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Note

Molecular Cloning of the Gene Encoding a Novel β-*N*-Acetylhexosaminidase from a Marine Bacterium, *Alteromonas* sp. Strain O-7, and Characterization of the Cloned Enzyme

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We have reported that the chitinolytic system of *Alteromonas* sp. strain O-7 consists of chitinases (ChiA, ChiB, and ChiC), a chitinase-like enzyme (ChiD), β-*N*-acetylglucosaminidases (GlcNAcasesA, GlcNAcaseB, and GlcNAcaseC), and a novel transglycosylative enzyme (Hex99). The gene encoding a β-hexosaminidase with an unusual substrate specificity (*hex86*), located upstream of the *hex99* gene, was cloned and sequenced. The gene encoded a protein of 761 amino acids with a calculated molecular mass of 86,758 Da. The deduced amino acid sequence of Hex86 showed sequence similarity with β-hexosaminidases belonging to family 20. The *hex86* gene was expressed in *Escherichia coli*, and the recombinant enzyme was purified to homogeneity. The enzyme rapidly cleaved *p*-nitrophenyl-β-*N*-acetyl-D-glucosaminide and slowly cleaved *p*-nitrophenyl-β-*N*-acetyl-D-galactosaminide. Unexpectedly, the enzyme did not hydrolyzed chitin oligosaccharides under the assay conditions for synthetic glycosides. However, after prolonged incubation with excessive quantities of the enzyme, Hex86 hydrolyzed chitin oligosaccharides. These results indicate that Hex86 is a novel enzyme that prefers *p*-nitrophenyl-β-*N*-acetyl-D-glucosaminide to chitin oligosaccharides as a substrate.

Key words: β-*N*-acetylhexosaminidase; chitin oligosaccharide; *Alteromonas* sp.

Chitin is an insoluble polysaccharide consisting of β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) units and is the second most abundant polymer after cellulose. Chitin is a major component of crustacean and insect exoskeletons and fungal cell walls. This polysaccharide is a particularly important nutrient source to maintain the ecosystem in the marine environment.[1] Chitinolytic marine bacteria play an important role in this chitin recycling process. To degrade chitin, chitinolytic microorganisms produce two classes of enzymes: chitinase (EC 3.2.1.14) and β-*N*-acetylglucosaminidase (EC 3.2.1.30). Chitinases cleave the insoluble chitin to give chitobiose [$(GlcNAc)_2$] as a major degradation end product. The resulting $(GlcNAc)_2$ is hydrolyzed by β-*N*-acetylglucosaminidases at the non-reducing end.

Alteromonas sp. strain O-7 is a Gram-negative, flagellated, motile, and aerobic rod-shaped bacterium of marine origin.[2] We have been studying the chitin degradation system of the strain as a model for defining the various components involved in chitin use. Chitinolytic enzymes of the strain consists of three chitinases (ChiA, ChiB, and ChiC) and three β-*N*-acetylglucosaminidases (GlcNAcaseA, GlcNAcaseB, and GlcNAcaseC).[3–7] We have clarified that these enzymes and the corresponding genes are essential for the chitinolytic system of the strain. In addition, we have recently cloned and characterized a novel chitinase-like enzyme (ChiD) with high activity only towards chitooligosaccharides from trimer to hexamer (unpublished data). In chitinase-producing microorganisms, chitin is a common inducer of chitinase production. However, since chitin is insoluble and impermeable to microorganisms, a soluble degradation product (s) such as GlcNAc, $(GlcNAc)_2$, or higher oligomers is considered to act as a direct inducer of chitinase. In *Altermonas* sp. strain O-7, β-(1→6)-$(GlcNAc)_2$ is one of the smallest molecules to induce chitinase production.[8] We have analyzed the gene encoding the enzyme (Hex99) which synthesizes β-(1→6)-$(GlcNAc)_2$ from β-(1→4)-$(GlcNAc)_2$.[8] Recently, we found that a gene (*hex86*), located upstream of the *hex99* gene, encoded a hexosaminidase with an unusual substrate specificity. In this study, we describe the cloning, expression, and the characterization of the recombinant enzyme.

