The Chitin Catabolic Cascade in the Marine Bacterium

*Vibrio furnissii*

MOLECULAR CLONING, ISOLATION, AND CHARACTERIZATION OF A PERIPLASMIC CHITODEXTRINASE*†

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Chitin catabolism in *Vibrio furnissii* comprises several signal transducing systems and many proteins. Two of these enzymes are periplasmic and convert chitin oligosaccharides to GlcNAc and (GlcNAc)2. One of these unique enzymes, a chitodextrinase, designated EndoI, is described here.

The protein, isolated from a recombinant *Escherichia coli* clone, exhibited (via SDS-polyacrylamide gel electrophoresis) two enzymatically active, close running bands (~mass of 120 kDa) with identical N-terminal sequences. The chitodextrinase rapidly cleaved chitin oligosaccharides, (GlcNAc)4 to (GlcNAc)2, and (GlcNAc)5,6 to (GlcNAc)2 and (GlcNAc)3. EndoI was substrate inhibited in the millimolar range and was inactive with chitin, glucosamine oligosaccharides, glycoproteins, and glycopeptides containing (GlcNAc)2. The sequence of the cloned gene indicates that it encodes an 112,690-kDa protein (1046 amino acids). Both proteins lacked the predicted N-terminal 31 amino acids, corresponding to a consensus prokaryotic signal peptide. Thus, *E. coli* recognizes and processes this *V. furnissii* signal sequence. Although inactive with chitin, the predicted amino acid sequence of EndoI displayed similarities to many chitinas, with 8 amino acids completely conserved in 10 or more of the homologous proteins. There was, however, no "consensus" chitin-binding domain in EndoI.

The marine bacterium *Vibrio furnissii* can utilize chitin as a sole source of carbon and nitrogen; the cascade involves several signal transduction processes and a multitude of proteins (1–4) whose expression is stringently regulated. These cells also rapidly catabolize GlcNAc and soluble chitin oligosaccharides, and the rate of GlcNAc utilization is comparable to that for the optimal rate of glucose catabolism by *Escherichia coli*.

The enzymatic hydrolysis of chitin has been studied for almost a century (5). In 1939, Zechmeister and Toth (6, 7) reported the key observation; the insoluble polymer was converted to its monomer, GlcNAc, by two enzymes, a "polysaccharidase" (chitinase), and a "disaccharidase," a β-N-acetylglucosaminidase or chitobiase. Chitinases and chitobases have since been purified from a wide variety of sources (8) including plants (9, 10), animals (11), yeast (12), and prokaryotes such as *Streptomyces* (13, 14), *Bacillus circulans* (15, 16), and *Vibrio parahaemolyticus* (17). In addition, recent reports describe similar enzymes in higher animals. Although their exact specificities and functions remain to be determined, enzymes capable of cleaving chitin oligosaccharides have been reported in human plasma (18), and in articular chondrocytes and synovial cells (19). Chitotriosidase activity is elevated more than 600-fold in the plasma of Gaucher patients (18).

As noted in the references and reviews cited above, many of the structural genes encoding these enzymes have been cloned and sequenced. In agreement with the early work, the chitinases yield (GlcNAc)2 as the major product of chitin hydrolysis, although higher oligomers are sometimes intermediates in the process.

We have previously reported (3) that the structural genes encoding two periplasmic enzymes were cloned from *V. furnissii* into *E. coli*. In this and the accompanying paper (20), the genes and gene products are characterized. Despite the extensive literature, the enzymes described here and in the accompanying paper, a chitodextrinase (EndoI) and a β-GlcNAcidase1 (ExoI) respectively, exhibit unique properties that are essential for functioning of the chitin catabolic cascade in *V. furnissii*.

EXPERIMENTAL PROCEDURES

Materials

The following chemicals were purchased from the indicated sources. GlcNAc, PNP-glycosides1 from Sigma; highly purified chitin (Chitin 1000), chitin oligosaccharides, and (GlcNAc), n = 2–6, were from Seikagaku America, Inc. (Rockville, MD); MUF-GlcNAc and MUF-(GlcNAc)2 were from Calbiochem; reagents for bacterial media were from Difco and J.T. Baker; HEPES was 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. Immobilon polyvinylidene difluoride and nitrocellulose transfer membranes were purchased from Millipore (Bedford, MA). Other buffers and reagents were of the highest purity available commercially. The structural gene that encodes the chitinase produced

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1 The following abbreviations are used: β-GlcNAcidase, β-N-acetylglucosaminidase; PNP, p-nitrophenol; (GlcNAc), chitin oligosaccharides, i.e. where the GlcNAc residues are linked β,1–4, and n = 2–6, as specified; MUF, 4-methylumbelliferone = 7-hydroxy-4-methyl-coumarin, a highly fluorescent compound; PNP- or MUF-(GlcNAc)n, the PNP or MUF β-glycoside of a chitin oligosaccharide; kb, kilobase pair(s); HPLC, high pressure liquid chromatography; DTT, dithiothreitol; αα, amino acid(s); Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.
by Aeromonas hydrophila has been cloned into E. coli (21), and the transformant was kindly made available to us by Dr. J. M. Pembridge. We thank Dr. C. Rowe, who purified the enzyme to homogeneity (22) and provided us with a sample.

