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# Molecular Cloning of the Gene Which Encodes β-*N*-Acetylglucosaminidase from a Marine Bacterium, *Alteromonas* sp. Strain O-7

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**The gene encoding the periplasmic β-*N*-acetylglucosaminidase (GlcNAcase B) from a marine *Alteromonas* sp. strain, O-7, was cloned and sequenced. The protein sequence of GlcNAcase B revealed a highly significant homology with *Vibrio* GlcNAcase and α- and β-chains of human β-hexosaminidase.**

Chitin, a polymer of *N*-acetyl-D-glucosamine (GlcNAc), exists abundantly in nature and serves as an important nutrient source of carbon and nitrogen in the marine and terrestrial environments. In these environments, chitin is converted to GlcNAc by the cooperative interaction of two enzymes, chitinase (EC 3.2.1.14) and β-*N*-acetylglucosaminidase (EC 3.2.1.30; GlcNAcase), which are produced by a number of chitinolytic bacteria. Bacterial chitinase genes have been cloned and characterized from *Serratia marcescens* (7, 8), *Serratia liquefaciens* (9), *Vibrio harveyi* (16), *Vibrio vulnificus* (24), *Vibrio furnissii* (1), *Aeromonas hydrophila* (4), *Bacillus circulans* (22, 23), and *Alteromonas* sp. strain O-7 (19), and the domain organization and its function of the enzyme have been actively investigated (2). On the other hand, the genes encoding GlcNAcase have been isolated from *S. marcescens* (5), *V. harveyi* (17), *V. vulnificus* (24), and *Vibrio parahaemolyticus* (25); however, in regard to the determination of nucleotide sequence the analysis of GlcNAcase genes from *V. harveyi* (17) is a solitary instance except for our recent characterization of GlcNAcase from *Alteromonas* sp. strain O-7 (18). Thus, little is known about the mechanism of hydrolysis, the relationship between structure and function, and the regulatory system involved in enzyme induction. Our interests in examining the mechanism of chitin degradation by a marine bacterium, *Alteromonas* sp. strain O-7, led to the present study of GlcNAcase, which plays a crucial role, together with chitinase, in the enzymatic degradation of chitin.

The same library previously constructed was used (19). Among 1,500 transformants tested, two colonies showed a bright yellow color, indicating the expression of GlcNAcase. One of the two clones, designated pNAG096, was most active towards trimers among the chitin oligosaccharides from dimer to hexamer. The other clone showed the activity only towards dimers. Further analysis was performed on pNAG096. This plasmid contained a 5.5-kb *Hin*dIII fragment from *Alteromonas* sp. strain O-7. To determine the location of GlcNAcase B gene, a restriction map was constructed and the various subclones were prepared. The results of subcloning showed that the 4.1-kb *Sac*I-*Hin*dIII fragment was the region necessary for the expression of GlcNAcase activity (Fig. 1). The nucleotide sequence of a 3.2-kb *Sac*I-*Pst*I fragment of pNAG096 and the deduced amino acid sequence of the gene are shown in Fig. 2. The GlcNAcase B-encoding sequence has an overall G+C content of 46.5%, which agrees well with that of *Alteromonas* sp. (43.5%) (20). An open reading frame of 2,322 bp starting at base 412 and ending at base 2,733 was found. The open reading frame encoded a protein of 773 amino acids with a molecular weight of 84,615. The deduced amino-terminal 19-amino-acid sequence showed the typical features of signal peptides, which are composed of a positively charged region, a hydrophobic region, and a signal sequence cleavage site. The N-terminal sequences of the native and cloned GlcNAcases B coincided precisely with the sequence starting from leucine residue 20 of the deduced amino acid sequence encoded by the gene. Thus, it was clarified that cleavage of the signal peptide occurred between alanine residue 19 and leucine residue 20, which is compatible with the −3, −1 rule of von Heijne (21). The amino acid sequence of GlcNAcase B was compared with available protein sequences from databases (PIR and Swiss-PROT) as well as those from the literature. The protein sequence of GlcNAcase B revealed a highly significant homology with *Vibrio* GlcNAcase (49.2%) (17), *Dictyostelium* β-hexosaminidase (25.0%) (6), and α- (25.8%) (11) and β- (27.3%) (13) chains of human β-hexosaminidase.

