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Catalytic function of a newly purified exo-β-D-glucosaminidase from the entomopathogenic fungus *Paecilomyces lilacinus*

Cheng-Fu Chao [a], Yi-Yun Chen [a], Chih-Yu Cheng [b,**], Yaw-Kuen Li [a,*]

[a] Department of Applied Chemistry, National Chiao Tung University, 1001 Ta-Hseh Road, HsinChu, Taiwan
[b] Department of Marine Biotechnology, National Kaohsiung Marine University, 142 Hai-Chuan Road, Kaohsiung, Taiwan

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ABSTRACT

An entomopathogenic fungus, *Paecilomyces lilacinus*, was found to grow on chitosanase-detecting plates. Besides an endo-chitosanase, an exo-β-D-glucosaminidase was purified by cation-exchange chromatography from this microorganism cultivated in M9 minimal media containing 0.5% chitosan as the sole carbon source. The molecular weight of the enzyme is 95 kDa; the optimum pH and temperature for activity are 6.0 and 45 °C, respectively. The purified exo-β-D-GlcNase promotes the hydrolysis of 95% deacetylated chitosan from its non-reducing end and liberates 2-amino-2-deoxy-D-glucopyranose (GlcN) as the sole product; however, 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) was not detected when chitin was used as the substrate. The cleavage pattern confirmed using real-time mass spectrometry shows that exo-β-D-glucosaminidase cleaves the glycosidic bonds between GlcN–GlcN and GlcN–GlcNAc but not between GlcNAc–GlcN or GlcNAc–GlcNAc. In the presence of a 10% solution of various alcohols, many alkyl-β-D-glucosaminides were obtained, indicating that exo-β-D-glucosaminidase is a retaining enzyme.

1. Introduction

Chitosan, the linear and partly acetylated (1–4)-2-amino-2-deoxy-β-D-glucan, is obtained from marine chitin, the second most abundant carbohydrate polymer after cellulose (Muzzarelli et al., 2012). In general, the biological degradation of these biopolymers consists of 2 processes (Nanjo, Katsumi, & Sakai, 1990; Nogawa et al., 1998). First, polymers are briefly digested into oligomers by an endo-type enzyme such as chitosanase, chitinase, or glucanase. Second, the resulting oligomers are then digested completely into their monomers by an exo-type enzyme such as β-D-glucosaminidase (β-D-GlcNase), β-*N*-acetylglucosaminidase, or glucosidase. The enzymatic degradation of cellulose and chitin has been extensively characterized, whereas the mechanism of chitosan cleavage is less well known because there is considerably less information available on its exo-type enzyme, namely exo-β-D-GlcNase.

Chitinous material is not only the second most abundant biomass but also the major source of amino sugars in nature. Hence, the biological degradation of chitosan is important for recycling nutrients in the environment. Therefore, by extension, exo-β-D-GlcNase plays an important role in the recycling of amino sugars, particularly with respect to the uptake of glucosamine.

Within the last few decades, endo-type chitosanase (EC 3.2.1.132) has been found in various organisms and has been classified into 8 glycosyl hydrolase families (GH-3, 5, 7, 8, 18, 46, 75, and 80 (http://afmb.cnrs-mrs.fr/CAZY/)). In contrast, the exo-type chitosanase exo-β-D-GlcNase (EC 3.2.1.165) is not as well characterized but is known to hydrolyze β-D-glucosaminide groups from the non-reducing end of chitosan. Exo-β-D-GlcNase was first characterized by Nanjo et al. (1990) but very few studies since then have been dedicated to this enzyme (Matsumura, Yao, & Toshima, 1999; Nogawa et al., 1998; Zhang et al., 2000). In 2003, related glycosyl hydrolase families, primary sequence information, and structural data of exo-β-D-GlcNases were presented (Ji, Yang, & Hur, 2003; Tanaka, Fukui, Atomi, & Imanaka, 2003; Tanaka, Fukui, Fujiwara, Atomi, & Imanaka, 2004). To date, only approximately 8 sequences of exo-β-D-GlcNase have been submitted (Fukamizo, Fleury, Cote, Mitsutomi, & Brzezinski, 2006; Ike et al., 2006; Liu et al., 2006). Based on primary sequence information, exo-β-D-GlcNases can be classified into 3 groups: 5 are classified into GH-2 (Cote et al., 2006; Fukamizo et al., 2006; Ike et al., 2006; Li, Wang, & Xia, 2009), 1 is classified into GH-9 (Honda, Shimaya, Ishisaki,

Abbreviations: GlcN, 2-amino-2-deoxy-D-glucopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; $(GlcN)_n$, ββ-1,4-linked oligomer of GlcN with a polymerization degree of *n*; exo-β-D-GlcNase, exo-β-D-glucosaminidase.

