The Chitin Catabolic Cascade in the Marine Bacterium

*Vibrio furnissii*

MOLECULAR CLONING, ISOLATION, AND CHARACTERIZATION OF A PERIPLASMIC

β-N-ACETYLGLUCOSAMINIDASE*

(Received for publication, March 22, 1996, and in revised form, August 19, 1996)

Nemat O. Keyhani‡ and Saul Roseman§

From the Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

We have described some steps in chitin catabolism by *Vibrio furnissii*, and proposed that chitin oligosaccharides are hydrolyzed in the periplasmic space to GlcNAc and (GlcNAc)2. Since (GlcNAc)2 is an important inducer in the cascade, it must resist hydrolysis in the periplasm.

Known V. furnissii periplasmic hydrolases comprise an endoenzyme (Keyhani, N. O. and Roseman, S. (1996) *J. Biol. Chem.* 271, 33414–33424), and the β-N-acetylglucosaminidase, ExoI, reported here. ExoI was isolated from a recombinant strain of *Escherichia coli*, and hydrolyzes aryl-β-GlcNAc, aryl-β-GalNAc, and chitin oligosaccharides. No other β-GlcNAc glycosides were cleaved. The pH optimum was 7.0 for (GlcNAc)n, n = 3–6, but 5.8 for (GlcNAc)2. At the pH of sea water (8.0–8.3), the enzymatic activity with (GlcNAc)2 is virtually undetectable. These results explain the stability of (GlcNAc)2 in the periplasmic space.

The cloned β-GlcNAcidase gene, *exoI*, encodes a 69,377-kDa protein (611 amino acids); the predicted N-terminal 20 amino acid residues matched those of the isolated protein. The protein amino acid sequence displays significant homologies to the α- and β-chains of human hexosaminidase despite their marked differences in substrate specificities and pH optima.

N-Acetylhexosaminidases are distributed throughout nature, from bacteria to man, and have been studied for many decades (1). Most of the exoenzymes in this class are β-N-acetylglucosaminidases (β-GlcNAcidases), although α-N-acetylglucosaminidases have been reported (1, 2).

In general, the β-GlcNACidases hydrolyze β-glycosides of GlcNAc and GalNAc, are not specific for the aglycon group although they prefer aryl substituents, and exhibit optimal activity between pH 4 and 6. The latter observation is expected, since many of these enzymes are of lysosomal origin. However, several bacterial enzymes have been reported that also show acid pH optima. Many β-GlcNAcidases have been cloned and/or purified and, as indicated under “Results” and “Discussion,” these include the genes and gene products from several marine *Vibrios* (3–6).

The marine bacterium *Vibrio furnissii* expresses a number of exo- and endohexosaminidases (7); characterizing these enzymes and the corresponding genes is essential for defining the chitin catabolic cascade in this organism. The accompanying papers describe a periplasmic chitodextrinase and an aryl β-GlcNAcidase (8, 9). This report is concerned with a second periplasmic enzyme, a β-GlcNAcidase designated ExoI to distinguish it from other β-GlcNAcidases produced by this organism. Although the enzyme can hydrolyze (GlcNAc)2, our studies suggest that (GlcNAc)2 is not significantly hydrolyzed under physiological conditions. Thus, the periplasmic β-GlcNAcidase is not a chitobiase in the usual sense. The unique properties of this enzyme are essential for the proper function and regulation of the chitin catabolic cascade, as explained in detail under “Discussion.”

**EXPERIMENTAL PROCEDURES**

**Materials**

The following chemicals, reagents and materials were purchased from the indicated sources: chitin, GlcNAc, and PNP-glycosides from Sigma; chitin oligosaccharides, (GlcNAc)n, n = 2–6, Seikagaku America, Inc. (Rockville, MD); MUF-GlcNAc and MUF-(GlcNAc)2 from Calbiochem; reagents for bacterial media from Difco and J.T. Baker; HEPES from Research Organics Inc. (Cleveland, OH). Reagents for molecular biology were obtained from New England Biolabs, U. S. Biochemical Corp., Life Technologies, Inc., Stratagene, and Boehringer Mannheim. Radioisotopes were purchased from DuPont NEN. Immobilized polyvinylidene difluoride and nitrocellulose transfer membranes were purchased from Millipore (Bedford, MA). Other buffers and reagents were of the highest purity available commercially.

**Methods**

N-Acetylglucosamine was determined by the Morgan-Elson method (10, 11), chitin oligosaccharides by HPLC chromatography as described (7), protein by the SDS-Lowry method (12), or, alternatively, by the Bio-Rad protein assay (13) with bovine serum albumin as the standard. SDS-PAGE was performed essentially as described (14). Western blots were prepared by electrophoretic transfer of the proteins from the SDS-PAGE gels to nitrocellulose (14). Samples for N-terminal amino acid sequence

This paper is available online at http://www-jbc.stanford.edu/jbc/
V. furnissii Periplasmic β-N-Acetylgalcosaminidase (ExoI)

determination were electroblotted onto Millipore Immobilon-P polyvinylidene difluoride membranes. The blots were then stained with 1× IPES using Coomassie Blue and the desired protein band on the membrane subjected to sequence analysis. N-terminus of the purified β-GlcNAcase was performed by Dr. Tomas Kempe, PNA Laboratory, Department of Chemistry and Biochemistry, University of Maryland, College Park. The N terminus was determined with the Edman (15) method using an Applied Biosystems model 477A protein sequencer. The phenylthiodydantoin derivatives were analyzed on a model 120A amino acid analyzer.

**Growth of V. furnissii**

Strain SR1514 was grown at 30°C in minimal-lactate medium (16) containing HEPE/ESCl-buffed 50% artificial sea water (lactate-50% ASW medium). The cells were induced to express ExoI by adding 0.6 mM (GlcNAc)2 (7).

