Chapter

Effect of LPMO on the Hydrolysis of Crystalline Chitin by Chitinase A and β-*N*-Acetylglucosaminidase from *Paenibacillus* sp.

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Abstract

We performed cloning and expression of chitinase A (Pb-ChiA), β-GlcNAcase (Pb-GlcNAcase), and lytic polysaccharide monooxygenase (Pb-LPMO) genes from *Paenibacillus* sp. The analysis of the hydrolysis products indicated Pb-ChiA to be an exo-type chitinase with 10-fold activity toward β-chitin as compared with α-chitin. The sequence of Pb-GlcNAcase was found to be similar to that of β-*N*-acetylhexosaminidase from *P. barengoltzii* (99%, WP_016313754.1). Pb-LPMO was expressed in the *Brevibacillus* expression system. Pb-ChiA was found to have affinity toward crystalline chitin higher than that of Pb-LPMO. Pb-LPMO boosted the activity of Pb-ChiA toward crystalline α-chitin but not toward crystalline β-chitin. When Pb-LPMO (3 μM) was added to the reaction mixture during the hydrolysis of crystalline α-chitin by Pb-ChiA, hydrolysis products at two-fold concentration were obtained. However, the hydrolysis products decreased upon addition of more than 3 μM Pb-LPMO to the reaction mixture.

Keywords: chitin, chitinase, lytic polysaccharide monooxygenase, β-D-*N*-acetylhexosaminidase, *Paenibacillus* sp.

1. Introduction

Chitin, which can be easily isolated from crab and shrimp shell waste, is the second most abundant biopolymer on earth. At least 10 gigatons (1×10^{13} kg) of chitin are synthesized and degraded each year in the biosphere. *N*-Acetylchitooligosaccharides (GlcNAc) and chitooligosaccharides (GlcN), which are hydrolyzed products of chitin and chitosan, have various biological roles and many possible future applications [1–3]. GlcNAc and glucosamine are ordinarily utilized for symptom relief in osteoarthritis patients [4]. Hyaluronic acid as heterogeneous polysaccharides contained GlcNAc, and addition of GlcNAc to cultured keratinocytes facilitates the production of hyaluronic acid in a dose-dependent manner [5].

Biotechnological tools for generating enzymes are required for environmentally sustainable production of chitin oligosaccharide, GlcNAc, or both. Multiple chitinases have been characterized and are categorized into four enzyme families, namely,

families 18, 19, 23, and 48 [6, 7]. The glycoside hydrolase (GH) families 18 and 19 chitinases have almost no amino acid sequence similarities and have entirely different three-dimensional structures. Ra-ChiC from *Ralstonia* sp. A-471 was the first chitinase of the GH family 23 [7] and has been categorized to goose-type lysozyme. However, although Ra-ChiC shows chitinase activity, no activity toward peptidoglycan has been described [7]. β-GlcNAcase (exo-type enzyme) belongs to GH family 20. Chitin oligosaccharides such as *N*-acetylchitobiose and *N*-acetylchitotriose are good substrates for β-GlcNAcase [8]. Lytic polysaccharide monooxygenases (LPMOs, auxiliary activity [AA] family 10) are copper-dependent and cleave polysaccharide chains embedded in the crystalline regions of the substrate that are generally inaccessible to glycoside hydrolases [9]. Hemsworth et al. reported that fungi in particular makes considerable use of LPMOs in biomass degradation, producing more LPMO enzymes than cellulases [10]. Enzyme cocktails contained cellulases and LPMO enzymes can be utilized in the bio-refinery for lignocellulose deconstruction [10].

To understand the synergic activity of LPMO during the hydrolysis of crystalline chitin by chitinase A (GH family 18) and β-GlcNAcase from *Paenibacillus* sp., cloning and expression of chitinase A (Pb-ChiA), β-GlcNAcase (Pb-GlcNAcase), and LPMO (Pb-LPMO) genes from *Paenibacillus* sp. were carried out in this study.

2. Materials and methods

2.1 Enzyme and protein measurements

The chitinase activity was assayed by determining the reducing sugars released from soluble chitin. The activity was assayed according to a previously paper [11].

The β-GlcNAcase activity was measured by determining the release of *p*-nitrophenol from *p*NP-GlcNAc. The enzyme activity required to form 1 μmol *p*-nitrophenol was regarded as one unit. The enzyme activity was measured according to a standard assay method. The reaction mixtures comprised 40 μL of 1 mM *p*NP-GlcNAc solution in 0.1 M acetate buffer (pH 6.0) and 20 μL of enzyme solution. After the reaction mixtures were incubated for 15 min at 37°C, the enzyme reaction was stopped by adding 200 μL of 0.5 M Na_2CO_3 solution.

The LPMO activity was measured by detecting the oxidized products released from chitin. Solutions of α or β-chitin (5 mg/mL), Pb-LPMO (1.0 mM), ascorbic acid (1.0 mM), and 20 mM acetate buffer (pH 5.0) were mixed to 1.0 mL volume. The reaction mixture was incubated at 37°C for 24 h. The oxidized products were then applied onto a TSK-gel Amide-80 column (4.6 × 250 mm, Tosoh Co., Tokyo, Japan) and eluted with 70% acetonitrile at a flow rate of 0.7 mL/min; the products were monitored by absorbance measurement at 210 nm.

Protein contents were assayed using Micro BCA protein assay kits (ThermoFisher Scientific) with bovine serum albumin as the standard. Protein concentrations of Pb-LPMO, Pb-ChiA, and Pb-GlcNAcase were calculated from absorbance measurement at 280 nm and the protein extinction coefficient according to the method of Gill and von Hippel [12].

