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Molecular cloning and characterization of a chitosanase from the chitosanolytic bacterium *Burkholderia gladioli* strain CHB101

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**Abstract** A chitosanase was purified from the culture fluid of the chitino- and chitosanolytic bacterium *Burkholderia gladioli* strain CHB101. The purified enzyme (chitosanase A) had a molecular mass of 28 kDa, and catalyzed the endo-type cleavage of chitosans having a low degree of acetylation (0–30%). The enzyme hydrolyzed glucosamine oligomers larger than a pentamer, but did not exhibit any activity toward *N*-acetylglucosamine oligomers and colloidal chitin. The gene coding for chitosanase A (csnA) was isolated and its nucleotide sequence determined. *B. gladioli* csnA has an ORF encoding a polypeptide of 355 amino acid residues. Analysis of the N-terminal amino acid sequence of the purified chitosanase A and comparison with that deduced from the *csnA* ORF suggests post-translational processing of a putative signal peptide and a possible substrate-binding domain. The deduced amino acid sequence corresponding to the mature protein showed 80% similarity to the sequences reported from *Bacillus circulans* strain MH-K1 and *Bacillus ehimensis* strain EAG1, which belong to family 46 glycosyl hydrolases.

**Introduction**

Chitosan, a deacetylated derivative of chitin (a linear polysaccharide of β-1,4-linked N-acetylglucosamine residues), has been found in the cell walls of a limited group of fungi belonging to the order Mucorales in nature (Bartnicki-Garcia 1968). Chitosan molecules having different degrees of acetylation (D.A.) can be obtained by chemical deacetylation of chitin extracted from abundant biomasses, such as shrimp or crab shells. Much attention has been paid to low-molecular-weight chitosan oligomers because of their beneficial biological activities, e.g. their inhibitory effect on the growth of fungi and bacteria (Allan and Hadwiger 1979; Hirano and Nagano 1989) and their ability to induce phytoalexin production in higher plants (Kendra et al. 1989). Chitosanases that catalyze the endo-type cleavage of chitosan polymers are potentially useful in the large-scale production of chitosan oligomers.

Chitosanases have been reported from various bacteria and fungi. Most of the bacterial chitosanases are induced by the substrate chitosan and play a role in the degradation and utilization of exogenous chitosan. We previously isolated a bacterial strain from soil that could assimilate chitosan as sole carbon source and tentatively identified the strain as a member of the genus *Acinetobacter* (Shimosaka et al. 1995). The strain secreted enzymes responsible for the degradation of both chitin and chitosan, even in the absence of chitin-related compounds in the growth media. This apparent constitutive production of enzymes can be exploited for the industrial production of chitin and chitosan oligomers.

Two enzymes, chitosanases I and II, catalyze the endo-type cleavage of chitosan having a moderate D.A. (30%) and have been previously purified and characterized from the culture fluid of *Burkholderia gladioli* strain CHB101 (Shimosaka et al. 1995). To clarify the mechanism of degradation and utilization of both chitin and chitosan by *B. gladioli* strain CHB101, characterization of all of the enzymes capable of degrading chitin-related compounds is required. Here, we describe the molecular cloning and characterization of a novel chitosanase that efficiently degrades chitosans having a low D.A. (0–30%), and we discuss a potential use for the enzyme in the production of chitosan oligomers.
Materials and methods

Strain and media

The bacterial strain used in this work was isolated from soil and was tentatively assigned to the genus *Acinetobacter* (Shimosaka et al. 1995). We identified and renamed the strain *Burkholderia gladioli* strain CHB101 after precise taxonomical analysis, as described later. For the purification of chitosanase, CHB101 cells were grown in M9 synthetic medium, as described previously (Shimosaka et al. 1995), using 0.2% glucose as sole carbon source. Chromosomal DNA was prepared from the cells grown in nutrient broth (1% peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.0).

Chitosanase assay

Chitosanase was assayed by measuring the reducing sugars liberated during the hydrolysis of chitosan (D.A. 30%) as described previously (Shimosaka et al. 1995). One unit of activity was defined as the amount of enzyme catalyzing the production of 1 µmol of the reducing sugar per min using glucosamine (GlcN) as the standard. The products of enzymatic hydrolysis of the GlcN oligomer or of fully deacetylated chitosan (D.A. 0%) were analyzed by thin-layer chromatography by the method of Sakai et al. (1991). A viscometric chitosanase assay was performed according to the method of Ohtakara (1988).

