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Heterologous Expression and Characterization of an N-Acetyl-β-D-hexosaminidase from *Lactococcus lactis* ssp. *lactis* IL1403

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ABSTRACT: The *lnbA* gene of *Lactococcus lactis* ssp. *lactis* IL1403 encodes a polypeptide with similarity to lacto-*N*-biosidases and *N*-acetyl-β-D-hexosaminidases. The gene was cloned into the expression vector pET-21d and overexpressed in *Escherichia coli* BL21* (DE3). The recombinant purified enzyme (LnbA) was a monomer with a molecular weight of approximately 37 kDa. Studies with chromogenic substrates including *p*-nitrophenyl *N*-acetyl-β-D-glucosamine (*p*NP-GlcNAc) and *p*-nitrophenyl *N*-acetyl-β-D-galactosamine (*p*NP-GalNAc) showed that the enzyme had both *N*-acetyl-β-D-glucosaminidase and *N*-acetyl-β-D-galactosaminidase activity, thus indicating that the enzyme is an *N*-acetyl-β-D-hexosaminidase. K_m and k_{cat} for *p*NP-GlcNAc were 2.56 mM and 26.7 s^{-1}, respectively, whereas kinetic parameters for *p*NP-GalNAc could not be determined due to the K_m being very high (>10 mM). The optimal temperature and pH of the enzyme were 37 °C and 5.5, respectively, for both substrates. The half-life of activity at 37 °C and pH 6.0 was 53 h, but activity was completely abolished after 30 min at 50 °C, meaning that the enzyme has relatively low temperature stability. The enzyme was stable in the pH 5.5−8 range and was unstable at pH below 5.5. Studies with natural substrates showed hydrolytic activity on chito-oligosaccharides but not on colloidal chitin or chitosan. Transglycosylation products were not detected. In all, the data suggest that LnbA's role may be to degrade chito-oligosaccharides that are produced by the previously described chitinolytic system of *L. lactis*.

KEYWORDS: *N-acetyl-β-D-hexosaminidase, Lactococcus lactis ssp. lactis IL1403, pNP-GlcNAc, pNP-GalNAc, chitin, chitinase*

■ INTRODUCTION

N-Acetyl-β-D-hexosaminidases (EC 3.2.1.52; β-NAHA) are found in glycosyl hydrolase families 3, 20, and 84 (CAZy, http://www.cazy.org). They cleave terminal *N*-acetyl-β-D-glucosamine (β-D-GlcNAc and *N*-acetyl-β-D-galactosamine (β-D-GalNAc) residues from the nonreducing end of *N*-acetyl-β-D-hexosaminides.[1] β-NAHA occur in most types of living organisms and have very diverse biological functions. β-NAHA is an autolytic enzyme in the cell cycle of *E. coli*.[2] It can act as a key enzyme in chitin degradation, where it converts chito-oligosaccharides liberated from chitin by chitinase(s) to *N*-acetyl-β-D-glucosamine.[3] In fungi, the enzyme plays an important role in vegetative growth[4] and antifungal action related to mycoparasitism.[5] In plants it can inhibit fungal growth as part of defense mechanisms[6] or act in seed germination[7,8] and textural softening during fruit ripening.[4,9,10] *N*-Acetyl-β-D-hexosaminidase A, located in lysosomes of human cells, plays a role in the brain and spinal cord by breaking down a substance called GM2 ganglioside. Lack of enzymatic activity causes accumulation of GM2 ganglioside, which leads to destruction of neurons, causing symptoms of Tay-Sachs disease.[11,12]

There is considerable interest in β-NAHAs for a variety of applications: determination of the structures of glycoproteins and glycolipids, utilization as biocontrol agents of plant pathogenic fungi and potential mycoinsecticides, or bioconversion of chitinous waste.[13] Fungal β-NAHAs can transglycosylate both β-D-GlcNAc and β-D-GalNAc residues in *N*-acetyl-β-D-hexosaminides to a variety of carbohydrate acceptors and have been applied for synthesis of new oligosaccharides[14] with various potential applications, including antitumor agents,[15] or immunomodulatory agents.[16,17]

There is increasing interest in applying enzymes from organisms that have GRAS status (are Generally Regarded As Safe), such as lactic acid bacteria, to produce novel oligosaccharides. These studies have mainly been focused on production of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS), because these are structurally related to human milk oligosaccharides and have prebiotic properties. Thus far, little attention has been paid to β-NAHA from lactic acid bacteria, despite indications from the studies cited above that the availability of a food-grade enzyme for GlcNAc- or GalNAc-containing glucoconjugates is of considerable interest.

The genome of *L. lactis* ssp. *lactis* IL1403 contains one gene coding for a glycosyl hydrolase family 20 β-NAHA. The protein lacks a typical signal peptide and is thus likely to have an intracellular location. One of the primary roles of the gene

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product could be in chitin degradation, since it is known that *L. lactis* has a functional chitinolytic enzyme system that would produce oligomers of *N*-acetylglucosamine that need further processing before they can be metabolized.[18] The aim of this work was to clone and express the *lnbA* gene and demonstrate the applicability of the resulting enzyme.