2.2 Cloning and expression of chitinase A (Pb-ChiA) gene from *Paenibacillus* sp.

Paenibacillus sp. A-471 was used as a DNA donor. *Paenibacillus* sp. A-471 is closely related to *Paenibacillus barengoltzii* G22 strain. The genome sequence of *P. barengoltzii* G22 strain has been identified [13]. To clone the chitinase, β-*N*-acetylglucosaminidase, and LPMO genes from *Paenibacillus* sp. A-471, we used

the nucleotide sequences of those genes from *P. barengoltzii* G22 (EOS56884.1) as templates. (The sequence of chitinase gene from *P. barengoltzii* G22 was similar to that of chitinase A from *Bacillus circulanse* WL-12 [AAA81528.1]).

First, forward (5′-ATGCATTCGAAGAGAACCCATAGCTTCAC-3′) and reverse (5′-TTATAGCGCCTGAAATAATGCGGGCAC-3′) primers for PCR were synthesized on the regions corresponding to amino acid residues 1–10 and 689–696 of Pb-ChiA, respectively. Second, to insert the nucleotide sequence corresponding to the mature Pb-ChiA in the pColdI expression vector (Takara Bio, Kyoto, Japan), forward (5′-TATACATATGCAACCGAAAGCCGCTGAGGC; the *Nde*I site is underlined) and reverse (5′-TATATCTAGATTATAGCGCCTGAAATAATGCGGG-3′; the *Xba*I site is underlined) primers for PCR were synthesized on regions corresponding to amino acid residues 33–39 and 690–696 of Pb-ChiA, respectively. PCR was carried out in a reaction mixture (20 μL) containing the *Paenibacillus* sp. A-471 genome DNA, 0.5 μM of each primer, and 10 μL of Takara PrimeSTAR Max premix (Takara Bio), according to the protocol of Takara Bio. A DNA fragment of 2.0 kb got by PCR was cloned into the *Nde*I and *Xba*I sites of the pCold I expression vector. The nucleotides of the amplified fragment were made more firm by sequencing after ligation. This expression plasmid coded mature Pb-ChiA was named pCold-Pb-ChiA.

pCold-Pb-ChiA was transformed into *Escherichia coli* DH5α, and the transformant was cultured in an LB medium (500 mL) containing ampicillin at 37°C to an OD_{600} value of 0.4 and established by induction with IPTG (0.1 mM) at 15°C overnight. The cultured *E. coli* cells were collected by centrifugation (9000 × *g*, 20 min, 4°C) and were resuspended in 20 mM Tris–HCl buffer (pH 7.5), sonicated, and centrifuged (9000 × *g*, 20 min, 4°C) again. The supernatant was put onto a HisTrap FF column (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, England) equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 20 mM imidazol and 300 mM NaCl. The enzyme was eluted with a gradient of 20–300 mM imidazol in the buffer. Active fractions were collected and utilized as the purified enzyme solution.

The enzyme activity was determined using soluble chitin (40% deacetylated chitin) as a substrate under various conditions of pH and temperature. The buffer systems were as follows: pH 2.0–4.0 0.2 M glycine-HCl, pH 4.0–6.0 0.2 M acetate buffer, pH 6.0–8.0 0.2 M KH_2PO_4-K_2HPO_4, pH 8.0–9.0 0.2 M Tris–HCl, and pH 9.0–11.0 0.2 M glycine-NaOH. The effect of temperature on the enzyme activity was examined at 10–80°C.

To recognize splitting patterns from hydrolysis products of the purified recombinant enzyme, 5.0 mM *N*-acetylglucosamine oligosaccharide substrates $(GlcNAc)_{2–6}$ were dissolved in 50 mM sodium acetate buffer (pH 6.0), and aliquots of the enzyme solution were added to 400 μL of each substrate solution. Enzyme reactions were carried out at 37°C for several times, and parts of the reaction mixture were then picked up and mixed with the same volume of chilled acetonitrile (−20°C) to stop the reaction.

Analysis of hydrolysis product using HPLC carried out according to a previously paper [13].

2.3 Cloning and expression of β-GlcNAcase (Pb-β-GlcNAcase) gene from *Paenibacillus* sp.

First, forward (5′-GAGGAGGAGGTTCACGGGAGGAGGAGGTTCACGG-3′) and reverse (5′-GCGAGCTGTTGGAGAAGTACTCGTA-3′) primers for PCR were respectively synthesized on the 5′-upstream and 3′-downstream regions of Pb-β-GlcNAcase. Second, to insert the nucleotide sequence of Pb-LPMO in the pCold I expression vector (Takara Bio), forward (5′-GAGCTCGGTACCCTCATGAAGCTTTTTTTT; the *Nde*I

site is underlined) and reverse (5′-GATTACCTATCTAGATTACATCGACAGCGA-3′; the *Xba*I site is underlined) primers for PCR were synthesized on the region corresponding to amino acid residues 1–5 and 638–641 of Pb-β-GlcNAcase, respectively. PCR was carried out in a reaction mixture (20 μL) containing the *Paenibacillus* genome DNA with first amplified PCR fragment, 0.5 μM of each primer, and 10 μL of Takara PrimeSTAR Max premix (Takara Bio), using the same thermocycling conditions as the Section 2.2. A DNA fragment of approximately 2.0 kb obtained by PCR was cloned into the *Xho*I and *Xba*I sites of the pCold I expression vector according to the protocol of InFusion cloning (Takara Bio). The nucleotides of the amplified fragment were confirmed by sequencing after InFusion cloning. This expression-plasmid-coded Pb-β-GlcNAcase was named pCold-Pb-β-GlcNAcase. Transformation and purification of Pb-β-GlcNAcase used the same method as those of Pb-Chi. Active fractions were dialyzed with 20 mM phosphate buffer (pH 7.0) and used as the purified enzyme solution.

The enzyme activity was determined using *p*NP-GlcNAc as a substrate under various conditions of pH and temperature. The buffer systems comprised x mL of 0.2 M boric acid and 0.05 mM citrate acid, and (200–x) mL of 0.1 M Na_3PO_4•$12H_2O$ (pH 2.0–12.0). The effect of temperature on enzyme activity was examined at 10–80°C.

