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Characterization of β-*N*-acetylglucosaminidase from a marine *Pseudoalteromonas* sp. for application in *N*-acetyl-glucosamine production

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ABSTRACT

The psychrotolerant *Pseudoalteromonas issachenkonii* PAMC 22718 was isolated for its high exo-acting chitinase activity in the Kara Sea, Arctic. An exo-acting chitinase (W-Chi22718) was homogeneously purified from the culture supernatant of PAMC 22718, the molecular weight of which was estimated to be approximately 112 kDa. Due to its β-*N*-acetylglucosaminidase activity, W-Chi22718 was able to produce *N*-acetyl-D-glucosamine (GlcNAc) monomers from chitin oligosaccharide substrates. W-Chi22718 displayed chitinase activity from 0 to 37°C (optimal temperature of 30°C) and maintained activity from pH 6.0 to 9.0 (optimal pH of 7.6). W-Chi22718 exhibited a relative activity of 13 and 35% of maximal activity at 0 and 10°C, respectively, which is comparable to the activities of previously characterized, cold-adapted bacterial chitinases. W-Chi22718 activity was enhanced by K^+, Ca^{2+}, and Fe^{2+}, but completely inhibited by Cu^{2+} and SDS. We found that W-Chi22718 can produce much more (GlcNAcs) from colloidal chitin, working together with previously characterized cold-active endochitinase W-Chi21702. Genome sequencing revealed that the corresponding gene (*chi22718_IV*) was 2,856 bp encoding a 951 amino acid protein with a calculated molecular weight of approximately 102 kDa.

KEYWORDS

β-*N*-Acetylglucosaminidase; arctic marine; cold-active; exochitinase; *N*-acetyl-glucosamine; psychrotolerant

Introduction

Chitin, a β-(1,4)-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc), is one of the most abundant natural biopolymers on earth.[1] Chitin is produced by many marine organisms and serves as a nutrient source for marine microbes. Chitinolytic bacteria play a critical role in the chitin recycling process.[2] The first step in chitin degradation is the hydrolysis of the β-(1,4)-glycosidic bonds between GlcNAc subunits catalyzed by chitinases. Until now, many chitinases have been isolated from various marine environments and studied for their biochemical and genetic characteristics.[2–4] In general, chitinases belong to glycosyl hydrolase families 18 and 19[5] and can be classified into two types based on their catalytic mechanism: endotype chitinase (EC 3. 2. 1. 14), which randomly cleaves chitin at internal sites into oligosaccharides (mainly dimeric chitobiose) and exotype chitobiase (EC 3. 2. 1. 52), also termed β-*N*-acetylglucosaminidase, which hydrolyzes the resulting oligosaccharides into monomeric GlcNAc.[6]

Bacteria of low temperature habitats, such as Arctic or Antarctic marine, are natural resources for cold-active enzymes with potential industrial applications. Cold-active chitinase is a useful biocatalyst for the production of pure GlcNAc monomers or oligomers through cold condition processing in industrial biotechnology. However, since only a few bacterial chitinases from polar regions have been characterized,[3,7,8] the available experimental data on cold-active, especially exo-acting, chitinases are insufficient for understanding their molecular mechanisms in cold environments. Here, we describe the purification and biochemical characterization of a cold-adapted β-*N*-acetylglucosaminidase from an Arctic marine bacterium.

Materials and methods

Screening for chitinase-producing bacteria and culture conditions

Forty-seven sediment samples were collected from the Kara Sea, Arctica, in August 2005. Each 100 mg of the sediments was suspended in 3-mL autoclaved seawater. After holding for 1 h at 4°C, the supernatant was spread on ZoBell plates (5 g of peptone, 1 g of yeast extract, 0.01 g of $FePO_4$, 15 g of agar, 750 mL of sea water, 250 mL of distilled water per liter) containing 0.4% swollen chitin (CZB) and cultured at 25°C for several days. A single colony producing a clearing zone on CZB plates was selected as a chitinolytic strain, transferred to liquid CZB, and further cultured at 25°C. To screen for bacterial strains having β-*N*-acetylglucosaminidase activity, the culture supernatants were incubated with chromogenic derivative *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*NP-GlcNAc). One strain producing *p*-nitrophenol from *p*NP-GlcNAc was finally selected as a putative GlcNAc producer.

Temperature effect on chitinase production

Strain PAMC 22718 was precultured by inoculating a small amount of glycerol stock in ZoBell medium containing 2% glucose and incubating for 1 d at 25°C. The preculture was

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transferred to CZB at a final concentration of 1% and incubated at various temperatures (5, 15, or 25°C). At appropriate time intervals, cell growth was examined by measuring the absorbance at 600 nm, and the chitinase activity of the culture was measured, as described below. The cell culture was centrifuged (12,000*g* for 3 min at 4°C) and 0.2 mL of supernatant was added to 0.8 mL of 50 mM sodium phosphate buffer (pH 7.6; standard buffer), containing *p*NP-GlcNAc substrate (final concentration 0.1 mM). After incubating the reaction mixture at 30°C for 3 min, absorbance was determined at 400 nm, and the enzyme activity was calculated on the basis of an extinction coefficient of 17,000 M^{-1} cm^{-1} for *p*-nitrophenol. One unit of chitinase activity was defined as the enzyme activity sufficient to produce 1 μmol of *p*-nitrophenol per hour, per milligram of protein at 30°C. To monitor a protein band, which increased in intensity with cell growth, SDS-PAGE was performed on 8% polyacrylamide slab gel using a Tris-glycine buffer system.