■ MATERIALS AND METHODS

Chemicals and Enzymes. Restriction enzymes and T4 DNA ligase were supplied by Fermentas (St. Leon-Rot, Germany). Isopropyl-*β*-D-thiogalactoside (IPTG) was from Roth (Karlsruhe, Germany). Chromogenic substrates (*p*NP-GlcNAc, *p*NPGlcNAc, *p*-nitrophenyl *β*-D-glucopyranoside, *p*-nitrophenyl *β*-D-galactopyranoside, *p*-nitrophenyl-*β*-D-arabinopyranoside, *p*-nitrophenyl-*β*-D-xylopyranoside), chitin, and chitosan were purchased from Sigma Aldrich (St. Louis, MO). Diacetyl-chitobiose, triacetyl-chitotriose, and tetraacetyl-chitotetraose were purchased from Megazyme (Bray, Ireland). Variants of *p*NP-GlcNAc, containing modifications at C-6 (*p*-nitrophenyl 6-*O*-sulfo-*N*-acetyl-*β*-D-glucosaminide, 2-acetamido-2-deoxy-*β*-D-glucopyranosylazide, 2-acetamido-2-deoxy-*β*-D-glucopyrano-urate),[19−21] were provided by the Laboratory of Biotransformation, Institute of Microbiology, Academy of Sciences of the Czech Republic. All other chemicals were reagent grade and obtained from commercial sources.

Bacterial Strains, Plasmids, and Media. *L. lactis* ssp. *lactis* IL1403 was kindly provided by the Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Science, and used to amplify *lnbA*. The strain was grown at 30 °C without aeration in M17 medium (Oxoid, Basingstoke, U.K.) supplemented with 0.2% (w/v) glucose (GM17) for isolation of genomic DNA.[18] Genomic DNA was isolated using the Bacterial Genomic DNA Isolation Kit (Norgen, Thorold, Canada). Plasmid pET-21d (Novagen, Merck, Darmstadt, Germany) was used as expression vector. *E. coli* BL21* (DE3) One Shot Chemically Competent (Invitrogen, Carlsbad, CA) was used as expression host.

Cloning and Expression of the *β*-NAHA-Encoding Gene. A forward primer (ggt ggt cca tgg aaa aag gat tat tag tcg) and reverse primer (ggt ggt ctc gag tga ttc atc att tcc tcc ata) containing *Nco*I and *Xho*I recognition sites (underlined), respectively, were designed based on the sequence of a gene putatively encoding a *β*-NAHA from *L. lactic* ssp. *lactis* IL1403 (GenBank Accession No. NC-002662). PCR standard reactions (final volume 25 *μ*L) contained approximately 100 ng of *L. lactis* genomic DNA as template, 10 pmol of each primer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, the appropriate buffer as supplied by the manufacturer, and 1 U Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). Amplification conditions for a standard PCR reaction were as follows: 1 cycle at 98 °C for 3 min, 30 cycles at 95 °C for 30s, 60 °C for 30s, and 72 °C for 2 min, with an extra extension at 72 °C for 5 min for the final cycle. The amplification product was purified from an agarose gel (0.8%) using the Wizard SV Gel PCR Cleanup system (Promega, Madison, WI) and digested with *Nco*I and *Xho*I before being ligated into the *Nco*I and *Xho*I sites of pET-21d. Insertion of the amplicon resulted in translational fusion of the *lnbA* open reading frame with the vector-encoded 6× His-tag (N-terminal tag). Plasmids were transformed into *E. coli* BL21* (DE3) by electroporation (MicroPulser, BioRad, Hercules, CA). Positive colonies were confirmed by colony-PCR and DNA sequencing. After culturing, cells were stored in 30% glycerol at −80 °C.

An overnight culture of *E. coli* BL21* (DE3) harboring pET-21d with the *β*-NAHA gene was used to inoculate 250 mL of fresh LB medium supplemented with 100 *μ*g/mL of ampicillin. The culture was incubated at 37 °C and 200 rpm until the OD600 reached 0.5. IPTG was added to the culture to a final concentration of 0.4 mM in order to produce the T7 RNA polymerase that transcribes the recombinant gene. Postinduction cultivation was done at 25 °C, 200 rpm for 16 h. Cells were harvested by centrifugation (4000*g*, 20 min, 4 °C) and washed two times with 50 mM citrate phosphate buffer pH 6.0. Biomass was stored at −20 °C for protein purification.

Protein Purification. Cells were disrupted 3 times in a French press (Amicon, Jessup, MD) in 50 mM citrate phosphate buffer pH 6.0. A cell-free lysate was obtained by ultracentrifugation at 60 000*g* for 30 min at 4 °C. Protein purification was done by immobilized metal affinity chromatography (IMAC) as follows: 10 mL of crude protein extract was loaded onto a 15 mL column of Profinity IMAC Ni-Charged Resin (BioRad) pre-equilibrated with buffer A (Na_2HPO_4 20 mM, NaCl 0.5 M, imidazol 20 mM, pH 6.5). After washing the column with two column volumes of buffer A the enzyme was eluted with a flow rate of 0.5 mL/min using a gradient from 0% to 100% of buffer B (Na_2HPO_4 20 mM, NaCl 0.5 M, imidazol 0.5 M, pH 6.5). Fractions containing the highest enzyme activity, detected using pNP-GlcNAc as substrate, were pooled, the buffer was exchanged to 50 mM citrate phosphate buffer pH 6.0, and samples were concentrated by 10 kDa cutoff Amicon Ultra Centrifugal filter tubes (Millipore, Billerica, MA). Purified enzyme in 50 mM citrate phosphate buffer pH 6.0 was stored at 4 °C and used for characterization

Protein Electrophoresis and Molecular Weight Determination. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions[22] was performed to verify the purity of the enzyme preparations and determine the molecular mass of the enzyme using 10% gels and a PerfectBlue vertical electrophoresis system (Peqlab, Erlangen, Germany). The molecular weight of the native recombinant protein was determined by gel filtration using a 180 mL Superose 12 column (16 × 1000 mm). The protein was applied in 20 mM sodium phosphate buffer pH 7.0 containing 150 mM NaCl at a flow rate of 0.5 mL/min; a Gel Filtration Molecular Weight Marker Kit was used as a standard (12−200 kDa; Sigma-Aldrich).