The location of the cloned GlcNAcase B in *Escherichia coli* JM109 was determined as for the previous paper (19). The GlcNAcase activity was located mainly in the periplasmic fraction (87% of the total activity). To ensure correct fractionation, we measured β-lactamase (15) and malate dehydrogenase (10) activities as marker enzymes in the periplasm and cytoplasm, respectively. When β-lactamase activity was determined, 95% was found in the periplasmic fraction and 85.3% of the malate dehydrogenase was present in the cytoplasmic fraction. GlcNAcase B was purified by the successive column chromatographies with DEAE-Toyopearl 650M (1.9 by 47 cm; Tosoh Co., Tokyo, Japan), Sephadex G-100 (2.7 by 91.5 cm; Pharmacia), Cosmogel QA (0.8 by 7.5 cm; Nacalai Tesque, Kyoto, Japan), Superdex 200 (1.5 by 30.5 cm; Pharmacia), and Cosmogel QA from the periplasmic fraction of *E. coli* carrying pNAG096. Tris-HCl buffer (50 mM, pH 8.0) was used in this procedure. Protein was measured by the method of Bradford (3) with bovine serum albumin as a standard. The final preparation gave a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12), and the molecular mass of the enzyme was estimated as 85 kDa (Fig. 3).

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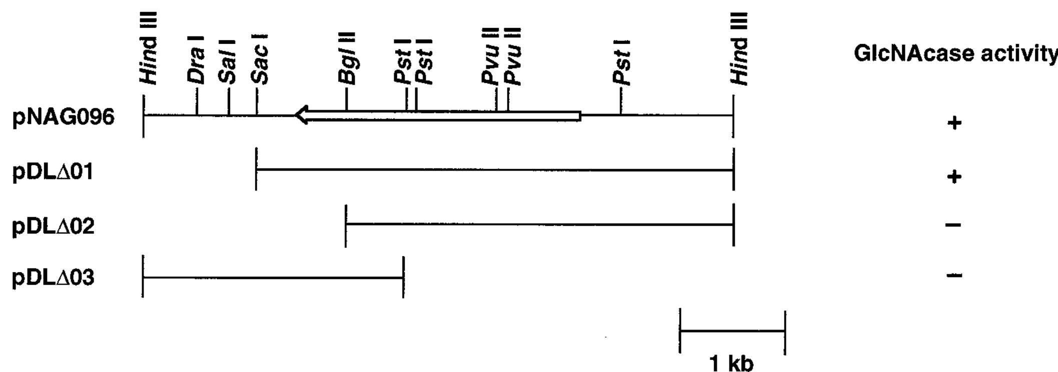


FIG. 1. Restriction map and deletion analysis of pNAG096. The transformants carrying the plasmids with appropriate deletions were transferred to an LB agar plate containing ampicillin (100 μg/ml). The transformants grown on LB agar plates containing ampicillin were sprayed with a 0.01 M solution of PNP-β-GlcNAc in 0.1 M sodium phosphate buffer, pH 7.5. GlcNAcase activity was judged by the production of a bright yellow color of the colonies. +, production of the color; −, no production of the color. The arrow indicates the coding sequence for the GlcNAcase protein and the direction of transcription of the gene.

The isoelectric point of the enzyme was 4.9. On the other hand, the purification of GlcNAcase from cellular extract of *Alteromonas* sp. strain O-7 was performed by the same procedure as that of the cloned GlcNAcase B. The native enzyme from the strain showed a single band in SDS-PAGE and the same molecular mass as that of the cloned GlcNAcase B (Fig. 3). The N-terminal amino acid sequences of the two enzymes were also found to be identical (LDQTAVNWLGQNLDVKYTLL-). The yield of cloned GlcNAcase B was better than that of the native one. From 6 g of *E. coli* cells (2 liters of LB medium containing 100 μg of ampicillin per ml) carrying pNAG096, 0.5 mg was obtained as homogeneous protein. In the case of the native enzyme, 0.13 mg was obtained from 20 g of *Alteromonas* cells (1 liter of Bacto Marine Broth 2216 containing 0.5% colloidal chitin) although the same purification procedure used for the cloned enzyme was used. To characterize the substrate specificity of GlcNAcase B, the rates of hydrolysis of various chitooligosaccharides and synthetic substrates were measured. GlcNAcase activity was measured as in the previous paper (18). Among the substrates tested, *p*-nitrophenyl-β-D-GlcNAc (PNP-β-GlcNAc) and acetylchitotriose were the best substrates. The enzyme was inactive against all alpha-linked *N*-acetylhexosamine glycosides, PNP-β-acetyl-1-thio-glucosamine, and PNP-β-glucose. The native enzyme also exhibited the same