Purification of chitinase

PAMC 22718 was cultivated in 4.0 L of CZB medium for 7 d at 25°C, and the culture was then centrifuged (12,000*g* for 30 min at 4°C). The culture supernatant was buffer exchanged with 40 L 20 mM Tris-HCl buffer (pH 7.6) and simultaneously concentrated through a lab-scale TFF System (Millipore) equipped with 10-kDa cutoff membrane. The concentrated protein solution was loaded onto a high Q ion-exchange column (Bio-Rad) on a FPLC system (Bio-Rad). Chitinolytic fractions were eluted from the column by applying the Tris-HCl buffer containing 1 M NaCl, pooled together, and then loaded onto a WorkBeads 17 SEC column (Bio Works). Elution was performed in Tris-HCl buffer, and the fractions having chitinase activity were pooled and concentrated with Vivaspin 20 (Sartorius Stedim Biotech). The molecular weight of purified chitinase (W-Chi22718) was estimated by Sephacryl S-100 size exclusion chromatography (1.6 × 62 cm, GE Healthcare) using a mixture of calibration materials (3 mg conalbumin, 75 kDa; 4 mg ovalbumin, 44 kDa; 3 mg carbonic anhydrase, 29 kDa; 3 mg ribonuclease A, 13.7 kDa; 3 mg aprotinin, 6.5 kDa) in standard buffer containing 0.15 M NaCl. The protein concentration was determined using a Bradford protein assay kit (Bio-Rad) in which bovine serum albumin served as the standard protein.

Analysis for hydrolysis of various substrates

To examine the substrate specificity of W-Chi22718, chromogenic derivatives *p*NP-GlcNAc, *p*-nitrophenyl-*β*-D-*N,N′*-diacetylchitobiose [*p*NP-$(GlcNAc)_2$], and *p*-nitrophenyl-*β*-D-*N,N′,N″*-triacetylchitotriose [*p*NP-$(GlcNAc)_3$] were used as substrates. Purified W-Chi22718 (13.2 μg) was added to 1 mL of standard buffer containing each substrate (final concentration 0.1 mM), and the reaction mixture was incubated at 30°C for 3 min.

Also, to analyze the pattern of chitin hydrolysis, W-Chi22718 (1.1 μg) was added to standard buffer containing each chitin oligosaccharide as a substrate at a final concentration of 3 mM GlcNAc, 2 mM *N,N′*-diacetylchitobiose ($(GlcNAc)_2$), 2 mM *N,N′,N‴*-triacetylchitotriose ($(GlcNAc)_3$), 2 mM tetra-*N*-acetylchitotetraose ($(GlcNAc)_4$), 1.5 mM penta-*N*-acetylchitopentaose ($(GlcNAc)_5$), or 1.5 mM hexa-*N*-acetylchitohexaose ($(GlcNAc)_6$). The reaction mixture was incubated at 30°C for various time intervals and then boiled for 5 min. An aliquot (10 μl) was then spotted on a silica gel plate (Merck) and developed with isopropanol/water/25% ammonia (34:15:1). Spots were detected with aniline–phthalate solution (2 mL of aniline, 3.3 g of phthalic acid per 100 mL of water-saturated butanol).

The hydrolysis of several polymeric chitins (swollen and colloidal chitin) and chitin derivatives (chitosan and glycol chitosan) by W-Chi22718 was examined, as described below. In brief, W-Chi22718 (11.0 μg) and each substrate (0.5%) were incubated in 1 mL standard buffer at 30°C for at least 3 h and then centrifuged (12,000*g*, 10 min, 4°C). An aliquot of the supernatant (500 μl) was then mixed with an equal volume of dinitrosalicylic acid (DNSA) reagent (1% DNSA, 0.2% phenol, 0.05% sodium sulfite, 1% sodium hydroxide) and boiled for 5 min. The amount of reducing sugar released from the substrates by W-Chi22718 was calculated from the absorbance at 575 nm.

Effects of temperature and pH on chitinase activity

The optimal temperature for W-Chi22718 reaction was determined by measuring the chitinolytic activity in reactions consisting of 0.1 mM *p*NP-(GlcNAc) and 1.3 μg of W-Chi22718 in 1 mL of standard buffer, performed for 1 h across a range of temperatures (0–60°C). To examine temperature stability, 1 mL of standard buffer, containing W-Chi22718 (1.3 μg), was preincubated at various temperatures (10–60°C) for 1 h, to which 0.1 mM *p*NP-(GlcNAc) was then added. After further incubating at 30°C for 1 h, the residual activity of W-Chi22718 was measured.

The optimum pH was determined by incubating at 30°C for 30 min and measuring the chitinolytic activity in a 1 mL of reaction mixture, which contained 0.1 mM *p*NP-(GlcNAc) and 2.6 μg W-Chi22718 in each buffer: 50 mM sodium acetate (pH 4.0–6.0) or 50 mM potassium phosphate (pH 6.0–9.5). To measure the stability of pH, 26.4 μg of W-Chi22718 was preincubated in 100 μl of different buffers with various pH ranges on ice for 1 h as follows: 50 mM sodium acetate (pH 2.0–6.0), 50 mM potassium phosphate (pH 7.0–9.5), or 50 mM sodium tetraborate (pH 10.0–11.0). An aliquot (10 μl) of the pretreated W-Chi22718 was then added to 1 mL of standard buffer containing 0.1 mM *p*NP-(GlcNAc) and incubated at 30°C for 30 min.

Effects of metal ions and detergents on chitinase

To examine the detrimental effects of metal ions and detergents, 1.3 μg W-Chi22718 was mixed with 2 mM KCl, $CuSO_4$, $MgSO_4$, $CaCl_2$, $BaCl_2$, $FeSO_4$, EDTA, NaN_3, or 5.7% SDS in 990 μl standard buffer and incubated for 1 h on ice. Subsequently, 10 μl of 10 mM *p*NP-(GlcNAc) was added to the reaction mixture, incubated at 30°C for 1 h, and the residual chitinase activity of W-Chi22718 was measured.