\[^{3}H\]Acetyl-labeled chitin was prepared as follows. Commercial chitosan was completely deacetylated by fusion with KOH (23), and the chitosan reacetylated with \(^{3}H\)-labeled acetic anhydride (24, 25). In the latter step, the solution is vigorously stirred, and as the chitin is formed it yields very fine particles, close to a colloidal dispersion that makes a good substrate for chitinases (25). It should be emphasized that there is extensive degradation of the polymer during the preparation, especially in the KOH treatment, producing, we believe, a highly polydisperse solution.

\[\text{PNP-(GlcNAc)}_{n} \text{ was adjusted to } 100 \text{mM} \]

was used to calculate \(p\)-nitrophenol produced/min of purified protein. Activity was monitored by the discontinuous spectrophotometric assay. The thermostability of the enzymes was measured by preincubating the enzyme (0.01–0.1 \(\mu\)g of purified protein) for 15 min at 50°C. The reaction mixture was then warmed to room temperature for 5 min before the reaction was started by adding substrate and followed by the continuous spectrophotometric assay at 37°C.

| Kinetic Parameters |
|-------------------|
| **Effect of Ionic Strength**—The effect of ionic strength on the purified recombinant \(V. \text{furnissii} \) chitodextrinase enzymes was determined using solutions of 330 \(\mu\)M PNP-(GlcNAc)\(_{3}\) in 10 mM Tris-HCl, pH 7.0, and in 10 mM Tris-HCl containing 2 mM NaCl or 2 mM KCl. The two solutions were mixed to give salt concentrations ranging from 0 to 1.0 M. Two solutions were then added to the cell culture at a 1:50 dilution, and this culture was grown to the desired density. Bovine serum albumin was used as the standard.

| **Bacterial Strains** |
|----------------------|
| Commercial E. coli strains HB101, BL21, and XL-Blue were stored as frozen cultures in LB. Typically, strains were grown overnight in LB media (plus appropriate antibiotics where indicated) with vigorous shaking. Fresh medium was inoculated with cells from the overnight culture at a 1:50 dilution, and this culture was grown to the desired density, usually mid-exponential (OD\(_{600}\) = 0.3–0.4).

**Protein Determination**

Protein concentrations were measured by the SDS-Lowry method (26) or, alternatively, by the Bio-Rad protein assay, which involves the binding of Coomassie Brilliant Blue G-250 to proteins (27). Bovine serum albumin was used as the standard.

| Molecular Analysis and Sequencing of DNA |
|-----------------------------------------|
| Preparation and analysis of DNA preparations, restriction enzyme digestions, ligations, and transformations were performed using standard techniques (28, 29). For DNA sequence analysis, the 6.1-kb HindIII insert containing the chitodextrinase gene in pBR322 (3) was subcloned into two single-strand producing phagemids, the pBluescript SK+ and SK− vectors and sequenced from these constructs (30, 31). The 6.1-kb insert was further subcloned to 3.6 kb using HindIII and AvaI. 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\textsuperscript{TM} 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/DOS 2.7, kindly performed by Michael Cleveland.

**Chitodextrinase Assays**

Enzyme activity was qualitatively determined from colonies growing on agar plates by use of the synthetic analogue, MUF-(GlcNAc)\(_{3}\), which, when hydrolyzed, yields a fluororescent product, 4-methylumbelliferone. Colonies were lifted onto sterile Whatman No. 1 paper, which was then sprayed with 0.6 mM MUF-(GlcNAc)\(_{3}\) 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 in 10 mM Tris-HCl containing 2M NaCl or 2M KCl. The two solutions were mixed to give salt concentrations ranging from 0 to 1.0 M. Assay reactions were started by adding 0.01–0.1 \(\mu\)g of purified protein and the absorbance of the solution was followed by the continuous spectrophotometric method.

**Effect of Temperature**—The temperature optimum and the thermostability of the chitodextrinase were investigated by incubating reaction mixtures (200 \(\mu\)l) containing 10 mM Tris-HCl, pH 7.0, 0.1 mM NaCl, and 330 mM substrate (PNP-(GlcNAc)\(_{3}\)) over the range 0°C to 75°C. Reaction mixtures were preincubated at the desired temperatures before 0.01–0.1 \(\mu\)g (1–5 \(\mu\)l) of purified protein were added. Activity was monitored by the discontinuous spectrophotometric assay. The thermostability of the enzymes was measured by preincubating the enzyme (0.01–0.1 \(\mu\)g) in 250 \(\mu\)l of buffer at the indicated temperature for 15 min. The reaction mixture was then warmed to room temperature for 5 min before the reaction was started by adding substrate and the activity measured by the continuous spectrophotometric assay at 37°C.

**SDS-PAGE**—Protein samples were heated to 100°C for 5–10 min in Laemmli buffer (65 mM Tris-Cl, pH 6.8, with 0.3% SDS, 10% glycerol, 5% \(\beta\)-mercaptoethanol, and 0.1 mM bromphenol blue) unless otherwise noted. Samples were electrophoresed in a vertical 8–10% polyacrylamide slab gel, pH 8.0, with a 6% stacking gel, pH 8.3 (29). Gels were stained with Coomassie Brilliant Blue G-250.