Inocula were grown overnight in LMB broth (10 g of Bacto-tryptone, 5 g of yeast extract, 20 g of NaCl/liter), diluted 1:50 into the lactate-50% ASW medium with or without 0.6 mM (GlcNAc)2, and grown to mid-exponential phase.

**Construction of β-GlcNAcase Overexpression Vector (pVex03.7) and DNA Sequence Analysis**

The β-GlcNAcase gene, *exoI*, cloned into pBR322 as a ClaI 4.5-kb insert (7) contained two internal NcoI sites separated by 0.8 kb. The 0.8-kb fragment was deleted from the insert by digesting the plasmid with NcoI followed by ligation of the residual plasmid DNA. Finally, the 3.7-kb insert was isolated by treating the plasmid with ClaI, “blunt-ended” by filling in the overhanging ends of the fragment with Klenow polymerase, and purified by gel electrophoresis. This fragment was inserted into the IPTG-inducible overexpression vector pVex (17). Since pVex does not contain a *ClaI* site in its multiple cloning site, the vector was digested with *Smal*, which produces blunt ends, followed by treatment with calf intestinal phosphatase to reduce the probability of regenerating the plasmid during ligation. The 3.7-kb V. furnissii DNA fragment was then ligated into pVex, transformed into *E. coli* HB101, and single colony purified. Two orientations of the *exoI* gene were expected in the vector, and indeed two plasmids were isolated from different clones. The plasmids were characterized by restriction mapping with NcoI and *BamHI* (pVex contains the *BamHI* and the Vibrio insert contains the *NcoI* site). One of the plasmids, designated pVex:exoI3.7, contained the insert in the desired orientation with respect to the T7 promoter.

The HB101-transformed cells harboring pVex:exoI3.7 produced 40 times more enzyme than did the pBR322:exoI transformants. It should be emphasized, however, that significant quantities of enzyme were also produced by cells harboring pVex:exoI3.7 in the opposite orientation, indicating that the *V. furnissii* DNA fragment carries its own promoter.

Maximal quantities of the enzyme were obtained as follows. (a) The plasmid was used to transform *E. coli* BL21 cells, which contains a genomic copy of the T7 polymerase gene under lac promoter control. IPTG is used to induce T7 polymerase production, which then stimulates transcription from the T7 promoter on the pVex plasmids (17). (b) IPTG induction of the T7 polymerase dramatically reduces growth of the cells. Therefore, the cells were grown for various periods of time in LB medium containing 100 μg of ampicillin/ml, and induced for 2 h with 1 mM IPTG. Cell density had little effect on the specific activity of the induced enzyme, and therefore the cells were grown to late exponential phase prior to induction. (c) The effect of induction time on the quantity of enzyme produced was studied, and maximum expression of ExoI was obtained at 2 h.

Double-stranded DNA prepared from the recombinant clone pVex:exoI3.7 was sequenced by the dyeoxy method using a Sequenase V2.0 sequencing kit from U. S. Biochemical Corp. (18, 19). The analyses of DNA and amino acid sequences were conducted with the GCG sequence analysis package (Version 7, Genetics Computer Group, Madison, WI). The data bases used for nucleotide and amino acid sequence similarities searches (using FASTA) were: GenBank™ Release 79, and Swiss Protein Release 26. Predicted amino acid sequences were aligned using CLUSTAL W version 1.5; similarities were shown by shading using BOXSHADE/DOGS 2.7, kindly provided by Michael Cleveland.

**Preparation of Polyclonal Antibodies to Purified β-GlcNAcase**

Polyclonal antibodies were prepared in Pasteurella free rabbits. Preimmune sera showed no reaction on Western blots with the purified β-GlcNAcase, or with crude extracts of *E. coli* and *V. furnissii*. The rabbit was subdermally immunized according to the following schedule: (a) 100 μl of pure β-GlcNAcase (0.5 ml) mixed with Freund’s incomplete adjuvant (1.5 ml); (b) after 15 days, 50 μg of β-GlcNAcase (0.25 ml) mixed with Freund’s incomplete adjuvant (1.75 ml); and (c) after 15 days, 25 μg of β-GlcNAcase (0.125 ml) in Freund’s incomplete adjuvant (1.85 ml). Ten days after the last injection, the rabbit was bled and the immune sera analyzed for reactivity with the β-GlcNAcase.

The IgG fraction of the antibody sera was purified by affinity chromatography on a Bioprobe International, Inc. Avidiprotein Protein A column using the manufacturer’s recommended procedure.

To study the subcellular distribution of the enzyme in *V. furnissii*, the cells were ruptured in a French pressure cell, the soluble and thoroughly washed membrane fractions isolated, and Western blots obtained as described (14), using the IgG fraction at 1:1000 dilution and horseradish peroxidase goat anti-rabbit IgG (Cappel Organon Teknika, Durham, NC).

**Enzyme Assay**

Enzyme activity was detected in colonies growing on agar plates by use of methylumbellifere-GlcNac (MUF-GlcNac). Colonies were lifted onto sterile Whatman No. 1 paper, which, when sprayed with 0.6 mM MUF-GlcNac in 10 mM sodium phosphate buffer, pH 7.5, the paper was incubated at 37°C for 10–20 min and subsequently sprayed with saturated sodium bicarbonate solution to enhance the fluorescence of the hydrolysis product, MUF. Colonies expressing the β-GlcNAcase gene product glowed bright blue when illuminated under UV light. Two quantitative enzyme assays were used when PNP-GlcNac was the substrate, and a third (Morgan-Elson) assay when the natural oligosaccharides were the substrates.