Determination of kinetic parameters

For kinetic study, 13.2 μg of W-Chi22718 was added to 1 mL of standard buffer containing 0.03–0.15 mM of *p*NP-(GlcNAc). Following incubation of the reaction mixture at various temperatures (0–30°C) for 10 min, k_{cat} and K_m values were experimentally obtained by fitting a linear Lineweaver–Burk plot to the experimental values from three independent experiments. The values of kinetic constants k_{cat} and K_m for *p*NP-$(GlcNAc)_2$ and *p*NP-$(GlcNAc)_3$ were determined at 30°C as mentioned above.

Hydrolysis of colloidal chitin

To determine the GlcNAc-polymer hydrolytic activity of W-Chi22718, colloidal chitin was used as a substrate. Purified W-Chi22718 or endochitinase W-Chi21702, which was previously purified and characterized in our lab,[9] was added to 1 mL of standard buffer containing colloidal chitin (final concentration 1.5%), and the reaction mixture was incubated at 30°C. After incubation, reaction mixture was boiled for 5 min and centrifuged (12,000*g*, 10 min, 4°C), and the supernatant was analyzed by thin layer chromatography (TLC) as mentioned above.

Results and discussion

Selection of β-N-acetylglucosaminidase-producing bacteria

In total, 136 bacterial strains were isolated from 47 sediment samples of the Kara Sea, Arctic, based on the ability to produce a clearing zone around a colony on CZB plates, a characteristic indicative of chitinolytic activity. To search for a strain which is able to produce GlcNAc monomers at cold temperatures, several strains, producing opaque clearing zone, were tested for their relative cold activities at 5°C and for their maximal activities against *p*NP-GlcNAc. One strain, designated PAMC 22718, from the sampling site was selected for the characterization of a cold-active exochitinase from an Arctic bacterium. Using a similarity-based search tool, EzTaxon server 2.1, the 16S rRNA gene sequence (GenBank accession number GQ885144) of PAMC 22718 was found to be matched with that of *Pseudoalteromonas issachenkonii* KMM 3549^T (100% similarity), a gram-negative, aerobic, marine bacterium of the Pacific Ocean.[10] Thus, PAMC 22718 was identified as a strain of *P. issachenkonii* and deposited at the Biological Resource Center, Korea, under the number KCTC 11415BP.

Temperature effect on chitinase production

The growth of PAMC 22718 was tested at different temperatures (5, 15, or 25°C) in CZB medium. Cell growth rapidly increased immediately after the culture was shifted to 25°C, with a maximum specific growth rate of 0.68 h^{-1}, which occurred after 9 h of incubation. Cell growth at 15°C increased after a short lag phase, showing a growth pattern similar to that at 25°C, but with a lower growth rate (0.38 h^{-1}). In contrast to growth at 15–25°C, PAMC 22718 required a much longer lag phase to reach an exponential phase and grew at a lower growth rate of 0.09 h^{-1} at 5°C (Figure 1). The growth

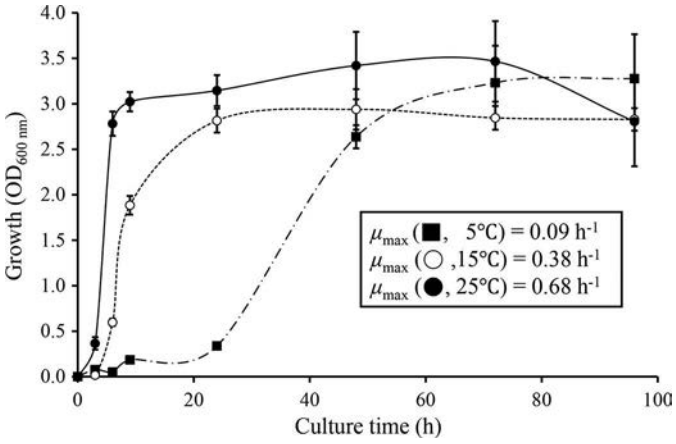


Figure 1. Growth of PAMC 22718 cells at different temperatures. A 500-μl aliquot of overnight culture grown in ZoBell containing 2% glucose (OD_{600} = 1.2) was transferred to 50 mL of CZB medium and was cultured at 5°C (■), 15°C (○), or 25°C (●). At the indicated time intervals, cell growth was determined by measuring the value of OD_{600}.

test indicates that PAMC 22718 is a psychrotolerant, capable of growth at low temperatures, but whose optimal growth temperature is above 20°C.

In our preliminary examination of the chitinase production and activity of PAMC 22718 at optimal 25°C, the culture volume increased by 10 times (500 mL). The chitinase activity against *p*NP-GlcNAc steadily increased concomitantly with the cell growth, approximately 25 h after culturing, and the maximal activity (4.5 U mg^{-1}) occurred after a 250 h of incubation, with the activity level sustained throughout the remainder of the experiment (Figure 2a). When the secreted soluble proteins in the culture supernatant, prepared at the same time intervals as above, were separated on a SDS-PAGE gel, a protein band of ~100 kDa was found to increase

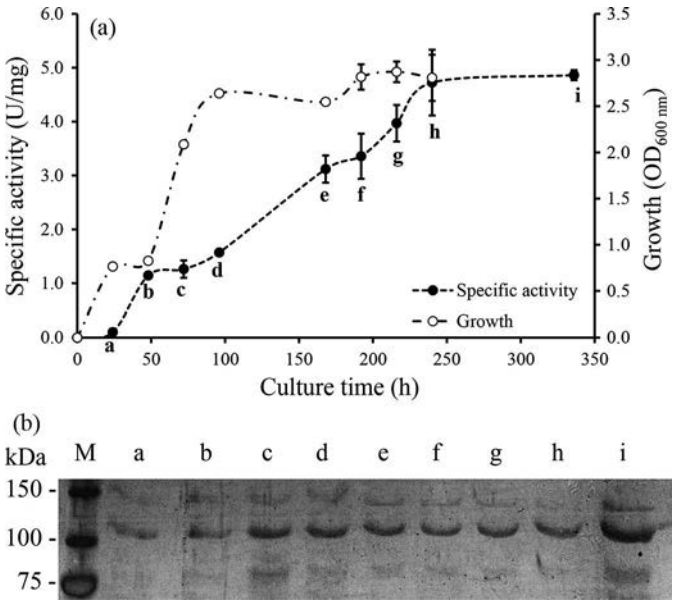


Figure 2. Production of β-*N*-acetylglucosaminidase from PAMC 22718. (a) β-*N*-acetylglucosaminidase secreted from PAMC 22718 grown in CZB medium. At appropriate time intervals during 24–336 h culture, the chitinolytic activity was determined by measuring the amount of *p*NP released from *p*NP-GlcNAc. (b) SDS-PAGE analysis of the culture supernatants at the same time intervals as in (a). Lane M, size standard; lanes a–i, culture supernatants at time intervals.