**Detection of Chitodextrinase in SDS-PAGE Gels**

The SDS-PAGE system was modified in order to resolve the two high molecular weight proteins in the purified chitodextrinase preparations described below. From 0.5 to 3.0 \(\mu\)g of purified protein/0.5-cm lane was used employing the same stacking gel, but with a 15-cm-long 5% resolving gel. Electrophoresis was continued for 1 h after the dye front had electrotetrated (3 h total). After electrophoresis, the gel was soaked.
details of the purification procedure are presented in the text. The 4-liter culture yielded about 10 g (wet weight) of washed cells. the protein sequencer.

### Purification of Chitodextrinase

**Step 1: Crude Extract**—Two liters each of LB medium supplemented with 75 μg/ml ampicillin in two 6-liter flasks were inoculated with 120 ml of an overnight culture of *E. coli* strain BL21 harboring the plasmid pBR-ENDO. The culture was shaken vigorously at 37 °C until A_{600} was about 3.0, and the cells harvested by centrifugation at 4000 g for 10 min. The following steps in the purification were conducted at 0–4 °C unless otherwise stated.

The cell pellet was washed twice with 800 ml of 50 mM Tris-HCl, pH 7.0, containing 0.1 M NaCl and 1 mM EDTA, resuspended in 50 mM Tris-HCl, pH 6.5, 1 mM EDTA, using 4.0 ml of buffer (wet weight) of cells, and the cells disrupted by three passages through a Wabash French press. Unlysed cell were removed by centrifugation at 12,000 × g for 15 min.

**Step 2: Streptomycin Sulfate Precipitation**—Nucleic acids were precipitated with a solution of streptomycin sulfate (160 μl of 10% stock/ml crude extract), which was added dropwise with stirring at 4°C. The white precipitate was removed by centrifugation at 235,000 × g for 60 min and discarded.

**Step 3: Ammonium Sulfate Fractionation**—Satuated ammonium sulfate solution (120 ml) was added dropwise, with stirring at 4 °C, to the streptomycin supernatant (50 ml) to a final concentration of 70%. After stirring for 12 h, the mixture was centrifuged at 235,000 × g for 1 h, the pellet resuspended in 10 ml Tris-HCl, pH 6.5, 1 ml EDTA, 0.05 M MgCl₂, and extensively dialyzed against the same buffer.

**Step 4: DEAE Chromatography**—The 70% ammonium sulfate fraction was transferred to a 30-ml DEAE-Sepharose CL-6B column equilibrated in the same buffer. The column was washed with 90 ml of the Tris-EDTA-DTT buffer, followed by 150 ml of buffer containing 0.2 M NaCl. A gradient (300 ml) from 0.2 M NaCl to 1.0 M NaCl in the buffer was applied, 3.0-ml fractions collected, and a broad peak of activity detected between 0.45 and 0.75 M NaCl. The active fractions were pooled and concentrated to 10 ml on a Millipore filter (30,000 molecular weight cutoff). To remove most of the salt, buffer without salt was added to bring the sample to 50 ml, the sample was concentrated, and the process was repeated.

**Step 5: Hydroxylapatite Column Chromatography**—The DEAE pool was transferred to a 30-ml hydroxyapatite column equilibrated with the Tris-DTT buffer (no EDTA). The column was washed with 90 ml of buffer containing 0.005 M MgCl₂, followed by 150 ml of buffer with 1.0 M MgCl₂. The 0.005 M MgCl₂ removes basic proteins, and the 1.0 M MgCl₂ elutes proteins with isoelectric points between 5.5 and 8.0 (32). The chitodextrinase activity was eluted with a 300-ml gradient of sodium phosphate, pH 7.0, from 0 to 50 mM PO₄. The activity eluted between 20–40 mM phosphate. Active fractions were pooled and concentrated as described above, and the salt exchanged for the Tris-NaCl buffer used in the next step.

**Step 6: Gel Filtration Chromatography**—An ACA-34 (2 × 83 cm) was equilibrated with 10 ml Tris-HCl, pH 7.0, 0.1 M NaCl. The pooled, concentrated fractions from Step 5 were transferred to the column, and eluted with the same buffer. The void volume of the column was about 80 ml, and the activity eluted between 80 and 100 ml.

**Step 7: HPLC Ion-exchange Column Chromatography**—The active, pooled fractions were concentrated to 1 ml and transferred to an HPLC-DEAE column equilibrated with 10 mM Tris-HCl, pH 6.5, containing 0.1 M NaCl. The column consisted of a Bio-Gel TSK-DEAE 5-PW HPLC column (7.5 × 75 mm) and was eluted at room temperature with 10 ml of the buffer, followed by a linear gradient, 0.1 to 1.0 M NaCl in 10 ml Tris-HCl buffer, pH 6.5. The active fractions eluted between 0.6–0.7 M NaCl.

Throughout the fractionation, samples were assayed both for activity and by SDS-PAGE. The final preparations were stable for at least several months at ~80 °C.