**Discontinuous Spectrophotometric Assay**—Cell extracts or protein fractions were incubated at 37°C for 5–20 min in 100 μl volumes containing the following: 0.66 mM PNP-GlcNac, 50 mM sodium phosphate buffer, pH 7.5, and 50 mM NaCl. The reaction was quenched with 3 ml of 1 M Tris base, pH 11, and the absorbance was measured at 400 nm. An extinction coefficient of 18,300 (× cm)−1 was used to calculate p-nitrophenol produced per minute. Production formation was proportional to time of incubation and the quantity of protein used in the assay. No hydrolysis of PNP-GlcNac was detected in the controls, containing boiled extracts of *E. coli* or *V. furnissii*.

**Continuous Spectrophotometric Assay**—Hydrolysis of PNP-GlcNac was quantitated by continuously monitoring the rate of PNP formation at A585 using a Perkin-Elmer Lambda 3 spectrophotometer and R-100A recorder. The temperature was maintained at 37°C with a Lauda circulator. Reactions were initiated by adding protein fractions or purified protein (2–5 μl) to 500 μl of the assay mixture described above. The rate of PNP formation (nmol/min/μg of enzyme) was determined using an extinction coefficient experimentally determined in the incubation buffer. Initial rates of PNP formation were proportional to protein concentration, and no detectable hydrolysis of the substrate was observed with the controls described above. One important control consisted of extracts of *E. coli* transformed with the pVex vector with no Vibrio DNA insert.

**Morgan-Elson Assay**—When the substrates consisted of GlcNAc-terminated oligomers, such as the chitin oligosaccharides, the product of hydrolysis, GlcNac, was determined by the Morgan-Elson assay (10, 11). In these cases, incubation mixtures contained 0.1–10 μg chitin oligosaccharides in the indicated buffers, and reactions were initiated by adding 0.01–0.1 μg of purified enzyme to the substrates at 37°C. Aliquots (0.1 ml) were taken at the indicated times, and the samples heated at 100°C to stop the reactions. GlcNac was determined by adding 0.1 ml of 0.8 M sodium borate, pH 9.2, heated at 100°C for 12 min, cooled to room temperature, and treated with 550 μl of freshly prepared, diluted Ehrlich reagent (10 g of p-dimethylaminobenzaldehyde in 90 ml of glacial acetic acid, 10 ml of concentrated HCl, diluted 1:10 in acetic acid before use). After 20 min at 37°C, the mixture was cooled to room temperature, and after 15 min the absorbance was determined at 585 nm.

**Effects of pH, Ionic Strength, and Temperature on Enzyme Activity**

The effects of these parameters on activity were studied with the purified enzyme at 37°C.

**Effects of pH**—The discontinuous assays were used with 330 μM PNP-GlcNac or 1 mM chitin oligosaccharide and the following buffer systems: McIlvaine’s sodium phosphate-citric acid broad-range buffer from pH 2.0–8.6, Bates’ and Bowers’ boric acid-KCl buffer (ranging from 8.0–10.0), sodium phosphate, Tris-HCl, Bis-Tris-HCl, and Hepes-
NaCl (20). Where possible, overlapping pH ranges were used with different buffers. At the same pH values, essentially the same rates were obtained with different buffers. Reactions were initiated by adding 0.01–0.1 μg of purified enzyme, and initial rates were plotted against pH provided that they were constant with time of incubation and proportional to protein concentration.

**Effect of Ionic Strength**—The effect of ionic strength on enzyme activity was determined as follows. A solution containing 330 μM PNP-GlcNAc was incubated at 37°C for 10 min at 4°C. The column was eluted at room temperature, and maintaining the solution at room temperature until an activity of 0.01–0.1 μg of purified protein, and PNP formation was followed by the continuous spectrophotometric assay. The temperature optimum and thermostability of the β-GlcNAcase were investigated using PNP-GlcNAc as substrate, and the discontinuous spectrophotometric assay. The temperature optimum was determined by incubating reaction mixtures (200 μl) containing 10 mM Tris-HCl, 0.1 mM NaCl, and 330 μM substrate PNP-GlcNAc over a temperature range from 0°C to 75°C. Reaction mixtures were preincubated at the desired temperatures before adding 0.01–0.1 μg (1 μl) of purified protein. The stability of the enzyme was measured by adding enzyme (0.01–0.1 μg) to 250 μl of buffer without substrate, incubating at the desired temperature for 15 min, cooling to room temperature, and maintaining the solution at room temperature for 5 min before assay by the continuous spectrophotometric method at 37°C.

**Purification of ExoI**

**Step 1: Crude Extract**—Three 6-liter flasks containing 2 liters each of LB medium (10 g of Bacto-tryptone, 5 g of yeast extract, 10 g of NaCl/liter) supplemented with 100 μg/ml ampicillin were inoculated with 40 ml of each of an overnight culture of E. coli strain BL21 harboring the plasmid pVexexoI. The cultures were shaken vigorously at 37°C until an A600 = 1.0 was attained, after which IPTG was added to a final concentration of 1 mM and the cultures returned to the shaker incubator for an additional 2 h. The cells were harvested by centrifuging at 4000 × g for 10 min at 4°C. All remaining steps were conducted at 0–4°C unless otherwise stated. The cell pellet was washed with 3 liters and then with 1 liters of 50 mM sodium phosphate, pH 7.5, containing 0.5% NaCl and 1 mM EDTA. The final cell pellet (10 g wet weight) was resuspended in 40 ml of the buffer without NaCl and disrupted by passage (two to three times) through a Wabash French press. Unlysed cells, as well as some cell debris, were removed by centrifugation at 12,000 × g for 15 min.

