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Purification and characterization of two types of chitosanase from *Aspergillus* sp. CJ22-326

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Abstract

Aspergillus CJ22-326, a fungi strain capable of utilizing chitosan as a carbon source, was isolated from soil samples. Two types of chitosanase (ChiA and ChiB) produced from the culture supernatant of *Aspergillus* CJ22-326 were purified to an apparent homogeneity identified by SDS–PAGE through ammonium sulfate precipitation, CM-Sepharose FF chromatography, and Sephacryl S-200 gel filtration. Molecular weights of the enzymes were 109 kDa (ChiA) and 29 kDa (ChiB). Optimum pH values and temperature of ChiA were 4.0 and 50 °C, respectively, those of ChiB were 6.0 and 65 °C. The enzyme activities of ChiA and ChiB were increased by about 0.5-fold and 1.5-fold, respectively, by the addition of 1 mM Mn^{2+}. However, 2.5 mM Ag^{+}, Hg^{2+} and Fe^{3+} strongly inhibited ChiA and ChiB activities. Viscosimetric assay and analysis of reaction products of these enzymes, using chitosan as a substrate, by TLC indicated endo- and exo-type cleavage of chitosan by ChiB and ChiA, respectively. ChiB catalysed the hydrolysis of glucosamine (GlcN) oligomers larger than pentamer, and chitosan with a low degree of acetylation (0–30%), and formed chitotriose with chitohexaose as the major products. ChiA released a single glucosamine residue from chitosan and glucosamine oligomers. Both of the activities of ChiA and ChiB increased with the degree of deacetylation of chitosan. The enzyme ChiB had a useful reactivity and a high specific activity for producing functional chitooligosaccharides with high degree of polymerization.

Keywords: Chitosanase; Chitooligosaccharides; *Aspergillus*; Chitosan; Endo-type; Exo-type

1. Introduction

Chitosan that have different degrees of deacetylation (DDA) can be readily obtained by *N*-deacetylating chitin that is extracted from an abundant natural source, shrimp and crab shells. It has high safety and peculiar physical properties because of its high cation content. Its biological properties, like compatibility and antimicrobial activity, are remarked. Actually, chitosan is applied widely to health foods such as for the prevention and treatment of hyperuricemia, and as an antimicrobial agent, preservative agent and edible film. But there is doubt about their level of absorption in the human intestine, and their high molecular weights and highly viscous nature may restrict their uses in in vivo systems. Recently much attention has been paid to converting chitosan to safe and functional chitooligosacchrides, because chitooligosaccharides with high degrees of polymerization (DP), especially those with six residues or more, show strong physiological activities, such as antitumor activities (Suzuki et al., 1986; Suzuki, Matsumoto, Tsukada, Aizawa, & Suzuki, 1989), immuno-enchancing effects (Suzuki, 1996), enhancing protective effects against infection with pathogens in mice (Tokoro, Kobayashi, Tatekawa, Suzuki, & Suzuki,

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1989; Yamada, Shibuya, Kodama, & Akatsuka, 1993), antifungal activity (Hirano & Nagao, 1989; Kendra, Christian & Hadwiger, 1989) and antimicrobial activity (Jeon, Park, & Kim, 2001; Savard, Beaulieu, Boucher, & Champagne, 2002; Uchida, Izume, & Ohtakara, 1989). Chitooligosaccharide functions have led to progressively increased utilization in the food and pharmaceutical fields for human health.