Enzyme Assays and Protein Determination. The standard enzyme assay consisted of 230 *μ*L of 10 mM *p*NP-GlcNAc in 50 mM citrate phosphate buffer, pH 6.0, and was started by adding 20 *μ*L of enzyme solution. Reaction mixtures were incubated at 37 °C in an Eppendorf Thermo Mixer (Hamburg, Germany) with agitation at 600 rpm. After 10 min the reaction was terminated by adding 750 *μ*L of 0.4 M Na_2CO_3. Enzyme activity was determined by measuring the absorption of liberated *p*-nitrophenol at 400 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 *μ*mol of *p*-nitrophenol per minute under the described conditions. Kinetic analysis of enzyme activity was carried out using the same assay conditions using *p*NP-GlcNAc and *p*NP-GalNAc as substrates at concentrations of 0.5−10 mM.

β-NAHA activity was also assayed by measuring the release of reducing sugars from colloidal chitin. The reaction mixture consisted of 250 *μ*L of 2% colloidal chitin in 50 mM citrate phosphate buffer (pH 6.0) and 250 *μ*L of enzyme solution. After incubation at 37 °C for 30 min in a thermomixer at 600 rpm the reaction was stopped by heating at 100 °C for 5 min, and residual colloidal chitin was removed by centrifugation at 16 000*g* for 5 min. Reducing sugars were determined by a modified dinitrosalicylic acid method as described previously.[23]

Liberation of reducing sugars from chitosan was also assayed by mixing 250 *μ*L of 1% (w/v) low molecular weight chitosan in 50 mM citrate phosphate buffer pH 6.0 and 250 *μ*L of enzyme solution. After incubation at 37 °C and 600 rpm for 30 min in a thermomixer the reaction was stopped by adding 500 *μ*L of DNS solution and heating at 100 °C for 15 min, followed by centrifugation at 16 000*g* for 5 min. Absorbance at 540 nm was measured, and enzyme activity was calculated as for the colloidal chitin assay.

Protein concentration was determined according to Bradford[24] using the Protein Assay Reagent (Bio-Rad) with bovine serum albumin as the standard.

Substrate Specificity. Substrate specificity of the recombinant enzyme was determined using various chromogenic substrates at a concentration of 5 mM under standard assay conditions as well as 2% colloidal chitin and 2% chitosan as described above. The chromogenic substrates tested were *p*NP-GlcNAc, *p*NP-GalNAc, *p*-nitrophenyl *β*-D-glucopyranoside, *p*-nitrophenyl *β*-D-galactopyranoside, *p*-nitrophenyl-*β*-D-arabinopyranoside, *p*-nitrophenyl-*β*-D-xylopyranoside), and variants of *p*NP-GlcNAc with modifications at C_6.

Table 1. Purification of Recombinant β-NAHA from *E. coli* (from 250 mL of LB Culture)[a]

step	activity[b] (U/mL)	mL	total activity (U)	protein conc (mg/mL)	spec activity (U/mg)	yield (%)
crude extract	53.7 ± 6.3	20	1196 ± 7.5	2.8 ± 0.3	19.1 ± 0.23	100
purified enzyme	418 ± 2.5	2	837.1 ± 4.8	11.3 ± 0.15	37 ± 0.78	70

[a]Values are means of three cultivations. [b]*p*NP-GlcNAc was used to determine enzyme activity.

Effect of Temperature and pH on Enzyme Activity. The temperature optimum of the recombinant enzyme was measured with the standard assay with *p*NP-GlcNAc and *p*NP-GalNAc as substrates in the range of 20−90 °C. The temperature stability of the enzyme was studied by incubating the purified enzyme in 50 mM citrate phosphate buffer pH 6.0 at various temperatures. At certain intervals, samples were withdrawn and residual activity was measured with *p*NP-GlcNAc as the substrate.

The pH optimum was determined by standard assay with 5 mM of *p*NP-GlcNAc and *p*NP-GalNAc in the pH range from 4.0 to 10.0 using Britton−Robinson buffers (50 mM sodium citrate, 50 mM sodium phosphate, and 50 mM sodium borate, pH adjusted to the required value with 1 M NaOH). To determine the pH stability of β-NAHA, the purified enzyme was incubated at various pH values using Britton−Robinson buffers as above and 37 °C for different times, and the remaining enzyme activity was measured at time intervals using *p*NP-GlcNAc as the substrate.

Effect of Various Chemicals on β-NAHA Activity. Purified β-NAHA was incubated in 10 mM Bis-Tris buffer pH 6.0 including various bivalent cations (Ca^{2+}, Zn^{2+}, Fe^{3+}, Mn^{2+} and Cu^{2+}, chloride forms) with final concentrations of 1.0, 10, and 50 mM at 37 °C for 10 min. Enzyme activity was measured and expressed as percentage of the activity without added cations under the same conditions.

Analysis of Transglycosylation Reactions. To determine the transglycosylation activity of β-NAHA, different amounts of purified β-NAHAs (0.5, 1, 2, 4 U) were added to 100 μL of substrate solution (*p*NP-GlcNAc, diacetyl chitobiose, and triacetyl chitotriose at high concentrations, 20, 50, and 100 mM) in 50 mM citrate phosphate buffer pH 6.0. Reaction mixtures were incubated at 37 °C and 600 rpm. Samples of 10 μL were withdrawn at different times, and the enzyme was inactivated by incubation at 100 °C for 10 min. Samples were loaded onto high-performance TLC silica plates (Kieselgel 60 F245, Merck) and run using a mobile phase of isopropanol/water/28% ammonia = 7:2:1, v/v/v. Plates were dried, sprayed with 5% H_2SO_4 in ethanol, and developed by baking in an oven at 220 °C for 10 min.