### RESULTS

**Purification of Chitodextrinase**

The enzyme was purified from *E. coli* BL21 harboring pBR-ENDO as described under “Experimental Procedures.” A summary of the results is given in Table I. The chitodextrinase was purified about 460-fold from the crude extract in 15% yield. SDS-PAGE of the crude extract and the final product are shown in Fig. 1A. The HPLC fraction appeared to be homogeneous, with an M₅₀ ~ 120,000 Da. When the lane contained 0.1–0.3 μg of protein instead of 3 μg as shown in Fig. 1A, it appeared that the single band might consist of a doublet. To clearly visualize the two protein bands,
SDS-PAGE conditions were optimized for resolving high molecular weight proteins. A 5% resolving gel and longer electrophoresis time separated the proteins (Fig. 1C), and both bands exhibited chitodextrinase activity after they were renatured in the gel (Fig. 1B).

**Determination of Chitodextrinase N-terminal Sequences**

The N-terminal sequences of each of the two protein bands on the SDS gels were determined as described under “Experimental Procedures.” The first 21 amino acids of the higher molecular weight protein exhibited the following sequence: Ala-Val-Asp-Cys-Ser-Ala-Leu-Ala-Trp-Gln-Ser-Asp-Thr-Ile-Tyr-Thr-Gly-Xaa-Xaa-Gln. The first 9 amino acids of the lower molecular weight product displayed the same sequence: Ala-Val-Asp-Cys-Ser-Ala-Leu-Ala-Glu. Since the N termini of the two protein bands are identical, the most likely explanation for the slight difference in molecular sizes is a change at the C termini, perhaps because of proteolysis, but this possibility was not further investigated.

**Properties of Chitodextrinase**

The following parameters were systematically investigated using PNP-(GlcNAc)₂ as the substrate for the enzyme:

**Ionic Strength**—The activity remained relatively constant over the range 50–250 mM NaCl (pH 7.0), but decreased significantly below and above these concentrations. In KCl in place of NaCl, the activity decreased 10–30%. There was no apparent effect of many different buffering ions on the activity (see “Experimental Procedures”).

**Divalent Cations**—Enzyme activity was not affected by 5 mM EDTA or EGTA, nor by the addition of up to 5 mM Ca²⁺ or Zn²⁺. Mg²⁺ was slightly stimulatory, with a 20–25% increase in activity at 2 mM, and Hg²⁺ acted as a weak inhibitor, decreasing activity by 25% at 5 mM.

**Temperature**—These effects were measured in 50 mM sodium phosphate, 0.1M NaCl, pH 7.0. The optimum assay temperature was 35–37 °C, and when the enzyme was pretreated for 15 min over the range 5–65 °C, some activity was lost between 40–50 °C, and the enzyme was completely inactivated at 52 °C.

**Effect of pH on Chitodextrinase Activity**

As shown in the accompanying paper (20), there is a large shift in the pH optimum of the periplasmic β-GlcNAcidase when the substrate is (GlcNAc)₂ compared to the other chitin oligosaccharides and the artificial substrate, PNP-GlcNAc. Furthermore, we believe that this shift is of great physiological significance because it permits the disaccharide to traverse the periplasmic space and act as an inducer for a variety of *V. furnissii* proteins. Thus, it was important to conduct similar studies with the periplasmic chitodextrinase.

The effects of pH on the activity with the synthetic and natural substrates are shown in Fig. 2. Different buffering ions had little effect on the activity with the synthetic substrate, PNP-(GlcNAc)₂, but this parameter could not be tested with the oligosaccharides. Hydrolysis of the oligosaccharides was followed by the HPLC procedure described under “Experimental Procedures.” Results are expressed as nanomoles of substrate cleaved/min/μg of EndoI. B, (GlcNAc)₄; C, (GlcNAc)₅; the initial rates of formation of (GlcNAc)₃ (●), (GlcNAc)₄ (●), and (GlcNAc)₅ (●) are shown. D, (GlcNAc)₆ disappearance (○), (GlcNAc)₅ (●), and (GlcNAc)₄ (●) formed.
Table II  
**Inhibition of PNP-(GlcNAc)₂ Hydrolysis by Chitodextrinase**

The rate of PNP-(GlcNAc)₂ hydrolysis was followed by the continuous spectrophotometric assay described in the text. Both the inhibitors and the substrate were tested over a range of concentrations. Some of the "inhibitors" are hydrolyzed by the enzyme. Lineweaver-Burk kinetic analyses were used to determine apparent Kᵢ values and the type of competition. In each case, the kinetics were consistent with competitive inhibition of the enzyme.

| Inhibitor or competitor | Substrate         | Kᵢ (µM) |
|-------------------------|-------------------|---------|
| PNP-GlcNAc              | No                | 650     |
| PNP-(GlcNAc)₃          | Yes               | 220     |
| (GlcNAc)₂              | No                | 1025    |
| (GlcNAc)₃             | No                | 570     |
| (GlcNAc)₄             | No                | 90      |
| (GlcNAc)₅             | Yes               | 88      |
| (GlcNAc)₆             | Yes               | 77      |

The deviation from typical Michaelis-Menten kinetics at higher substrate levels suggested the possibility of potent product, or possibly substrate inhibition.