Chitooligosaccharides can be made by hydrolyzing chitosan with chitosanase. Chitosanase (EC 3.2.1.132) catalyses the hydrolysis of the glycosidic bonds of chitosan, and has been found in a variety of microorganisms, including bacteria (Akiyama et al., 1999; Chiang, Chang, & Sung, 2003; Omumasaba, Yoshida, Sekiguchi, Kariya, & Ogawa, 2000; Yoon et al., 2000), actinomycetes (Boucher, Dupuy, Vidal, Neugebauer, & Brzezinski, 1992; Sakai, Katsumi, Isobe, & Nanjo, 1991), and fungi (Shimosaka, Nagawa, Ohno, & Okazaki, 1991; Zhang et al., 2000). Chitosanases from individual organisms differ in their hydrolytic action pattern. However, most chitosanases from the isolated microorganisms intend to make dimers, trimers and tetramers rather than oligomers above DP 4, so the utility of these enzymes is not good. To obtain a novel chitosanase which could be used for large-scale production of chitosan oligomers above DP 4, we screened various types of microorganisms. An *Aspergillus* CJ22-326 produced high chitosanase activities when grown on medium with wheat bran and chitosan as carbon source. In this paper, we describe purification and characterization of two different chitosanolytic enzymes, and one of the endo-type chitosanase ChiB is potentially valuable for industrial applications for producing functional chitooligosaccharides.

2. Materials and methods

2.1. Strain and culture conditions

The strain used in this study is one of the fungal strains isolated from marine soil in China. The culture medium composed of 1.0% soluble chitosan, 2.0% wheat bran, 0.2% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, and 0.05% $MgSO_4$ per liter (pH 5.6). The cultivation was carried out in a 500 ml baffle flask with 150 ml of the culture medium at 30 °C for 96 h with agitation at 150 rpm.

2.2. Chemicals and substrates

CM-Sepharose FF, Sephacryl S-200 and Phenyl CL-4B were from Pharmacia. Chitosan and chitin were purchased from the local suppliers in China. Glucosamine (GlcN), chitobiose $(GlcN)_2$, chitotriose $(GlcN)_3$, chitotetraose $(GlcN)_4$, chitopentose $(GlcN)_5$ chitohexaose $(GlcN)_6$ were purchased from Seikagaku Co. All other reagents were of analytical grade.

2.3. Enzyme purification

Ammonium sulfate precipitation – After cultivation, the cells were removed from the medium by centrifugation at 4000 rpm for 30 min. Solid ammonium sulfate was added to the culture filtrate to 80% saturation. After standing overnight, the precipitate was collected by centrifugation and dissolved in 50 ml of 20 mM sodium acetate buffer, pH 5.6 (buffer A). The enzyme solution was dialyzed against the same buffer.

Chromatography on CM-Sepharose FF – The dialyzed solution was put on a CM-Sepharose FF column (2.6×50 cm) that had been equilibrated with 20 mM sodium acetate buffer. The column was washed with the same 500 ml buffer, and then eluted with a 600 ml linear gradient of 0–1.0 M NaCl in buffer A, at a flow rate of 30 ml/h, and 5 ml fractions were collected. The two active fractions were pooled and concentrated separately by ultrafiltration.

Gel filtration on Sephacryl S-200 – Fractions from each peak were pooled separately and put on a Sephacryl S-200 column (2.0×100 cm) equilibrated with buffer A containing 0.1 M NaCl (buffer B). The proteins were eluted with buffer B at a flow rate of 12 ml/h, and the resulting active fractions were collected and used as the purified enzyme preparations throughout this study.

All purification steps were performed at 4 °C.

2.4. Enzyme assay

Unless indicated otherwise, chitosan with a DDA of 83% was used as the substrate in the chitoanase assay. The incubation mixture contained 1 ml of 0.5% soluble chitosan and 1 ml of diluted enzyme solution (pH 5.6). The incubation was carried out at 37 °C for 15 min, with shaking. The amount of reducing sugar in the supernatant was measured using the modified dinitrosalicyclic acid (DNS) method (Miller, 1959). One enzyme unit was defined as the amount of enzyme required to produce 1 μmol of reducing sugar as glucosamine per min.

2.5. Protein assay and electrophoresis

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as the standard. During purification, protein was estimated by measuring absorbance at 280 nm, with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis (SDS–PAGE) was used to determine protein purity and the molecular mass of the purified enzyme under denaturing conditions using a 12% acrylamide gel, as described by Laemmli (1970). Protein was stained by Coomassie blue.