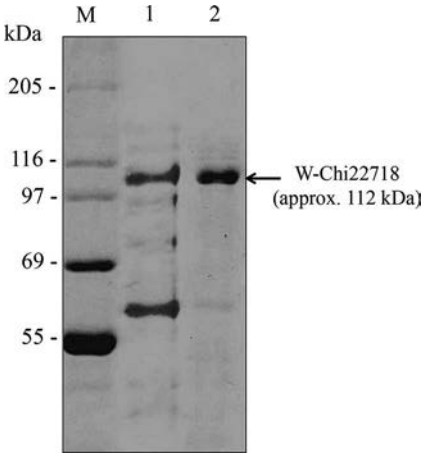


Figure 3. Purification of W-Chi22718 from PAMC 22718. Lane M, size standard; lane 1, W-Chi22718 partially purified through High *Q* ion-exchange chromatography; lane 2, W-Chi22718 homogeneously purified through gel filtration chromatography.

in its intensity with cell growth and chitinolytic activity (Figure 2b). This indicated that the ~100 kDa protein (designated W-Chi22718) was responsible for the chitinolytic activity in PAMC 22718, and that it is a *β*-*N*-acetylglucosaminidase capable of producing glucosamine monomer, a useful biocompound.

Purification of W-Chi22718

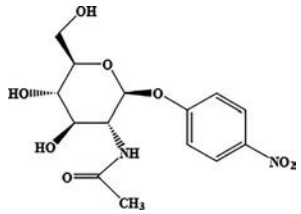
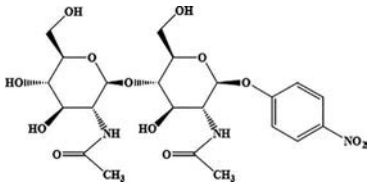
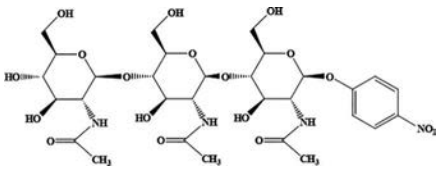
W-Chi22718 was produced by 4.0 L pilot-scale fermentation for 7 d at 25°C. After buffer exchange of the culture supernatant, the concentrated enzyme solution was subjected to a High Q ion-exchange column, and the bound proteins started to elute with the addition of 1 M NaCl. One major protein peak exhibiting chitinolytic activity against *p*NP-GlcNAc eluted between 550 and 800 mM NaCl. When the active fraction containing the protein was separated on an 8% SDS-PAGE gel, the ~100 kDa W-Chi22718 band was a major component of the fraction, along with other protein species of similar or lesser abundance. The partially purified W-Chi22718 was purified to apparent homogeneity by gel filtration chromatography (Figure 3). The homogeneously purified W-Chi22718 was demonstrated to have *β*-*N*-acetylglucosaminidase activity, producing *p*-nitrophenol and *β*-*N*-acetylglucosamine from *p*NP-GlcNAc. The purification of W-Chi22718 yielded 2.8 mg in total protein content and was determined to have a specific activity of 21.8 U mg^{-1} toward *p*NP-GlcNAc, with a purification fold of 8.0 (data not shown). The molecular weight of purified W-Chi22718 was determined to be approximately 112 kDa through Sephacryl S-100 size exclusion chromatography.

Analysis for hydrolysis pattern by W-Chi22718

W-Chi22718 activity against glycosidic bond hydrolysis of chromogenic GlcNAc derivatives was measured using a rapid assay for the function-based classification of target chitinase: *p*NP-GlcNAc for *β*-*N*-acetylglucosaminidase, *p*NP-$(GlcNAc)_2$ for chitobiosidase (exochitinase), and *p*NP-$(GlcNAc)_3$ for endochitinase. In this assay, W-Chi22718 produced the highest specific activity for *p*NP-GlcNAc, indicating that it possesses an exo-acting *β*-*N*-acetylglucosaminidase activity (Table 1).

The W-Chi22718 hydrolysis products of chitin oligosaccharides [GlcNAc (G1)-$GlcNAc_6$ (G6)] were analyzed by TLC (Figure 4). Immediately after addition of W-Chi22718, substrate G2 began to be hydrolyzed into G1, and most G2

Table 1. Substrate specificity of W-Chi22718 toward chromogenic GlcNAc derivatives.

Substrate	Molecular formula	Specific activity (U mg^{-1})[a]
*p*NP-GlcNAc		21.8 ± 0.1
*p*NP-$(GlcNAc)_2$		5.6 ± 0.3
*p*NP-$(GlcNAc)_3$		1.3 ± 0.1

[a]Specific activity was calculated based on the amount of *p*NP released from *p*NP-$(GlcNAc)_n$.

*p*NP-GlcNAc, *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide; *p*NP-$(GlcNAc)_2$, *p*-nitrophenyl-*β*-D-*N*,*N*′-diacetylchitobiose; *p*NP-$(GlcNAc)_3$, *p*-nitrophenyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose.

