Molecular Cloning and Characterization of a Novel β-N-Acetyl-d-glucosaminidase from Vibrio furnissii*

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Edith Chitlaru‡ and Saul Roseman§

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

The accompanying papers (Keyhani, N. O., and Roseman, S. (1996) J. Biol. Chem. 271, 33414–33424; Keyhani, N. O., and Roseman, S. (1996) J. Biol. Chem. 271, 33425–33432) describe two unique β-N-acetylglucosaminidases from Vibrio furnissii. A third, ExoII, is reported here. The gene, exoII, was cloned into Escherichia coli, sequenced, and ExoII purified to apparent homogeneity (36 kDa). The molecular weight and N-terminal 16 amino acids of the protein conform to the predicted sequence. ExoII exhibited unique substrate specificity. It rapidly cleaved p-nitrophenyl and 4-methylumbelliferyl β-GlcnAc, was slightly active with p-nitrophenyl-β-GalNac, and was inactive with all other GlcNac derivatives tested, including N,N'-diacetylchitobiose and (GlcNac)_n, n = 3–6. Unlike GlcNac (K_p, 210 μM), (GlcNac)_n are poor inhibitors of ExoII.

The predicted protein sequence is unique among β-N-acetylglucosaminidases excepting Cht60, recently cloned from a marine Alteromonas (Tsujido, H., Fujimoto, K., Tanno, H., Miyamoto, K., Imada, C., Okami, Y., and Inamori, Y. (1994) Gene (Amst.) 146, 111–115). Cht60, a chitobiase, is 26.9% identical to ExoII in a 182-amino acid overlap, but the two enzymes differ in substrate specificity and other properties. ExoII shares similarity with five bacterial and yeast β-glucosidases, up to 44% identity in the 25-amino acid catalytic domain. By analogy, ExoII may play a role in signal transduction between invertebrate hosts and V. furnissii.

The chitin catabolic cascade of the marine bacterium Vibrio furnissii comprises a complex array of genes and gene products required for the utilization of chitin. The accompanying papers describe the molecular cloning and characterization of two hexosaminidases that are part of this cascade (1, 2).

In this report we describe the molecular cloning of a third β-N-acetylglucosaminidase (GlcNacidase) from V. furnissii.

This novel enzyme differs from all reported β-GlcnAcidases, possibly excepting one, in its substrate specificity and amino acid sequence. The enzyme is designated ExoII to differentiate it from the periplasmic exohexosaminidase, Exol (2); the corresponding structural genes are exol and exoII, respectively.

EXPERIMENTAL PROCEDURES

Materials

The following chemicals were purchased or were gifts from the indicated sources. Chitin, GlcnAc, p-nitrophenyl (PNP) glycosides, and GlcnAc-6-P were from Sigma; chitin oligosaccharides, (GlcNac)_n (n = 2–6), were from Seikagaku America, Inc. (Rockville, MD); MUF-β-GlcnAc and MUF-β-(GlcNac)_n were from Calbiochem; phenyl-β-GlcnAc, benzyl-β-GlcnAc, p-methylphenyl-β-GlcnAc, 6-aminohexyl-β-GlcnAc, β-GlcnAc-Asn, and ThrSer-Asn-GlcNac-GlcNac-AspMan_α were kindly provided by Dr. Y. C. Lee (Department of Biology, The Johns Hopkins University, Baltimore, MD). [14C]GlcNac-O-Thr-Ser-Thr-Ser-Phe-Ser-Asp-Ser-Tyr and PUNGC (O-(2-acetamido-2-deoxy-ν-gluco-pyranosylidene)-amino-ν-phenylcarbamate) were kindly provided by Dr. Gerald Hart (Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham). 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. Vector pVEX-11 was a kind gift from Dr. Chaudhary (Laboratory of Molecular Biology, National Institutes of Health, Bethesda, MD). Primers T7, T3 were purchased from Stratagene. Additional primers were synthesized in the laboratory of Dr. Robert Schleif (Department of Biology, The Johns Hopkins University, Baltimore, MD). Radioisotopes were purchased from DuPont NEN. Immobilon polyvinylidene difluoride and nitrocellulose transfer membranes were purchased from Millipore (Bedford, MA). SSS, the buffer used to wash hybridized DNA on these membranes, is 15 mM sodium citrate, pH 7.0, containing 150 mM NaCl. The concentration of SSC used is indicated as follows: 1 × SSC is the concentration defined above, 4 × SSC is four times that concentration, etc.