**Step 2: Streptomycin Sulfate**—To precipitate nucleic acids, streptomycin sulfate (160 μg of a 10% stock/ml of crude extract) was added dropwise with stirring at 4°C. The mixture was stirred for an additional 30 min and centrifuged at 235,000 × g for 60 min. Adding more streptomycin sulfate did not give any detectable precipitate.

**Step 3: Ammonium Sulfate Fractionation**—Proteins in the streptomycin sulfate-treated supernatant (55 ml at 25 mg/ml) were precipitated by the dropwise addition of saturated ammonium sulfate (22 ml) to a final concentration of 30% of saturation. The solution was stirred for 1 h and centrifuged at 235,000 × g for 30 min. The resulting supernatant was treated with 110 ml of saturated ammonium sulfate (to 70% saturation) and the mixture stirred for 12 h before the pellet was recovered by centrifugation at 235,000 × g for 1 h. The 30–70% ammonium sulfate pellet was resuspended in approximately 20 ml of 10 mM Tris-Cl, pH 7.5, containing 1 mM EDTA and 0.1 mM dithiothreitol and extensively dialyzed against the same buffer.

**Step 4: DEAE-Sepharose Chromatography**—The 30–70% ammonium sulfate fraction was transferred to a 30-ml DEAE-Sepharose CL-6B column that had been equilibrated with 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 0.1 mM dithiothreitol. After placing the sample on the column, it was washed with 90 ml of the same buffer. A gradient was then applied to elute the column; the linear gradient consisted of 0.01–0.5 M NaCl in the same buffer, 300 ml total volume. The activity eluted as a sharp peak between 0.25 and 0.30 M NaCl. The active fractions were pooled and concentrated to 10 ml in an Amicon concentrator with a Millipore PPTK membrane (30,000 molecular weight cutoff). Buffer without salt was added to bring the sample to 50 ml, and the sample was concentrated.

**Step 5: ACA-34 gel filtration chromatography**—A 1.8 × 85-ml gel filtration ACA-34 column was equilibrated with 10 mM Tris-Cl, pH 7.5, 0.1 M NaCl. The active pool from Step 4 was placed on the column in two batches, and the column was eluted with the Tris/NaCl solution. The active peaks from the two runs were combined and concentrated to 1.0 ml as described above.

**Step 6: HPLC Ion-exchange Chromatography**—The pooled sample from Step 5 was transferred to a Bio-Rad (7.5 × 75 mm) Bio-Gel TSK-DEAE-5-FW HPLC column equilibrated with 10 mM Tris-Cl, pH 7.0, containing 1 mM EDTA. The column was eluted at room temperature with 10 ml of the same buffer, followed by a 0–0.4 M NaCl gradient in the buffer (0.5-ml fractions). Purified β-GlcNAcase eluted at 0.3 M NaCl. Purity was monitored throughout the fractionation by SDS-PAGE and enzyme activity.

**Results**

**Purification and Properties of β-GlcNAcase**—The gene exoI, which encodes the β-GlcNAcase ExoI, was subcloned from the original isolate (7) into the overexpression vector pVex, and the enzyme purified from the transformed cells. A summary of the purification procedure is given in Table I. The enzyme was isolated in about 22% yield and purified about 40-fold to apparent homogeneity. The estimated molecular weight from SDS-PAGE was 70 kDa. Preliminary experiments with gel filtration columns suggest that the enzyme may dimerize (data not shown). ExoI is stable both at room temperature and 37°C for at least 24 h.

The effect of ionic strength on enzymatic activity was measured at the optimum pH (see below) and in the NaCl and KCl concentration ranges: 0–2.0 M. Activity was maximal over a broad range, 0.05–0.5 M salt; the enzyme retained 80% of its activity at 1 M, and 50% of its activity at 2 M salt. Thus, ExoI would be fully active in the periplasmic space of the organism, regardless of extreme variations in salinity found in estuarine and marine waters.

Sea water also contains significant concentrations of divalent cations, especially Mg2+ and Ca2+. However, the enzyme retained >90% of its activity in 1–10 mM EDTA/EGTA. The effects of Mg2+, Ca2+, Zn2+, and Hg2+ were examined in reaction mixtures containing 0.0–5.0 mM concentrations of these cations. Mg2+, Ca2+, and Zn2+ had no effect on enzyme activity.

**TABLE I**

| Step | Total protein | Total activitya | Specific activityb | Purification factor |
|------|---------------|----------------|-------------------|--------------------|
|      | mg            | units          | units/mg          |                    |
| 1. Crude extract | 1625          | 7962           | 4.9               | 1.0                |
| 2. Streptomycin | 1375          | 7425           | 5.4               | 1.1                |
| 3. Ammonium sulfate | 600       | 5760           | 9.5               | 1.9                |
| 4. DEAE-Sepharose | 100           | 4400           | 44                | 8.9                |
| 5. ACA-44 gel filtration | 20         | 2660           | 133               | 26.6               |
| 6. HPLC-DEAE | 8.75          | 1760           |                   |                    |

a PNP-GlcNAc hydrolyzed (μmol/min) in the continuous spectrophotometric assay.
b PNP-GlcNAc hydrolyzed, μmol/min · mg protein.
up to 5.0 mM; however, 1 mM Hg$^{2+}$ (330 µM PNP-GlcNAc) inhibited β-GlcNAcIDase activity by 50%, and only 10% of the enzyme activity remained at 5 mM Hg$^{2+}$. The enzyme may contain sensitive -SH groups.

