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ORIGINAL PAPER

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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 $\beta$-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.

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## 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 *Sau*3A1, 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 *Bam*HI 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*-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*-hydroxybutyrate-accumulation-positive, *ortho*-cleavage-of-protocatechuate-positive, utilized D-xylose, D-tartrate, and mesaconate but did not utilize L-rhamnose, levulinate, 2,3-butylene 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).

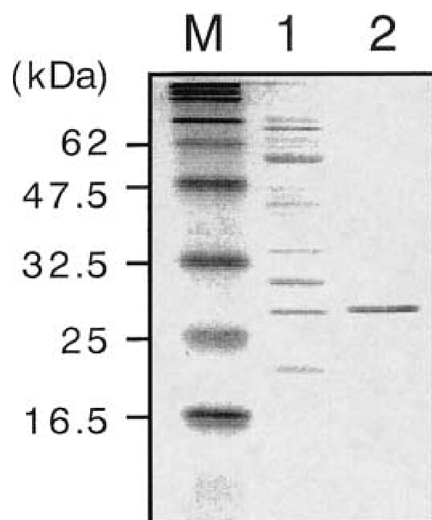


**Fig. 1** Analysis of the purified chitosanase A by SDS-PAGE. Proteins from culture fluid (*lane 1*) and the purified chitosanase A (*lane 2*) were electrophoresed and stained with Coomassie brilliant blue. Molecular mass markers (*lane M*) were broad-range protein markers (New England Biolabs)

The overall purification was 80-fold, with recovery of 1.2% of the original activity (Table 1). The molecular mass of the purified enzyme was estimated to be 28 kDa by SDS-PAGE. The purified enzyme eluted from Sephadex G-100 gel sieving corresponded to the position of the protein with the estimated molecular weight by SDS-PAGE, indicating that it was monomeric. The N-terminal amino acid sequence of the purified protein was determined to be $NH_2$-ALDHDANFSPATLQFL-KDNTGLDGE. We named the enzyme chitosanase A after characterization of the kinetic properties as described below.

## Kinetic properties of chitosanase A

*Effects of pH and temperature on activity.* Chitosanase A exhibited the same level of activity over a broad pH range (pH 5–7.5) when glycol chitosan was used as the substrate. The enzyme exhibited maximum activity at 55 °C under standard assay conditions toward chitosan (D.A. 30%) in sodium acetate buffer (pH 5.6).

*Substrate specificity.* The activity of chitosanase A toward various substrates is summarized in Table 2. It effectively degraded chitosans having a low D.A. (0–30%), but did not hydrolyze glycol chitin, colloidal chitin or carboxymethyl cellulose.

*Reduction in the viscosity of chitosan solution.* Chitosanase A had the ability to drastically reduce the viscosity of chitosan (D.A. 30%) solution (Fig. 2).

*Analysis of the reaction products.* The reaction products were analyzed by thin-layer chromatography after a prolonged reaction, using chito-oligosaccharides (GlcN oligomers) as the substrates (Fig. 3). The enzyme could not hydrolyze chitobiose, chitotriose, or chitotetraose. Chitopentaose was hydrolyzed to chitobiose and chitotriose. Chitohexaose was hydrolyzed to a mixture of chitobiose, chitotriose and chitotetraose. When chitosan (D.A. 0%) was used as the substrate, a mixture of chitobiose, chitotriose and chitotetraose was produced after prolonged reaction. The enzyme did not hydrolyze *N*-acetyl-chito-oligosaccharides (GlcNAc oligomers) from dimers to pentamers to even the slightest extent (data not shown).

## Isolation of the chitosanase A gene (*csnA*) and sequence analysis

The gene (*csnA*) encoding chitosanase A was isolated by expression screening of a plasmid library constructed in *E. coli* on selection plates containing chitosan (D.A. 30%). Twenty positive clones exhibiting a clear zone around the white colony were selected out of total of 20,000 clones tested. After analysis of these clones by restriction endonuclease digestion, 18 were found to contain a common DNA fragment. One of the clones was chosen for further analysis, and a precise restriction map of the insert was constructed (Fig. 4). Construction of subclones revealed that the 1.7-kb *Eco*RI–*Sal*I fragment was the minimum region necessary for clear zone formation on chitosan-containing plates. The nucleotide sequence of this region was determined by combining the partial sequences of both strands from various deleted clones.