Growth of Bacteria and Preparation of Subcellular Fractions

V. furnissii 7225 was grown at 25 °C in Luria-Bertani medium (LB broth) supplemented with 2% NaCl. Inocula were grown overnight to stationary phase, diluted 1:50 into growth medium, grown to mid-exponential phase, and harvested by centrifugation. Extracts for immunoblotting procedures were obtained from bacteria grown in minimal lactate (0.5% w/v-lactate) medium containing 50 mM Hepes and 50% artificial sea water adjusted to pH 7 (3). Escherichia coli strains DH11S (4), HB101, and BL21 (5) were grown at 37 °C in LB broth. E. coli transformants were grown with 50 mg/ml ampicillin in LB broth or on LB agar plates.

For the isolation of subcellular fractions from V. furnissii, all steps were conducted at 0–4 °C. Minimal lactate medium (100 ml) prepared as described (3) was inoculated with an overnight culture, grown in complex media (1:50 dilution). The cells were harvested at mid-exponential phase of growth, washed twice with buffer A (sodium phosphate

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‡ Present address: Dept. of Biochemistry, Weizmann Institute of Science, Rehovot 76100, Israel.

§ To whom correspondence should be addressed: Dept. of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Mudd Hall, Rm. 214, 3400 N. Charles St., Baltimore, MD 21218.

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50 mm, pH 7, and 0.25 M NaCl), and suspended in about 1.5 ml of the same buffer without NaCl (buffer B). The suspension was then passed twice through a French press and centrifuged at low speed (12,000 × g, 5 min). This pellet, designated “low speed pellet,” was washed twice and resuspended in about 0.1 ml of buffer B.

The supernatant fraction, “crude extract,” was centrifuged at high speed (148,000 × g for 1 h), yielding a “high speed” supernatant and pellet fraction. The pellet was washed twice and resuspended in one fifth the volume of buffer B used for disruption of the cells. In recovery experiments, unlabeled ExoII, and 125I-ExoII were added back to the French press extracts. The labeled protein was kindly prepared by Dr. R. Lee (Department of Biology, The Johns Hopkins University, Baltimore, MD) by the chloramine-T method (6).

SDS-PAGE was performed as described (7) and protein bands stained with Coomassie Blue. Protein was determined by the Bradford method (8), using the reagent kit from Bio-Rad.

**Assays for β-GlcNAcIdase**

The enzyme was assayed by modifications of the procedures used for assaying ExoI (2). Briefly, enzyme activity was detected in colonies growing on agar plates with MUF-GlcNAc. Two quantitative enzyme assays were used when FPN-GlcNAc was the substrate, and a third, the Morgan-Elson colorimetric method, when the substrates were oligosaccharides containing terminal GlcNAc residues. The modifications of the assays are as follows.

**Discontinuous Spectrophotometric Assay**—Cell extracts or protein fractions were incubated at 22°C for up to 40 min in 100 μl volumes containing the following: 1 mM PNP-GlcNAc, 50 mM Hepes buffer, pH 7.0, and 400 mM NaCl. The reaction was quenched with 2 ml of 1.8 M Tris base, pH 11, and absorbance measured at 400 nm.

**Continuous Spectrophotometric Assay**—Hydrolysis of PNP-GlcNAc was quantitated by continuously monitoring the rate of PNP formation at 400 nm. The temperature was maintained at 22°C with a Hauser circulator, and reactions were initiated by adding protein fractions or purified protein (μg) to 400 μl of the assay mixture described above.

**Morgan-Elson Assay**—GlcNAc was determined by a modification of the method for ExoI assay (9). In these cases, standard incubation mixtures contained the following (in 100 μl): 50 mM Hepes buffer, pH 7, 400 mM NaCl, 1–5 mM substrate, and 0.5 ng of enzyme. After incubating at 25°C for 1 h to 16 h, the reaction was stopped by adding 100 μl of 0.8 M sodium borate, pH 9.2, and the mixtures were heated for 12 min at 100°C. The samples were rapidly cooled, adjusted to 25°C, and treated with 0.5 ml of freshly prepared Ehrlich reagent (10 g of p-dimethylaminobenzaldehyde in 90 ml of glacial acetic acid and 10 ml of concentrated HCl diluted 1:10 with acetic acid before use). After 20 min at 37°C, absorbances were determined at 585 nm.