Plasmid pTG3 contains a 4.8-kb *Pst*I-*Eco*RI fragment of the *Alteromonas* sp. strain O-7 chromosome carrying the *hex99* gene encoding a transglycosylating enzyme.[8] Sequencing analysis of the 0.35-kb region immediately upstream of *hex99* revealed a partial open reading frame (ORF) encoding a protein similar to the C-terminal part of hexosaminidases

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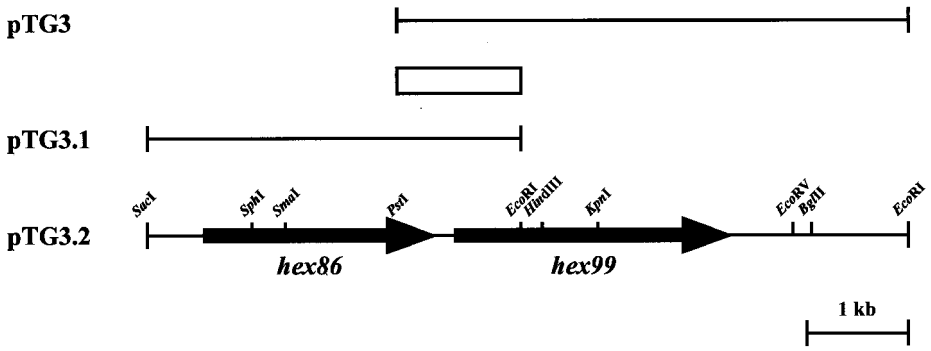

Fig. 1. Restriction Map of pTG3.2.
The hybridization probe is represented by the box. The arrows indicate ORFs and directions of transcription.

belonging to family 20 of glycosyl hydrolases.[9–11] Therefore, cloning of the 5′ upstream region of the gene, termed *hex86*, was done by colony hybridization using the 1.2-kb *Pst*I-*Eco*RI fragment as a probe (Fig. 1). Southern hybridization against total DNA digested with various restriction enzymes showed that the probe strongly hybridized with a 3.6-kb *Sac*I-*Eco*RI fragment (data not shown). Colony hybridization and Southern hybridization were done as in the previous paper.[8] Chromosomal DNA of the strain was digested with *Sac*I and *Eco*RI and electrophoresed on a 0.6% agarose gel. The fragments corresponding to the size of 3.6 kb were excised from the gel and purified with a Sephaglas BandPrep kit (Amersham Pharmacia Biotech). These were ligated into the corresponding sites of pUC19 and the recombinant plasmids were introduced into *E. coli* JM109. The library was screened by colony hybridization with the labelled probe. Among about 850 transformants, only one clone (pTG3.1) was isolated (Fig. 1). Analyses by restriction enzyme digestion and sequencing of the fragment showed that the insert of pTG3 and that of pTG3.1 shared a 1.2-kb *Pst*I-*Eco*RI region. The 2.4-kb *Sac*I-*Pst*I fragment of pTG3.1 and 4.8-kb *Pst*I-*Eco*RI fragment of pTG3 were ligated together. The resulting 7.2-kb fragment was inserted into the corresponding sites of pUC19. The plasmid was named pTG3.2.

The entire nucleotide sequence of pTG3.2 indicated that *hex86* was transcribed from the same strand as *hex99* and the two genes were separated by 142 nucleotides (Fig. 2). The ORF has an ATG start codon which is preceded by a plausible ribosome-binding site (AGGAAG) at a distance of seven nucleotides. The *hex86* gene consists of 2,286 nucleotides encoding a protein of 761 amino acids with a calculated molecular mass of 86,758 Da. There is no apparent N-terminal secretory signal sequence, which is composed of a positively charged amino terminus followed by a hydrophobic core and a string of polar residues, downstream from the start site, suggesting that Hex86 is an intracellular enzyme.

Comparison of the deduced amino acid sequence of Hex86 with the BLAST database showed that the gene encoded a protein homologous to the several β-hexosaminidases belonging to family 20. Hex86 showed similarity with β-*N*-acetylglucosaminidase (52% identity) from *Pseudoalteromonas* sp. S9,[12] β-*N*-acetylhexosaminidase (40% identity) from *Porphyromonas gingivalis*,[13] putative sugar hydrolase (40% identity) from *Streptomyes coelicolor* (GenBank accession no. CAB72189), β-hexosaminidase (37% identity) from *Xylella fastidiosa* (GenBank accession no. B82755), and β-*N*-acetylglucosaminidase (35% identity) from *Caulobacter crescentus* (GenBank accession no. AAK22434). Tews *et al.* reported that an *N*-acetylglucosaminidase from *Serratia marcescens* uses an acid-base reaction mechanism with glutamic acid 540 as the catalytic amino acid, which is well conserved in all members of family 20 glycosyl hydrolases.[14] Hex86 also contains the glutamic acid residue (Glu 339) proposed to be the proton donor essential for catalytic activity (Fig. 3). These results indicate that Hex86 is a member of family 20 of the glycosyl hydrolases.