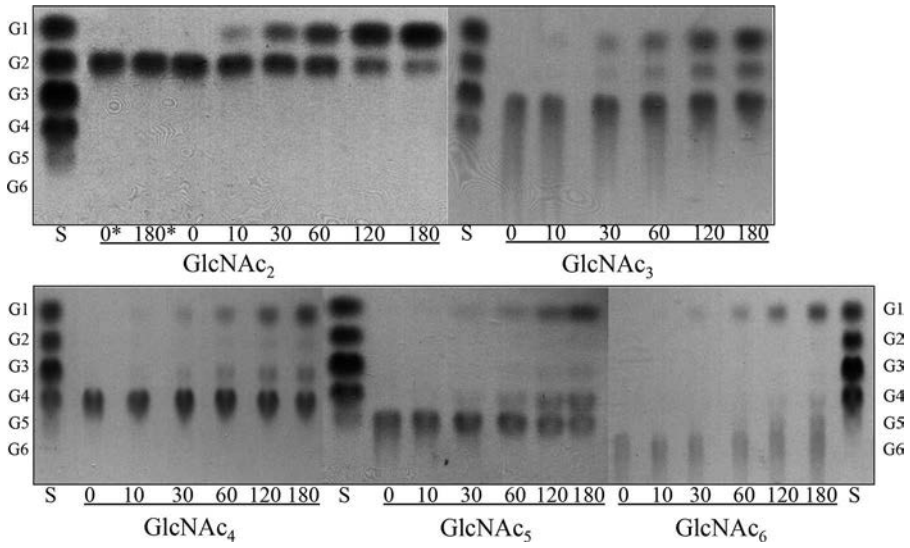


Figure 4. Thin layer chromatography analysis of the hydrolyzed products of chitin oligosaccharides by W-Chi22718. The standard buffer containing W-Chi22718 was incubated with each substrate at the optimal temperature, 30°C, for up to 3 h, and then analyzed at the indicated times. Lane S, mixture of chitin oligosaccharides from GlcNAc (G1) to $GlcNAc_6$ (G6). The asterisk (*) indicates the control reaction without W-Chi22718.

was converted into G1 during the 3 h of incubation. G3 was cleaved into G1+G2 and the resulting G2 was further hydrolyzed to G1 with longer incubation. G4, G5, and G6 were also hydrolyzed, and some intermediate products were finally converted to G1. In all the hydrolytic reactions tested, G1 accumulated as the end product and was not further degraded by W-Chi22718.

Effects of temperature, pH, metal ions, and detergents on W-Chi22718 activity

The chitinolytic activity of W-Chi22718 was high in temperatures ranging from 20 to 37°C, with an optimum temperature at 30°C, but rapidly decreased at temperatures above 40°C. Interestingly, W-Chi22718 exhibited a relative activity of 13 and 35% at 0 and 10°C, respectively, relative to its maximal activity (30°C) (Figure 5a). The relative activities measured at 0–10°C are comparable to those of the previously characterized, cold-adapted chitinases from other bacterial strains: *Sanguibacter antarcticus* (40–60%),[9] *Alteromonas* sp. (28%),[11] and *Moritella marina* (10%).[4] When W-Chi22718 was pretreated at different temperatures for 1 h, the enzymatic stability was not maintained at temperatures above the optimal 30°C and slowly decreased (Figure 5b).

When assayed in various pH-adjusted buffers, W-Chi22718 exhibited maximum reactivity between pH 7.6 and 8.0 and was reduced to less than 10% of its maximal activity below pH 6.0 (Figure 5c). Accordingly, W-Chi22718 was stable only in near neutral conditions (pH 7.0–9.0) (Figure 5d).

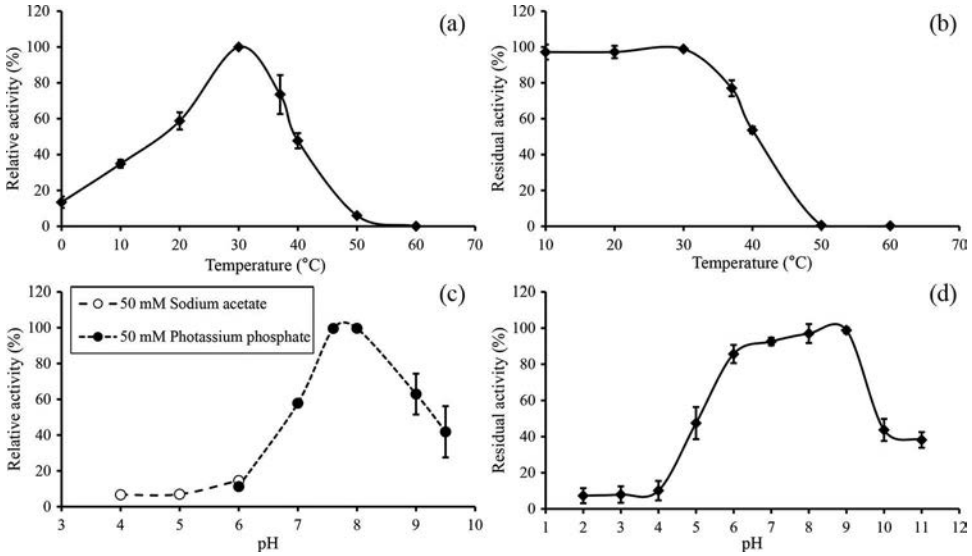


Figure 5. The effects of temperature and pH on W-Chi22718 activity. The optimal temperature (a) and pH (c) for W-Chi22718 activity and stability across a broad range of temperatures (b) and pHs (d) were measured as described in materials and methods.

Table 2. The effects of metal ions and chemical reagents on W-Chi22718.

Compound	Concentration	Residual activity (%)[a]
None	–	100 ± 0
SDS	5.8%	0 ± 0
EDTA	2 mM	97.8 ± 0.8
NaN_3	2 mM	164.1 ± 3.2
$CuSO_4$	2 mM	0 ± 0
$MgSO_4$	2 mM	100.5 ± 15.6
$CaCl_2$	2 mM	139.7 ± 13.7
$BaCl_2$	2 mM	92.6 ± 10.8
KCl	2 mM	194.6 ± 3.6
$FeSO_4$	2 mM	133.1 ± 8.3

[a]Residual chitinolytic activities were calculated relative to the activity of an untreated control reaction.