The determined nucleotide sequence contains a single ORF encoding a protein of 355 amino acids with a calculated molecular mass of 37,600 Da (Fig. 5). The N-terminal amino acid sequence determined from the purified chitosanase A coincides precisely with the sequence starting from Ala-96 in the deduced amino acid sequence of the *csnA* ORF. The preceding 95 amino acid residues are probably removed by post-translational processing. Removal of these 95 residues would yield a protein of 260 amino acids with a calculated molecular mass of 28,200 Da, which is in good agreement with the molecular mass of purified chitosanase A (28 kDa). The

**Table 1** Purification of chitosanase A from culture fluid of *Burkholderia gladioli* CHB101

| Step | Total activity[a] (U) | Total protein (mg) | Specific activity (U/mg) | Purification (-fold) | Recovery (%) |
|---|---|---|---|---|---|
| Culture fluid | 386 | 190 | 2.0 | 1 | 100 |
| 0–80% $(NH_4)_2SO_4$ | 149 | 6.5 | 23 | 12 | 39 |
| 1st CM-Sepharose | 13.5 | 0.16 | 84 | 42 | 3.5 |
| 2nd CM-Sepharose | 4.8 | 0.03 | 160 | 80 | 1.2 |

[a] Chitosan (D.A. 30%) was used as substrate for the enzyme assay

**Table 2** Substrate specificities of *B. gladioli* chitosanase A

| Substrate[a] | Relative activity (%)[b] |
| --- | --- |
| Chitosan (D.A. 30%) | 100 |
| Chitosan (D.A. 0%) | 80 |
| Glycol chitosan | 46 |
| Glycol chitin | < 1 |
| Colloidal chitin | < 1 |
| Carboxymethyl cellulose | < 1 |

[a] Each reaction mixture contained substrate at a concentration of 0.25%
[b] The amount of reducing sugar liberated after a 15-min reaction relative to the largest amount

putative −35 and −10 regions, which showed homology with the consensus sequence of typical *E. coli* promoters, were found upstream of the initial codon of the *csnA* ORF. Chromosomal DNA of *B. gladioli* CHB101 was digested with various restriction endonucleases and subjected to Southern blot analysis using the *csnA* gene (1.7-kb *Eco*RI–*Sal*I fragment) as the probe. A single band was detected for almost all digestions, indicating that *B. gladioli* CHB101 possesses only one copy of *csnA* (data not shown).

## Discussion

A chitosanase was purified from the culture fluid of the chitino- and chitosanolytic bacterium *B. gladioli* strain CHB101. The purified chitosanase A catalyzed the endo-type cleavage of chitosans with a low D.A. (0–30%), judging by the rapid decrease in the viscosity of the chitosan solution (Fig. 2) and the production of a mixture of chitobiose to chitotetraose after prolonged reaction (Fig. 3). *B. gladioli* chitosanase cleaves the bonds between two GlcN residues and also between GlcN and GlcNAc residues of the chitosan polymer, since the enzyme hydrolyzed chitosan (D.A. 30%) to a higher extent than chitosan (D.A. 0%) (Table 2). Most microbial chitosanases have been reported to show preference for substrates with a certain degree of acetylation.

We previously reported the purification and characterization of two enzymes (chiotsanases I and II) catalyzing the endo-type cleavage of chitosan with a moderate D.A. (30%) from a *B. gladioli* CHB101 culture fluid (Shimosaka et al. 1995). These two enzymes were produced in an apparently constitutive manner (even in a medium not containing any chitin or chitosan) and