* Corresponding author. Tel.: +886 3 5731985; fax: +886 3 5723764.
** Corresponding author. Tel.: +886 7 3617141x3817; fax: +886 7 3011171.
E-mail addresses: cycheng@mail.nkmu.edu.tw, cycheng@webmail.nkmu.edu.tw (C.-Y. Cheng), ykl@cc.nctu.edu.tw (Y.-K. Li).

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Ebihara, & Taniguchi, 2011), and 2 are classified into GH-35 (Liu et al., 2006). The most well studied family is GH-2; crystal structures of enzymes in this family have been solved (Sakamoto et al., 2010) and the essential amino acid residues (Asp^{469} and Glu^{541}) have been identified (Fukamizo et al., 2006).

Paecilomyces lilacinus is a fungus found in soils and vegetation; this fungus rarely causes human infection. As an entomopathogenic fungus necessary for the biological control of root-knot disease (Siddiqui, Qureshi, Sultana, Ehteshamul-Haque, & Ghaffar, 2000), it is the most widely tested fungus for agricultural use with food and non-food crops under field conditions (Wei et al., 2009). Because chitin is the major structural component in insects, it is thought that the entomopathogenic property of *P. lilacinus* is related to its high chitinolytic capacity (Davila, Acosta, Betancourt, & Negron, 1999), which is attributed to the activity of endo-chitinase and *N*-acetylglucosaminidase that are both expressed in the fungus (Gupta, Leathers, & Wicklow, 1993). Two additional *Paecilomyces* chitosanolytic enzymes, endo-chitosanase (Chen, Cheng, Haung, & Li, 2005) and β-D-GlcNase, have been successively discovered by our group in the past few years. Because these are chitinolytic-related enzymes, they might also contribute to the entomopathogenic ability of this fungus. In this study, *P. lilacinus* was engineered to secrete higher levels exo-β-D-GlcNase, which was then purified and identified as a retaining glycohydrolase. The catalytic properties and splitting patterns of the exo-β-D-GlcNase-mediated catalytic reaction are also discussed herein.

2. Materials and methods

2.1. Chitosan and its derivatives

Chitooligomers (DP2-DP6) were purchased from Yaizu Suisankagaku Industry (Yaizu, Japan). Chitosan with various degrees of deacetylation (DDA) and chitin were obtained from a local supplier in Taiwan. The chitosan (MW 200–400 kDa) used for most of this study was 85% deacetylated, unless otherwise specified. *N*-carboxymethyl chitosan were prepared according to procedures described in the literature (Muzzarelli, 1988). Chitosan with different DDAs, *N*-carboxymethyl chitosan, chitin, and $(GlcN)_6$ were used to investigate substrate specificity.

2.2. Microorganism screening and identification

A microorganism strain was screened and isolated from soil by using chitosan as the sole carbon source. The screening process, as described previously (Cheng & Li, 2000), was performed on a chitosanase detection agar plate by visualizing a clear zone surrounding the microorganism colony. The isolated strain was further identified by Centraalbureau voor Schimmelcultures (CBS, the Netherlands).

2.3. Preparation of the chitosan solution and colloidal chitin

To prepare a 1% chitosan solution, 1 g of chitosan was dissolved in 90 mL of acetic acid solution (0.33%, *v*/*v*). The acidity of the solution was adjusted to pH 6.0 by slowly adding NaOH solution (1 M). Water was then added to the resultant solution to a final volume of 100 mL. Colloidal chitin (in 50 mM acetate buffer, pH 6.0) was prepared as described previously (Wen, Tseng, Cheng, & Li, 2002).

2.4. Domestication of P. lilacinus

The culture medium used for the enzyme induction was 0.5% chitosan at pH 6.0 in M9 medium containing Na_2HPO_4 (1.3 g/L), KH_2PO_4 (3.0 g/L), NaCl (0.5 g/L), NH_4Cl (1.0 g/L), Mg_2SO_4 (0.24 g/L), and $CaCl_2$ (0.01 g/L). One liter of the medium was inoculated with 10^9 spores, and the flask was incubated at 28 °C and 140 rpm on a rotary shaker for 4 days. After the 4-day incubation period, the mycelia were spun down and transferred to fresh chitosan-M9 medium for another incubation cycle of 4 days. During the domestication process, the amount of exo-β-D-GlcNase within the crude extract was determined by SDS-PAGE.