Other substrates were assayed as follows. After 16 h of incubation of the enzyme with the compound Thr/Ser-Asn-GlcNAc-GlcNAc-(Man)9, 37°C, absorbances were determined at 585 nm.

**Isolation of Transformants**

A ClaI-restricted *F. vernissii* DNA bank was ligated into pBR322 and transformed into *E. coli* HB101 by the CaCl2 or electroporation procedures (10). Three thousand *E. coli* transformant colonies were screened for β-GlcNAcIdase activity as described for ExoI (2). Colonies expressing ExoII were distinguishable from those expressing ExoI by their relative intensities; ExoII colonies exhibited much less fluorescence when treated with MUF-GlcNAc.

**Southern Blots**

DNA was blotted from agarose gels onto nitrocellulose (Immobilon-NC transfer membranes) and probed as described (11). In brief, DNA was cross-linked to the membrane by exposure to long wave UV light for 3 min. The probe was 32P-labeled by the random priming method (Boehringer Mannheim). Hybridization was performed at low stringency, in the presence of 60% formamide at 22°C. Following hybridization, the blot was twice washed extensively with 200 ml each of the following solutions: (i) 4 × SSC (see “Experimental Procedures”), 50 mM KPO4, and 0.5% SDS at 65°C; (ii) 1 × SSC, 12 mM KPO4, and 0.5% SDS at 65°C; (iii) 0.2 × SSC, 2.4 mM KPO4, and 0.1% SDS at 65°C; (iv) 0.1 × SSC, 1.2 mM KPO4, and 0.1% SDS at 22°C; (v) 0.005 × SSC at 22°C.

**Western Blots (Immunoblots)**

Protein samples were separated by SDS-PAGE, electroblotted onto nitrocellulose, and probed with monoclonal antibodies, IETD4 and 1E9H10, at a dilution of 1:10,000. The monoclonal antibodies were prepared against homogeneous ExoII. Bound antibody on the nitrocellulose membranes was detected with the luminescent ECL kit from Amersham, according to the manufacturer’s directions.

**DNA Sequencing**

Sequencing of both strands of DNA was conducted by the dideoxy chain termination method of double- or single-strand template with a USB Sequenase 2 kit and [α-35S]dATP. For the rescue of single strand DNA, the 1.8-kb fragment containing the β-GlcNAcIdase gene was excised from pXE18 by BamHI and KpnI digestion and ligated to pBR322 DNA with SmaI restriction sites. Single stranded DNA was labeled with [32P]dCTP and isolated using the phage YCSM31 as a helper vector and *E. coli* DH11S (4). Template for double-stranded DNA sequencing (pXE18) was purified with a Qiagen Plasmid Maxi Kit column and extracted twice with chloroform/phenol/isoamyl alcohol.

Sequencing reactions were performed as recommended by U. S. Biochemical Corp. with minor modifications in the annealing step. Double-stranded DNA (5 μg) was denatured with 0.5 M NaOH and ethanol-precipitated. Double-stranded (denatured) or single-stranded (3 μg) DNA were mixed with 10 ng of primer and Sequenase buffer in a total volume of 10 ml. The mixture was warmed 5 min at 44°C, followed by a 20-min incubation at 37°C. Compressions were resolved by sequencing both with dGTP and dTTP on the same gel.

Discontinuous DNA 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/DOS 2.7, kindly performed by Michael Cleveland.

**Purification of Recombinant ExoII**

A 1-liter culture of transformed *E. coli* BL21 (DE3) (12) harboring pXE28 was grown to late exponential phase (OD600 = 1.5) in LB broth containing 50 μg/ml ampicillin. (The enzyme was also purified by the same procedures using the transformant harboring pXE18, and gave the same results.)

The cells were harvested and washed twice with Buffer C (0.5% NaCl, 1 mM EDTA, 0.2 mM dithiothreitol, 50 mM sodium phosphate, pH 7.0) resuspended in 10 ml of the same buffer without NaCl (Buffer D), and passed three times through a French press at 0–5°C. All remaining steps were conducted in the cold.

Streptomycin sulfate was added to a final concentration of 1.4%, and the mixture centrifuged at 180,000 × g for 2 h.

Fifteen ml of a saturated ammonium sulfate solution was added dropwise with stirring to 10 ml of the streptomycin sulfate supernatant fraction. After 16 h, the 60% ammonium sulfate precipitate was collected by centrifugation, and the pellet dissolved in Buffer D and exhaughtially dialyzed against the same buffer.