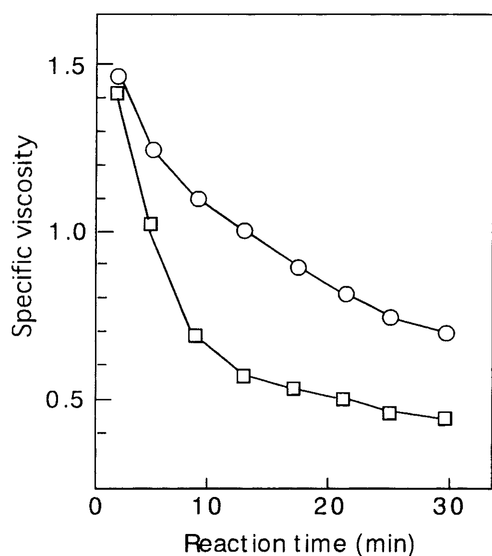


**Fig. 2** Reduction in the viscosity of chitosan solution. The reaction mixture (7 ml), consisting of 0.1% chitosan (D.A. 30%) in 25 mM sodium acetate buffer (pH 5.6) with various amounts of the purified chitosanase A, was kept at 37 °C in an Ostwald viscosimeter (Shibata model 1). The flow time of the mixture was determined at appropriate intervals. Specific viscosity = (the flow time of the reaction mixture/the flow time of distilled water)−1. *Open circles* 5 mU, and *open squares* 20 mU of the purified chitosanase A

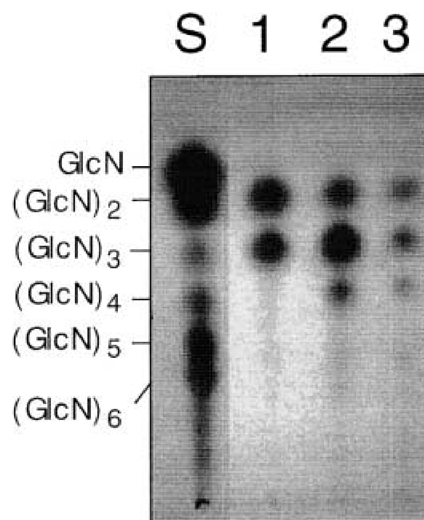


**Fig. 3** Analysis of enzymatic hydrolysates by thin-layer chromatography. The substrates chitopentaose (*lane 1*), chitohexaose (*lane 2*) and chitosan D.A. 0% (*lane 3*) were hydrolyzed completely with the purified chitosanase A. *Lane S* contained a mixture of chitooligosaccharides ranging from glucosamine (GlcN) to chitohexaose $(GlcN)_6$

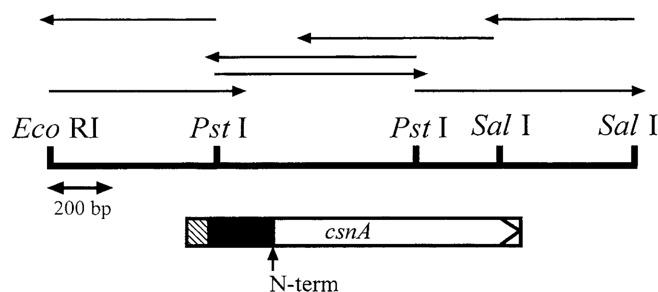


**Fig. 4** Restriction map of the DNA fragment containing *csnA*. *Box* represents the *csnA* ORF with the direction of transcription. *Hatched box* indicates a plausible signal peptide. *Closed box* indicates the region that is homologous to the chitin-binding domain of bacterial chitinases. *N-term* is the position of the N-terminus of the purified chitosanase A. The determined sequences shown by *arrows* were combined to obtain the entire nucleotide sequence of *csnA*

**Fig. 5** Nucleotide sequence and deduced amino acid sequence of *csnA*. *Box* indicates the amino acid sequence determined from the purified chitosanase A using the protein sequencer. Two sequences similar to promoter consensus sequences (−35 and −10) are *underlined*