2.5. Enzyme purification

After removing the mycelia, the crude filtrate was concentrated (approximately 10-fold) using a vacuum-equipped rotary evaporator. The concentrated solution was then dialyzed using a 10 kDa cutoff membrane against 1 L of acetate buffer (20 mM NaOAc, pH 5.5) overnight at 4 °C. The resultant solution was applied to a cation exchange column (GE Healthcare, SP Sepharose Fast Flow) that had been equilibrated with acetate buffer (20 mM NaOAc, pH 5.5). Elution was performed using a linear NaCl gradient (0–500 mM) at a flow rate of 2 mL/min. Fractions were monitored at A_{280} nm, collected, and assayed for chitosanase activity. Active fractions were identified using SDS-PAGE and then pooled, concentrated, and stored for subsequent studies.

2.6. Determination of protein content

The protein content of the enzyme preparation was determined using the bicinchoninic acid method as described in the manufacturer's protocol (BCA-1, kit for protein determination, Sigma–Aldrich).

2.7. Enzyme activity assay

The activity of chitosanases, including exo-β-D-GlcNase and endo-chitosanase, were analyzed by estimating the reducing end of the sugars derived from the catalytic hydrolysis of chitosan. The assay was performed by mixing 0.3 mL of 1% chitosan at pH 6.0 and 0.3 mL of suitably diluted enzyme. After incubation for 3 h at 37 °C, the hydrolysis reaction was terminated and analyzed by adding 0.6 mL of a dinitrosalicylic acid reagent (Miller, 1959). The mixture was boiled for 10 min, chilled, and then centrifuged to remove insoluble chitosan. The resultant adducts of reducing sugars were measured spectrophotometrically at 540 nm. The extinction coefficient of the resultant adducts was determined to be 788 M^{-1} cm^{-1} at a 540 nm wavelength when D-glucosamine was used as the reducing sugar. One unit of chitosanase activity was defined as the amount of enzyme required to release 1 μmol of detectable reducing sugar at 37 °C in 1 min.

2.8. Hydrolysis products analysis

The reaction was performed using 2 μg/μL $(GlcN)_6$ and a suitably diluted enzyme (approximately 0.45 mU) at 37 °C. A 10 μL aliquot withdrawn from the reaction mixture at 15 min intervals for up to 6 h was treated with an equal volume of acetonitrile and centrifuged; the supernatant was then analyzed using a mass spectrometer (Q-TOF, Micromass) as described in Section 2.9.

2.9. Electrospray ionization mass spectrometry analysis

The mass spectra were recorded using a quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK). The quadrupole mass analyzer was scanned over a mass-to-charge ratio range of 100–2000 atomic mass units (*m*/*z*), with a scan step of 2 s and an interscan of 0.1 s/step. In the electrospray ionization mass spectrometry (ESI-MS) experiments, the quadrupole scan mode was under an electrospray voltage of 3 kV at the tip of a stainless steel capillary needle, with a source block temperature of 80 °C.

The elution conditions were 50% acetonitrile containing 0.1% formic acid at a rate of 2 μL/min.

2.10. Transglycosylation reaction and stereochemical study

The transglycosylation reaction was performed using a suitably diluted enzyme and 1% chitooligomers in 10% alcohol (methanol, ethanol, isopropanol, or isoamyl alcohol) for 6 h at 37 °C. The chitooligomers mixture (DP2-DP10) was obtained from the hydrolysates of chitosan using *Aspergillus* chitosanase (Cheng & Li, 2000). The reaction was terminated by adding an equal amount of acetonitrile and then analyzed using MS. Some transglycosyl products were lyophilized and exchanged twice against D_2O for further stereochemistry studies and then later identified using ^1H NMR performed at 25 °C in a Bruker Avance 500 spectrometer.

2.11. Preparation of GlcN–GlcNAc–butyl and partial acetylation of the chitooligomer mixture

Chitosan (1%) with 38% DDA was hydrolyzed using *Serratia marcescens* chitinase A for 24 h (Wu, Cheng, & Li, 2009) in 10% *n*-butanol for GlcN–GlcNAc–butyl preparation or in an aqueous solution for preparation of the partially acetylated chitooligomer mixture. The supernatant of the reaction mixture was lyophilized, extracted using 95% ethanol, lyophilized again, redissolved in water, and assayed using ESI-MS.

2.12. Real-time ESI-MS

The mass spectra were recorded using the method described earlier, except that the elution system comprised the enzymatic reaction itself (Dennhart, Fukamizo, Brzezinski, Lacombe-Harvey, & Letzel, 2008); that is, the enzymatic reaction mixture flowed directly and continuously into the mass spectrometer (MS). Partial acetylation of the chitooligomer mixture prepared earlier with additional commercial chitobiose $(GlcN)_2$ was digested using a suitable amount of enzyme in 10% ethanol. Changes in the components of the reaction mixture (100 μL) were assayed via a direct flow into the MS at a rate of 2 μL/min. The ion counts for GlcN–GlcN, GlcN–GlcNAc, GlcNAc–GlcNAc, GlcNAc–GlcN–GlcNAc, and GlcNAc–GlcN–GlcN–GlcNAc were set at $m/z = 341$, $m/z = 383$, $m/z = 447$, $m/z = 586$, and = m/z 747, respectively.