The sample (about 9 ml) was transferred to a 30-ml DEAE-Sepharose CL-6B column (Pharmacia Biotech Inc.) that had been equilibrated with Buffer B. The column was washed with 36 ml of Buffer B, which eluted considerable inactive protein, and the column was then eluted with a 0–1 M NaCl linear gradient (80 ml) in Buffer B at a rate of 1 ml/min. ExoII activity was detected in the 0.15 to 0.2 M NaCl fractions, which were pooled. The active fractions displayed three widely separated protein bands on SDS-PAGE.

The pooled active fractions (12 ml) from the previous step was concentrated to 1.7 ml in an Amicon 30 ultrafiltration apparatus, and transferred to a 1.8 × 85-mm Ultrigel ACA34 gel filtration column (a polyacrylamide/agarose, IBF Biotechnics, Savage, MD). The column was pre-equilibrated with Buffer D containing 0.1 M NaCl, and eluted with the same buffer/NaCl mixture. Fractions were collected at 0.7 ml/min. Activity was detected in fractions 37–45 (3 ml each), with the peak in fractions 40–41. The specific activity was constant across the peak, and the pooled fractions 37–39, 40–42, and 43–45 showed only one band on SDS-PAGE.

A DEAE-HPLC column gave no additional purification, and was not incorporated into the purification procedure. The purified protein was stored at −80°C at concentrations of 0.5 mg/ml or less in Buffer D containing 0.1 M NaCl. The enzyme precipitated at higher concentra-
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Preparation of Monoclonal Antibodies

The homogeneous recombinant protein was used to raise monoclonal antibodies in mice by a described injection schedule and procedure (10). Antibodies were detected by Western blotting of the recombinant protein. Two hybridomas were isolated, 1E9-H10 and 1E7-D4. They secreted specific antibodies of the IgG3 class.

RESULTS

Molecular Cloning of exoII—A V. furnissii ClaI genomic library was constructed in pBR322, and the colonies were screened for expression of β-GlcNAcidase with MUF-GlcNAc. Five of 3,000 colonies yielded positive results. Plasmid DNA from each of the positive colonies was subjected to restriction digestion and Southern blot analysis, using the V. furnissii periplasmic β-GlcNAcidase gene (exoI) as a probe (2). Three of the clones were identical to exoI, but two clones were different. These harbored plasmids containing identical 10-kb inserts, designated pRE100.

The gene, exoII, was subcloned in two steps from pRE100. Digestion of the 10-kb V. furnissii DNA fragment with SphI gave three fragments. One of these (2.8 kb) contained exoII, was gel-purified (10), and ligated into the high copy number plasmid pVEX11, giving pXE28. The fragment was further subcloned by digestion with SalI, giving two smaller DNA fragments, of 1.8 and 1 kb, respectively. The 1.8-kb SphI/SalI fragment was blunt-ended and ligated in both orientations in the Smal site of pVEX11. The transformants isolated from each of the plasmids (pXE18, pXE81) expressed active enzyme (data not shown), indicating that exoII in the 1.8-kb fragment carries its own promoter.

Detection of exoII in V. furnissii Chromosomal DNA—Southern blots showed that the cloned gene is a component of the V. furnissii genome. Complete SphI digests of V. furnissii and E. coli HB101 chromosomal DNA were probed with the 1.8-kb DNA fragment from pXE18 (Fig. 1). Under highly stringent hybridization conditions, the probe hybridized only to a DNA fragment in the Vibrio digest. Furthermore, the V. furnissii DNA fragment containing exoII was the predicted size, 2.8 kb, for a SphI complete digest.

Purification of ExoII—To minimize protein degradation during the course of the purification, the E. coli strain BL21 (DE3) was chosen as a host for pXE28 and pXE18 because this strain is deficient in several proteases (5). Activity was observed in the soluble protein fraction of the BL21 transformants harboring pXE28 and pXE18, whereas no activity was present in cells transformed with the vector plasmid alone. The enzyme was purified in three steps from crude extracts of the transformant as shown in Table I, giving a 72-fold purification, and a total recovery of 80%.

Only one protein band was detected on SDS-PAGE (Fig. 2). The apparent molecular mass of the protein is 36 kDa; this value agrees with the molecular mass calculated from the predicted amino acid sequence (see below).