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   1  TGCTGCAGGAGA GCAGGCAACTTA AGCTATCTTGAT GGAATTATTGAA GATTATGCTTCA GGACAATTCTTT GGGCGACAAGGA ATTGAAGGGAGG TGGTTGGCCGAA CAAGCACGCAGT   120
 121  GCGACCCATTTG CACTACAACAAT TTGAGACTTTTG GCAGTCACGTTG GTCATGCACTCG CGCAAGTAGTCA TGGTGCTGAATC CAGATGTCATTG TACTTGGCGGCT CCGTGACGCATT   240
 241  CATATGACTATT TTAACGCTGCAC TTGAAGCCAAGA TGCAGCAGTTGC TTGGCAATGCTT TATATCAATCCG TTCGTGTCATTC CAAGCAGCTTAA ATCACGCGCCAC TTTTAGGGGCAT   360
 361  ACGGATTGTTTC AACACAATAAAA TTAACCATGATA ACAAGGAACTAG CCAATGAAGTTT AATCGGCTTATG GCCCTACTTTTT GGGGTGTCATCC CCCCTTTATGCG CTCGACCAAACC   480
                                                           M  K  F   N  R  L  M    A  L  L  F    G  V  S  S    P  L  Y  A ↓L  D  Q  T      23
 481  GCAGTAAACTGG TTAGGCCAGAAT CTTGACGTGAAA TACACCTTGCTA GATACTAAGCCA ACCACCTGCCCC AAGGCACAACAA AAATGCTATTAC TCAGAGCTGAGT TTTAGCGTACGT   600
  24  A  V  N  W   L  G  Q  N   L  D  V  K   Y  T  L  L   D  T  K  P   T  T  C  P   K  A  Q  Q    K  C  Y  Y    S  E  L  S    F  S  V  R      63
 601  AAAGAAAATACC AAAGCAAATAAT GACTTTGCTATC TTTTTTAGCCAA CTGATGCCGATT TACCATGTTGAG GGCGATAACTTT GCCATCACCCAT ATCAATGGTGAT ATTCACAAAATA   720
  64  K  E  N  T   K  A  N  N   D  F  A  I   F  F  S  Q   L  M  P  I   Y  H  V  E   G  D  N  F    A  I  T  H    I  N  G  D    I  H  K  I     103
 721  ACGCCTGCGGCA GGGTTTAGTGGG TTTTCAAGTGCC CCCACTACGGTG CGCTTTTATACC AAGGATTCTCAG GTTACGCGCTCT GAAGTTTATGCC AAATTATGTTGT GAACGGACGCGC   840
 104  T  P  A  A   G  F  S  G   F  S  S  A   P  T  T  V   R  F  Y  T   K  D  S  Q   V  T  R  S    E  V  Y  A    K  L  C  C    E  R  T  R     143
 841  AGCTTGAAGCTC ACCCCACAAGTG ATAAAAAGCACG CAAACCCAGCGC GATAACGACACC GGACTTGACTGC AACCTTAATTTG ACGCCATTTGTT ACGCTCAACCAG CTGCAAACCAGC   960
 144  S  L  K  L   T  P  Q  V   I  K  S  T   Q  T  Q  R   D  N  D  T   G  L  D  C   N  L  N  L    T  P  F  V    T  L  N  Q    L  G  T  S     183
 961  AGTAAAGATGAC ACGCCGTGGATG GGCAGCGAATAC TTATATCAACAT CAAGTGAAGCCA ACACTTGATGCT GCAATTGGGCTG ATACCAAAACCT AAACAACTGACT GTGCTGTCCGAT  1080
 184  S  K  D  D   T  P  W  M   G  S  E  Y   L  Y  Q  H   Q  V  K  P   T  L  D  A   A  I  G  L    I  P  K  P    K  Q  L  T    V  L  S  D     223
1081  AAACGACTCAAT CTAGCAGCCGGT ATTAATCTGCAA CTTTCAGGTATT TCAGCTGATGCT ATGGCCATGGCA CAACAGCGACTC AATACCCTTGGG GTAAAAAGCACC AAGGAGGGGCTT  1200
 224  K  R  L  N   L  A  A  G   I  N  L  Q   L  S  G  I   S  A  D  A   I  A  M  A   Q  Q  R  L    N  T  L  G    V  K  S  T    K  E  G  L     263
1201  GTAGTCAATGTC GCTGTAAAGCCA AATAAGCAATCA AGTCCACATTAT CAACTGACAGTC GCCGAAAATAAT ATCTCAATCCAA GGCAATAACAGC GCCGCTGCATTT TATGCGCTACAA  1320
 264  V  V  N  V   A  V  K  P   N  K  Q  S   S  P  H  Y   Q  L  T  V   A  E  N  N   I  S  I  Q    G  N  N  S    A  A  A  F    Y  A  L  Q     303