RESULTS

Overexpression of a Putative β-NAHA-Encoding Gene from *L. lactis* ssp. *lactis* IL1403 in *E. coli*. The *lnbA* gene of *L. lactis* ssp. *lactis*, which putatively encodes a β-NAHA and is thus potentially involved in chitin degradation, was successfully cloned into pET-21d, and the protein could be overexpressed in *E. coli* BL21* (DE3) driven by the T7 RNA polymerase promoter. Cells from a 250 mL culture induced with 0.4 mM IPTG and grown for 16 h at 25 °C after induction were disrupted, and LnbA was purified from the crude extract by one-step IMAC with a yield of 70% (Table 1). Typically, close to 90 mg of pure recombinant enzyme was obtained from 1 L of culture with a total activity of around 3300 U and a specific activity of about 37 U/mg of protein.

Characterization of Purified Recombinant β-NAHA. SDS-PAGE (Figure 1) revealed a single polypeptide band with a molecular mass of ∼37 kDa, which is close to the calculated mass of 37.6 kDa. Gel filtration on a Superose 12 column against molecular weight standards resulted in a molecular weight of 38.8 kDa, indicating that the β-NAHA encoded by *lnbA* from *L. lactis* ssp. *lactis* IL1403 is active as a monomer.

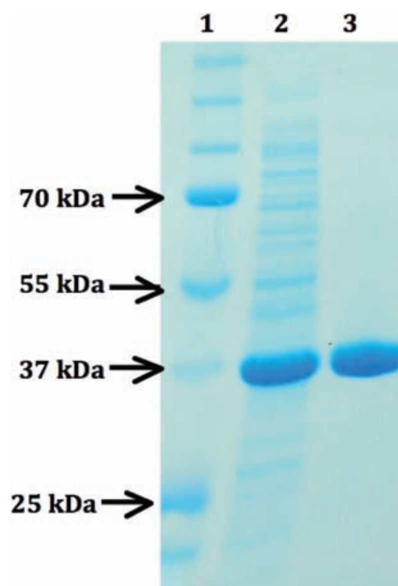

Figure 1. SDS-PAGE analysis of purified β-NAHA. Lane 1, size marker; lane 2, crude extract; lane 3, purified enzyme.

Several chromogenic substrates were used in the standard assay at a concentration of 5 mM. No significant activity (i.e., less than 0.5% of activity with *p*NP-GlcNAc) was detected with *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl β-D-arabinopyranoside, *p*-nitrophenyl β-D-xylopyranoside, several structurally modified variants of *p*NP-GlcNAc (see Materials and Methods), colloidal chitin, and chitosan. The only substrates for which activity was detected were *p*-nitrophenyl *N*-acetyl-β-D-glucosamine (*p*NP-GlcNAc; 38 U/mg specific activity) and *p*-nitrophenyl *N*-acetyl-β-D-galactosamine (*p*NP-GalNAc; 3.1 U/mg specific activity). The *N*-acetyl-β-D-glucosaminidase activity was 12 times higher than the *N*-acetyl-β-D-galactosaminidase activity, which is quite typical for most of *N*-acetyl-β-D-hexosaminidases.[25]

Both *p*NP-GlcNAc and *p*NP-GalNAc were used as substrates to determine the temperature and pH optimum of β-NAHA. The optimal temperature and pH of the purified enzyme were 37 °C (Figure 2A) and 5.5 (Figure 2B), respectively, for both *p*NP-GlcNAc and *p*NP-GalNAc. The enzyme was stable in the range of 20−37 °C, and the half-life of the enzyme at 37 °C (assessed by measuring residual activity in the standard assay) was 53 h (Figure 3A). However, enzyme activity quickly decreased after incubation at temperatures above 40 °C; there was no residual enzyme activity after incubation for 30 min at 50 °C (results not shown). The enzyme was quite stable in the pH range from 6.0 to 7.0; the residual activity of the enzyme after 24 h of incubation at pH 6.0, 6.5, and 7.0 and 37 °C was 83%, 86%, and 84%, respectively (Figure 3B). Figure 3B shows that the pH-stability profile is rather broad at alkaline values, but that stability very rapidly drops at pH values below 5.5. The residual activity after 30 min incubation at pHs from 4.0 to 5.0 was below 20%.