3. Results

3.1. Enzyme induction and purification

A chitosan-degrading fungus was isolated and identified as *P. lilacinus*. An effective method for inducing exo-β-D-GlcNase was to incubate this fungus in M9 medium containing 0.5% chitosan, pH 6.0, at 28 °C for 4 days. Under this cultivating condition, *P. lilacinus* produced endo-chitosanase (Chen et al., 2005) and exo-β-D-GlcNase. During domestication, the amount of exo-β-D-GlcNase in the crude extract was gradually increased, and it became the predominant protein (data not shown).

The purification of exo-β-D-GlcNase can be achieved via SP column chromatography. Before loading onto an SP column, the culture filtrate was concentrated using a rotary vacuum evaporator, centrifuged to remove any suspended particles, and further dialyzed to eliminate excess salts. The resulting crude extracellular protein is shown in Fig. 1. There were 2 chitosanase-active regions in the chromatogram from the SP column separation (data not shown). One fraction with chitosanase activity was eluted from 150 to 170 mM NaCl, and it has been identified as endo-chitosanase (Chen et al., 2005). We identified the second fraction with chitosanase activity that was eluted from 230 to 250 mM NaCl.

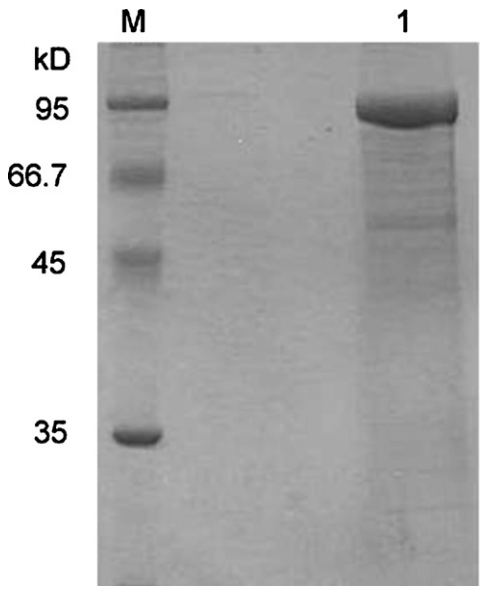


Fig. 1. SDS-PAGE analysis of the purified exo-β-D-GlcNase. The molecular weight of the purified β-D-GlcNase was estimated to be 95 kDa.

When the activity of the purified enzyme was assayed, we noted that that, even with a long reaction time, the viscosity of the chitosan solution (substrate) was not significantly decreased, while the sugar-reducing end was constantly increased. These data indicate that the enzyme was an exo-type chitosanase and was further identified as an exo-β-D-GlcNase based on our results (discussed below). By SDS-PAGE (Fig. 1), the enzyme was judged to be >80% homogeneous with a molecular weight of 95 kDa. The gel filtration analysis on a Sephadex G-200 column showed that the enzyme was eluted at a position corresponding to a molecular weight of approximately 96 kDa, indicating that the native enzyme is a monomeric protein. Periodate staining of the SDS-PAGE gel was negative for the presence of glycoproteins.

3.2. Characterization of the enzyme stability and catalytic function

When chitosan is used as the substrate, the solution acidity is a major factor for optimizing enzymatic function. The catalytic outcome influenced by pH conditions may be due to 2 possible effects: the solubility of chitosan and the intrinsic power of enzymatic function. The enzyme showed strong activity and good stability near pH 6 but displayed weak activity at other pH values (data not shown). Further thermostability studies showed that the purified enzyme was stable at temperatures lower than 55 °C for at least 90 min at pH 6.0.

3.3. Substrate specificity

Chitosan with different DDAs, *N*-carboxymethyl chitosan, chitin, and $(GlcN)_6$ were used to investigate substrate specificity. Results showed that the purified enzyme displayed high activity toward chitosan and $(GlcN)_6$ but no significant activity for *N*-carboxymethyl chitosan, or chitin. A time course study on $(GlcN)_6$ hydrolysis catalyzed by the purified enzyme was analyzed using mass spectrometry (Fig. 2). The results show that $(GlcN)_6$ ($m/z = 985$) was sequentially cleaved into $GlcN_5$ ($m/z = 824$), $GlcN_4$ ($m/z = 663$), $GlcN_3$ ($m/z = 502$), $GlcN_2$ ($m/z = 341$), and GlcN ($m/z = 162$; $M-H_2O+H^+$) ($m/z = 180$; $M+H^+$). After 6 h, GlcN was the only product. The liberation of the reducing sugar from chitosan with different DDA values was examined. Chitosan with higher