Kinetic Characterization of ExoII—The continuous spectrophotometric assay was used for kinetic studies involving PNP-β-GlcNAc, except for the pH studies, where the discontinuous method was used.

The enzyme exhibited a fairly sharp peak of activity at pH 7.0 both in Hepes and McIlvaine buffer. There was only slight activity at pH 6.0 and 8.0. The optimal temperature was 45°C, and about half of the maximal activity was observed at 25°C, the temperature used for growth of V. furnissii and for the routine enzyme assays. Increasing the ionic strength had a small stimulatory effect on the activity. The optimum NaCl concentration was 0.4–0.7 M, and in this range, the enzyme was about 70% more active than in the absence of NaCl. There was no apparent divalent ion requirement, and 0.04 M EDTA did not inhibit the activity.

The enzyme exhibited typical Michaelis-Menten kinetics. The apparent kinetic constants with PNP-β-GlcNAc, calculated from a Lineweaver-Burk plot (Fig. 3A), were: $K_{m}$, 440 μM; $V_{max}$, 1.1 μmol/min × mg protein at 22°C.

Effect of GlcNAc on Enzyme Activity—Given sufficient time, the rate of the reaction decreases, and this was first observed in the continuous spectrophotometric assay. Several possibilities were tested, and it appeared that we were observing product inhibition. Indeed, GlcNAc inhibits the β-GlcNAcidase.

A Dixon plot is shown in Fig. 3B and suggests that GlcNAc is a noncompetitive inhibitor of ExoII, with an apparent $K_i$ of 210 μM.

Substrate Specificity—The cloned enzyme is an exo-β-GlcNAcidase and hydrolyzes PNP-β-GlcNAc and MUF-β-GlcNAc. The substrate specificity of the enzyme, however, shows that it is not a classical β-GlcNAcidase but is unique.

Unlike most β-GlcNAcidases, the enzyme had only a slight, albeit detectable effect with PNP-β-GalNAc. Under standard assay conditions (1 μg of pure enzyme in 400 μl of buffer containing 0.66 mM substrate), PNP-β-GalNAc was hydrolyzed at a rate of 356 pmol/min, whereas PNP-β-GlNAc was hydrolyzed at a rate of 10 pmol/min, or a ratio of about 36-fold in favor of PNP-β-GlcNAc.

It was possible that the GalNAc derivative might be contaminated with small amounts of PNP-β-GlcNAc. Therefore, the following mixture (440 μl) was incubated at 22°C: 330 μM PNP-β-GalNAc, 5 μg of enzyme, 10 mM Hepes buffer, pH 7.0. After 16 h, 9% of the substrate was cleaved. This value far exceeded the quantity of PNP-β-GlcNAc that could be a contaminant (based on chromatography of the derivatives).

The enzyme was tested for its ability to hydrolyze a variety of p-nitrophenyl β-D-glycopyranosides, including β-GlcNAc, β-Glc, β-GalNAc, and β-GalNAc. The enzyme showed specific activity against β-GlcNAc and β-GalNAc, with β-GalNAc being a slightly better substrate.

![Fig. 1. Detection of exoII in the genome of V. furnissii by Southern blot analysis. Genomic DNA from E. coli and V. furnissii was extracted, purified, and digested to completion with the restriction enzyme SphI. Equal amounts of DNA (3 μg) from E. coli (lane 2), V. furnissii (lane 3), and V. furnissii DNA supplemented with the cloned 1.8-kb fragment (lane 1) were electrophoresed in an agarose gel (0.8%). The gel was blotted onto a nitrocellulose membrane and probed with the 1.8-kb DNA fragment containing exoII.](image-url)