To investigate the enzymatic properties of Hex86, the *hex86* gene was overexpressed by using a plasmid, pET-20b(+) (Novagen). Primers for amplification of the gene, (5′-GTTCACACCACTTTGTACAAAG-CTTCAG-3′, 5′-GTATTAGGGCTCGAGTGATT-GATATACC-3′) were designed to facilitate cloning in frame into pET-20b(+). The *hex86* gene was amplified by the polymerase chain reaction (PCR) with these primers with pTG3.2 as the template. PCR amplification was done out for 30 cycles (30 s at 94°C, 30 s at 59°C, 2.5 min at 68°C) with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The amplified DNA was digested by *Hind*III and *Xho*I, and the resulting fragment was inserted into the corresponding sites of pET-20b(+). This plasmid was designated pET-HEX86.

E. coli BL21 (DE3) cells carrying pET-HEX86 were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at the mid-exponential phase and further incubated for 2 h at 37°C. Cells were harvested by centrifugation and resuspended in phos-

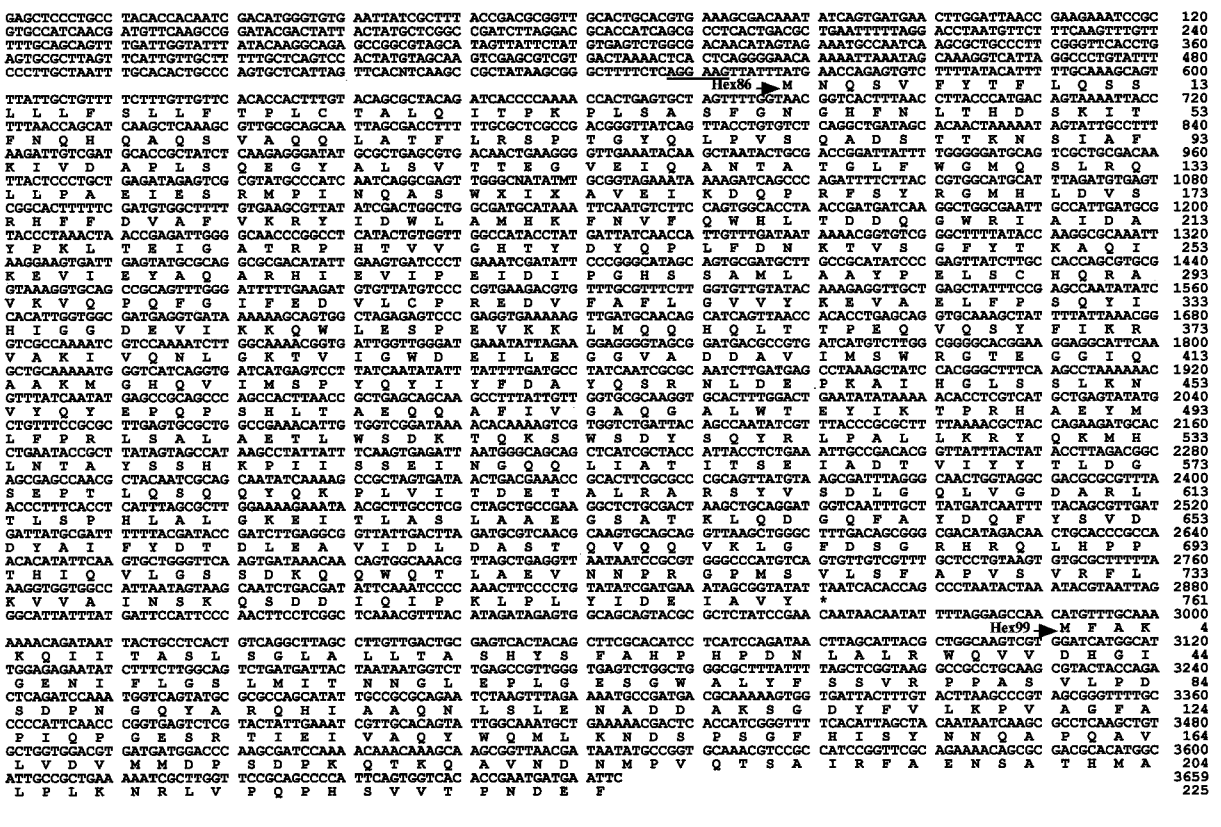

Fig. 2. Nucleotide Sequence of *hex86*.