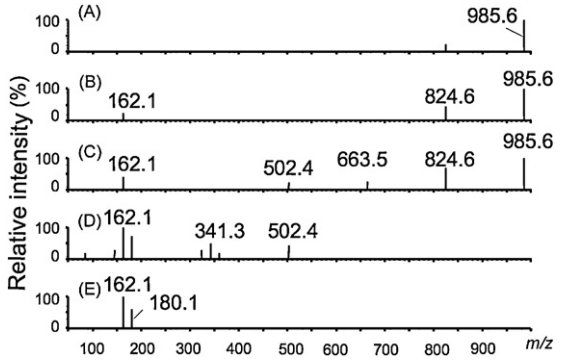


Fig. 2. Monitoring the enzymatic hydrolysis of $(GlcN)_6$ by ESI-MS. An aliquot was removed for analysis after incubation with exo-β-D-GlcNase at (A) 0 min, (B) 15 min, (C) 30 min, (D) 2 h, and (E) 6 h. $(GlcN)_6$ (m/z = 985) was sequentially cleaved into $GlcN_5$ (m/z = 824), $GlcN_4$ (m/z = 663), $GlcN_3$ (m/z = 502), $GlcN_2$ (m/z = 341), and GlcN (m/z = 162; M−H_2O+H^+, m/z = 180; M+H^+, m/z = 202; M+Na^+).

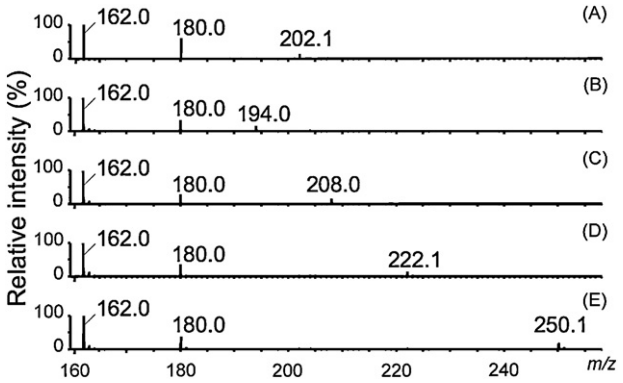


Fig. 4. ESI-MS spectra of the products derived from transglycosylation. The m/z = 180, 194, 208, 222, and 250 corresponded to GlcN and methyl-, ethyl-, isopropyl-, and isoamyl-β-D-glucosaminide, respectively. These reactions were separately performed in (A) water, (B) methanol, (C) ethanol, (D) isopropanol, and (E) isoamyl alcohol by using chitooligosaccharides as the substrate.

DDA values was hydrolyzed more efficiently than chitosan with lower DDA values. Fig. 3 shows the time course reaction of chitosan with 95% and 38% DDA. When 95% DDA chitosan was used as the substrate, the initial rate of the reaction was approximately 1.6-fold faster than that of 38% DDA chitosan. The 38% DDA chitosan became indigestible after 130 min of incubation with the enzyme, while the catalysis continued with 95% DDA chitosan as the substrate. A similar reaction profile was observed with exo-β-D-GlcNase from different microorganisms (Nanjo et al., 1990; Nogawa et al., 1998). In summary, these results indicate that the purified enzyme is an exo-β-D-GlcNase with a catalytic preference for cleavage of GlcN–GlcN linkages. Since chitin is not a substrate for this enzyme, GlcNAc–GlcNAc could not be hydrolyzed. However, catalysis of GlcN–GlcNAc and GlcNAc–GlcN linkages by this enzyme remains unknown.

3.4. Transglycosylation of exo-β-D-GlcNase and its anomeric configuration

Glycohydrolases are classified as retaining or inverting enzymes on the basis of the stereochemistry of the catalytic mechanism. Time-dependent ^{1}H NMR spectroscopy is commonly employed to investigate the anomeric configuration of the C-1H bond of the released sugar. Since the α/β ratio of the reducing sugar tends to approach the equilibrium state quickly via mutarotation, our preliminary study on time-dependent ^{1}H NMR investigation did not provide a clear conclusion. As an alternative technique, after the reaction has occurred, analyzing the anomeric configuration of the transglycosylation product can provide useful stereochemical information. Scheme 1 depicts the catalytic function and possible transglycosylation of a retaining-type glycoside hydrolase. In general, retaining-type enzymes carry out the catalysis with a two-step, double-displacement mechanism that typically involves formation of a glycosyl-enzyme intermediate upon glycosylation followed by deglycosylation with decomposition of the intermediate. Transglycosylation may occur if alcohols are present as the glycosyl acceptor at the decomposition step of the glycosyl-enzyme intermediate. The anomeric configuration of the product should be retained. In this study, the transglycosylation reaction catalyzed by the purified enzyme was confirmed using various alcohols, including methanol, ethanol, isopropanol, and isoamyl alcohol as the glycosyl acceptor. The reaction products were analyzed by positive-mode ESI/Mass (Fig. 4). The common product with m/z = 180 was determined to be GlcN, and the other products with m/z = 194, 208, 222, and 250 were methyl-, ethyl-, isopropyl-, and isoamyl glucosaminide, respectively. Fig. 5 was further employed to investigate the stereochemistry of this enzyme by analyzing the anomeric configuration of the transglycosylation product by ^{1}H NMR. Fig. 5 shows a partial ^{1}H NMR spectrum (4.7–5.7 ppm) of the products