The effects of various cations and chemical reagents on W-Chi22718 activity were tested (Table 2). W-Chi22718 was activated in the presence of K^+, Ca^{2+}, Fe^{2+}, or NaN_3 up to 130–160% compared to nontreated control (100%), but scarcely affected by Mg^{2+}, Ba^{2+}, or EDTA. In contrast, W-Chi22718 completely lost activity by the addition of SDS, an anionic protein detergent, and the denatured form could not recover its activity even after washing out SDS. W-Chi22718 activity was also inhibited by Cu^{2+}.

Kinetic study of W-Chi22718

The values of kinetic constants of W-Chi22718 activity against *p*NP-GlcNAc were calculated at temperatures ranging from 0 to 30°C (Table 3). Even at low temperatures, W-Chi22718 exhibited relatively high turnover rate (k_{cat}) and catalytic efficiency (k_{cat}/K_m), which correspond approximately 10 and 20% at 0 and 10°C, respectively, relative to their maximal values at optimal 30°C. Its binding affinity (K_m) for *p*NP-GlcNAc was determined to be constant irrespective of the reaction temperature.

The kinetic parameters and catalytic efficiency of W-Chi22718 for *p*NP-$(GlcNAc)_2$ and *p*NP-$(GlcNAc)_3$ were measured at optimal 30°C and compared with those against *p*NP-GlcNAc. W-Chi22718 had approximately 3–8-fold higher k_{cat} against *p*NP-GlcNAc ($k_{cat} = 0.8\ s^{-1}$) than *p*NP-$(GlcNAc)_2$ ($k_{cat} = 0.3\ s^{-1}$) and *p*NP-$(GlcNAc)_3$ ($k_{cat} = 0.1\ s^{-1}$). This supports the previous result that W-Chi22718 exhibited the highest specific activity for *p*NP-GlcNAc and confirms that W-Chi22718 is a *β-N*-acetylglucosaminidase that acts on $GlcNAc_2$ and higher analogues in exosplitting fashion to produce GlcNAc monomers.

Table 3. Kinetic parameters of W-Chi22718 activity against *p*NP-GlcNAc at various temperatures.

Temperature (°C)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
0	2.40 ± 0.33	0.07 ± 0.01	0.16 ± 0.01	0.41 ± 0.05
10	5.66 ± 0.31	0.16 ± 0.01	0.17 ± 0.02	0.91 ± 0.15
20	14.71 ± 1.11	0.41 ± 0.03	0.18 ± 0.02	2.26 ± 0.11
30	29.67 ± 3.09	0.82 ± 0.09	0.19 ± 0.01	4.31 ± 0.13

These experiments for kinetic parameters were independently performed 3 times ($n = 3$). V_{max}, maximal velocity; k_{cat}, turnover number; K_m, Michaelis constant; k_{cat}/K_m, catalytic efficiency. k_{cat} and k_{cat}/K_m were calculated after V_{max} and K_m were determined by Lineweaver–Burk plot.
*p*NP-GlcNAc, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide.

Production of GlcNAc monomers by W-Chi22718

When analyzed by TLC, W-Chi22718 could not hydrolyze the substrate colloidal chitin, and thus not produce GlcNAc in 3 h reaction (Figure 6, lane 3). Such results are also shown in many exo-acting chitinases. For example, *N*-acetylglucosaminidase ChiP from *Pseudoalteromonas* sp. strain S91 could not degrade colloidal chitin, although it exhibited a hydrolytic activity against fluorescent substrates 4-MU-$(GlcNAc)_{1-3}$.[2] We previously purified and characterized the cold-active endochitinase (W-Chi21702) from Antarctic bacterium *S. antarcticus* KOPRI 21702.[9] W-Chi21702 was able to concomitantly produce a mixture of monomer, dimer, and oligomer of GlcNAc from colloidal and swollen chitin polymers. We tested whether the mixture of W-Chi22718 and W-Chi21702 can produce more GlcNAc monomers than W-Chi21702 alone. As results, the combination could produce much more GlcNAc monomers than did only W-Chi21702 (Figure 6, lanes 2 and 4). Based on the above results, one can reasonably propose that the GlcNAc dimers and oligomers produced from colloidal chitin by W-Chi21702 endoactivity were further hydrolyzed into GlcNAc monomers due to W-Chi22718 exoactivity. To confirm the attribution of W-Chi22718, an additional test was performed. The endochitinase reaction by W-Chi21702 was processed for 90 min and then boiled for 5 min to completely inhibit the endoactivity (Figure 6, lane 5). After addition of W-Chi22718, the reaction was further incubated for 90 min and analyzed. As shown in Figure 6, lane 6, the quantity of GlcNAc monomers increased after the addition of exo-acting W-Chi22718, which clearly shows that W-Chi22718 catalyzes the formation of monomers.

Sequence analysis of W-Chi22718 coding gene

Previously, the genome sequence of PAMC 22718 was deposited by Park et al.[12] in NCBI Genome Database (BioProject Accession No. PRJNA159457). To search for the corresponding gene, purified W-Chi22718 was separated on an 8% polyacrylamide gel by SDS-PAGE, blotted on a PVDF membrane, and subjected to *N*-terminal amino acid sequencing. Also, W-Chi22718 was separated by isoelectric focusing using an immobilized pH gradient (4–10 NL) and then on a