\[ \text{\( \beta \)-Gal, and \( \beta \)-celllobioside. The following glycosides of \( \beta \)-GlcNAc were also assayed: phenyl, benzyl, methyl, and 6-aminohexyl. Only PNP-\( \beta \)-GalNAc was slowly hydrolyzed, as described above. Negative results were also obtained with O- and N-linked glycopeptides (all \( \beta \), GlcNAc-Asn, (Man)\(_n\)-GlcNAc-(\( \beta \),1–4)-GlcNAc-Asn-Ser/Thr and \([14\text{C}]\text{GlcNAc-O-Thr-Ser-Thr-Phe-Ser-Asp-Ser-Tyr. In the case of the labeled O-linked [14C]GlcNAc, the sensitivity of the assay would have detected a trace of hydrolysis. In view of the chitinolytic properties of \( V. \) furnissii, the most important set of derivatives tested for enzyme specificity were the chitin oligosaccharides, (GlcNAc)\(_n\), \( n = 2–6 \), and these were studied extensively. For example, each of the oligosaccharides (1 mM) was incubated at 37 °C with 3.4 mg of ExoII in 100 \( \mu \)l of 10 mM phosphate or Heps buffer, pH 7.0. After 16 h, there was no detectable GlcNAc (by the Morgan-Elson reaction) in any of the incubation mixtures. In addition, recovery experiments with GlcNAc added to the incubation mixtures showed no detectable loss of the monosaccharide. Given the sensitivity of the assay, the quantity of enzyme used, and its specific activity with PNP-\( \beta \)-GlcNAc, we conclude that ExoII hydrolyzes (GlcNAc)\(_n\), the rate must be less than 1.5 pmol/min \( \times \mu \)g ExoII, or, less than 0.15% of the rate observed with PNP-\( \beta \)-GlcNAc. We especially wish to emphasize the lack of activity with the disaccharide, (GlcNAc)\(_2\), since the cloning experiments were aimed at isolating the gene that encodes the cytoplasmic chitobiase expressed by \( V. \) furnissii (13).}

**Enzyme Inhibitors**—As noted above, GlcNAc is a very effective (apparent noncompetitive) inhibitor of the enzyme with \( K_\text{m} = 210 \mu \text{M, significantly less than the } K_\text{m} \text{ for PNP-\( \beta \)-GlcNAc, 440 } \mu \text{M. Other potential inhibitors (none of which were hydrolyzed) were also screened because they might shed light on the unexpected specificity of the enzyme. The continuous assay was used with PNP-\( \beta \)-GlcNAc as the substrate (Table II). The most effective inhibitor in this screen was PUGNAC (for structure, see “Experimental Procedures”), a GlcNAc derivative, which was also found to be the most effective inhibitor of a recently reported rat spleen cytosolic \( \beta \)-GlcNAc (14).**

**Table I**

*Purification of ExoII*  
The enzyme was purified from the *E. coli* transformant as described under “Experimental Procedures.”

| Fraction          | Total protein | Specific activity | Purification Factor | Yield |
|-------------------|---------------|-------------------|---------------------|-------|
|                   | mg            | n mol/mg × min   | -fold              | %     |
| Crude extract     | 116           | 10.0              | 1                   | 100   |
| Ammonium sulfate  | 100           | 10.4              | 1.2                 | 90    |
| DEAE-Sepharose    | 9.5           | 105               | 10.5                | 86    |
| Ultrogel ACA34    | 1.3           | 716               | 71.6                | 80    |

**Fig. 2.** SDS-PAGE of purified ExoII. The crude extract of *E. coli* BL21 (DE3) harboring pXE28 (33 \( \mu \)g of protein, lane 1) and purified ExoII (10 \( \mu \)g, lane 2) were electrophoresed in a 10% SDS-polyacrylamide gel and stained with Coomassie Blue. Positions of molecular size markers are indicated by arrows.

**Fig. 3.** Effect of PNP-\( \beta \)-GlcNAc concentration on the activity of ExoII, and inhibition by GlcNAc. Panel A, Lineweaver-Burk plot of initial rate of PNP released as a function of PNP-\( \beta \)-GlcNAc concentration. Each reaction mixture consisted of 50 mM Heps buffer, pH 7.0, supplemented with 0.4 M NaCl and the indicated concentrations of PNP-\( \beta \)-GlcNAc in a 400-\( \mu \)l total volume. The rates of PNP release were recorded continuously at 400 nm, 22 °C, following the addition of 1 \( \mu \)g of the purified enzyme. Panel B, Dixon plot of inhibition of ExoII by GlcNAc. The reaction conditions were the same as in panel A except that GlcNAc was present at the indicated concentrations in solutions containing 73, 162, 325, and 650 \( \mu \)M PNP-\( \beta \)-GlcNAc.
TABLE II
Inhibition studies

Unles otherwise indicated, potential inhibitors were assayed by the continuous spectrophotometric method at 400 nm and 22 °C; the assay mixture contained 1 µg of enzyme, PNP-β-GlcNAc (50 µM), Hepes buffer, pH 7.0 (50 mM), 0.4 mM NaCl, and the indicated inhibitors in 0.4 ml. The discontinuous assay method was used with asparaginyl- and naphthyl-β-GlcNAc. Percent inhibition values were obtained with inhibitors at 5 mM concentration except for those of low solubility: compounds 4 (1 mM), 5 (2 mM), 10 (2 mM), and 20 (4.5 mM).