1321  AGCCTTGCAGGA CTGTTGGATATT AATGATTTGCGT ATTCCCATGGTG GATATTATTGAT ACACCAAGGTAT GACTTTCGTGGT TTACACGTCGAT GTTGCCCGCAAC TTCCGCTCCAAA  1440
 304  S  L  A  G   L  L  D  I   N  D  L  R   I  P  M  V   D  I  I  D   T  P  R  Y   D  F  R  G    L  H  V  D    V  A  R  N    F  R  S  K     343
1441  GCCTTTATTTTA CAAACCATTGAA CAGATGGCGGCA TATAAGCTAAAT AAACTGCACCTC CATTTAGCGGAT GATGAAGGCTGG CGCTTAGCCATC GACGGTCTTGAT GAACTCACCTCT  1560
 344  A  F  I  L   Q  T  I  E   Q  M  A  A   Y  K  L  N   K  L  H  L   H  L  A  D   D  E  G  W    R  L  A  I    D  G  L  D    E  L  T  S     383
1561  GTCGGCGCTTAT CGCTGCTTTGAT TTGACCGAAACT CGCTGCCTATTG CCGCAATTAGGC GCAGGGAATGAT AAAAACGCGCAA GTGAATGGCTTT TACTCTGCAGAG GATTATATTGAG  1680
 384  V  G  A  Y   R  C  F  D   L  T  E  T   R  C  L  L   P  Q  L  G   A  G  N  D   K  N  A  Q    V  N  G  F    Y  S  A  E    D  Y  I  E     423
1681  ATCCTGCGCTAT GCCAAGGCGCAT CATATCGAGGTG TTACCCTCATTA GATATGCCTGGT CACTCACGCGCT GCAATCATCGCC ATGGAGGCGCGA TATAAAAAGTTG ATGGCACAAGGC  1800
 424  I  L  R  Y   A  K  A  H   H  I  E  V   L  P  S  L   D  M  P  G   H  S  R  A   A  I  I  A    M  E  A  R    Y  K  K  L    M  A  Q  Q     463
1781  AAACCTGAGGAT GCGCAGAAATAT CGATTAGTAGAA ACTGCAGATAAG ACGCGTTATAGC TCCATTCAACAT TACAACGACAAC ACGCTAAATGTC TGTATTGCAAAC ACTTACACCTTT  1920
 464  K  P  E  D   A  Q  K  Y   R  L  V  E   T  A  D  K   T  R  Y  S   S  I  Q  H   Y  N  D  N    T  L  N  V    C  I  A  N    T  Y  F  I     503
1921  ATCGACAAAGTA TTAAGCGAAGTA AAAGTGCTGCAT GACCGTGCAGGT GTACCACTCAAC ACTTACCATATA GGTGCAGATGAA ACCGCAGTGCTT TGGCTAGAATCG CCGGCTTGTAAA  2040
 504  I  D  K  V   L  S  E  V   K  V  L  H   D  R  A  G   V  P  L  N   T  Y  H  I   G  A  D  E    T  A  V  L    W  L  E  S    P  A  C  K     543
2041  AAGTTGCAGGCA AGTGTGAAAGAT TTTACCAACTTT AACGGTTACTTC ATTGAACGGGTC GCAAAGTTACTA GATAAAAAAGGA ATACAGGTTGCA GGGTGGAGCGAC GGTTTAGGTGAT  2160
 544  K  L  Q  A   S  V  K  D   F  T  N  F   N  G  Y  F   I  E  R  V   A  K  L  L   D  K  K  G    I  Q  V  A    G  W  S  D    G  L  G  D     583
2161  GTACGTGCTGCG AATATGCCAGCA AACATACAGAGC AATGGCTTGGGC GACATTAAGCGA AAACGGGCACCG GTCGCGCATCGT TTCGCCAATCAG GGCTGGCAGGTT GCTTTGTCTAGC  2280
 584  V  R  A  A   N  M  P  A   N  I  Q  S   N  G  L  G   D  I  K  R   K  R  A  P   V  A  H  R    F  A  N  Q    G  W  Q  V    V  L  S  S     623
2281  CCTGATGTAACC TACTTCGACTTT CCATATCAGTCT CACCCAGAGGAG CGCGGTAATCAC TGGGCAAGCCGT GCTATCGAAAGC AAAAAGATGTTT GAGTTTATGCCA GACAACCTTCCT  2400
 624  P  D  V  T   Y  F  D  F   P  Y  Q  S   H  P  E  E   R  G  N  H   W  A  S  R   A  I  E  S    K  K  M  F    E  F  M  P    D  N  L  P     663
2401  GCGCACGCAGAG ATCTGGAAAAAT ACCAATAACCAC GCCTACATAGCC AACGACAGCGAC TCATCACTAAAC AAAGGGGTTCAG TTTGCCGGGCTC CAAGGGCATTTG TGGAGTGAAATG  2520