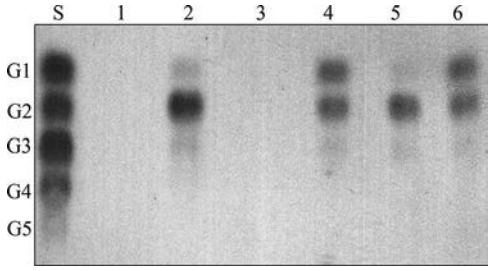


Figure 6. Thin layer chromatography analysis of GlcNAc production from colloidal chitin by W-Chi22718. Each enzyme solution was added to the standard buffer containing 1.5% colloidal chitin and incubated at 30°C for proper time. Lane S, a mixture of chitin oligosaccharides from GlcNAc (G1) to $GlcNAc_5$ (G5); lane 1, control reaction without enzyme for 3 h; lane 2, reaction with 5.5 μg W-Chi21702 for 3 h; lane 3, reaction with 5.5 μg W-Chi22718 for 3 h; lane 4, reaction with 2.75 μg W-Chi21702 and 2.75 μg W-Chi22718 for 3 h; lane 5, reaction with 2.75 μg W-Chi21702 for 90 min; lane 6, after boiling of lane 5 sample for 5 min, 2.75 μg W-Chi22718 was added to the reaction and further incubated for 90 min.

10–15% polyacrylamide gradient gel. The separated protein spots were excised and applied to Ettan MALDI-TOF, and the amino acid sequence of the peptide was obtained. The *N*-terminal "EEGAEKVERIQVT" (100%) and internal "IYGTNAVAGVVNNNTR" (81%) sequences were matched well with the amino acid sequence deduced from a PAMC 22718 gene against NCBI BLASTP (protein–protein blast) database. The W-Chi22718 coding gene (designated *chi22718_IV*, GenBank accession no. KT819916) was composed of 2,856 bp encoding 951 amino acids, having a calculated molecular weight of approximately 102 kDa. When the amino acid sequence of *chi22718_IV* was analyzed through NCBI BLASTP and CD-Search programs, the sequence matched well with those of TonB-dependent receptors from *Pseudoalteromonas* sp. TonB-dependent receptors are presumably involved in the transport of GlcNAc and chitin oligosaccharides across the outer membrane and cell growth,[13] but their chitinolytic activities in chitinases have not yet been reported.

In *Escherichia coli*, TonB protein interacts with outer membrane receptor proteins that perform high-affinity binding and uptake of specific substrates.[14] In PAMC 22718, it is supposed that TonB is involved in chitin utilization pathway,[15] and carbohydrates including GlcNAc are transported by various TonB-dependent transport mechanisms. While the specific roles of TonB-dependent receptors are poorly understood, some bacterial membrane proteins have been known to have enzymatic functions.[16,17]

Conclusion

The Arctic psychrotolerant *P. issachenkonii* PAMC 22718 produced an exo-acting chitinase (*β-N*-acetylglucosaminidase), which was cold active even at 0–10°C. It hydrolyzed chitin oligosaccharides ($GlcNAc_2$–$GlcNAc_6$) into GlcNAc monomers, which are an expensive and valuable natural material used in a wide variety of applications such as medicine, food, and agriculture. However, it was unable to hydrolyze other polymeric chitins (swollen or colloidal chitin). When W-Chi22718 was mixed with the endochitinase W-Chi21702 from Antarctic bacterium *S. antarcticus*, this enzyme mixture could produce much more GlcNAc monomers from colloidal chitin than did W-Chi21702 alone.

N-Acetyl-D-glucosamine is produced at a low yield (below 65%) by acid hydrolysis of chitin extracted from crab and shrimp shells.[18] Currently, however, bioconversion using microbial enzymes is used as an alternative method for pure GlcNAc production, owing to its environmentally friendly process and high product purity. To solve the problems with low production yield and limited availability of enzymes, new strategies have been applied to the bioconversion process.[19] For example, crude chitinase (mixture of endochitinase and *β-N*-acetylhexosaminidase) from each of three soil fungal strains hydrolyzed colloidal chitin into GlcNAc (~80%) and a small amount of $GlcNAc_4$ (~20%) for 48-h-reaction time.[20] A crude enzyme mixture (five chitinases and one *β-N*-acetylglucosaminidase) derived from a marine bacterium *Aeromonas* sp. produced GlcNAc in 66–77% of yield from α-chitin after 10 d.[18] In our preparative practice, the mixture of two purified chitinases (exo-acting W-Chi22718 and endo-acting W-Chi21702) from two different marine bacteria was able to hydrolyze colloidal chitin to GlcNAc (~45%) and $GlcNAc_2$ (~45%) for 3-h-reaction time.

When screened or cultured on ZoBell medium containing each chitin polymer, PAMC 22718 could hydrolyze them, with producing clearing zone. This discrepancy between these data could be explained by a possibility that except the exo-acting W-Chi22718, PAMC 22718 possesses other chitinases which are able to hydrolyze chitin polymer. The multiplicity of chitinase genes (4–6) per chitinolytic species has been reported from several bacteria. It is assumed that the number of chitinase enzymes may be larger than that of chitinase genes because proteolytic processing of translated chitinases is common, thereby producing additional chitinases.[21] Now, we are attempting to try to find an endo-acting chitinase, if any, and produce the active recombinant protein of W-Chi22718 in *E. coli* or yeast cell. In future, the overproduction of the recombinant exo- and endo-acting chitinases will be a research subject for GlcNAc monomer production at a commercial scale.