The deduced amino acid sequence of Hex86 is shown below the nucleotide sequence. The putative ribosome-binding site is underlined. The stop codon is indicated by an asterisk. The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB067646.

Hex86	**288**	SCHQ**RA**V**KV**QPQ**FGIFEDVLCP**R-**E**D**VFAFLG**V**VY**K**EVAELFPSQYIHIGGDEVIK**K**QWLE**	**347**
ChiQ	**309**	GCKNQTLA**V**EGN**FGIFE**P**VLCP**T-EQT**FAFL**KN**VY**S**EVA**ALFPSQYIHIGGDEVIK**T**QWLE**	**368**
NahA	**283**	RCFP**R**EF**K**PRIIW**G**VEQ**DV**YC**A**GKDS**VF**R**F**ISD**V**ID**EVA**P**LFP**GT**YF**HIGGDE**CP**K**DR**W**KA**	**343**
Pshy	**268**	VIDTT**A**LS**V**WDTW**G**VSPN**VLAP**T-**E**NTLR**F**YEG**V**FE**EVLELFPS**EFV**HIGGDE**CP**K**D**QW**RA**	**327**
Hex	**341**	GVTGTTPP**V**SVDW**G**VNPYLFDTA-TPSLD**FIGNV**LD**EV**LT**LFPS**PY**IHIGGDE**AV**K**D**QW**EA**	**400**
Nah	**274**	GTAPPDASKMGDW**GIF**PWLYNTD-DAT**FAFL**DD**V**LN**EV**MDI**FPS**TF**IH**V**GGDE**A**IK**D**QW**KA**	**333**

Fig. 3. Alignment of the Active Site of Hex86 and Other β-Hexosaminidases.

Numbers on the left are the residue numbers of the first amino acid in each line. Residues that are identical are indicated by bold letters. The putative active-site glutamic acid rsidues are marked by an asterisk. Hex86, β-hexosaminidase from *Alteromonas* sp. strain O-7; ChiQ, β-*N*-acetylglucosaminidase from *Pseudoalteromonas* sp. S9; NahA, β-*N*-acetylglucosaminidase from *Porphyromonas gingivalis*; Pshy, putative sugar hydrolase from *Streptomyces coelicolor*; Hex, β-hexosaminidase from *Xylella fastidiosa*; Nah, β-*N*-acetylglucosaminidase from *Caulobacter crescentus*.

phate-buffered saline (PBS). The cells were disrupted by sonication, and the lysate was centrifuged at 10,000 × *g* for 20 min. The pellet containing insoluble Hex86 was solubilized with PBS containing 8 M urea. The solution was centrifuged , and dialyzed against a series of 50 mM Tris-HCl buffers (pH 7.0) with stepwise decreases in the urea concentration from 6 to 0 M. The soluble protein was put on a chelating Sepharose HP column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The active fraction was further purified by Mono Q column chromatography (Amersham Pharmacia Biotech). The molecular mass of the recombinant Hex86 calculated from the deduced amino acid sequence was in reasonable agreement with the 86 kDa estimated by SDS-PAGE (Fig. 4).

Enzyme activity was measured using *p*-nitrophenyl-β-*N*-acetyl-D-glucosaminide (PNP-β-GlcNAc) in 50 mM acetate buffer, pH 5.5. The reaction mixture, composed of 2.5 mM PNP-β-GlcNAc, 50 mM acetate buffer (pH 5.5), and enzyme solution in 0.15 ml of reaction volume, was incubated at 37°C for 10 min. The reaction was stopped by adding 0.5 ml of 0.2 M Na_2CO_3 and the *p*-nitrophenol liberated was measured at 420 nm. One unit of β-*N*-acetylhexosaminidase was defined as the amount of

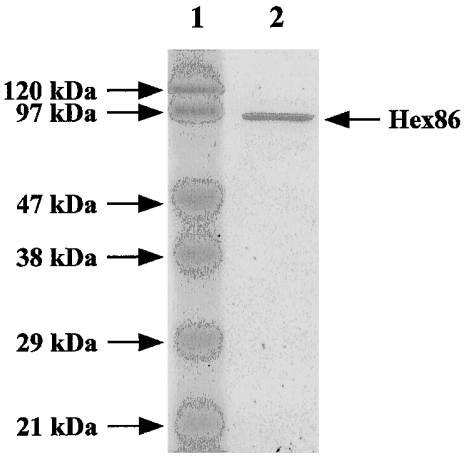

Fig. 4. SDS-PAGE of Hex86.
Lanes: 1, marker proteins; 2, Hex86.