| Glycoside    | Aglycon group of glycosides | K<sub>i</sub> | Inhibition |
|--------------|------------------------------|--------------|------------|
| 1. GlcNAc   |                             | 210          | 96         |
| 2. β-GlcNAc | (N)-Asparagine               | 400          | 100        |
| 3. β-GlcNAc | 6-Aminohexyl                 | 75           |            |
| 4. β-S-GlcNAc | (S)-Aminohexanoyl-      | 350          | 50         |
| 5. β-S-GlcNAc | p-Nitrophenyl                | 50           |            |
| 6. β-GlcNAc | Phenyl                       | 49           |            |
| 7. β-GlcNAc | Naphthyl                     | 45           |            |
| 8. β-GlcNAc | Benzyl                       | 47           |            |
| 9. β-GlcNAc | Methyl                       | 12           |            |
| 10. α-GlcNAc | p-Nitrophenyl               | 16           |            |
| 11. PUGNAC (see *Experimental Procedures*) | 0.3 | |

Chitin oligosaccharides

12. β-(GlcNAc)<sub>2</sub> | p-Nitrophenyl | 33 |
13. GlcNAc<sub>2</sub> | 25 |
14. GlcNAc<sub>4</sub> | 0 |

GlcNAc metabolites

15. GlcNAc-6-P | 50 |
16. GlcNH<sub>2</sub>-6-P | 70 |
17. GlcNH<sub>2</sub> | 25 |

Glucosides<sup>a</sup>

18. β-Glucosyl | p-Hydroxyphenyl (arbutin)<sup>b</sup> | 65 |
19. β-Glucosyl | Coniferyl (coniferin)<sup>b</sup> | 20 |
20. β-Glucosyl | p-Nitrophenyl | 0 |

<sup>a</sup> Galactose derivatives exhibited no activity.
<sup>b</sup> Arbutin is p-hydroxyphenyl-β-D-glucopyranoside. Coniferin is 2-methoxy-4-(6-hydroxy)propenylphenyl-β-D-glucopyranoside.

An extensive series of experiments were conducted using cells grown under different conditions, e.g., lactate medium ± (GlcNAc)<sub>2</sub> or chitin as potential inducers. The immunoblots did not reveal any anti-ExoII reactive protein in any of the fractions, including inner and outer membranes (15). When pure ExoII was added back to crude extracts of V. furnissii, the same negative result was obtained, i.e., the antibody was unable to detect exogenously added ExoII. Finally, 125I-labeled ExoII was prepared and added to the extract. The 125I-ExoII was detected at the proper position on the blot by autoradiography, but again the monoclonal antibody yielded no immunoblot.<sup>3</sup> Therefore, for reasons that we have been unable to determine, substances in the crude extracts co-migrate with ExoII on SDS-PAGE and interfere with immunoprecipitation by the monoclonal antibodies. The interfering substance(s) have not been identified.

**DNA Sequence of exoII**—The 1.8-kb fragment from pXE18 was subcloned into pBluescript SK<sup>Ⅱ-</sup> to generate the plasmid pBLE18, and the gene sequenced by the dideoxy method from both single- and double-stranded DNA. The V. furnissii ExoII nucleotide sequence and predicted amino acid translation has been deposited in the GenBank™ data base with the accession number U52818. There is a single long open reading frame of 984 base pairs. The start codon (residue 202) is preceded by a potential ribosomal binding site at residue 191 (15). Two regions, upstream of the start codon, correspond to a consensus sequence specifying the Pribnow box (residue 184) and the −35 box (residue 166), respectively (16, 17). A region with dyad symmetry composed of 22 base pairs was found following the translational termination signal, and is a potential Rho-independent termination signal.

**Predicted Amino Acid Sequence of ExoII**—The 984-base pair open reading frame in pBLE18 encodes a 36,016-Da protein composed of 328 amino acids. The transcriptional start site was confirmed by sequencing the first 16 amino acids in the N-terminal region in the pure recombinant protein. No apparent N-terminal secretory signal sequence is present downstream from the start site (18). A computer search of protein sequences in the Swiss Protein Data Bank showed no similarity to the predicted sequences of any other cloned β-GlcNAcidase with one exception. Recently, Tsujiho et al. (19) reported cloning the gene cht60 encoding a β-GlcNAcidase from the marine bacterium Alteromonas sp. strain O-7. The predicted amino acid sequence of this enzyme, designated Cht60 (from its migration on SDS-PAGE), is 26.9% identical in a 182-amino acid overlap with ExoII. Despite this similarity, there are significant differences between ExoII and Cht60.

