract. Ammonium sulfate was added to the crude extracts to 35% saturation. After 120 min, the solution was centrifuged at $18{,}000\times g$ for 30 min. Ammonium sulfate was added to the supernatant to 80% saturation again. After 18 h, the precipitated proteins were collected by centrifugation at $20{,}000\times g$ for 30 min.

The precipitated proteins were dissolved in distilled water (10 ml). The insoluble material was removed by centrifugation at $20{,}000\times g$ for 30 min and the supernatant was then applied to a Sephadex G-25 column (16 mm diam × 80 cm). The column was eluted with 50 mM Tris–HCl, pH 7.5. The fractions were monitored by UV assay (at 280 nm) and for chitin deacetylase and those enzymically active were combined and applied to a Sephadex G-100 column (16 mm diam × 60 cm). The column was eluted with 50 mM Tris–HCl, pH 7.5, monitoring the fractions (5 ml) at 280 nm. The fractions containing chitin deacetlyase activity were combined and dialyzed overnight against distilled water (5 l). After dialysis, the enzyme was lyophilized with and suspended in distilled water again (5 ml).

2.4. Enzyme activity assay

A chitin deacltylase enzyme activity assay based on the literature method (Kauss & Bauch, 1988; Martinou, Kafetzopoulos, & Bouriotis, 1995; Ride & Drysdale, 1972) was performed in glass tubes using 50 mM Tris–HCl buffer pH 7.5 (50 μl), hexa-*N*-acetylchitohexaose (100 μg) in water (100 μl), and enzyme preparation (50 μl). Incubation time was 15 min at 55 °C, and the reaction was terminated by the addition of 5% (w/v) $KHSO_4$ (250 μl).

For color formation 5% w/v $NaNO_2$ (250 μl) was added, and the tubes capped immediately and allowed to stand with occasional shaking for 15 min, and 12.5% w/v aqueous 3-methy1-2-benzothiazolinonehydrazone hydrochloride (250 μl, freshly prepared each day) added and the mixture heated at 100 °C for 3 min. After cooling to room temperature, 0.5% w/v $FeCl_3$ (250 μl) was added and the developing color was read after 30 min at 650 nm. Standard curves were prepared with D-glucosamine·HCl standard (0–6 μg).