enzyme that liberated 1 μmol of *p*-nitrophenol in 1 min under the conditions described above. The optimun pH and temperature of Hex86 were 5.5 and 37°C, respectively. We investigated the substrate specificity of the enzyme by using various substrates. Among synthetic glycosides, the enzyme was most active towards PNP-β-GlcNAc. The enzyme also showed a low activity towards PNP-β-*N*-acetyl-D-galactosaminide (7.6% of the reactivity towards PNP-β-GlcNAc). However, the enzyme did not hydrolyze PNP-β-D-glucopyranoside or PNP-β-D-xylopyranoside. To investigate whether Hex86 is involved in the chitin degradation system of *Alteromonas* sp. strain O-7, chitin oligosaccharides from dimer to hexamer were used as a substrate. The assay system for chitin oligosaccharides from dimer to hexamer consisted of 0.1 ml each of enzyme solution, 50 mM acetate buffer (pH 5.5), and 5 mM substrate. After incubation at 37°C for an appropriate period, the GlcNAc produced was measured by the method of Reissig *et al.*[15] When each of the oligosaccharides was incubated with 28 mU of Hex86 at 37°C for 10 min, there was no detectable GlcNAc in any of the reaction mixtures. Under the same assay conditions as those for oligosaccharides, Hex86 rapidly hydrolyzed PNP-β-GlcNAc. These results indicate that Hex86 prefers PNP-β-GlcNAc to chitin oligosaccharides as a substrate. Recently, ExoII of *Vibrio furnissii*[16] and NagZ of *E. coli*[17] were shown to have an unusual substrate specificity. These enzymes showed no activity against chitin oligosaccharides, but rapidly cleaved PNP-β-GlcNAc and slowly cleaved PNP-β-GalNAc. Therefore, Hex86 has a similar substrate specificity with those of ExoII and NagZ under the standard assay conditions described above. However, after prolonged incubation (5 h) with 0.5 U of Hex86, the enzyme hydrolyzed chitin oligosaccharides from dimer to hexamer. Among the substrates tested, $(GlcNAc)_2$ was the best substrate, and the activity showed a tendency to decrease with increases in the degree of polymerization (Table). On the other hand, ExoII and NagZ had no activity against chitin oligosaccharides despite prolonged incubation with excessive quantities of these enzymes.[16,17] Hex86, belonging to family 20, showed no sequence similarity with ExoII and NagZ, belonging to family 3. The difference in amino acid sequences of these enzymes might reflect the difference of the reactivity towards chitin oligosaccharides. There are many reports on hexosaminidases from a wide variety of organisms, however, there is no report of a family 20 hexosaminidase with the unusual specificity of Hex86.

Table. Hydrolysis Rate of Chitin Oligosaccharides by the Purified Hex86

Substrate	Relative rate (%)[a]
Di-*N*-acetylchitobiose	100.0
Tri-*N*-acetylchitotriose	74.8
Tetra-*N*-acetylchitotetraose	46.6
Penta-*N*-acetylchitopentaose	41.1
Hexa-*N*-acetylchitohexaose	27.6

[a] Rate relative to activity with *N*-acetylchitobiose as a substrate, which was taken as 100%.

What is the physiological role of Hex86 in *Alteromonas* sp. strain O-7 ? Chitlaru and Roseman speculated that ExoII cleaves phenolic β-GlcNAc derivatives from chitin-producing organisms, such as fungi and invertebrates, and the resulting phenol derivatives may induce *V. furnissii* to invade its host.[16] On the other hand, NagZ, the predicted amino acid sequence of which is 57% identical to that of ExoII, hydrolyzed the β-1,4 glycosidic bond between GlcNAc and anhydro-*N*-acetylmuramic acid in cell wall degradation products, suggesting that the enzyme might participate in the cell wall recycling of *E. coli*.[17] In *Alteromonas* sp. strain O-7, three chitinases (ChiA, ChiB, and ChiC), a chitinase-like enzyme (ChiD), and three β-*N*-acetylglucosaminidases (GlcNAcaseA, GlcNAcaseB, and GlcNAcaseC) are involved in the chitinolytic system. However, judging from the substrate specificity of Hex86, unlike GlcNAcaseA,[7] GlcNAcaseB,[6] and GlcNAcaseC,[5] the enzyme does not appear to take part in the degradation of chitin oligosaccharides. To clarify the physiological role of Hex86 in *Alteromonas* sp. strain O-7, it remains to be investigated whether the enzyme cleaves phenolic compounds from chitin-producing organisms or cell wall degradation products such as anhydro-muropeptides.

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