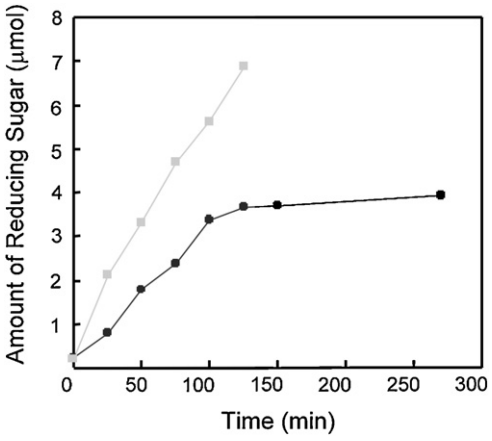


Fig. 3. Time course of the enzymatic liberation of the reducing sugars from chitosan with a DDA of 95% (■) and 38% (♦).

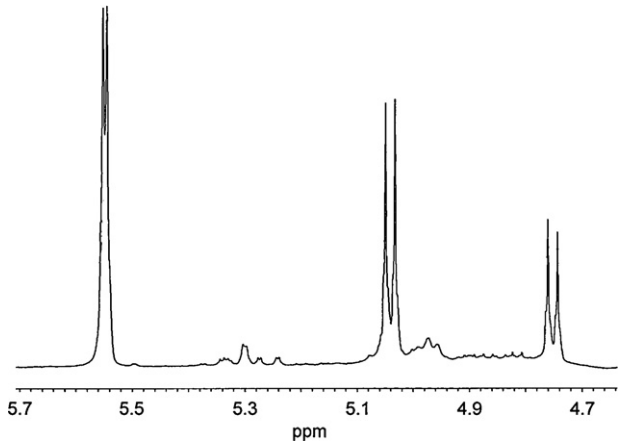
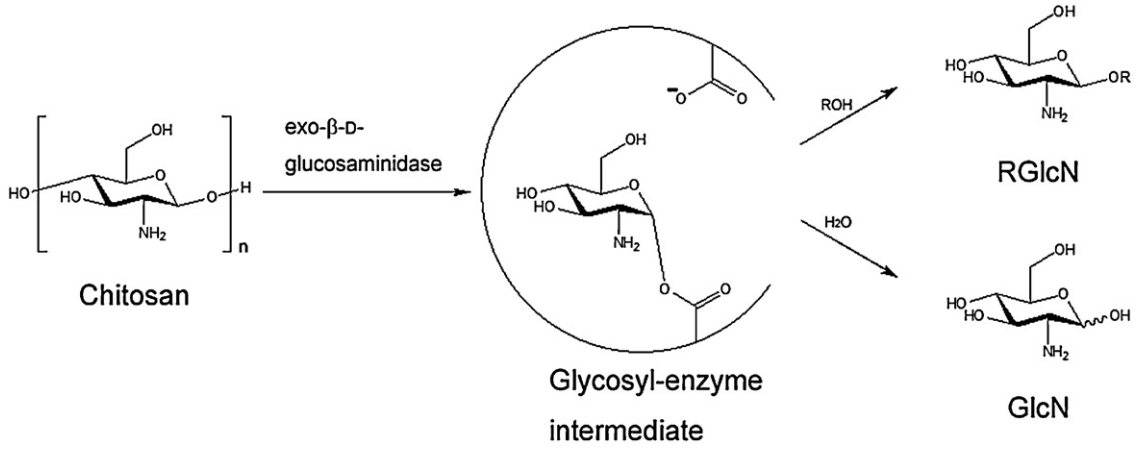


Fig. 5. ^{1}H NMR analysis of the products derived from exo-β-D-GlcNase catalysis. The partial spectrum shows the region of C-1 protons of methyl-β-D-glucosaminide and GlcN with α- and β- configurations. Doublets centered at δ = 5.55 ppm (J = 3.5 Hz), δ = 5.04 ppm (J = 8.5 Hz), and δ = 4.75 ppm (J = 8.5 Hz) were assigned to be the C-1 proton of α-GlcN, β-GlcN, and methyl-β-D-glucosaminide, respectively.