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   1  TTCGTTATCGCGGATTTCCCGGCGTTGTATCCCGATCGGCTGCCACCCGGCCGATCGAGTCGAACGCCGAGTTTCAGGTCGTCACAAAAA   90
  91  GACGCTGTATCCGGCCAGCCTGGCCGGGCGGGACGGTCTTGCACCGCCTGCCTTCCGCATCATTCCGCCATGCCGAACCGTTTGTGCGCC  180
 181  GATTTTGAATCGCCGCGCAAGGGGGACCGTTTTGCCTGGATTTCAGTAAATCGAGTTCGGTGCGAACGATTGAGCGGTGCATAAAACAAT  270
 271  GAAGCAACTCGCGTGGTTCGTTTGTCTGTCGAAAATAATTAACCGGAGAATTTCATCATGAAAATCGTCTCGACGAAATGCTGGATTCAT  360
 361  GCCGCGCTCTGCGCGGCCGTGATGGCGACCCTGCCGCTGGCCGCGGCCGACGCCTCGGCGCGCACCGTGCACGCGGCGCGCGCCCCGCAA  450
   1                      M  A  T  L  P  L  A  A  A  D  A  S  A  R  T  V  H  A  A  R  A  P  Q    23
 451  GCGGCCGCTGCCGCCGCCTCGCCCGAAATCTGCAGCTCGCCCTGGACGGCAGCGCGCATCTACGAAGCCGGCAACGTGGTCAGCTTCGAC  540
  24  A  A  A  A  A  A  S  P  E  I  C  S  S  P  W  T  A  A  R  I  Y  E  A  G  N  V  V  S  F  D    53
 541  GGCCACGACTATACGGCCGCCTACCGCAGCCAGGGCAACGCGCCCGCGACGGCCAGCGGCGAGGCCGGCAGCGGCCAGCCCTGGGTGGCC  630
  54  G  H  D  Y  T  A  A  Y  R  S  Q  G  N  A  P  A  T  A  S  G  E  A  G  S  G  Q  P  W  V  A    83
 631  GGCGAGGCCTGCAAGCCGGCCAAGCTGAGCAAGGCGGCGCTCGACCACGACGCCAACTTCTCGCCCGCCACGCTGCAATTCCTGAAGGAC  720
  84  G  E  A  C  K  P  A  K  L  S  K  A [A  L  D  H  D  A  N  F  S  P  A  T  L  Q  F  L  K  D   113
 721  AACACCGGTCTGGACGGCGAGCAGTGGGACAACATCATGAAGCTGGTCAACAAGCCGGAGCAGGATTCGCTCGACTGGACCAAGTTCTAC  810
 114  N  T  G  L  D  G  E] Q  W  D  N  I  M  K  L  V  N  K  P  E  Q  D  S  L  D  W  T  K  F  Y   143
 811  GGCTACTGCGAGGACATCGGCGACGATCGCGGCTACACCATGGGCATCTTCGGCGCCACCACGGGCGGGCCGAACGACGGCGGCCCGGAC  900
 144  G  Y  C  E  D  I  G  D  D  R  G  Y  T  M  G  I  F  G  A  T  T  G  G  P  N  D  G  G  P  D   173
 901  GGCCCGGCCCTGTTCAAGGCCTACGACGCGGCCAGCGGCGCCAGCAATCCCTCGGTGCAGGGCGGCCTGGCGCGGATCGGCGCGCACGGC  990
 174  G  P  A  L  F  K  A  Y  D  A  A  S  G  A  S  N  P  S  V  Q  G  G  L  A  R  I  G  A  H  G   203
 991  TCGATGCAGGGTTCGATCCTGAAGATCACCGACAGCGAGAAGGTGTTCTGCGGCAAGGTCAAGGGCCTGCAGAACGACGCGGCCTGGCGC 1080
 204  S  M  Q  G  S  I  L  K  I  T  D  S  E  K  V  F  C  G  K  V  K  G  L  Q  N  D  A  A  W  R   233
1081  GAGGCGATGTGGCGCACGTTCTACTCGGTCTACATCCAGTACAGCGTGCAGCAGGCGCGGTCGCGCGGCTTCGGCAGCGCACTGACGATC 1170
 234  E  A  M  W  R  T  F  Y  S  V  Y  I  Q  Y  S  V  Q  Q  A  R  S  R  G  F  G  S  A  L  T  I   263
1171  GGCTCCTTCGTCGATACGGCCTTGAACCAGGGCGCCGACGGCGGTAGCAACACGCTGCAAGGTCTGCTCTCGCGCTCGGGCAACAGCACC 1260
 264  G  S  F  V  D  T  A  L  N  Q  G  A  D  G  G  S  N  T  L  Q  G  L  L  S  R  S  G  N  S  T   293
1261  GACGAGAAGACCTTCATGACGAGCTTCTACGCGCAGCGCACCAAGGTGGTCGACACGCACGACTTCAACCAGCCGCCCAACGGCAAGAAC 1350
 294  D  E  K  T  F  M  T  S  F  Y  A  Q  R  T  K  V  V  D  T  H  D  F  N  Q  P  P  N  G  K  N   323
1351  CGGGTGAAGCAGTGGAGCACCCTGATGAGCCAGGGCATCACCAGCCTCAAGAACTGTGACGCCGATATCGTCAAGGTGACCAGCTGGACG 1440