The optimum temperature and the thermal stability of the β-GlcNAcIDase activity at pH 7.5 was studied as described under “Methods.” The temperature optimum for assay was 35–40 °C, and the β-GlcNAcIDase was stable for at least 15 min from 4–42 °C. Above 42 °C, however, the enzyme decreased in stability until a 15-min incubation at 65 °C resulted in complete loss of activity.

**Effect of pH**—The effect of pH on activity was studied by the continuous spectrophotometric assay with PNP-GlcNAc as substrate, and by the Morgan-Elson discontinuous method with (GlcNAc)$_n$, $n = 2$–6, as the substrates.

With PNP-GlcNAc, the pH was varied from 2.5 to 12, and the results are shown in Fig. 1. The pH optimum was between 7.0 and 8.0, but the range was broad. The enzyme exhibited about 50% of its activity at pH 6.0 and 9.0. The enzyme also exhibited full activity at pH 7.5 after preincubation for 15 min at 25 °C in the pH range 5.5–9.5.

The most intriguing and potentially important results were obtained in pH studies with the natural substrates, (GlcNAc)$_n$, $n = 2$–6. The data are shown in Fig. 2. With the tri- through hexasaccharides, a broad peak was observed with an optimum close to pH 7. At the pH of sea water, 8.0–8.3, the enzyme exhibits 50–60% of maximal activity.

With the disaccharide, however, quite different results were obtained. Again the peak was relatively broad, but the optimum was now pH 5.8, 1.2 pH units below the optimum for (GlcNAc)$_n$, $n = 3$–6. At the present time, there is no obvious explanation for these results.

At pH 8.0–8.3, the rate of hydrolysis of the disaccharide is not detectable. The potential physiological implications of this phenomenon are considered below.

**Effect of Substrate Concentration**—Kinetic constants were determined at pH 7.5 (the pH of the growth medium) for all substrates tested except (GlcNAc)$_2$. The dependence of the β-GlcNAcIDase activity on substrate concentration was measured by the continuous spectrophotometric assay with two artificial substrates, PNP-GlcNAc and PNP-GalNAc. $K_m$ and $V_{max}$ were calculated from Woolf-Augustinson ($v$ versus $1/[S]$) plots, and are given in Table II. PNP-GlcNAc is the more effective substrate, with about half the $K_m$ and 3 times the $V_{max}$ of PNP-GalNAc.

The $K_m$ and $V_{max}$ for the natural oligosaccharides, (GlcNAc)$_n$, $n = 2$–6, are also shown in Table II. The $K_m$ values for these oligosaccharides are 10–40-fold greater than the $K_m$ for the artificial substrate. However, the $V_{max}$ values for (GlcNAc)$_n$, $n = 3$–6, are about 30% higher than the $V_{max}$ for PNP-GlcNAc.

For reasons discussed in the Introduction, we were particularly interested in the kinetic constants for (GlcNAc)$_2$. At pH 5.8, the $K_m$ for the disaccharide was in the same range as the other oligosaccharides, but the $V_{max}$ was 6–7-fold lower.

As indicated under “Discussion,” the natural environment for marine organisms is generally above pH 7.5. At pH 8.0–8.3, the pH of sea water, (GlcNAc)$_2$ hydrolysis was so low as to be questionable. At pH 7.5, a slow hydrolysis appeared to take place, but it was not reproducible from one experiment to another, did not remain constant sufficiently long to obtain an initial rate, and was not proportional to protein concentration. For this reason, we can only conclude that the maximum rate of hydrolysis of the disaccharide at pH 7.5 is ≤2% of the hydrolysis rate of the higher oligosaccharides.

**Substrate Specificity and Inhibition of PNP-GlcNAc Hydrolysis**—The purified β-GlcNAcIDase showed no detectable activity with PNP-β-Glc, PNP-β-Gal, PNP-α-GlcNAc, or PNP-β-S-GlcNAc, although some of these were potent inhibitors (competitors) of the PNP-GlcNAc activity (see below).

The following GlcNAc terminated glycopeptides were kindly provided by Dr. Y. C. Lee: (a) ovalbumin glycopeptide, which is biantennary with one terminal GlcNAc; (b) asialo, agalacto triantennary fetuin glycopeptide, where the oligosaccharide is...
Ki and $V_{\text{max}}$ values determined at pH 6.0.

linked to Asn-Asp-Ser, and contains three terminal GlcNAc residues. Neither glycopeptide was a substrate for ExoI.

A number of compounds were tested as potential inhibitors of ExoI by following PNP-GlcNAc hydrolysis in the continuous assay.

GlcNAc, a product of the enzymatic reaction, was a poor inhibitor, <10% when used at concentrations below 2 mM. At high concentrations, 10 mM, GlcNAc did show significant inhibition (40–50%) but the same results were obtained with the following sugars: Glc, Gal, Glc-6-P, GlcNH₂-6-P, maltose, cellobiase, and lactose. GlcNAc-6-P showed a somewhat greater effect, a 50% inhibition being observed at 5 mM.

The non-hydrolyzable artificial analogues were more potent inhibitors of PNP-GlcNAc hydrolysis, and their effects were analyzed kinetically using a concentration range for each inhibitor at several substrate concentrations. In these experiments, initial rate measurements were obtained under conditions where less than 5% of the substrate was hydrolyzed. The data were analyzed using Lineweaver-Burk plots (1/v versus 1/[S]) to calculate both the type of inhibition and the respective $K_i$ values, and the results are shown in Table III.

It is interesting to note that none of the synthetic analogues tested were hydrolyzed with the exception of phenyl-β-glucosaminidase, but that the other four compounds (PNP-β-Glc, PNP-β-Gal, PNP-α-GlcNAc, and PNP-β-S-GlcNAc) were effective inhibitors. As a first interpretation, the data suggest an aromatic ring binding site close to the catalytic site of the enzyme, and that this binding is not affected by the sugar (Glc, Gal, and GlcNAc), or anomeric configuration of the glycosyl unit (e.g. PNP-α-GlcNAc).