Scheme 1. The two-step mechanism and transglycosylation of a retaining glycoside hydrolase.

obtained from the enzymatic catalysis of chitooligomer containing 10% methanol. Three doublets were found to be centered at $\delta = 5.55$ ppm ($J = 3.5$ Hz), $\delta = 5.04$ ppm ($J = 8.5$ Hz), and $\delta = 4.75$ ppm ($J = 8.5$ Hz). The first 2 downfield signals were assigned to be the α and β anomeric C-1 protons of GlcN. The third signal ($\delta = 4.75$ ppm) corresponded to the C1 proton of methyl-D-glucosaminide. The coupling constant ($J = 8.5$ Hz) further suggested it to be a β-form glycoside. Taken together, these data confirm that the purified exo-β-D-GlcNase is a retaining-type enzyme. With the present reaction conditions, the production of methyl-β-D-glucosaminide was estimated to be 20%.

3.5. Identification of the cleavage of GlcN–GlcNAc–butyl

To further identify the cleavage of GlcN–GlcNAc, a partially acetylated chitooligomer mixture was prepared as described in Section 2. When chitosan with 38% DDA was hydrolyzed using chitinase A, the major product was GlcN–GlcNAc, with minor components of GlcNAc–GlcNAc, GlcN–GlcN–GlcNAc, GlcNAc–GlcN–GlcNAc, and GlcNAc–GlcN–GlcN–GlcNAc (Aiba, 1994). When this reaction was carried out in 10% *n*-butanol, it generated GlcN–GlcNAc ($m/z = 405.2$; $M + Na^+$), GlcN–GlcNAc–butyl ($m/z = 461.3$; $M + Na^+$), and GlcNAc–GlcNAc–butyl ($m/z = 503.3$; $M + Na^+$) as shown in Fig. 6A. The major product GlcN–GlcNAc–butyl was further analyzed by ESI-MSMS as shown in Fig. 6B. When using this hydrolysate as the substrate, both GlcN–GlcNAc and GlcN–GlcNAc–butyl were hydrolyzed efficiently by exo-β-D-GlcNase. GlcN ($m/z = 162.1$; $M-H_2O+H^+$) ($m/z = 202.0$; $M+Na^+$), GlcNAc–butyl ($m/z = 300.1$; $M+Na^+$), and GlcNAc ($m/z = 244.1$; $M+Na^+$) were found after the enzymatic treatment (Fig. 6C). These results strongly suggest that exo-β-D-GlcNase can promote the cleavage of the glycosidic bond of GlcN–GlcNAc.

3.6. Identification of cleavage specificity using real-time ESI-MS

To obtain specific insight into the cleavage specificity, chitobiose $(GlcN)_2$ mixed with the chitooligomers (as described in Section 2) was used as the substrate for the exo-β-D-GlcNase assay. Changes in ion counts of various components in the substrate were continuously monitored by ESI-MS after the addition of enzyme. The results were plotted as shown in Fig. 7. GlcN–GlcNAc was slowly hydrolyzed by exo-β-D-GlcNase catalysis, whereas the enzymatic hydrolysis of GlcN–GlcN was much more efficient. No significant enzymatic catalysis or transglycosylation was observed with GlcNAc–GlcNAc, GlcNAc–GlcN–GlcNAc, or GlcNAc–GlcN–GlcN–GlcNAc substrates. These results confirmed that the enzyme preference on the (−1) site was specific for GlcN, and the (+1) site was concessionary for GlcN and GlcNAc. Although exo-β-D-GlcNase enhances the hydrolysis of GlcN–GlcN and GlcN–GlcNAc, the synthetic *p*-nitrophenyl-β-D-glucosaminide was not hydrolyzed. Similar results were observed for the exo-β-D-GlcNase from *Nocardia* (Nanjo et al., 1990), *Penicillium* (Matsumura et al., 1999), *Actinomycetes* (Cote et al., 2006), and *Photobacterium* (Honda et al., 2011). However, we found that *Paecilomyces* exo-β-D-GlcNase could accelerate the hydrolysis of *p*-nitrophenyl-β-D-*N*-acetylglucosaminide, *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobioside, and *p*-nitrophenyl-β-D-*N*-acetylgalactosaminide to release the corresponding sugar (data not shown). To the best of our knowledge, this feature of diverse substrate specificity has not yet been reported for exo-β-D-GlcNases.