 324  R  V  K  Q  W  S  T  L  M  S  Q  G  I  T  S  L  K  N  C  D  A  D  I  V  K  V  T  S  W  T   353
1441  ATGAAGTAAGTCCGGGGCGGCGCCGGCCGCCGCGAGCGCGCCCGGGAGCCGCCCACTATCTGGGCGCCTCGCACGCGCTTTTGCATTTCC 1530
 354  M  K  *                                                                                      355
1531  GGGCCGGACCGTGTATCAGAGCGAAACGGCGTCGGCGCCGGCGCCGCCCGGCCGCATCGGCGGCCGGTGGCGGCACATGGCGCCGGCCGA 1620
1621  TCCTCAACCGCCAGCCGACCGAAATCCGATGACCGACACGACTGCCCAGCCCGCCCCGAACGCCACCGCCACCCTGCTGGTCGAC  1705
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comprised more than 90% of the total activity in the culture fluid when assayed using chitosan (D.A. 30%) as the substrate. The novel chitosanase A purified in this work was also found to be produced in an apparently constitutive manner, albeit as a minor component (less than 10% of the total activity). This is the reason why the final yield of purified chitosanase A from the culture fluid was remarkably low (1.2%; Table 1). It is noteworthy that chitosanase A could hydrolyze fully deacetylated chitosan (D.A. 0%), chitopentaose, and chitohexaose, whereas the previously reported chitosanases I and II could not hydrolyze these substrates to even the slightest extent. This result indicates that *B. gladioli* chitosanase A is responsible for the degradation and utilization of GlcN oligomers produced from partially acetylated chitosan by the action of chitosanases I and II in this bacterium. The enzyme nomenclature of glycosyl hydrolases is sometimes confusing, particularly when based on the substrate specificity alone. Most chitinases, which cleave the bond between two GlcNAc residues in chitin polymers, can also significantly hydrolyze partially acetylated chitosan because they can cleave the bond between GlcNAc and GlcN residues. Moreover, some of the purified chitosanases were reported to exhibit hydrolyzing activity toward $\beta$-1,3-glucan or $\beta$-1,4-glucan (Mitsutomi et al. 1998; Pedraza-Reyes and Gutierrez-Corona 1997). One method of assigning glycosyl hydrolases is to compare the primary amino acid sequence of the enzyme with those of other glycosyl hydrolases. Henrissat (1996) proposed classification of glycosyl hydrolases into families using the criteria of similarity of the amino acid sequence in the catalytic center. It must be noted that, in some cases, enzymes belonging to the same family exhibit different substrate specificities or different cleavage patterns (endo- or exo-type). The two previously reported enzymes (chitosanases I and II) should be subjected to comprehensive analysis, including gene cloning and nucleotide sequencing, to assign their exact nomenclature, taking into consideration their evolutionary relationships with other microbial glycosyl hydrolases.