The natural substrates (GlcNAc)$_n$, $n = 3$–6, were also tested as competitive inhibitors of PNP-GlcNAc hydrolysis. Table III gives the apparent $K_i$ values, and these are approximately the same as the $K_m$ values for these compounds when used as substrates (Table II).

(GlcNAc)$_3$ is again of special interest. While it is virtually not hydrolyzed at pH 7.5, it is a non-competitive inhibitor of the enzyme, and the $K_i$ value is 2.4 mM, only 2–4-fold higher than the corresponding $K_i$ values for the oligosaccharides that are substrates of the enzyme. If the apparent $K_i$ (or $K_m$) values represent affinity constants, then the disaccharide binds to the enzyme almost as well as the other oligomers at a pH where it is a very poor substrate.

**ExoI Is Not a Processive Enzyme—**When the β-GlcNAcIdase catalyzes the hydrolysis of (GlcNAc)$_n$, it could conceivably act processively. Two types of experiments were performed to test this idea.

The rate of hydrolysis of PNP-(GlcNAc)$_2$ by the β-GlcNAcIdase was approximately 15 μmol of PNP released/min/mg of protein. However, Fig. 3 shows that GlcNAc release from PNP-(GlcNAc)$_2$ preceded PNP formation, despite the fact PNP-GlcNAc is the most effective substrate thus far tested with this enzyme. Thus, the enzyme must cleave the terminal GlcNAc from PNP-(GlcNAc)$_n$, releasing the products GlcNAc and PNP-GlcNAc into the medium. PNP is only formed when sufficient PNP-GlcNAc accumulates.

In a second set of experiments, the hydrolyzable (GlcNAc)$_n$ oligosaccharides were tested, samples collected at early time points, and analyzed by the HPLC method. In the case of every substrate tested, only GlcNAc and (GlcNAc)$_n-1$ were observed at these kinetic time points.

From these data we conclude that the enzyme is not processive, i.e. when it hydrolyzes (GlcNAc)$_n$, both products, (GlcNAc)$_n-1$ and the terminal GlcNAc, are released into the medium before the enzyme attacks a second molecule of (GlcNAc)$_n$ or (GlcNAc)$_n-1$. 

### Table II

| Substrate | $K_m$ (mM) | $V_{\text{max}}$ (μmol/min/mg) |
|-----------|-----------|-----------------------------|
| PNP-GlcNAc | 0.090 | 270 |
| PNP-GalNAc | 0.33 | 130 |
| GlcNAc | 1.7 | 53.6 |
| GlcNAc$_2$ | 0.83 | 335 |
| GlcNAc$_3$ | 0.78 | 376 |
| GlcNAc$_4$ | 3.7 | 385 |
| GlcNAc$_6$ | 3.5 | 376 |

### Table III

| Inhibitor type | Substrate / Inhibitor (×) | Inhibitor type | $K_i$ (mM) |
|----------------|--------------------------|----------------|-----------|
| Competitive    |                          | Non-competitive | 2.4       |
| Non-competitive|                          | Competitive     | 0.73      |
| Non-competitive|                          | Competitive     | 0.65      |
| Competitive     |                          |                 | 1.42      |
| Competitive     |                          |                 | 1.22      |

**FIG. 3. Hydrolysis of PNP-(GlcNAc)$_n$ by purified β-GlcNAcIdase.** The rates of formation of GlcNAc and β-nitrophenol were followed. Duplicate assay mixtures contained the following components in 1.0 ml: 1 mM PNP-(GlcNAc)$_n$, 10 mM sodium phosphate buffer, pH 7.5, 0.1 mM NaCl. The reaction was started by adding 0.1 μg of β-GlcNAcIdase, and incubations were conducted at 37°C. Aliquots (0.2 ml) were removed at the indicated times from one of the duplicate mixtures, heated at 100°C for 5 min, and GlcNAc determined by the Morgan-Elson method (●). PNP formation was followed by the continuous spectrophotometric method in the second incubation mixture. The values represent nanomoles of product per incubation mixture.
It is especially interesting to compare the rates of hydrolysis of PNP-(GlcNAc)₂ with the two most effective substrates and with (GlcNAc)₃. Under the conditions described in Fig. 3, the rates are as follows. The terminal GlcNAc is released from PNP-(GlcNAc)₂ at a rate of 250 nmol/min/µg protein; PNP-GlcNAc, 200; (GlcNAc)₃, 150; (GlcNAc)₄, virtually nil. Although extensive kinetics were not performed with PNP-(GlcNAc)₂, it appears to be the most effective substrate for the enzyme and suggests that ExoI contains a hydrophobic pocket at or near the position occupied by the reducing sugar in the trisaccharide.

Subcellular Location of β-GlcnAcidase in V. furnissii—In our earlier paper, evidence was presented that the β-GlcnAcidase, ExoI, is a periplasmic enzyme. Enzymatic activity is expressed by intact, induced cells, in contrast to the cytoplasmic chitobiase (7). The availability of antibodies directed against the homogenous protein permitted a more detailed analysis of the subcellular distribution of the enzyme in V. furnissii. The study was conducted using Western blots to detect proteins with the same epitopes as the recombinant ExoI. Only one antigenic band was detected in crude extracts of V. furnissii, and (a) the band migrated with the same mobility as the isolated enzyme, and (b) it appeared only when the cells were induced with (GlcNAc)₃, consistent with previous results.