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References

[1] Kurita, K. Controlled Functionalization of the Polysaccharide Chitin. *Prog. Polym. Sci.* **2001**, *26*, 1921–1971.
[2] Techkarnjanaruk, S.; Goodman, A.E. Multiple Genes Involved in Chitin Degradation from the Marine Bacterium *Pseudoalteromonas* sp. Strain S91. *Microbiology* **1999**, *145*, 925–934.
[3] Mavromatis, K.; Lorito, M.; Woo, S.L.; Bouriotis, V. Mode of Action and Antifungal Properties of Two Cold-Adapted Chitinases. *Extremophiles* **2003**, *7*, 385–390.
[4] Stefanidi, E.; Vorgias, C.E. Molecular Analysis of the Gene Encoding a New Chitinase from the Marine Psychrophilic Bacterium *Moritella Marina* and Biochemical Characterization of the Recombinant Enzyme. *Extremophiles* **2008**, *12*, 541–552.
[5] Bhattacharya, D.; Nagpure, A.; Gupta, R.K. Bacterial Chitinases: Properties and Potential. *Crit. Rev. Biotechnol.* **2007**, *27*, 21–28.
[6] Lan, X.; Zhang, X.; Kodaira, R.; Zhou, Z.; Shimosaka, M. Gene Cloning, Expression, and Characterization of a Second *β-N*-Acetylglucosaminidase from the Chitinolytic Bacterium *Aeromonas Hydrophila* Strain SUWA-9. *Biosci. Biotechnol. Biochem.* **2008**, *72*, 492–498.
[7] Bendt, A.; Hüller, H.; Kammel, U.; Helmke, E.; Schweder, T. Cloning, Expression, and Characterization of a Chitinase Gene from the Antarctic Psychrotolerant Bacterium *Vibrio* sp. Strain Fi:7. *Extremophiles* **2001**, *5*, 119–126.
[8] Fenice, M.; Selbmann, L.; Giambattista, R.D.; Federici, F. Chitinolytic Activity at Low Temperature of an Antarctic Strain (A3) of *Verticillium Lecanii*. *Res. Microbiol.* **1998**, *149*, 289–300.
[9] Park, H.J.; Kim, D.; Kim, I.H.; Lee, C.E.; Kim, I.C.; Kim, J.Y.; Kim, S. J.; Lee, H.K.; Yim, J.H. Characteristics of Cold-Adaptive Endochitinase from Antarctic Bacterium *Sanguibacter Antarcticus* KOPRI 21702. *Enzyme Microb. Technol.* **2009**, *45*, 391–396.
[10] Ivanova, E.P.; Sawabe, T.; Alexeeva, Y.V.; Lysenko, A.M.; Gorshkova, N.M.; Hayashi, K.; Zukova, N.V.; Christen, R.; Mikhailov, V.V. *Pseudoalteromonas Issachenkonii* sp. nov., a Bacterium that Degrades the Thallus of Brown Alga *Fucus Evanescens*. *Int. J. Syst. Evol. Microbiol.* **2002**, *52*, 229–232.

[11] Orikoshi, H.; Baba, N.; Nakayama, S.; Kashu, H.; Miyamoto, K.; Yasuda, M.; Inamori, Y.; Tsujibo, H. Molecular Analysis of the Gene Encoding a Novel Cold-Adapted Chitinase (ChiB) from a Marine Bacterium, *Alteromonas* sp. Strain O-7. *J. Bacteriol.* **2003**, *185*, 1153–1160.
[12] Park, H.J.; Shin, S.C.; Kim, D. Draft Genome Sequence of Arctic Marine Bacterium *Pseudoalteromonas Issachenkonii* PAMC 22718. *J. Bacteriol.* **2012**, *194*, 4140.
[13] Eisenbeis, S.; Lohmiller, S.; Valdebenito, M.; Leicht, S.; Braun, V. NagA-Dependent Uptake of *N*-Acetyl-Glucosamine and *N*-Acetyl-Chitin Oligosaccharides Across the Outer Membrane of *Caulobacter Crescentus*. *J. Bacteriol.* **2008**, *190*, 5230–5238.
[14] Chimento, D.P.; Kadner, R.J.; Wiener, M.C. The *Escherichia Coli* Outer Membrane Cobalamin Transporter BtuB: Structural Analysis of Calcium and Substrate Binding, and Identification of Orthologous Transporters by Sequence/Structure Conservation. *J. Mol. Biol.* **2003**, *332*, 999–1014.
[15] Yang, C.; Rodionov, D.A.; Li, X.; Laikova, O.N.; Gelfand, M.S.; Zagnitko, O.P.; Romine, M.F.; Obraztsova, A.Y.; Nealson, K.H.; Osterman, A.L. Comparative Genomics and Experimental Characterization of *N*-Acetylglucosamine Utilization Pathway of *Shewanella Oneidensis*. *J. Biol. Chem.* **2006**, *281*, 29872–29885.
[16] Vandeputte-Rutten, L.; Kramer, R.A.; Kroon, J.; Dekker, N.; Egmond, M.R.; Gros, P. Crystal Structure of the Outer Membrane Protease OmpT from *Escherichia Coli* Suggests a Novel Catalytic Site. *EMBO J.* **2001**, *20*, 5033–5039.
[17] Baaden, M.; Meier, C.; Sansom, M.S. A Molecular Dynamics Investigation of Mono and Dimeric States of the Outer Membrane Enzyme OMPLA. *J. Mol. Biol.* **2003**, *331*, 177–189.
[18] Sashiwa, H.; Fujishima, S.; Yamano, N.; Kawasaki, N.; Nakayama, A.; Muraki, E.; Hiraga, K.; Oda, K.; Aiba, S. Production of *N*-Acetyl-D-Glucosamine from α-Chitin by Crude Enzymes from *Aeromonas Hydrophila* H-2330. *Carbohydr. Res.* **2002**, *337*, 761–763.
[19] Liu, L.; Liu, Y.; Shin, H.D.; Chen, R.; Li, J.; Du, G.; Chen, J. Microbial Production of Glucosamine and *N*-Acetylglucosamine: Advances and Perspectives. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 6149–6158.
[20] Suresh, P.V.; Anil Kumar, P.K. Enhanced Degradation of α-Chitin Materials Prepared from Shrimp Processing Byproduct and Production of *N*-Acetyl-D-Glucosamine by Thermoactive Chitinases from Soil Mesophilic Fungi. *Biodegradation* **2012**, *23*, 597–607.
[21] Cottrell, M.T.; Moore, J.A.; Kirchman, D.L. Chitinases from Uncultured Marine Microorganisms. *Appl. Environ. Microbiol.* **1999**, *65*, 2553–2557.