Purification of chitosanase

The supernatant of a 2-L culture at the early stationary phase was obtained and used as the enzyme source. Ammonium sulfate was added to the culture supernatant to achieve 80% saturation, and the resultant precipitate was collected by centrifugation and dissolved in 50 ml of 20 mM sodium acetate buffer, pH 5.6 (buffer A). After desalting by dialysis against buffer A, the sample was loaded onto a CM-Sepharose CL-6B (Pharmacia) column (2.2 × 25 cm) equilibrated with buffer A. Washing the column with a linear gradient of buffer A containing 0–1 M KCl resulted in the elution of proteins with chitosanase activity in a single peak. The active fractions were collected, desalted by dialysis, and applied again to a CM-Sepharose CL-6B column. The active enzyme was eluted with a linear gradient of buffer A containing 0–0.7 M KCl and characterized as the purified enzyme.

Amino acid sequencing

The N-terminal amino acid sequence of the purified chitosanase was determined using an automated protein sequencer (Shimadzu PPSQ-21).

General DNA manipulation

Standard DNA recombination techniques were used for DNA manipulation (Sambrook et al. 1989). To isolate chitosanase genes from *B. gladioli* strain CHB101, chromosomal DNA was partially digested with *Sau3A1*, and fragments > 5 kb in size were collected by sucrose density gradient centrifugation. The resultant DNA fragments were ligated to the plasmid vector pUC119, previously cut with *BamHII* and dephosphorylated. The ligation reaction mixture was used to transform *Escherichia coli* JM109. Transformant clones expressing chitosanase activity were selected on Luria broth (LB) color selection plates containing 0.25% chitosan (D.A. 30%) with ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG). The nucleotide sequence was determined by the dideoxy termination method using a Shimadzu DNA sequencer DSQ-2000L. Sequence data were analyzed with the Genetyx software (Software Development). The nucleotide sequence of chitosanase A has been deposited in the DDBJ/GenBank/EMBL database under accession number AB029336.

Chemicals

Chitosan 10B (D.A. 0%) and chitosan 7B (D.A. 30%) were obtained from Funakoshi. Glycol chitosan (degree of polymerization ≥400) and GlcN oligomers (dimer to hexamer) were purchased from Wako Junyaku.

Results

Taxonomic analysis

*Burkholderia gladioli* strain CHB101 had been tentatively identified as a member of the genus *Acinetobacter* by brief taxonomic tests; however, we found that the G+C content of DNA (67%) obviously differed from the reported value for the genus *Acinetobacter* (38–47%). Precise taxonomic analysis was then done based on Bergey’s Manual of Systematic Bacteriology, and the strain could be identified as *Burkholderia gladioli* (Yabuuchi et al. 1992). This taxonomic identification was based on the following criteria: the organisms were gram-negative, rod-shaped with polar flagella, showed no spore formation, were motile, aerobic, oxidase-positive, catalase-positive, arginine-dihydorolase-negative, reduction-of-nitrate-negative, gelatin-liquefaction-positive, starch-hydrolysis-negative, denitrification-negative, diffusible-pigment-production-positive, poly-β-hydroxypabutyrate-accumulation-positive, or*tho-cleavage-of-protocatechuic-positive, utilized D-xylose, D-tartrate, and mesaconate but did not utilize L-rihamnose, levulinate, 2,3-butylen glycol, tryptamine, and Q-8 quinone; the mol% G + C of DNA was 67%. Thus, we hereafter refer to the strain as *B. gladioli* strain CHB101.

Purification of chitosanase A

*Burkholderia gladioli* strain CHB101 secreted chitin- and chitosan-degrading enzymes in a synthetic medium containing glucose or N-acetylglucosamine (GlcNAc) as sole carbon source. We previously purified two major enzymes that hydrolyzed chitosan with a moderate D.A. (30%) in an endo-type manner; however, neither enzyme could hydrolyze fully deacetylated chitosan (D.A. 0%) to even the slightest extent, even after a prolonged reaction. The production of a third enzyme that preferentially hydrolyzed chitosan (D.A. 0%) was expected, since the crude proteins in the culture fluid hydrolyzed chitosan (D.A. 0%) into a mixture of GlcN dimers and trimers. When crude enzymes were applied to a CM-Sepharose CL-6B column and washed with 20 mM sodium acetate buffer (pH 5.6), two proteins corresponding to the previously purified enzymes were eluted from the column. Successive washing with a linear gradient of KCl resulted in the elution of a third protein capable of degrading chitosan (D.A. 0%). We purified this enzyme to homogeneity by PAGE analysis for proteins (Fig. 1).