In a second set of experiments, the V. furnissii crude extract was separated into soluble and membrane fractions and analyzed by the same method. Both fractions contained the antigen, and the intensity of the band was about the same in each fraction. We do not know the origin of the soluble antigen. It could be located either in the cytoplasm or in the periplasmic space, as is the membrane-bound protein. In light of the homologies shown by ExoI with other Vibrio chitobiases, the antigenic soluble protein could conceivably be the cytoplasmic chitobiase; this would require that it have a cross-reactive epitope and the same mobility on SDS-PAGE.

N-terminal Amino Acid Sequence—The N-terminal sequence of the purified enzyme was determined as described under “Experimental Procedures.” The sequence of the first 20 amino acids is: Met-Asn-Tyr-Arg-Ile-Asp-Phe-Ala-Val-Leu-Ser-Glu-His-Pro-Gln-Phe-Cys-Arg-Phe-Gly.

Sequence of exoI Gene—The nucleotide sequence of the V. furnissii insert in pVex and the deduced amino acid translation for the region encoding the β-GlcnAcidase has been deposited in GenBank with the accession number U41417.

The start codon of the gene was found at 844 nucleotides from the SmaI cloning site of pVex, clockwise from the T7 promoter, and contained its own putative promoter including a −10 and −35 consensus region and a ribosome binding site. The start codon of the gene was found to be at nucleotide 844, and it extends to nucleotide 2677, the beginning of a stop codon.

An exact alignment was observed between the first 20 amino acids of the N terminus determined by amino acid sequencing of the purified protein and that predicted by the DNA sequence of the open reading frame 844-2677. The protein encoded by exoI contains 611 amino acids, and the molecular mass is 69,377 Da (70 kDa was the estimate from SDS-PAGE).

A search of the Swiss Protein Data Bank identified six proteins with significant similarities to the translated open reading frame of the β-GlcnAcidase gene. These proteins include: (a) the Vibrio harveyi chitobiase (3, 4), 883 amino acids long, and with a 33.0% identity in a 276-amino acid overlap with the β-GlcnAcidase described here; (b) the α-chain of human β-hexosaminidase (21), 529 amino acids, and with 31.1% identity in a 182-amino acid overlap; (c) the β-chain of human β-hexosaminidase (22), 556 amino acids, and 28.8% identical in a 125-amino acid overlap; (d) the α-chain of mouse β-hexosaminidase (23), 528 amino acids, 33.3% identity in a 171-amino acid overlap; (e) the β-chain of mouse β-hexosaminidase (24), 536 amino acids, and 28.7% identical in a 265-amino acid overlap; (f) Dictyostelium discoideum (slime mold) β-hexosaminidase (25), 532 amino acids, and with 31.5% identity in a 111-amino acid overlap. The individual regions of identity and similarity are given in Fig. 4.

The mouse hexosaminidase α-chain, the human hexosaminidase α- and β-chains, and the Dictyostelium hexosaminidase are all similar to a region spanning amino acids 200–400 in the V. furnissii β-GlcnAcidase. The V. harveyi chitobiase homology extended from amino acid 120 to 400 in the V. furnissii gene. The mouse β-chain hexosaminidase is noticeably different from the other sequences, and the similarity to exoI extended from amino acid 340 to 610 of the V. furnissii enzyme.

A chitobiase has also been reported in V. parahemolyticus (6), and although the DNA sequence of this gene has not yet been reported, the sequence of the 46 amino acids at the N terminus of the protein has been determined. A comparison of the V. furnissii and V. parahemolyticus N termini shows a complete match in 32 of 46 amino acids (approximately 70% sequence identity). It remains to be seen whether this large homology will extend to the whole protein. This preliminary comparison may, in fact, be somewhat misleading; it is possible that the two enzymes share a similar Vibrio leader sequence, since both are thought to be periplasmic in the corresponding Vibrio strains.

Thus, as has been reported elsewhere (3, 4), there is considerable conservation of β-hexosaminidases throughout evolution, from Vibrios to mouse and man.

DISCUSSION

The chitin catabolic cascade in V. furnissii is a complex process involving a minimum of three signal transduction systems and many enzymes and proteins. These cells rapidly catabolize chitin and chitin oligosaccharides (7), and the periplasmic space plays a key role in this process. All available data suggest that the oligosaccharides, (GlcNAc)₃, are hydrolyzed by β-N-acetylglucosaminidases in the periplasm to (GlcNAc)₂ and GlcNAc. These sugars are then taken up by separate transport systems (26, 27). One periplasmic enzyme, EndoI, is described in the accompanying paper (9), and here we report the properties of the second enzyme, ExoI.

Three exo-β-N-acetylglucosaminidases have been cloned from Vibrio, and all are chitobiases. The first such enzyme reported (3, 4) is an outer membrane protein in V. harveyi and is translocated to the outer membrane of the E. coli transformant. The unprocessed enzyme is 97.8 kDa (883 amino acid residues). A similar enzyme was cloned from Vibrio vulnificus (5). The third enzyme, from Vibrio parahemolyticus (6), appears to be unrelated to the first two according to its N-terminal amino acid sequence. This enzyme is also quite large, 80 kDa on SDS-PAGE, and is also a chitobiase, although its physical and biochemical properties are different from all other reported chitobiases. As explained below, we do not classify ExoI (611 amino acids, 69,377 kDa) as a chitobiase, although it can display this activity.

ExoI exhibits some of the characteristics expected of a β-N-acetylglucosaminidase or, more generally, of exoglycosidases. It actively hydrolyzes PNP-β-GlcnAc and the corresponding umbelliferyl derivative, displays a lower activity with PNP-GalNAc, slight to negligible activity with phenyl β-GlcnAc, and no detectable activity with aliphatic β-GlcnAc glycosides.