4. Discussion

The entomopathogenic fungus *P. lilacinus* is commonly used as a biological control in the field. Its entomopathogenic ability originates not only from its high chitinolytic capacity (Davila et al.,

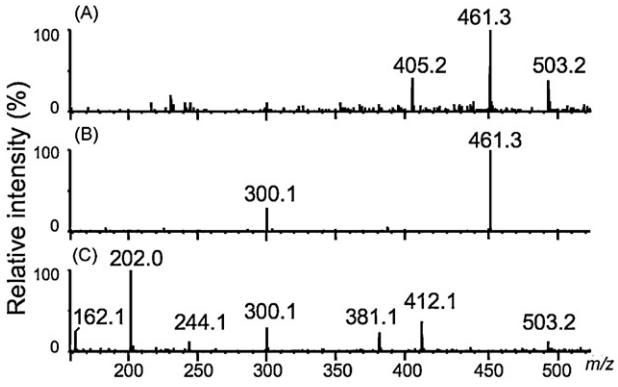


Fig. 6. Positive-mode ESI-MS analysis of GlcN–GlcNAc hydrolysis catalyzed by exo-β-D-GlcNase. (A) ESI-MS spectra of 38% DDA chitosan hydrolyzed by chitinase A in the solution containing 10% *n*-butanol, (B) ESI/MSMS analysis of GlcN–GlcNAc–butyl ($m/z = 405.2$; $M + Na^+$). GlcNAc–butyl ($M + Na^+$) was calculated to be $m/z = 300.1$, and (C) The ESI-MS spectra of the products derived from exo-β-D-GlcNase catalysis. Note that both $m/z = 405.2$ (GlcN–GlcNAc + Na^+) and $m/z = 461.3$ (GlcN–GlcNAc–butyl + Na^+) disappeared after enzymatic treatment. New peaks corresponding to GlcN ($m/z = 162.1$; $M-H_2O+H^+$) ($m/z = 202.0$; $M+Na^+$), GlcNAc–butyl ($m/z = 300.1$; $M+Na^+$), and GlcNAc ($m/z = 244.1$; $M+Na^+$) were found.

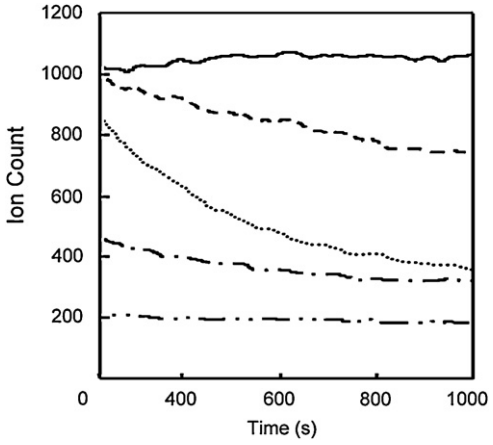


Fig. 7. Time-course MS analysis to monitor substrate degradation. The relative rate of substrate decomposition catalyzed by β-D-GlcNase was continuously monitored via the change of ion counts: GlcNAc–GlcNAc (–), GlcN–GlcNAc (—), GlcN–GlcN (. . .), GlcNAc–GlcN–GlcNAc (-.-.), and GlcNAc–GlcN–GlcN–GlcNAc (-.-.).

1999), but also from its chitosanolytic capacity. The possession of chitinolytic and chitosanolytic capacities has been observed in various microorganisms. Several of them are native biocontrol agents (Najar, Anwar, Masoodi, & Khar, and, 2011). However, it is rare to find a microorganism such as *P. lilacinus* that possesses all 4 of these enzymes: chitinase, β-*N*-acetylglucosaminidase, chitosanase, and exo-β-D-GlcNase, which could make *P. lilacinus* a popular entomopathogenic fungus for testing under field conditions.

The diversity of substrate specificity, which includes natural and artificial substrates, is noticeable with regard to *Paecilomyces* exo-β-D-GlcNase. Similar to other β-D-GlcNases (Matsumura et al., 1999; Nanjo et al., 1990), *Paecilomyces* exo-β-D-GlcNase liberates GlcN from chitosan, but not from *p*-nitrophenyl-β-D-glucosaminides. Interestingly, nearly all published β-D-GlcNases cannot release GlcNAc from either chitin or *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (Nanjo et al., 1990; Nogawa et al., 1998). Nevertheless, *Paecilomyces* β-D-GlcNase, which cannot liberate GlcNAc from chitin, is surprisingly capable of decomposing *p*-nitrophenyl-β-D-*N*-acetylglucosaminide and *p*-nitrophenyl-β-D-*N,N′*-diacetylchitobioside. The diversity of specificity between natural and artificial substrates has also been identified for β-glucosidase (Czjzek et al., 2001) and hexosaminidase (Pennybacker et al., 1996). It is believed that the mechanism regarding the enzyme's substrate specificity is likely attributed to its affinity and specificity for particular substrate-binding motifs. Indeed, *Paecilomyces* exo-β-D-GlcNase may possess motifs different from other β-D-GlcNases and can provide a good reference for further studies of substrate-binding domains.

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