(a) The predicted sequences suggest that unprocessed Cht60 contains 598 amino acid residues, compared to 328 for ExoII. (b) Cht60 contains an 11-amino acid N-terminal signal sequence, which is recognized by and is processed by the E. coli host so that the enzyme is found in the periplasmic space. (c) Most importantly, Cht60, unlike ExoII, is a chitobiase, i.e., it cleaves (GlcNAc)<sub>2</sub> at the same rate as PNP-β-GlcNAc. Cht60 shows almost no activity with the higher chitin oligosaccharides, and these findings led the authors to suggest that it is similar to the cytosolic β-GlcNAcidase from V. furnissii (13).

While ExoII showed sequence similarity to only a single β-GlcNAcidase, the computer search revealed significant similarity to nine bacterial and yeast β-glucosidases. The highest degree of similarity was found with a β-glucosidase from Agrobacterium tumefaciens (20). The two proteins share 26% identity in a stretch of 153 amino acids. This stretch of amino

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<sup>3</sup> 125I-ExoII was also added to crude cell extracts, and the extracts were treated with the monoclonal antibodies bound to Protein A beads. The labeled protein was recovered from the controls, extracts of the E. coli transformants, but not from crude extracts of V. furnissii.
Various non-hydrolyzable GlcNAc derivatives also inhibit the enzyme, but there is little to no inhibition by chitin oligosaccharides. The covalent linkage region of N-linked glycoproteins, β-GlcNAc-Asn, is a potent inhibitor, perhaps as potent as GlcNAc itself, but the physiological significance of this observation is unclear.

Glycosides of other sugars were generally inactive as inhibitors except for arbutin (p-hydroxyphenyl β-glucoside). The rationale for testing arbutin is given below.

A comparison of the predicted amino acid sequence of ExoII with all available sequences in the Swiss Protein Data Bank showed no similarity to any cloned hexosaminidase excepting Cht60, a chitobiase recently cloned from the marine bacterium Alteromonas sp. strain O-7 (19). Cht60 and ExoII are 26.9% identical in a 182-amino acid overlap, but, as discussed above (see “Results”), the enzymes differ in several important respects (substrate specificity, size, signal sequence, etc.).

The more surprising finding was that ExoII shared similarity with five bacterial and yeast β-glucosidases. The closest similarity was to a β-glucosidase from Agrobacterium tumefaciens (20), 26% identity in a 153-amino acid overlap, and 40% identity in the 30-amino acid catalytic domain of the glucosidase. We suppose that this is why arbutin and coniferin inhibit ExoII, although we cannot explain why PNP-β-Glc neither is hydrolyzed nor acts as an inhibitor.

We can only speculate on the function(s) of ExoII. Microbial invasion of plants, whether symbiotic (nodulation of root hairs) or pathogenic (tumor growth), involves complex signal transducing systems that affect both organisms. Flavonoids, which are often glycosides, induce nitrogen fixing bacteria to synthesize chitooligosaccharides that in turn signal the plant to promote nodulation (23). Coniferin is an aryl β-glucoside that is split by the A. tumefaciens β-glucosidase to coniferyl alcohol, which in turn induces virulence genes in the bacterium that are responsible for tumor induction (20). By analogy, we speculate that ExoII performs a similar function. In chitin producing organisms, such as fungi and invertebrates, we assume that phenolic β-GlcNAc derivatives are also formed. (Invertebrate cuticles are very rich in cross-linked polyphenolic compounds (24, 25).) One or more of these is predicted to be hydrolyzed by ExoII. Indeed the β-GlcNAc glycosides may induce expression of exoII, and the hydrolysis product, phenol derivatives may then induce V. furnissii to invade its host. One experimental approach to this hypothesis will be to clone a repressor of exoII, followed by characterization of the inducer.

Acknowledgments—We are very grateful to Drs. Y. C. Lee and R. Lee for their help in the preparation of the 125I-ExoII and in the analysis of hydrolysatess for GlcNAc by HPLC (Dionex) chromatography, and to Michael Cleveland for help in preparing Fig. 4.

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