The gene encoding *B. gladioli* strain CHB101 chitosanase A (*csnA*) was isolated and its complete nucleotide sequence determined. The *csnA* ORF encodes a polypeptide of 355 amino acid residues, and its sequence starting from Ala-96 corresponds to the N-terminal amino acid residue of the purified chitosanase A. Computer analysis of the amino acid sequence of *B. gladioli* strain CHB101 chitosanase A revealed a high degree of similarity (80%) to sequences of chitosanases from *Bacillus circulans* MH-K1 (Ando et al. 1992) and from *Bacillus ehimensis* EAG1 (Akiyama et al. 1999). Multi-alignment of the amino acid sequences of these chitosanases and other bacterial chitosanases with lower similarity (20–30%) (Masson et al. 1994, 1995) are shown in Fig. 6. A highly conserved sequence (consisting of about 50 amino acid residues) was found to be located at the N-terminal end of the mature chitosanase proteins. This region is assumed to be the catalytic domain, in which two completely conserved carboxylic

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                                                               *                  *
BUR_HOLD  96 -ALDHDANFSPATLQFLKDNTGLDGEQWDNIMK-LVNKPEQDSLDWTKFYGYCEDIGDDRGYTMGIFGAT--TGGPNDGGPDGPALFK-A
BAC_CIRC  43 ASP--DDNFSPETLQFLRNNTGLDGEQWNNIMK-LINKPEQDDLNWIKYYGYCEDIEDERGYTIGLFGAT--TGGSRDTHPRWPGPLSKA
BAC_EHIM  44 ASP--DENFSPETLQFLRDRTGLDGEQWNNIMK-LINKPEQDDLNWIKYYGYCEDINDERGYSIGIFGAT--TGGPRDTHPDGPELF-KA
STR_N174  41 -----------------AGAGLDDPHKKEIAMELVSSAENSSLDWKAQYKYIEDIGDGRGYTGGIIGFCSGTGDMLELVQHYTDLEPGN
NOC_N106  42 -----------------AAVGLDDPHKKDIAMQLVSSAENSSLDWKSQYKYIEDIKDGRGYTAGIIGFCSGTGDMLDLVADYTDLKPGN

BUR_HOLD 181 YDAASGASNPSVDGGLARIGAHGSMDGSILKITDSEKVFCGKVKGLQNDAAWREAMWRTFYSVYIQYSVQDARSRGFGSALT-IGSFVDT
BAC_CIRC 128 YDAAKGASNPSADDALKRLGINGKMKGSILEIKDSEKVFCGKIKKLQNDAAWRKAMWETFYNVYIRYSVEQARHA-FTSAVT-IGSFVDT
BAC_EHIM 128 YDAAKGAGNPSVEGALKRLGINGKMKGSILEIKDSEKVFCGKIKKLQNDPAWRKAMWETFYNVYIRYSVEQARQRGFTSALT-IGSFVDT
STR_N174 113 ILAKYLPALKKVNGSASHSGLGTPFTKDWATAAKD-TVFQQAQN---DERDR---VY---FDPAV---S-DAKADGLRA-LGQFAYY-DA
NOC_N106 114 ILAKYLPALRKVNGTESHAGLASAFEKDWATAAKD-SVFQQAQN---DERDR---SY---FNPAV---N-DAKAS-LRA-LGQFAYY-DA

BUR_HOLD 270 A-LNQGADGGSNTLQGLLS----RSGNSTDEK---TFMTSFYAQRTKVVDTHDFNQPPNGKNRVKQWSTLMSQGITSLKNCDADIVKVTS
BAC_CIRC 216 A-LNQGATGGSDTLQGLLA----RSGSSSNEK---TFMKNFHAKRTLVVDTNKYNKPPNGKNRVKQWDTLVDMGKMNLKNVDSEIAQVTD
BAC_EHIM 217 A-LNQGATGDSNTLQGLLA----RSGSSTNEK---TFLKKFHAKRTLVVDTNEYNQPPNGKNRVKQWDTLLDMGKMNLKNVDAEIAQVTN
STR_N174 187 IVM-HGPGNDPTSFGGIRKTAMKKARTPAQGGDETTYLNAFLDARKAAMLTEAAHDDTS-RVDTEQRVFLKAGNLD-LNPPLKWKTYGDP
NOC_N106 187 IVM-HGPGDSSDSFGGIRKAAMKKAKTPAQGRDEATYLKAFLAARKTVMLKEEAHSDTS-RVDTEQTVFLNAKNFD-LNPPLKWKVYGDS

BUR_HOLD 352 WTMK-
BAC_CIRC 298 WEMK-
BAC_EHIM 299 WEMK-
STR_N174 274 YVINS
NOC_N106 274 YAINS
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**Fig. 6** Amino acid alignments for bacterial chitosanases. All sequences correspond to the mature proteins of chitosanases. *Numbers* show the number of amino acid residue initiated from the N-terminus of the total ORF. *Asterisks* indicate two conserved carboxylic amino acid residues. Amino acids identical in at least three sequences are enclosed in a *black box*. *BUR HOLD*, *Burkholderia gladioli* CHB101 (AB029336); *BAC CIRC Bacillus circulans* MH-K1 (D10624), *BAC EHIM Bacillus ehimensis* EAG1 (AB008788), *STR N174 Streptomyces* sp. N174 (L07779), *NOC N106 Nocardioides* sp. N106 (L40408)