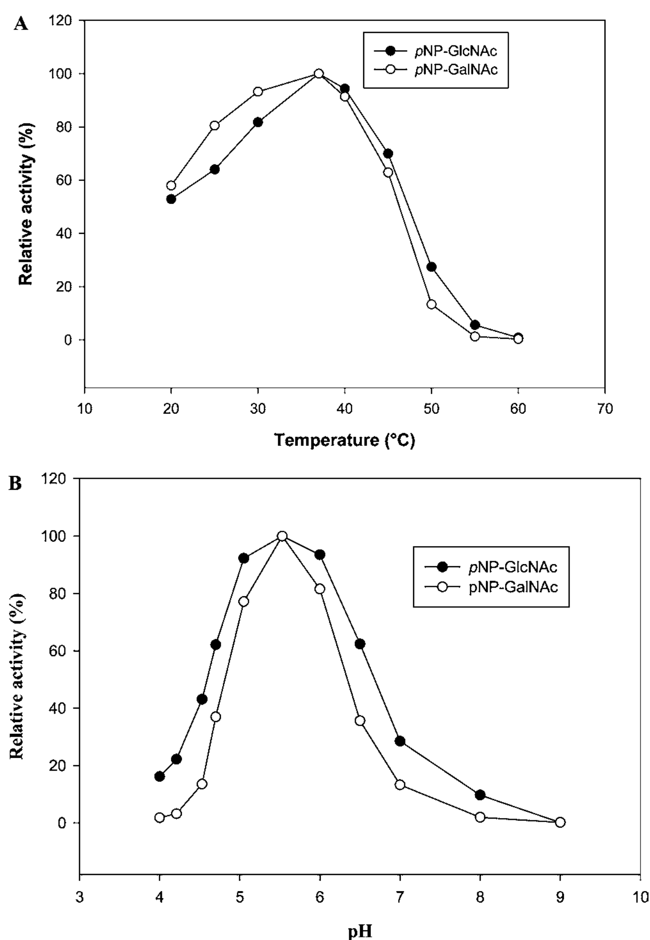

Figure 2. Temperature optimum (A) and pH optimum (B) of recombinant β-NAHA: (●) pNP-GlcNAc as substrate and (○) pNP-GalNAc as substrate.

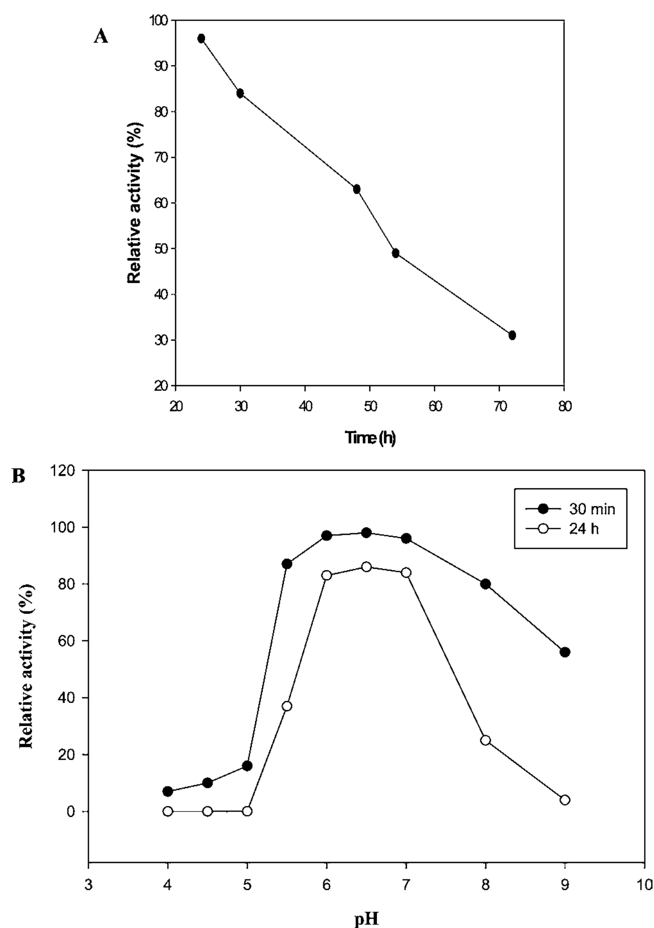

Figure 3. Stability of the enzyme over time at 37 °C (A) and at different pHs (B). pNP-GlcNAc was used as substrate for the enzyme assay. For conditions, see Materials and Methods.

The effect of metal ions on enzyme activity was assayed using 5 mM pNP-GlcNAc as the substrate and is presented in Table 2. Activity was not affected by the presence of monovalent Na^+ or K^+ ions up to concentrations of 100 mM at pH 6.0; however, various bivalent metal ions present in the same concentration range (1−100 mM) had a relatively moderate (e.g., Mg^{2+} and Zn^{2+}) to quite strong effect (Mn^{2+}, Cu^{2+}, Fe^{3+}). Mn^{2+}, Cu^{2+}, and Fe^{3+} inhibited activity by 65%, 85%, and 100%, respectively, at a concentration as low as 1 mM.

The kinetic constants of the enzyme were determined with pNP-GlcNAc and pNP-GalNAc as substrates. K_m and v_{max} values were determined using a Lineweaver−Burk plot; k_{cat} values were calculated based on v_{max} values by nonlinear regression and using a molecular mass of 37 kDa for the enzyme. K_m and k_{cat} were 2.56 mM and 26.7 s^{-1}, respectively, in the case of pNP-GlcNAc as substrate. Experiments with pNP-GalNAc showed an almost linear correlation between the substrate concentration and enzyme speed even up to the highest tested substrate concentration of 10 mM, which was the limit of substrate solubility in the relevant buffers. This indicated that the substrate concentrations used were clearly below K_m; consequently, K_m and k_{cat} values could not be determined for pNP-GalNAc.

Figure 4 shows that LbnA is capable of converting chitobiose and chitotriose to N-acetylglucosamine, and a similar result was obtained with chitotetraose (not shown). Despite the fact that high (50 mM) substrate concentrations were used, no new

Table 2. Effect of Metal Ions on the Activity of β-NAHA

cation	relative activity (%)[b] 1 mM	10 mM	50 mM
control[a]	100	100	100
Na^+	109	107	107
K^+	105	107	104
Mg^{2+}	92	73	44
Ca^{2+}	63	13	27
Zn^{2+}	58	23	13
Fe^{3+}	35	4	0
Mn^{2+}	15	15	13
Cu^{2+}	0	0	0

[a]Standard assay conditions without pretreatment as described in Materials and Methods. [b]Calculated based on the activity of control sample.

oligosaccharides derived from transglycosylation were detected on the TLC plates.