The activity of the purified recombinant protein was tested using soluble chitin, *p*NP-GlcNAc, *p*NP-GalNAC, and $(GlcNAc)_{2–6}$. In the case of *p*NP substrates, the released *p*-nitrophenol was detected by absorbance measurement at 405 nm. The hydrolysis products of $(GlcNAC)_{2–6}$ were assayed by HPLC and measured as described above.

HPLC analysis of the hydrolysis products of chitin oligosaccharides $(GlcNAc)_{2–6}$ was performed by the same method as described in Section 2.2. To investigate the cleavage patterns from the hydrolysis products of the purified enzyme (130 units/mL substrate: pNP-GlcNAc), 5.0 mM $(GlcNAc)_{2–6}$ was dissolved in 50 mM sodium acetate buffer (pH 6.0), and aliquots of the enzyme solution were added to 400 μL of each substrate solution.

2.4 Cloning and expression of lytic polysaccharide monooxygenase (Pb-LPMO) gene from *Paenibacillus* sp.

pBIC3 forward (5′-AGTTCCGCATTCGCTCACGGCTGGGTGGACGG-3′) and pBIC reverse (5′-CATCCTGTTAAGCTTTTATTTCAGCAGCCACAA-3′) primers for PCR were synthesized on the regions corresponding to amino acid residues 38–43 and 445–449 of Pb-LPMO, respectively. PCR was carried out in a reaction mixture (20 μL) containing the *Paenibacillus* genome DNA, 0.5 μM of each primer, and 10 μL of Takara PrimeSTAR Max premix (Takara Bio), using the same thermocycling conditions as the Section 2.2. A DNA fragment of approximately 1.4 kb obtained by PCR was cloned into the pBIC3 expression vector. The nucleotides of the amplified fragment were confirmed by sequencing after ligation. This expression-plasmid-coded Pb-LPMO was named pBIC-Pb-LPMO.

pBIC-Pb-LPMO was transformed to *Brevibacillus choshinensis* according to the protocol of the *Brevibacillus* expression system (Takara Bio). The transformant was cultured using TMNm medium (10 g/L glucose, 10 g/L Bacto Soytone, 5 g/L tuna extract, 2.0 g/L Bacto Yeast Extract, 10 mg/L $FeSO_4$•$7H_2O$, 10 mg/L $MnSO_4$•$4H_2O$, and 1.0 mg/L $ZeSO_4$•$7H_2O$) with neomycin (final concentration of 50 μg/mL) at 30°C for 2 days. After cultivation, the culture medium was centrifuged at 20,000 × *g* for 15 min at 4°C. The supernatant was added to 80% saturated $(NH_4)_2SO_4$. A pellet was recovered by centrifugation at 20,000 × *g* for 15 min at 4°C.

The pellet was then redissolved in 20 mM Tris-HCl buffer (pH 7.5) and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) for 1 day. The supernatant was applied to a chitin resin column (1 × 4 cm, Biorad Laboratory, America) equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted with 20 mM acetic acid. Active fractions were dialyzed with 20 mM acetate buffer (pH 5.0) and used as the purified enzyme solution.

2.5 Optimum ratio of Pb-LPMO and Pb-ChiA during the hydrolysis of crystalline chitin

Pb-LPMO (0.01, 0.1, 0.5, 1.0, 3.0, 5.0, 10, and 30 μM), 1 μM Pb-ChiA, and 1.0 mM ascorbic acid in 20 mM acetate buffer (pH 5.0) were incubated with α-chitin at 37°C for 24 h. After the reaction, the reaction mixture was boiled for 10 min to terminate the enzyme reaction. The samples were mixed with the same volume of acetonitrile. The resultant solution was applied onto a column of TSK-GEL Amide 80 (4.6 × 250 mm, Tosoh Co., Tokyo, Japan) and eluted with 70% acetonitrile at a flow rate of 0.7 mL/min, and the products were monitored by absorbance measurement at 210 nm. In this experiment, we estimated the concentration of GlcNAc, $(GlcNAc)_2$, and total sugars (GlcNAc). The concentration of $(GlcNAc)_2$ in total sugars was calculated as twice the concentration of GlcNAc.

2.6 Optimum ratio of Pb-LPMO, Pb-ChiA, and Pb-GlcNAcase during the hydrolysis of crystalline chitin

We incubated 3.0 μM Pb-LPMO, 1 μM Pb-ChiA, Pb-GlcNAcase (0, 0.1, 1.0, 3.0, 5.0, and 10 μM), and 1.0 mM ascorbic acid in 20 mM acetate buffer (pH 5.0) with α-chitin at 37°C for 24 h. The reaction mixture was then boiled for 10 min to terminate the enzyme reaction. The samples were mixed with the same volume of acetonitrile. The resultant solution was applied onto the same column and conditions as mentioned in Section 2.4. In this experiment, we estimated the concentration of GlcNAc, $(GlcNAc)_2$, and total sugars (GlcNAc). The concentration of $(GlcNAc)_2$ in total sugars was calculated as the concentration of two GlcNAc.

2.7 Additional effect of Pb-LPMO during the hydrolysis of crystalline chitin by Pb-ChiA

We incubated 1 μM Pb-ChiA and 1.0 mM ascorbic acid with or without 3 μM Pb-LPMO in 20 mM acetate buffer (pH 5.0) with α-chitin at 37°C for 24 h. The reaction mixture was then boiled for 10 min to terminate the enzyme reaction. The samples were mixed with the same volume of acetonitrile. The resultant solution was applied onto the same column and conditions mentioned in Section 2.4. In this experiment, we investigated the total concentration of GlcNAc, $(GlcNAc)_2$, and total sugars (GlcNAc). The concentration of $(GlcNAc)_2$ in total sugars was calculated as twice the concentration of GlcNAc.