**Inhibition of PNP-(GlcNAc)₂ Hydrolysis**

Chitodextrinase-catalyzed hydrolysis of PNP-(GlcNAc)₂ was measured in the presence of various monosaccharides to test them as potential inhibitors. Neither glucose, galactose, GlcNAc, nor GlcNAc-6-PO₄ showed any appreciable inhibition at concentrations up to 3 mM. Likewise, there was little inhibition by disaccharides, including lactose, maltose, and cellobiose. Other PNP glycosides that were neither hydrolyzed to PNP nor acted as inhibitors included: PNP-β-cellobioside, PNP-β-maltoside, and PNP-α-N-acetylglucosaminide.

GlcNAc derivatives were found to inhibit the enzyme, and the data are summarized in Table II. In each case, Kᵢ values were derived by assaying the enzyme at different PNP-(GlcNAc)₂ concentrations and a range of potential competitor/inhibitor concentrations.

Perhaps the most important result shown in the table is that the products of the enzymatic reactions, (GlcNAc)₂ and/or (GlcNAc)₃ (depending on the substrate) are poor inhibitors. Thus, the deviation from Michaelis-Menten kinetics in the hydrolysis of PNP-(GlcNAc)₂ is not because of inhibition by (GlcNAc)₃.

All of the compounds shown in Table II act as competitive inhibitors, which is expected for those that are substrates, but non-hydrolyzable compounds behaved similarly, i.e., PNP-GlcNAc, (GlcNAc)₂, and (GlcNAc)₃.

PNP-(GlcNAc)₃ was a good competitor with a Kᵢ of 220 µM. While PNP-(GlcNAc)₂ is not hydrolyzed to PNP by the chitodextrinase, it is possible that this compound is cleaved to PNP-(GlcNAc)₂ and (GlcNAc)₃, both of which would resist further hydrolysis.

Finally, the natural substrates (GlcNAc), were potent competitors, as expected, exhibiting Kᵢ values of 70–90 µM.

**Effect of Concentration of (GlcNAc)ₙ on Chitodextrinase Activity**

The quantitative HPLC method (3) modified as described under "Experimental Procedures" was used to follow the kinetics of hydrolysis of the chitin oligosaccharides. There was no detectable hydrolysis of (GlcNAc)₂ and (GlcNAc)₃, even after prolonged digestion with excessive quantities of enzyme. (GlcNAc)₄, (GlcNAc)₅, and (GlcNAc)₆, on the other hand were rapidly hydrolyzed. In every case the products consisted solely of the di- and/or trisaccharide. That is, (GlcNAc)₄ gave (GlcNAc)₂ as the sole product, (GlcNAc)₅ yielded equimolar quantities of (GlcNAc)₂ and (GlcNAc)₃, while (GlcNAc)₆ gave variable quan-
tities of (GlcNAc)$_2$ and (GlcNAc)$_3$, depending on the conditions of the assay (e.g. pH, Fig. 2).

Initial rates of hydrolysis of (GlcNAc)$_n$, $n = 4-6$, as a function of substrate concentrations were determined, and are shown in Fig. 4. The three substrates gave virtually identical results. At low concentrations, below about 0.1 mM, the compounds were rapidly cleaved. However, as the concentrations of the three substrates were increased above about 0.2 mM, the rates of hydrolysis rapidly declined. An explanation for these kinetics was therefore sought.

Possible product inhibition effects were ruled out by adding the quantities of (GlcNAc)$_2$ and (GlcNAc)$_3$ formed at the early stage (initial rate) of 10 mM (GlcNAc)$_5$ hydrolysis to reaction mixtures containing 0.1, 0.5, 1.0, or 10 mM of the substrate, (GlcNAc)$_n$. There was no detectable effect of these additions on the kinetics of hydrolysis of (GlcNAc)$_n$.

Conceivably, inhibition results from a contaminant(s) in the oligosaccharide preparations. But this possibility appeared to be eliminated by the following. (a) The substrates were analyzed and found to be 97% pure (minimally) by the Amide-80 HPLC system. The HPLC system was then used to purify enough (GlcNAc)$_n$ to repeat the kinetics measurements, and the latter gave similar results to those presented in Fig. 4. There remains the possibility that the hypothetical contaminant co-eluted with the (GlcNAc)$_n$ during the “purification.” If so, it was also present in the (GlcNAc)$_4$ and (GlcNAc)$_6$ preparations and at the same levels in each substrate. (b) The hypothetical contaminant was absent from the (GlcNAc)$_4$ and (GlcNAc)$_6$ preparations since they are poor inhibitors. (c) Finally, (GlcNAc)$_n$, $n = 4-6$, are potent competitive inhibitors of the enzyme when the substrate is PNP-(GlcNAc)$_2$ (Table II). Much higher concentrations of each of the oligomers is required for 50% inhibition of their own hydrolyses (Fig. 4).