■ DISCUSSION

In recent years there has been increasing interest in the characterization and application of chitin hydrolyzing enzymes;[26,27] however, not much attention has been paid to such enzymes from lactic acid bacteria (LAB) or to the ability of LAB to degrade chitin. It has previously been shown that intestinal bacteria, including some lactic acid bacteria, can

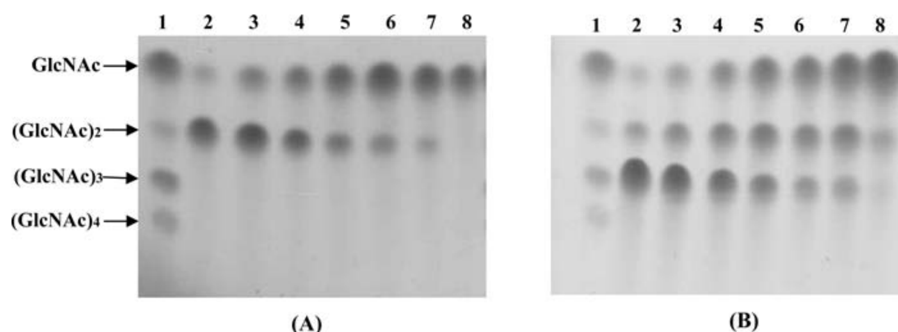

Figure 4. Thin layer chromatography of β-NAHA hydrolysis products obtained upon incubation of 50 mM diacetyl chitobiose (A) and 50 mM triacetyl chitotriose (B) with 4U of enzyme in a total volume of 100 μL: lane 1, standard mixture; lanes 2, 3, 4, 5, 6, 7, and 8, 1 μL of sample taken after 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h of incubation, respectively.

utilize products of chitin hydrolysis (GlcNAc, chito-oligosaccharides) as main carbon sources.[28] This suggests that at least some LAB contain enzyme machineries needed to convert chitin into GlcNAc. Indeed, Vaaje-Kolstad and co-workers[18] demonstrated the presence of a functional chitinolytic enzyme system *L. lactis* comprising an endochitinase and an accessory CBM33 enzyme; however, preliminary growth experiments indicated that *L. lactis* does not grow well on chitin. On the other hand, convincing extracellular chitinase activity was detected upon carbon starvation.[18]

The genome of *L. lactis* ssp. *lactis* IL1403 contains only three genes putatively encoding proteins that are potentially involved in chitin hydrolysis, namely, LlChi18A, LlCBP33A, and LnbA.[29] LlChi18A is an endochitinase, whereas LlCBP33A is a chitin-binding protein currently classified as CBM33, which acts synergistically with the chitinase in the degradation of chitin.[18] Subsequent studies have shown that these CBM33 proteins in fact are oxidative enzymes that cleave chitin chains in their crystalline context, thus increasing substrate accessibility.[18]

In the present study, the third protein, LnbA, has been studied, aiming at further unraveling the ability of *L. lactis* to metabolize chitin and chitin-derived products and at finding a potentially useful food-grade β-NAHA. The gene was cloned into the expression vector pET-21d and overexpressed in *E. coli* BL21* (DE3), a commercially available standard expression system. Physical and biochemical characteristics of the resulting recombinant enzyme were comparable with those of recombinant β-NAHA from *L. casei* ATCC 27092[30] but quite different to dominant β-NAHAs from bacteria such as StrH from *Streptococcus pneumonia*, which is a multidomain secretory protein of 140 kDa putatively anchored to the cell wall,[31] or GcnA from *Streptococcus gordonii*, a cytoplasmic homodimeric protein composed of two 72 kDa subunits.[32]

LnbA can hydrolyze *N*-acetyl-chitobiose as well as *N*-acetyl-chitotriose, the principal degradation products of LlChi18A when degrading chitin or chito-oligomers. It would thus seem that LlChi18A, LlCBP33A, and LnbA are sufficient for complete hydrolysis of chitin to GlcNAc.

The studies with other substrates show that LnbA specifically cleaves the β-linkage of *N*-acetyl-β-D-hexosaminides. The 2-acetamido group of the substrates was found to be essential for activity, as no activity could be detected with substrates such as *p*-nitrophenyl β-D-glucopyranoside or *p*-nitrophenyl β-D-galactopyranoside, which lack that group. No activity was detected when using GlcNAc substrates that were modified at C_6. This narrow substrate specificity indicates a rather specific function of the enzyme, which in part is due to the fact that family 20 enzymes have a substrate-assisted catalytic mechanism that involves the acetamidogroup of the sugar bound in the −1 subsite[33,34] The enzyme was capable of acting on both GlcNAc and GalNAc, but it should be noted that the kinetic data indicated that the affinity for GalNAc is low. Judged by k_{cat}/K_m, LbnA has a clear preference for GlcNAc.

LbnA did not show transglycosylation activity, which can be readily detected in fungal β-NAHAs belonging to the GH Family 20. The transglycosylating activity of fungal β-glucosidases (which also cleave disaccharides and release monosaccharides from short oligosaccharides in an exofashion) is thought to play a regulatory role in carbohydrate metabolism.[35] Since LbnA lacks transglycosylation activity it is unlikely that the enzyme plays a similar regulatory role in chitin metabolism in *L. lactis* ssp. *lactis*. More generally, there are reasons to wonder whether the apparently complete chitinolytic machinery of *L. lactis* might have other roles in addition to harvesting chitin. The fact that *L. lactis* does not grow well on chitin is puzzling. In addition, there are several recent studies in the literature that suggest a role for chitinolytic enzymes in (hitherto nondefined) host−microbe interactions.[36,37] These issues await further studies, e.g., phenotypical studies of knockout mutants.