3. Results and discussion

3.1 Cloning and sequencing of Pb-ChiA

The length of the Pb-Chi gene was determined to be 2091 bp. It encodes a protein of 697 amino acids. The mRNA sequence was deposited in the GenBank

database (LC573547). The amino acid sequence of Pb-Chi was similar to that of the chitinases from *P. barengoltzii* (99%, AIT70967), *Paenibacillus* sp. FPU-7 (93%, BAM67142.1), and *Brevibacillus* sp. AG162 (71%, WP_142063498.1). These chitinases belong to the GH family 18, and Pb-Chi may belong to this enzyme family. In addition, all catalytically important residues of chitinases from the GH family 18 [13] were conserved in Pb-Chi (Asp199, Asp201, and Glu203), as shown in *P. barengoltzii, Paenibacillus* sp. FPU-7, and *Brevibacillus* sp. AG162. Moreover, Pb-Chi contains a signal peptide, a catalytic domain, fibronectin type III domains, and a chitin-binding domain, as reported for chitinase A from *Bacillus circulans* WL-12 and the chitinase from *Paenibacillus* sp. FPU-7 [14, 15].

The amino acid sequence of the chitin-binding domain from Pb-Chi was similar to that of the carbohydrate-binding module (CBM) family 12. Chitinase II from *Aeromonas* sp. no. 10S-24, GH family 23 chitinase from *Ralstonia* sp. A-471, and chitinase A from *Bacillus circulans* WL-12 also belong to CBM 12 [16–18]. The aromatic amino acids (tryptophan, phenylalanine, and tyrosine) are present in the binding site of a number of sugar-binding proteins. The interaction of the saccharide with the aromatic residue is determined by their relative position and orientation, which have been found to vary in different sugar-binding proteins [19]. It is reported that CBM family 12 containing GH family 23 chitinase from *Ralstonia* sp. A-471 interacts with the insoluble chitin, but not with soluble chitin [17]. This binding domain can improve the hydrolysis ability against insoluble chitin. Watanabe et al. reported that W687 and Q679 are important amino acid residues during the interaction of saccharides with CBD from *B. circulans* WL-12 [20].

3.2 Expression of Pb-ChiA gene and characterization of recombinant protein

The molecular mass of the protein rPb-ChiA was 70 kDa, as determined by SDS-PAGE (data not shown), in good agreement with that predicted from the amino acid sequence. The activity of rPb-ChiA at various temperatures and pH values were determined in enzyme assays using soluble chitin as the substrate. The optimum pH and temperature of rPb-Chi were 6.0 (**Figure 1A**) and 50°C (**Figure 1B**), respectively.

To recognized the action modes of purified rPb-Chi, enzyme assays were carried with chitin oligosaccharides of several lengths, such as *N*-acetylchitobiose $(GlcNAc)_2$, *N*-acetylchitotriose $(GlcNAc)_3$, *N*-acetylchitotetraose $(GlcNAc)_4$, *N*-acetylchitopentaose $(GlcNAc)_5$, and *N*-acetylchitohexaose $(GlcNAc)_6$; buffer

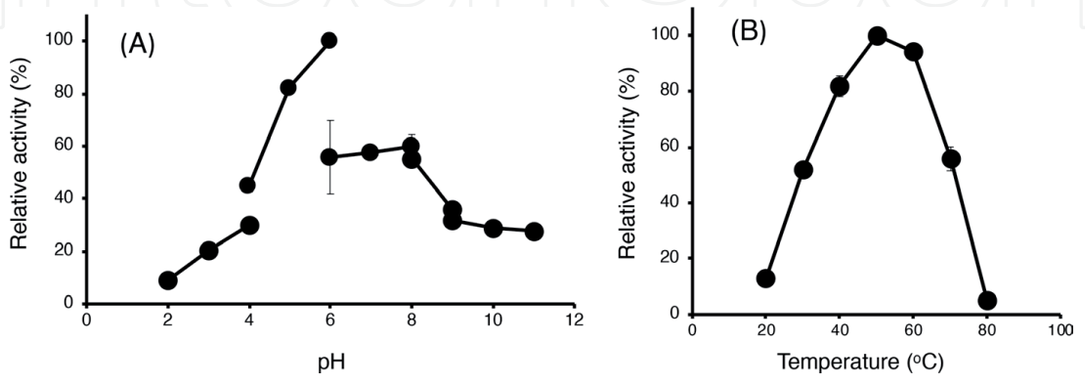

Figure 1.
Functional properties of purified Pb-ChiA. All reactions were conducted with purified enzyme and soluble chitin as the substrate. (A) Effect of pH on enzyme activity at 37°C in 0.2 M of the following buffers: Glycine-HCl (pH 2.0–4.0), sodium acetate (pH 4.0–6.0), KH_2PO_4-K_2HPO_4 (pH 6.0–8.0), Tris-HCl (pH 8.0–9.0), and glycine-HCl (pH 9.0–11.0). (B) Effect of temperature on enzyme activity measured at 20–80°C. The average values of triplicate measurements were used as each activity values.

aliquots were picked up over time and analyzed by HPLC (**Figure 2**). The main hydrolysis products from $(GlcNAc)_6$ were $(GlcNAc)_2$ (**Figure 2A**), and those from $(GlcNAc)_5$ were $(GlcNAc)_3$ and $(GlcNAc)_2$ (**Figure 2B**). It was suggested that $(GlcNAc)_3$ is further degraded to $(GlcNAc)_2$ and GlcNAc. The major hydrolysis products from $(GlcNAc)_4$ was $(GlcNAc)_2$ (**Figure 2C**) and those from $(GlcNAc)_3$ were $(GlcNAc)_2$ and GlcNAc (**Figure 2D**). In these experiments, $GlcNAc_2$ was not degraded, and the major hydrolysis product of colloidal chitin was $GlcNAc_2$ (data not shown). Mainly, rPb-Chi hydrolyzed the second glycosidic bond from the nonreducing end and/or reducing end, and hydrolysis patterns and products made by rPB-Chi were resemble to those described for chitinase A from *Ralstonias* sp. A-471, chitinase A from *Serratia marcescens*, and chitinase A1 from *B. circulans* WL-12 [14, 21]. The present data suggest that this enzyme is a processive exo-type chitinase.