These results led to the conclusion that the chitodextrinase displays potent substrate inhibition kinetics. The initial portions of the curves in Fig. 4 show an approximate linear increase of the reaction rates with substrate concentrations, and these were analyzed in Woolf-Augustinsson plots. The apparent $K_m$ and $V_{max}$ values are summarized in Table III, along with the corresponding values for PNP-(GlcNAc)$_2$. The natural substrates are evidently significantly better substrates of the enzyme than is the synthetic analogue. In addition, the apparent substrate $K_m$ values are in the 20–90 $\mu$m range for the oligosaccharides, which approximates the $K_i$ values (77–90 $\mu$m) when the oligomers act as inhibitors of the hydrolysis of the synthetic analogue.

Is the Enzyme a Chitinase?

Unlike bacterial chitinases, which generally contain a chitin binding domain (8, 34), the chitodextrinase gene does not contain this domain (see below). Furthermore, we were unsuccessful in repeated attempts to bind the chitodextrinase to chitin columns (for affinity chromatography).

Bacterial chitinases generally yield (GlcNAc)$_2$ as the major product of hydrolysis of chitin (35). The homogeneous enzyme from A. hydrophila (see “Experimental Procedures”) also yields (GlcNAc)$_2$, along with small amounts of (GlcNAc)$_3$ and GlcNAc. The chitinase rapidly hydrolyzes the higher oligosaccharides, at about 10-fold the rate of labeled chitin, and PNP-(GlcNAc)$_2$ at about twice the rate (22). The enzyme is product inhibited by the disaccharide ($K_i$, 0.26 mM), although the chitinase can virtually completely digest the polysaccharide when the hydrolysis products are continuously removed by dialysis.

The chitodextrinase was compared to the chitinase in two types of experiments. To use similar quantities of activity, the homogeneous protein preparations were normalized with

| Substrate       | $K_m$ ($\mu$M) | $V_{max}$ (pmol/min/mg) |
|-----------------|---------------|-------------------------|
| PNP-(GlcNAc)$_2$| 170           | 34                      |
| (GlcNAc)$_4$    | 40            | 28                      |
| (GlcNAc)$_5$    | 20            | 71                      |
| (GlcNAc)$_6$    | 90            | 86                      |

Fig. 4. Effect of oligosaccharide concentrations on chitodextrinase activity. Assay conditions (37 °C) were the indicated concentrations of substrates in mixtures containing 0.05 $\mu$g of purified enzyme, 10 mM Na$_2$PO$_4$, pH 7.0, and 0.1 mM NaCl. After heating at 100 °C, aliquots were analyzed by the HPLC method, and the initial rate at each concentration is shown. A, (GlcNAc)$_4$. The inset gives an expanded view of the 0 to 1 mM concentration range. B, (GlcNAc)$_5$. C, (GlcNAc)$_6$.
Characterization of V. furnissii Periplasmic Chitodextrinase

![Graph](image)

**Fig. 5. Comparison of EndoI and chitinase activities on chitin.** The experiment is described under "Is The Enzyme a Chitinase?" A. purified commercial chitin (Seigaku Chitin 1000) was extensively washed with the buffer that gave the maximal rate of hydrolysis of \((\text{GlcNAc})_n\), with each of the enzymes. The "chitodextrinase buffer" was 10 mM sodium phosphate buffer, pH 7.0, 0.1M NaCl, while the "chitinase buffer" was 10 mM phosphate, pH 7.5, 0.1M NaCl, 0.1% bovine serum albumin. Ten-mg samples of the washed chitin were then incubated at 37 °C in the respective buffers with 167 units of chitinase (●) or with 142 units of the chitodextrinase (○). Aliquots were removed at the indicated times, heated to 100 °C, and the supernatants analyzed by the HPLC method. A separate incubation mixture contained chitodextrinase assayed with chitin in the chitodextrinase buffer, and gave the same results (○) shown for the chitodextrinase buffer, i.e., no detectable hydrolysis. B. chitodextrinase activity with \(^{3}H\)chitin. Commercial chitodextrin was completely deacylated by fusing with KOH (180 °C, 30 min, under N₂) and then reacylated using labeled acetic anhydride. The number of counts/min in the supernatant, solubilized by each of the enzymes, are shown as nanomoles of GlcNAc equivalent. The incubation mixtures (37 °C) contained the labeled chitin in the respective buffers with 5 units of chitinase (●) or with 7 units of chitodextrinase (○) added to each of several replicate samples. At 6 h, 5 units of chitinase (○) was added to one of the chitodextrinase replicate samples, and an additional 7 units of chitodextrinase (□) to another. At 10 h, the chitodextrinase had hydrolyzed 2.5% of the labeled chitin. There was little to no increase in the solubilized counts/min after overnight incubation.

\((\text{GlcNAc})_n\), an excellent substrate for each enzyme: *A. hydrophila* chitinase, \(V_{\text{max}}\), 167 nmol cleaved/µg/min; chitodextrinase, \(V_{\text{max}}\), 71 nmol cleaved/µg/min. For purposes of the following experiments, a unit of activity is defined as 1 nmol of \((\text{GlcNAc})_n\) hydrolyzed/min.