E. coli expression systems where plasmid stability is achieved by antibiotic resistance are undesirable for enzymes used in food-related applications both for economic reasons (cost of fermentation medium) as well as consumer perception. Enzymes that are themselves derived from LAB such as LnbA can also be produced in food-grade cell factories such as LAB engineered according to self-cloning principles, as recently reviewed.[38]

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Slamova, K.; Bojarova, P.; Petraskova, L.; Kren, V. *β-N*-Acetylhexosaminidase: What's in a name... ? *Biotechnol. Adv.* **2010**, *28*, 682−693.

(2) Yem, D. W.; Wu, H. C. Purification and properties of *β-N*-acetylglucosaminidase from *Escherichia coli*. *J. Bacteriol.* **1976**, *125*, 324−331.

(3) Lee, Y. S.; Park, I. H.; Yoo, J. S.; Chung, S. Y.; Lee, Y. C.; Cho, Y. S.; Ahn, S. C.; Kim, C. M.; Choi, Y. L. Cloning, purification, and characterization of chitinase from *Bacillus* sp. DAU101. *Bioresour. Technol.* **2007**, *98*, 2734−2741.

(4) Rast, D. M.; Horsch, M.; Furter, R.; Gooday, G. W. A complex chitinolytic system in exponentially growing mycelium of *Mucor rouxii* - Properties and function. *J. Gen. Microbiol.* **1991**, *137*, 2797−2810.

(5) Lorito, M.; Harman, G. E.; Hayes, C. K.; Broadway, R. M.; Tronsmo, A.; Woo, S. L.; Dipietro, A. Chitinolytic enzymes produced by *Trichoderma harzianum* - antifungal activity of purified endochitinase and chitobiosidase. *Phytopathology* **1993**, *83*, 302−307.

(6) Schlumbaum, A.; Mauch, F.; Vögeli, U.; Boller, T. Plant chitinases are potent inhibitors of fungal growth. *Nature* **1986**, *324*, 365−367.

(7) Oikawa, A.; Itoh, E.; Ishihara, A.; Iwamura, H. Purification and characterization of *β-N*-acetylhexosaminidase from maize seedlings. *J. Plant Physiol.* **2003**, *160*, 991−999.

(8) Jin, Y. L.; Jo, Y. Y.; Kim, K. Y.; Shim, J. H.; Kim, Y. W.; Park, R. D. Purification and characterization of *β-N*-acetylhexosaminidase from rice seeds. *J. Biochem. Mol. Biol.* **2002**, *35*, 313−319.

(9) Jagadeesh, B. H.; Prabha, T. N.; Srinivasan, K. Activities of glycosidases during fruit development and ripening of tomato (*Lycopersicum esculantum* L.): implication in fruit ripening. *Plant Sci.* **2004**, *166*, 1451−1459.

(10) Jagadeesh, B. H.; Prabha, T. N. *β*-Hexosaminidase, an enzyme from ripening bell capsicum (*Capsicum annuum* var. *variata*). *Phytochemistry* **2002**, *61*, 295−300.

(11) Lemieux, M. J.; Mark, B. L.; Cherney, M. M.; Withers, S. G.; Mahuran, D. J.; James, M. N. Crystallographic structure of human *β*-hexosaminidase A: interpretation of Tay-Sachs mutations and loss of GM2 ganglioside hydrolysis. *J. Mol. Biol.* **2006**, *359*, 913−929.

(12) Cohen, C. M.; Weissmann, G.; Hoffstein, S.; Awasthi, Y. C.; Srivastava, S. K. Introduction of purified hexosaminidase A into Tay-Sachs leukocytes by means of immunoglobulin-coated liposomes. *Biochemistry* **1976**, *15*, 452−460.

(13) Scigelova, M.; Singh, S.; Crout, D. H. G. Glycosidases - a great synthetic tool. *J. Mol. Catal. B: Enzym.* **1999**, *6*, 483−494.

(14) Rauvolfova, J.; Kuzma, M.; Weignerova, L.; Fialova, P.; Prikrylova, V.; Pisvejcova, A.; Mackova, M.; Kren, V. *β-N*-Acetylhexosaminidase-catalysed synthesis of non-reducing oligosaccharides. *J. Mol. Catal. B: Enzym.* **2004**, *29*, 233−239.

(15) Singh, S.; Packwood, J.; Samuel, C. J.; Critchley, P.; Crout, D. H. G. Glycosidase-catalysed oligosaccharide synthesis: Preparation of *N*-acetylchitooligosaccharides using the *β-N*-acetylhexosaminidase of *Aspergillus oryzae*. *Carbohydr. Res.* **1995**, *279*, 293−305.

(16) Weignerova, L.; Pelantova, H.; Manglova, D.; Michalkova, K.; Kren, V. Condensation reactions catalyzed by *α-N*-acetylgalactosaminidase from *Aspergillus niger* yielding *α-N*-acetylgalactosaminides. *Biocatal. Biotransform.* **2010**, *28*, 150−159.

(17) Rauvolfova, J.; Weignerova, L.; Kuzma, M.; Prikrylova, V.; Mackova, M.; Pisvejcova, A.; Kren, V. Enzymatic synthesis of *N*-acetylglucosaminobioses by reverse hydrolysis: characterisation and application of the library of fungal *β-N*-acetylhexosaminidases. *J. Mol. Catal. B: Enzym.* **2004**, *29*, 259−264.