 664  A  H  A  E   I  W  K  N   T  N  N  H   A  Y  I  A   N  D  S  D   S  S  L  N   K  G  V  Q    F  A  G  L    Q  G  H  L    W  S  E  M     703
2521  CTGCGCAGTGAT GCACAAGCCGAA TACATGCTTTAT CCACGGCTATTG GCCCTTGCAGAG CGTGCATGGCAC CATGCCGAGTGG GAATTACCCTAC CAAGCCGGTCGT ATATACAGCCAA  2640
 704  L  R  S  D   A  Q  A  E   Y  M  L  Y   P  R  L  L   A  L  A  E   R  A  W  H   H  A  E  W    E  L  P  Y    Q  A  G  R    I  Y  S  Q     743
2641  TCTAGCGGATAT TTTACGGCTAAG TTACAGGCGCAA CGTGAGGCTGAT TGGCAACGATTC GTGGCTATTTTG GGCAATTCAAGA ACTACCTAAGTT AGCACAAGCCGG AATTGAATTTCG  2760
 744  S  S  G  Y   F  T  A  K   L  Q  A  Q   R  E  A  D   W  Q  R  F   V  A  I  L   G  N  S  R    T  T  *                                    773
2761  CATTCCCGTCGT CGCCGCAAAACA GCTATCTGGAGC AAAGCTCGACGC TTTTACCGCCAT TCCGGGTTTTGT TATCGAAGCACA GCTTGAAAACGG CAACTGGGTGCC CTTCAATGCCTC  2880
2881  ACTTAATCAAGT ACAGGCGCTTAG AGCGGTGCACCC AAACTCTGATCG CAAAGGACGAGC TTTGAGTTTAGA GAAAAAATAACG AATAAGATCAAC ATGCGATGCTAA GCCCTACTCAGT  3000
3001  TCTTAGCATCTC TATCACCATTAT CAGCCGCAAAAG AAACATTGAGAA AATGTAATTAAT AGCAAAATGTTG AAAAAGTAATAA ATATGTTTTAGC TTAGTTGTTATG TTACTTTTTTGT  3120
3121  TCACTGAAGAAT AAATCAGATGAC ATAACTACAATA TAATGAGCTC                                                                                     3166
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FIG. 2. Nucleotide sequence of a 3.2-kb DNA fragment from pNAG096. The putative ribosome binding site (AGGAA) is underlined. The deduced amino acid sequence of GlcNAcase B is given below the nucleotide sequence. The amino-terminal amino acid sequence of GlcNAcase B from *Alteromonas* sp. strain O-7 was determined by using an Applied Biosystems model 477A gas-phase sequencer and is underlined. The signal peptide cleavage site is shown by an arrow, and the stop codon is shown by an asterisk. DNA sequencing was done by the dideoxy chain-termination method (14).

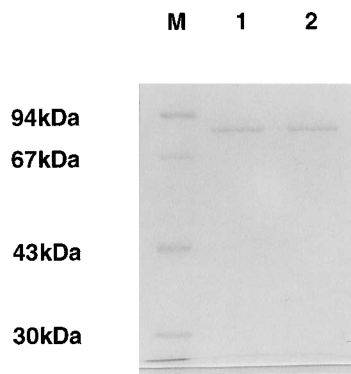


FIG. 3. SDS-PAGE of cloned and native GlcNAcase B. Lane M, molecular size standards: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa). Lane 1, GlcNAcase B from *E. coli* carrying pNAG096. Lane 2, GlcNAcase B from *Alteromonas* sp. strain O-7.

substrate specificity as that of the cloned enzyme. When PNP-β-GlcNAc was used as a substrate, the optimum pH and temperature of the native and cloned GlcNAcases B were 9 and 40°C, respectively, and the proteins showed identical specific activities (913.6 U/mg of protein).

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper (Fig. 2) will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D29665.

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