Fig. 5A shows the results with unlabeled commercial highly purified chitin (Seikagaku 1000). The chitinase rapidly produced \((\text{GlcNAc})_n\), about 1 µmol in 2 h at 37 °C, at which point product inhibition resulted in a decrease in the rate. By sharp contrast, neither \((\text{GlcNAc})_n\) nor any other oligosaccharide was detected in the chitodextrinase incubation mixture throughout the 72-h incubation. Aliquots of the mixtures were assayed with PNP-(GlcNAc)₂ over the 72-h experiment, and both enzymes retained at least 75% of their respective activities.

In the second experiment (Fig. 5B), partially degraded, labeled chitin was used as substrate. The results show that 5 units of the chitinase solubilized the labeled chitin at a constant rate over the time course of the experiment. The chitodextrinase (7 units) solubilized some of the label, but the reaction virtually stopped after 4 h of incubation. At 6 h, fresh chitodextrinase was added to one incubation, and the chitinase to another. The chitinase produced an immediate reaction, whereas the additional chitodextrinase had a slight to negligible effect on the labeled chitin.

In the experiment shown in Fig. 5B, a maximum of 2.5% of the radioactive substrate was solubilized by the chitodextrinase, even after prolonged incubation, whereas the chitinase continues to hydrolyze the substrate at a constant rate until sufficient product is formed to inhibit the enzyme.

In view of the drastic conditions used for making the chitosan, it should be emphasized that the labeled chitin consists of a polydisperse mixture of GlcNAc chains. Indeed, there is a slight but continuous leeching of soluble \(^{3}H\) from the final product for an indeterminate period of time, and this is despite prolonged dialysis against dilute acetic acid and water. In fact, we suspect that shorter, soluble GlcNAc oligomers may be trapped in the chitin particles and are slowly eluted. Furthermore, during the N-acetylation step we do not know what fraction of the polymer actually forms the sheets of hydrogen bonded cross-linked chains typical of native chitin. Thus, the slight solubilization effect shown in Fig. 5B does not, we think, represent the action of a true chitinase, but rather they result from polydispersity of the substrate.

**Nucleotide Sequence and Deduced Amino Acid Sequence**

The *V. furnissii* genomic DNA fragment containing the chitodextrinase coding region was subcloned into the single-strand producing sequencing vector pBluescript (pBS), in both the ‘+’ and ‘−’ strand, and sequenced by the dideoxy chain termination method (30, 31).

The DNA sequence of the 3.6-kb subclone of the *V. furnissii* insert is given in Fig. 6, with the predicted amino acid sequence for the region encoding the chitodextrinase.

The start codon of the gene was found to be at nucleotide 34, and the sequence extends to a stop codon beginning at nucleotide 3172. The deduced sequence of the protein consists of 1046 amino acid residues, with a total molecular mass of 112,690; the estimated molecular mass of the isolated protein(s) from SDS-PAGE was 120 kDa.

Although the purified chitodextrinase was resolved into two protein bands on SDS-PAGE, they exhibited identical N-terminal sequences. However, the observed N-terminal amino acid sequences corresponded to amino acid residue 31 deduced from the start codon of the DNA open reading frame. Thus, the purified chitodextrinase (albeit a doublet on SDS-PAGE) is probably a processed product, with a 31-amino acid peptide cleaved from its N terminus.

An analysis of the first 31 amino acids predicted by the DNA sequence translation is given until the stop codon at 3172. The putative −10 region and a potential termination loop structure (at the end of the reading frame) are underlined. The deduced protein sequence consists of 1046 amino acids, with a molecular mass of 112,690 Da.
sequence fulfills the conserved criteria described for a signal peptide (36) in \textit{E. coli} and other enteric bacteria. The first 10 predicted amino acids contain 5 with positive charges: 2 Arg, 2 Lys, and 1 His. This region is followed by neutral or hydrophobic amino acids ending at residue 27. Residues 28–31 are Ser–Tyr–Ala–Ala, and the last Ala (residue 31) is the observed N-terminus of the purified protein. These data indicate that the \textit{E. coli} host recognizes and cleaves the \textit{V. furnissii} chitodextrinase signal sequence. Osmotic shock experiments (data not shown) with the recombinant \textit{E. coli} cells resulted in the release of at least 50% of the chitodextrinase, suggesting that it is located in the periplasm as is the case in \textit{V. furnissii}.

**Fig. 7. Alignment of \textit{V. furnissii} chitodextrinase primary sequence with chitinases.** The primary sequence of the chitodextrinase gene (\textit{Vfendo}) is aligned with those of \textit{Bacillus circulans} chitinase A1 (\textit{BcichA}), \textit{Alteromonas} sp. chitinase (\textit{Altchi}), \textit{Serratia marcescens} chitinase B (\textit{SmachB}), \textit{Steptomyces placei} chitinase 6A (\textit{Splc63}), \textit{Bacillus circulans} chitinase D (\textit{BchichD}), \textit{Kluyveromyces lactis} killer plasmid toxin (\textit{Klatox}), and \textit{Brugia malayi} chitinase (\textit{Bmachi}). Identical residues are indicated by white on black, similar residues by white on gray.
Characterization of V. furnissii Periplasmic Chitodextrinase