3.3 Cloning and sequencing of Pb-GlcNAcase

The length of the Pb-GlcNAcase gene was determined to be 1926 bp. It encodes a protein of 642 amino acids. The mRNA sequence was deposited in the GenBank database (LC573548). The amino acid sequence of Pb-GlcNAcase was similar to that of the β-*N*-acetylhexosaminidase from *P. barengoltzii* (99%, WP_016313754.1), β-*N*-GlcNAcase from *Fontibacillus panacisegetis* (63%, WP_091231148.1), and β-*N*-acetylhexosaminidase from *Gorillbacterium massiliense* (49%, WP_040950886.1). These β-*N*-GlcNAcase and β-*N*-acetylhexosaminidases belong to the GH family 20, and so Pb-GlcNAcase may belong to this enzyme family. In addition, all catalytically important residues of GlcNAcase from the GH family 20 [22] were conserved in Pb-GlcNAcase (Asp228 and Glu229).

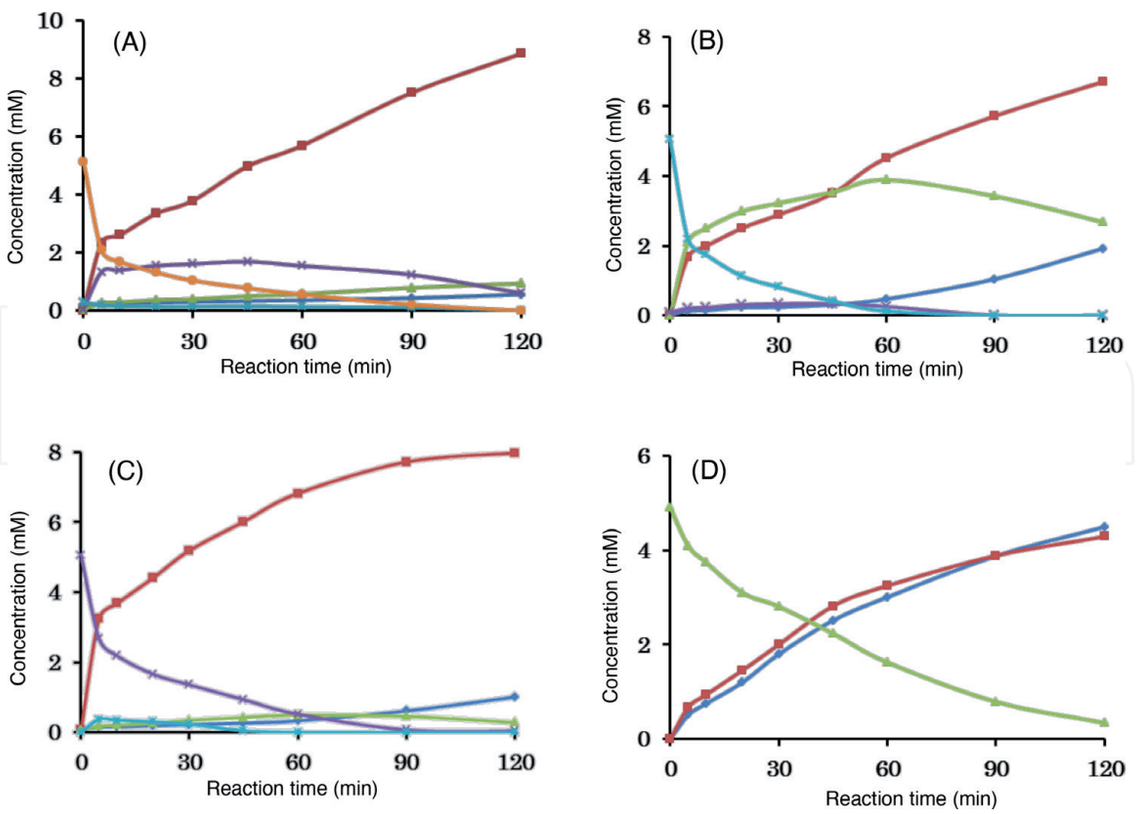

Figure 2.
HPLC analysis of products of hydrolysis of chitin oligosaccharides $(GlcNAc)_3$ to $(GlcNAc)_6$ by recombinant Pb-ChiA. The hydrolysis products of (A) $(GlcNAc)_6$, (B) $(GlcNAc)_5$, (C) $(GlcNAc)_4$, and (D) $(GlcNAc)_3$ by the enzyme were detected by HPLC, as described in the materials and methods. Lines: GlcNAc (dark-blue line), $(GlcNAc)_2$ (red line), $(GlcNAC)_3$ (yellowish olive-green line), $(GlcNAc)_4$ (purple line), $(GlcNAc)_5$ (light blue line), and $(GlcNAc)_6$ (mustard-yellow line).

3.4 Expression of Pb-GlcNAcase gene and characterization of recombinant protein

The molecular mass of the recombinant protein rPb-GlcNAcase was 70 kDa, as determined by SDS-PAGE (data not shown), in good agreement with that predicted from the amino acid sequence. The activity of rPb-GlcNAcase at various temperatures and pH values were determined in enzyme assays using *p*NP-GlcNAc as the substrate. The optimum pH of rPb-GlcNAcase was 6.0 (**Figure 3A**), while the optimal temperature for rPb-Chi activity was 50°C (**Figure 3B**); these values were similar to those of rPb-ChiA.