amino acid residues (Glu and Asp) were demonstrated to be responsible for the catalytic activity of the enzyme (Boucher et al. 1995). The two residues (Glu-133 and Asp-152) corresponding to these essential carboxylic amino acid residues were also conserved in *B. gladioli* strain CHB101 *csnA*. The alignment of amino acid sequences clearly shows that the gene belongs to family 46 glycosyl hydrolases, in which other bacterial chitosanases are also grouped. Fukamizo and Brzezinski (1997) proposed that bacterial chitosanases can be classified into two clusters at the total primary structure level. *B. gladioli* strain CHB101 *csnA* obviously belongs to cluster II, which is represented by chitosanase from *Bacillus circulans* MH-K1. On the other hand, unique sequences of chitosanases showing no homology with these family 46 chitosanases have been reported from the bacterium *Matsuebacter chitosanotabidus* (Park et al. 1999, accession number AB010493), and the fungus *Fusarium solani* (Shimosaka et al. 1996, accession number D85388). The primary sequences of chitosanases from various kinds of microorganisms must be determined to facilitate further characterization of microbial chitosanases, taking into consideration their evolutionary relationships.

The mature chitosanase A protein begins at Ala-96 of the *csnA* ORF. The size of the preceding 95 residues is fairly long compared to the length of a usual bacterial signal peptide (20–40 residues). Computer analysis of the amino acid sequence revealed that the region from Cys-34 to Ala-90 showed significant similarity with the substrate-binding domain of bacterial chitinases essential for the efficient hydrolysis of insoluble chitin (Fig. 7). The preceding N-terminal amino acid sequence coincides in its structure with the prokaryotic signal peptide, and it could be removed by cutting at a stretch of six alanines (Ala-24–Ala-29) (Von Heijne 1986). Therefore, the initial product from *csnA* was possibly secreted as a polypeptide containing a substrate-binding domain at the N-terminus, and this domain was then removed by proteolysis to yield a truncated form corresponding to the chitosanase A purified in this work. A substrate-binding domain has not yet been reported in microbial chitosanases. We are now attempting to optimize culture conditions for the accumulation of chitosanase A containing the plausible substrate-binding domain in order to examine the role of this domain in the binding of this enzyme to insoluble chitosan and for hydrolytic reactions.

The overexpression of *B. gladioli* strain CHB101 *csnA* in *E. coli* is now being undertaken to obtain a large amount of chitosanase for chitosan oligomer production. The three-dimensional structures of chitosanases from *Streptomyces* sp. strain N174 (Marcotte et al. 1996) and *B. circulans*. MH-K1 (Saito et al. 1999) have been reported, and six binding subsites accommodating glucosamine residues were identified. This information on the structure and function of chitosanase will be helpful to design a protein-engineered chitosanase that

**Fig. 7** Amino acid alignments of plausible substrate-binding domain. *Numbers* show the number of amino acid residue initiated from the N-terminus of the total ORF. Amino acids identical in at least four sequences are enclosed in a *black box*. BUR HOLD *Burkholderia gladioli* CHB101 (AB029336), *JAN LIVI Janthinobacterium lividum* chitinase (U07025), *AER 10S Aeromonas* sp. 10S-24 chitinase (AB014768), *STR GRIS Streptomyces griseus* HUT 6037 chitinase C (AB009289), *PSE S9 Pseudoalteromonas* sp. S9 chitinase B (AF007895), *VIB HARV Vibrio harveyi* chitinase A (U81496), *ALT O-7 Alteromonas* sp. O-7 chitinase (AB004557)

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BUR_HOLD  34 CSSPWTAARIYEAGNVVSFDGHDYTAAYRSDGNAPATASGEAGSG----QPWVAGEACKPA--  90
JAN_LIVI 150 CALAWAAGTAYSAGATVSYAGTNYRANYWTQGDNPSTSSGGAGTG---K-PWTSQEIC------ 203
AER_10S  108 -AAAWNSSTAYSGGAVVSYNGHTYTAKWWTQGNVPSSSTGDQ---------PWTDNGPC------ 157
STR_GRIS  32 CATAWSSSSVYTNGGTVSYNGRNYTAKWWTQNERPGTS----D--------VWADKGACGTGGE  83
PSE_S9   439 ----WDASKAYNAGDKVSYLGTVYKAKWWVQGEKPNSSDAWQSLSQSTDDAWLSSKA------ 491
VIB_HARV 510 CAAAWDANTVYVEGDQVSHDGATWVAGWWTRGEEPGTT------G---E--WG---------- 551
ALT_O7    30 ----WQSQQVYTGGDAVTYQSAKYTAKWWTQNQNPAQNSNTYD-------VWQANGSCDPA--  79
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can be utilized for efficient production of low-molecular-weight chitosan oligomers.

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