Unlike most β-GlcnAcidases, however, the Vₘₐₓ values obtained with ExoI and the natural substrates, (GlcNAc)n, n = 3–6, are larger than the Vₘₐₓ for PNP-β-GlcnAc, although the Kₘ for PNP-β-GlcnAc is much lower. Furthermore, no other GlcnAc terminated oligosaccharide tested served as a sub-
strate for this enzyme. But there is another reason to conclude that ExoI is different from other β-N-acetylglucosaminidases.

Many proteins and enzymes of the chitin cascade are induced by (GlcNAc)2. Thus, it is essential that the disaccharide escape hydrolysis in the periplasm. At the same time, it is important that the higher oligosaccharides, such as the trisaccharide, be hydrolyzed in the same compartment. *V. furnissii* has developed an unexpected mechanism for dealing with this apparent paradox, and it is this mechanism that makes ExoI unique.

ExoI can act as a chitobias, but only at non-physiological pH values. The pH optimum is 7.0 for (GlcNAc)n, n ≥ 3, but is shifted 1.2 pH units, to pH 5.8 for (GlcNAc)2 (Fig. 2). Furthermore, under optimum conditions for each substrate, the Vmax values for the higher oligosaccharides are 6.3–7 fold greater than for (GlcNAc)2 (Table II). But the crucial point is that at the pH of marine waters, 8.0–8.3, hydrolysis of the disaccharide is
not detectable. At the pH of the growth medium, 7.5, the maximum rate of hydrolysis of the disaccharide is less than 2% of that of the higher oligosaccharides (Fig. 2). Thus, a simple shift in the pH optimum has these potent physiological implications. How this effect is achieved remains to be determined, but it is incorrect, we believe, to characterize ExoI as a “chitobiase.”

To summarize, the kinetic properties of the two *V. furnissii* periplasmic enzymes described in these reports, EndoI and ExoI, explain the fate of the higher chitin oligosaccharides when they enter the periplasm. The oligomers are converted to GlcNAc and (GlcNAc)₂, which are then taken up by their specific transport systems. But how do the higher oligosaccharides enter the periplasmic space? Present experiments are designed to answer this question.

REFERENCES
1. Walker, P. G. (1966) in *The Amino Sugars* (Balazs, E. A., and Jeanloz, R. W., eds) pp. 155–169, Academic Press, New York
2. Roseman, S., and Dorfman, A. (1951) *J. Biol. Chem.* **191**, 607–620
3. Soto-Gil, R. W., and Zyskind, J. W. (1989) *J. Biol. Chem.* **264**, 14778–14783
4. Jannatipour, M., Soto-Gil, R. W., Childers, L. C., and Zyskind, J. W. (1987) *J. Bacteriol.* **169**, 3785–3791
5. Somerville, C. C., and Colwell, R. R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6751–6755
6. Zhu, B. C. R., Lo, J., Li, Y., Li, S., Jaynes, J. M., Gildemeister, O. S., Laine, R. A., and Ou, C. (1992) *J. Biochem. (Tokyo)* **112**, 163–167
7. Bassler, B. L., Yu, C., Lee, Y. C., and Roseman, S. (1991) *J. Biol. Chem.* **266**, 24276–24286
8. Chitiara, E., and Roseman, S. (1996) *J. Biol. Chem.* **271**, 33433–33439
9. Keyhani, N. O., and Roseman, S. (1996) *J. Biol. Chem.* **271**, 33414–33424
10. Reisig, J. L., Strominger, J. L., and Leloir, L. F. (1955) *J. Biol. Chem.* **217**, 959–966
11. Morgan, W., and Elson, L. (1934) *Biochem. J.* **28**, 988
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
13. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–252
14. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1996) *Current Protocols in Molecular Biology*, Vols. I–III, and supplements, John Wiley & Sons, New York
15. Edman, P. (1949) *Arch. Biochem. Biophys.* **22**, 475
16. Yu, C., Lee, A. M., Bassler, B. L., and Roseman, S. (1991) *J. Biol. Chem.* **266**, 24260–24267
17. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 61–63
18. Sanger, F., Nklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
19. U. S. Biochemical Corp. (1993) *Protocols for DNA Sequencing with Sequenase (V 2.0)* 7th Ed., U. S. Biochemical Corp., Cleveland, OH
20. Perrin, D. D., and Dempsey, B. (1987) *Buffers for pH and Metal Ion Control*, pp. 126–156, Chapman and Hall, London
21. Korneluk, R. G., Mahuran, D. J., Neote, K., Klavins, M. H., O'Dowd, B. F., Trepak, M., Willard, H. F., Anderson, M.-J., Lowden, J. A., and Gravel, R. A. (1986) *J. Biol. Chem.* **261**, 8407–8413
22. Neote, K., Babat, B., Dumbrille-Ross, A., Truxel, C., Shuster, S. M., Mahuran, D. J., and Gravel, R. A. (1988) *Genomics* **3**, 279–286
23. Berceri, T., Hoade, J., Orlachio, A., and Stirling, J. L. (1992) *Biochem. J.* **265**, 593–596
24. Bapat, B., Ethier, M., Neote, K., Mahuran, D., and Gravel, R. A. (1998) *FEBS Lett.* **237**, 191–195
25. Graham, T. R., Zassenhaus, P., and Kaplan, A. (1988) *J. Biol. Chem.* **263**, 16823–16829
26. Bouma, C., and Roseman, S. (1996) *J. Biol. Chem.* **271**, 33468–33475
27. Keyhani, N. O., Wang, L. X., Lee, Y. C., and Roseman, S. (1996) *J. Biol. Chem.* **271**, 33469–33473