To recognized the action modes of purified rPb-GlcNAcase, enzyme assays were carried with chitin oligosaccharides of several lengths, such as $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$; buffer aliquots were picked up over time and analyzed by HPLC (**Figure 4**). The main hydrolysis products of $(GlcNAc)_6$ were GlcNAc and $(GlcNAc)_5$ (**Figure 4A**), those of $(GlcNAc)_5$ were GlcNAc and $(GlcNAc)_4$ (**Figure 4B**), and those of $(GlcNAc)_4$ and $(GlcNAc)_3$ were GlcNAc (**Figure 4C, D**). In these experiments, $(GlcNAc)_2$ was degraded to GlcNAc (**Figure 4E**). In addition, the specific activities of $(GlcNAc)_2$ and $(GlcNAc)_3$ were higher than those of $(GlcNAc)_{4–6}$ (**Table 1**). The present data indicate that this enzyme is an exo-type carbohydrate hydrolase.

3.5 Cloning and sequencing of Pb-LPMO

The length of the Pb-LPMO gene was determined to be 1350 bp. It encodes a protein of 449 amino acids. The amino acid sequence of Pb-LPMO was the same or very similar to that of the LPMO from *Paenibacillus* sp. (100%, WP_028538775) and *P. barengoltzii* (99%, WP_016312786) and the chitin-binding domain from *Paenibacillus* sp. oral taxon 786 (96%, WP_009222736). As these LPMOs belong to the AA family 10, Pb-LPMO may also belong to this enzyme family. In addition, catalytically important residues of LPMO from the AA family 10 [9] were conserved in Pb-LPMO (His38, His123) as well as the residues of LPMO from *P. barengoltzii* (WP_016312786), *Paenibacillus* sp. (WP_028538775), and *S. marcescens* BJL200 (AAU882020.1). Moreover, Pb-LPMO contains a signal peptide, an LPMO domain, two fibronectin type III domains, and a chitin-binding domain, as reported for the LPMOs of *P. barengoltzii* (WP_016312786) and *Paenibacillus* sp. (WP_028538775).

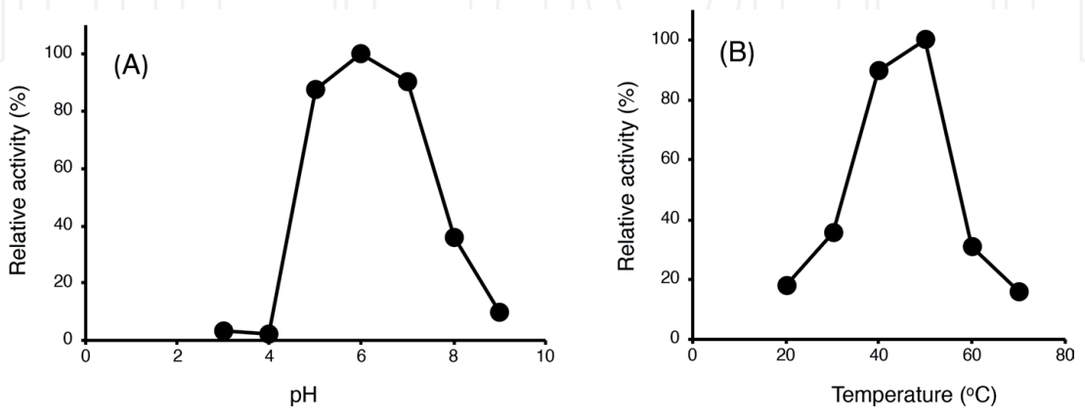

Figure 3.
Functional properties of purified Pb-GlcNAc. All reactions were conducted with purified enzyme and pNp-GlcNAc as the substrate. (A) Effect of pH on enzyme activity at 37°C in various buffers. The buffer systems comprised x mL of 0.2 M boric acid and 0.05 mM citrate acid, and (200–x) mL of 0.1 M Na_3PO_4•$12H_2O$ (pH 3.0–9.0). (B) Effect of temperature on enzyme activity measured at 20–70°C. The average values of triplicate measurements were used as activity values.