(18) Vaaje-Kolstad, G.; Bunaes, A. C.; Mathiesen, G.; Eijsink, V. G. H. The chitinolytic system of *Lactococcus lactis* ssp *lactis* comprises a nonprocessive chitinase and a chitin-binding protein that promotes the degradation of alpha- and beta-chitin. *FEBS J.* **2009**, *276*, 2402−2415.

(19) Loft, K. J.; Bojarova, P.; Slamova, K.; Kren, V.; Williams, S. J. Synthesis of sulfated glucosaminides for profiling substrate specificities of sulfatases and sungal *β-N*-acetylhexosaminidases. *ChemBioChem* **2009**, *10*, 565−576.

(20) Bojarova, P.; Petraskova, L.; Ferrandi, E. E.; Monti, D.; Pelantova, H.; Kuzma, M.; Simerska, P.; Kren, V. Glycosyl azides - An alternative way to disaccharides. *Adv. Synth. Catal.* **2007**, *349*, 1514−1520.

(21) Bojarova, P.; Slamova, K.; Krenek, K.; Gazak, R.; Kulik, N.; Ettrich, R.; Pelantova, H.; Kuzma, M.; Riva, S.; Adamek, D.; Bezouska, K.; Kren, V. Charged hexosaminides as new substrates for *β-N*-acetylhexosaminidase-catalyzed synthesis of immunomodulatory disaccharides. *Adv. Synth. Catal.* **2011**, *353*, 2409−2420.

(22) Laemmli, U. K. Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(23) Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426−428.

(24) Bradford, M. M. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(25) Weignerova, L.; Vavruskova, P.; Pisvejcova, A.; Thiem, J.; Kren, V. Fungal *β-N*-acetylhexosaminidases with high *β-N*-acetylgalactosaminidase activity and their use for synthesis of *β*-GalNAc-containing oligosaccharides. *Carbohydr. Res.* **2003**, *338*, 1003−1008.

(26) Aam, B. B.; Heggset, E. B.; Norberg, A. L.; Sorlie, M.; Varum, K. M.; Eijsink, V. G. H. Production of chitooligosaccharides and their potential applications in medicine. *Mar. Drugs* **2010**, *8*, 1482−1517.

(27) Hoell, I. A.; Vaaje-Kolstad, G.; Eijsink, V. G. H. Structure and function of enzymes acting on chitin and chitosan. *Biotechnol. Genet. Eng. Rev.* **2010**, *27*, 331−366.

(28) Chen, H. C.; Chang, C. C.; Mau, W. J.; Yen, L. S. Evaluation of *N*-acetylchitooligosaccharides as the main carbon sources for the growth of intestinal bacteria. *FEMS Microbiol. Lett.* **2002**, *209*, 53−56.

(29) Bolotin, A.; Wincker, P.; Mauger, S.; Jaillon, O.; Malarme, K.; Weissenbach, J.; Ehrlich, S. D.; Sorokin, A. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp *lactis* IL1403. *Genome Res.* **2001**, *11*, 731−753.

(30) Senba, M.; Kashige, N.; Nakashima, K.; Miake, F.; Watanabe, K. Cloning of the gene of *β-N*-acetylglucosaminidase from *Lactobacillus casei* ATCC 27092 and characterization of the enzyme espressed in *Escherichia coli*. *Biol. Pharm. Bull.* **2000**, *23*, 527−531.

(31) Clarke, V. A.; Platt, N.; Butters, T. D. Cloning and expression of the *β-N*-acetylglucosaminidase gene from *Streptococcus pneumoniae* - generation of truncated enzymes with modified aglycon specificity. *J. Biol. Chem.* **1995**, *270*, 8805−8814.

(32) Harty, D. W. S.; Chen, Y. J.; Simpson, C. L.; Berg, T.; Cook, S. L.; Mayo, J. A.; Hunter, N.; Jacques, N. A. Characterisation of a novel homodimeric N-acetyl-*β*-D-glucosaminidase from *Streptococcus gordonii*. *Biochem. Biophys. Res. Commun.* **2004**, *319*, 439−447.

(33) Tews, I.; Perrakis, A.; Oppenheim, A.; Dauter, Z.; Wilson, K. S.; Vorgias, C. E. Bacterial chitobiase structure provides insight into catalytic mechanism and the basis of Tay-Sachs disease. *Nat. Struct. Biol.* **1996**, *3*, 638−648.

(34) Tews, I.; van Scheltinga, A. C. T.; Perrakis, A.; Wilson, K. S.; Dijkstra, B. W. Substrate-assisted catalysis unifies two families of chitinolytic enzymes. *J. Am. Chem. Soc.* **1997**, *119*, 7954−7959.

(35) Aro, N.; Pakula, T.; Penttila, M. Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiol. Rev.* **2005**, *29*, 719−739.

(36) Chaudhuri, S.; Bruno, J. C.; Alonzo, F. III; Xayarath, B.; Cianciotto, N. P.; Freitag, N. E. Contribution of chitinases to *Listeria monocytogenes* pathogenesis. *Appl. Environ. Microbiol.* **2010**, *76*, 7302−7305.

(37) Sanchez, B.; Gonzalez-Tejedo, C.; Ruas-Madiedo, P.; Urdaci, M. C.; Margolles, A. *Lactobacillus plantarum* extracellular chitin-binding

protein and its role in the interaction between chitin, Caco-2 cells, and cucin. *Appl. Environ. Microbiol.* **2011**, *77*, 1123−1126.
(38) Peterbauer, C. K.; Maischberger, T.; Haltrich, D. Food-grade gene expression in lactic acid bacteria. *Biotechnol. J.* **2011**, *6*, 1147−1161.