Similarity of V. furnissii Chitodextrinase to Other Proteins

A search of the Swiss Protein Data Bank identified several proteins with significant similarity to regions of the translated open reading frame of the chitodextrinase gene. These proteins included: (a) the Bacillus circulans WL-12 chitinase A1 (chiA) gene (15, 16, 34, 37), consisting of 699 amino acids, with a 29.3% identity in a 328-aa overlap spanning residues 320–640 of the chitodextrinase gene; (b) the Alteromonas sp. chitinase (38), 820 aa, with a 21.5% identity in a 451-aa overlap spanning residues 310–750 of the chitodextrinase; (c) the Serratia marcescens chitinase B (chiB) (39), 499 aa, with a 28.9% identity in a 204-aa overlap (spanning residues 450–640); (d) the Alphanocladium album chitinase 1 (chiA) (40), 423 aa, with a 24.6% identity in a 167-aa overlap (spanning residues 510–670); (e) the Serratia marcescens chitinase A (chiA) (41), 561 aa, with a 22.2% identity in a 108-aa overlap (spanning residues 700–810); (f) the Trichoderma harzianum endochitinase (42), 518 aa, 31.2% identity in an 138-aa overlap (spanning residues 510–650); (g) Streptomyces pilatus chi (chiA) (43), 610 aa, with a 25% identity in a 132-aa overlap (spanning residues 520–650); (h) the Bacillus circulans chitinase D (16), 488 aa, with a 24.1% identity in a 232-aa overlap (spanning residues 15–280), (i) the Kluyveromyces lactis killer plasmid toxin (44), 1146 aa, with a 28% identity in a 118-aa overlap (spanning residues 450–570); and (j) the Brugia malayi chitinase (45), 504 aa, with a 22% identity in a 209-aa overlap (spanning residues 500–700). A summary of all the alignments is given in Fig. 7. Most of the proteins are homologous to a region spanning from residues 300–700 of the chitodextrinase gene; (k) several proteins with significant similarity to region of the translated protein sequence, which had homology to residues 700–810 of the chitodextrinase gene. These proteins were selected because they showed high similarity with the chitinase from a typical marine bacterium, Aeromonas hydrophila (21). Both enzymes hydrolyze (GlcNAc)n, n = 4–6, and both are substrate inhibited (44). How then do the two enzymes differ?

(a) The chitinase rapidly hydrolyzes chitin, at about one-tenth the rate of the oligosaccharides, and will ultimately cleave virtually all of the polysaccharide to (GlcNAc)2 and small quantities of (GlcNAc)3 and GlcNAc. The enzyme described here, however, was inactive with a commercial chitin preparation, which was a good substrate for the chitinase, and only slightly active with [1H]acetyl-labeled chitin for a short period of time, after which there was no further hydrolysis. After prolonged incubation, only 2.5% of the labeled chitin was hydrolyzed by the chitodextrinase, unlike the chitinase, which hydrolyzes this substrate until it is product-inhibited at about 0.25 mM (GlcNAc)2. We attribute the slight activity of Endo I on the labeled chitin to the polydispersity of the preparation, i.e., the chitodextrinase may act on “frayed” ends of the molecule, or on labeled oligomers that are trapped in the polydispersite chitin preparations.

(b) The chitinase displays slow but detectable activity with (GlcNAc)2, but the chitodextrinase (Endo I) does not. Endo I is unable to cleave either the first or last glycosidic bonds in the oligosaccharide chains; therefore, (GlcNAc)2 and (GlcNAc)3 are the hydrolysis products.

The chitodextrinase exhibits homology to a large number of chitinases, and several mechanisms have been proposed to explain this effect (45). The most likely explanation for the chitodextrinase is that it contains an elongated binding site (viz., lysozyme) that can accommodate two or more substrate molecules. Binding of one molecule in the appropriate orientation yields a productive complex, whereas binding of two or more substrate molecules clogs the active site, yielding unproductive complexes. Another possibility is that there is a catalytic site and other (allosteric) binding sites, and at mM substrate concentrations the allosteric sites are filled.2

At this point we can only speculate on the physiological significance of the substrate inhibitory effects on the chitodextrinase. At low concentrations of (GlcNAc)2, n ≥ 3, the disaccharide pathway (3) will predominate, whereas at high concentrations, the monosaccharide pathway will be the dominant route for catabolism because the chitodextrinase is inhibited. The importance of this change cannot be overemphasized. For example, when the cells grow on GlcNAc, they cannot be induced to express the chemotactic apparatus for the oligomers (2). Thus, at millimolar concentrations of the oligosaccharides, the cells will remain where they are, regardless of (GlcNAc)3. The artificial substrate PNP-(GlcNAc)2 appears to be an anomaly in that substituting PNP for the reducing GlcNAc residue in (GlcNAc)2 permits efficient catalysis (Table III). Thus, a productive enzyme-substrate complex is formed with PNP-(GlcNAc)2 (Km, 170 μM), whereas the complex with (GlcNAc)2 is non-productive, although it competes (K, 570 μM, Table II) with PNP-(GlcNAc)2. The results suggest a catalytic site in the chitodextrinase analogous to that of lysozyme (46, 47), comprising six subsites. Some of the subsites in lysozyme accept the PNP group in place of GlcNAc, and some of the complexes are productive.

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