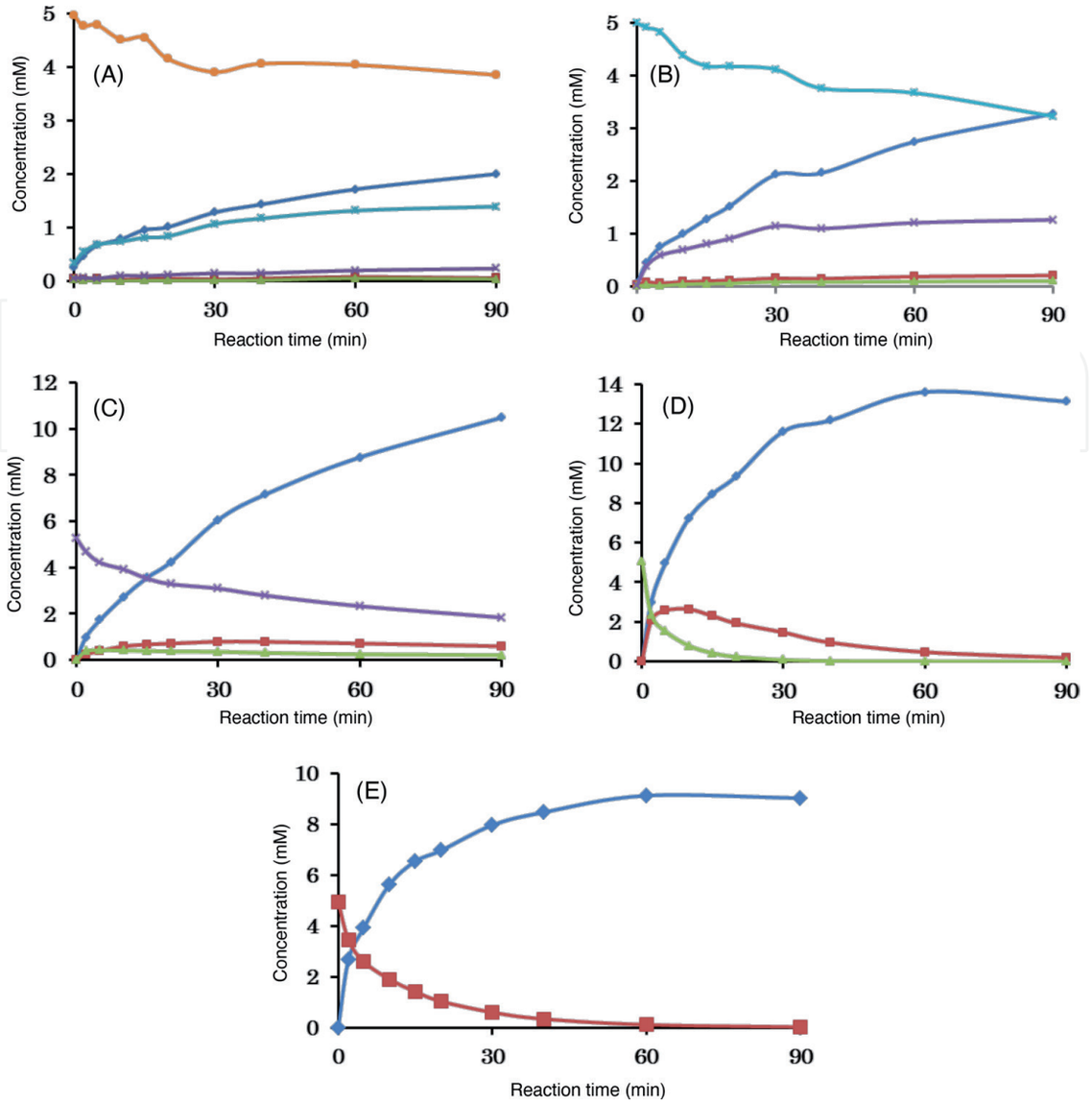

Figure 4.
HPLC analysis of products of hydrolysis of chitin oligosaccharides $(GlcNAc)_3$ to $(GlcNAc)_6$) by recombinant Pb-GlcNAcase. The degradation products of (A) $(GlcNAc)_6$, (B) $(GlcNAc)_5$, (C) $(GlcNAc)_4$, (D) $(GlcNAc)_3$, and (E) $(GlcNAc)_2$ by the enzyme were detected by HPLC, as described in the materials and methods. Lines: GlcNAc (dark-blue line), $(GlcNAc)_2$ (red line), $(GlcNAC)_3$ (yellowish olive-green line), $(GlcNAc)_4$ (purple line), $(GlcNAc)_5$ (light-blue line), $(GlcNAc)_6$ (mustard-yellow line).

Substrate	Activity (units/ mg protein)
Soluble chitin	ND
pNP-GlcNAc	111×10^{-3}
pNP-GalNAc	73.7×10^{-3}
$(GlcNAc)_2$	10.2
$(GlcNAc)_3$	12.7
$(GlcNAc)_4$	5.8
$(GlcNAc)_5$	3.8
$(GlcNAc)_6$	3.7

The average values of triplicate measurements were used as each activity value.

Table 1.
Substrate specificity of Pb-GlcNAcase.

3.6 Expression of Pb-LPMO gene and characterization of recombinant protein

The molecular mass of the recombinant protein rPb-LPMO was 45 kDa, as determined by SDS-PAGE (data not shown), in good agreement with that predicted from the amino acid sequence. The activity of rPb-LPMO was determined in enzyme assays using α-chitin or β-chitin as the substrate. After incubation, oxidized products were detected by HPLC (data not shown).

3.7 Optimum ratio of Pb-LPMO and Pb-ChiA during the hydrolysis of crystalline chitin

To understand the effect of Pb-LPMO on the hydrolysis of crystalline chitin, we added 0.01–30 μM Pb-LPMO to the reaction solution containing 1 μM Pb-ChiA. (**Figure 5**). With increasing concentration of Pb-LPMO, the concentration of the hydrolysis products (GlcNAc and $(GlcNAc)_2$) increased. When 1–10 μM of Pb-LPMO was added to the reaction mixture, products at higher concentration were obtained as compared with other concentrations. However, the addition of more than 10 μM Pb-LPMO decreased the concentration of the hydrolysis products in the reaction mixture. For efficient hydrolysis of crystalline chitin, addition of 3 μM Pb-LPMO to the reaction mixture containing 1 μM Pb-ChiA is optimal. The released products were approximately two-fold higher upon the addition of Pb-LPMO. Vaaje-Kolstad et al. reported that CBP21 (LPMO) strongly promotes hydrolysis of crystalline β-chitin by chitinase A and C from *S. marcescens* [23]. In this study, Pb-LPMO was found to efficiently promote hydrolysis of crystalline chitin.

3.8 Optimum ratio of Pb-LPMO, Pb-ChiA, and Pb-GlcNAcase during the hydrolysis of crystalline chitin

To understand the effect of Pb-GlcNAcase on the hydrolysis of crystalline chitin, we added 0.1–10 mM Pb-LPMO to the reaction solution containing

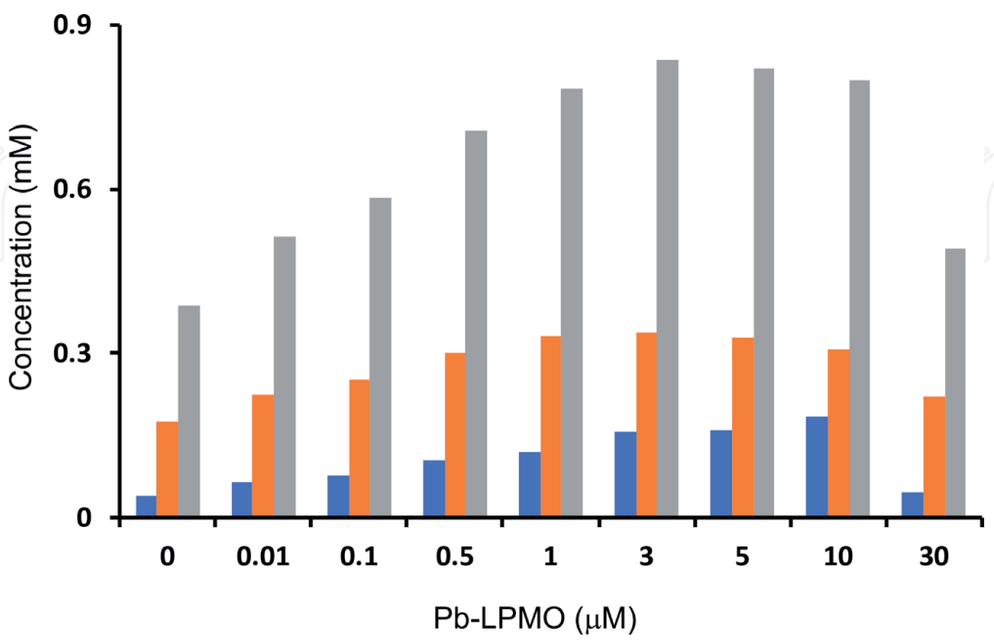

Figure 5.
Optimum ratio of Pb-LPMO and Pb-ChiA during the hydrolysis of crystalline chitin. Blue bar: GlcNAc content; reddish brown bar: $(GlcNAc)_2$ content; gray bar: Total GlcNAc content. Total GlcNAc content: $(GlcNAc)_2$ (reddish brown bar) content was converted to twice the GlcNAc content. Total GlcNAc content = GlcNAc (blue bar) content + converted GlcNAc content.

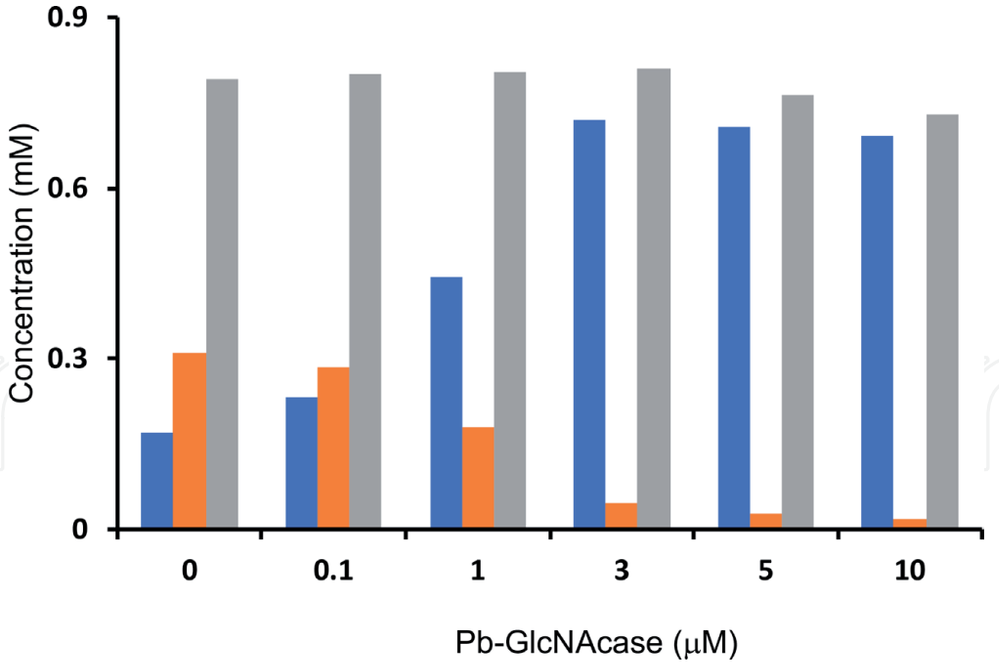

Figure 6.
Optimum ratio of Pb-LPMO, Pb-ChiA, and Pb-GlcNAcase during the hydrolysis of crystalline chitin. Blue bar: GlcNAc content; reddish brown bar: $(GlcNAc)_2$ content; gray bar: Total GlcNAc content. Total GlcNAc content: $(GlcNAc)_2$ (reddish brown bar) content was converted to twice the GlcNAc content. Total GlcNAc content = GlcNAc (blue bar) content + converted GlcNAc content.

1 mM Pb-ChiA and 3 mM Pb-LPMO (**Figure 6**). With increasing concentration of Pb-GlcNAcase, the concentration of released GlcNAc increased, but the concentration of released $(GlcNAc)_2$ decreased. It is possible that $(GlcNAc)_2$ was hydrolyzed by Pb-GlcNAcase. The concentration (approximately 0.8 mM) of released products (**Figure 6**, gray bar) when Pb-GlcNAcase was added was almost the same as that obtained from the reaction of only Pb-ChiA and Pb-LPMO (**Figure 5**). Therefore, Pb-GlcNAcase has no effect on the hydrolysis of crystal chitin but only contributes to the degradation of chitin oligosaccharides. Wang et al. reported that only β-*N*-GlcNAcase plays a role in the hydrolysis of chitin-oligosaccharides and transglycosylation [24]. Hemsworth et al. reported that it will become increasingly important to understand how cellulase and LPMO enzymes synergize with one another in biomass breakdown [10]. It found that synergization of chitinase and LPMO enzyme is also very important in the crystalline chitin hydrolysis.

4. Conclusion

Pb-ChiA had a higher affinity toward crystalline chitin than Pb-LPMO. Pb-LPMO boosts the activity of Pb-ChiA toward crystalline α-chitin but not toward crystalline β-chitin. For the efficient hydrolysis of crystalline chitin, addition of 3 μM Pb-LPMO to the reaction mixture containing 1 μM Pb-ChiA was optimal. When Pb-LPMO (3 μM) was added to the reaction mixture during the hydrolysis of crystalline α-chitin by Pb-ChiA, two-fold higher concentration hydrolysis products were obtained. It was found that Pb-GlcNAcase is involved only in the degradation of chitin oligosaccharides. Synergization of Pb-ChiA and Pb-LPMO was shown during crystalline chitin decomposition. LPMO contributes to the efficiency of chitinase used in the conversion of chitin biomass.

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