Elucidation of exo-β-D-glucosaminidase activity of a family 9 glycoside hydrolase (PBPRA0520) from Photobacterium profundum SS9

Yuji Honda1, Nozomi Shimaya, Kana Ishisaki, Mitsuru Ebihara, and Hajime Taniguchi
Department of Food Science, Ishikawa Prefectural University, 1-308 Suematsu, Nonenchi, Ishikawa 921-8836, Japan

Received on July 28, 2010; revised on November 5, 2010; accepted on November 11, 2010

A glycoside hydrolase (GH) gene from Photobacterium profundum SS9 (PBPRA0520) belonging to GH family 9 was expressed in Escherichia coli. The protein was expressed with the intact N-terminal sequence, suggesting that it is an intracellular enzyme. The recombinant protein showed hydrolytic activity toward chitobiose [(GlcN)2] and cellobiose (CG2) in various disaccharides. This protein also released 4-nitrophenol (PNP) from both 4-nitrophenyl-β-D-glucosaminide (GlcN-PNP) and 4-nitrophenyl-β-D-glucoside (Glc-PNP). The hydrolytic pattern observed in chitooligosaccharides and cellooligosaccharides suggested that the reaction proceeded from the nonreducing end in an exo-type manner. Time-dependent 1H-nuclear magnetic resonance (NMR) analysis of the anomeric form of the enzymatic reaction products indicated that the protein is an inverting enzyme. $k_{\text{eq}}/k_m$ of (GlcN)$_2$ hydrolysis was 14 times greater than that of CG$_2$ hydrolysis. These results suggested that the protein is an exo-β-D-glucosaminidase (EC 3.2.1.165) rather than a glucan 1,4-β-D-glucosidase (EC 3.2.1.74). Based on the results, we suggest that the function of conserved GH proteins in the chitin catabolic operon is to cleave a (GlcN)$_2$-phosphate derivative by hydrolysis during intracellular chitooligosaccharide catabolism in Vibrioaceae.

Keywords: chitin / exo-β-D-glucosaminidase / GH family 9 / Photobacterium / Vibrio

Introduction

Chitin is a N-acetyl-β-D-glucosamine (GlcNAc) polysaccharide, the monomers of which are linked by β-1,4-glycosidic bonds. This insoluble polysaccharide is the main component of the exoskeleton of arthropods (crustaceans and insects) as well as the cell walls of fungi. In an aquatic environment, chitin is generated by molting of cuticles in the exoskeleton of crustaceans (Keyhani and Roseman 1999). Marine bacteria utilize chitin as an important source of carbon and nitrogen (Zobell and Rittenberg 1938). Investigation of chitin catabolism in Vibrioaceae suggests that many functional proteins are produced from the bacteria during this process (Keyhani and Roseman 1999; Li and Roseman 2004; Meibom et al. 2004). Recently, the genomic sequences of Vibrio and Photobacterium species have been elucidated, and the genes for various enzymes involved in chitin catabolism were identified (Heidelberg et al. 2000; Chen et al. 2003; Kim et al. 2003; Makino et al. 2003; Meibom et al. 2004; Ruby et al. 2005).

Chitin catabolic systems of various Vibrio species have been well studied. Based on evidence from microarray expression profiling studies of V. cholerae, the chitin catabolic operon (VC0611–VC0622 genes) was suggested to be regulated by chitooligosaccharides (Li and Roseman 2004; Meibom et al. 2004). In the chitin catabolic system, the chitin sensor (ChiS, VC0622) plays an important role in regulating this gene cluster, which was also found in the genomic sequences of other Vibrio and Photobacterium species (Heidelberg et al. 2000; Chen et al. 2003; Kim et al. 2003; Makino et al. 2003; Meibom et al. 2004; Ruby et al. 2005). Consequently, the proteins encoded by this cluster have been identified and characterized as a β-N-acetylglucosaminidase (Keyhani et al. 1996), a N,N'-diacetylchitobiose phosphorylase (Park et al. 2000; Hidaka et al. 2004; Honda et al. 2004), a glucosamine kinase (Park et al. 2002b), and an exo-β,1,4-glucosidase (other name was glucan 1,4-β-D-glucosidase (EC 3.2.1.74)) (Park et al. 2002a).

Nevertheless, the role of glucan 1,4-β-D-glucosidase in the chitin catabolism cascade in Vibrioaceae remains unclear. According to the Carbohydrate-Active EnZymes (CAZy) database, this enzyme belongs to glycoside hydrolase (GH) family 9 (Cantarel et al. 2009). Park et al. (2002a) reported that the VC0615 protein is a β,1,4-glucosidase having no exo-β-D-glucosaminidase activity [EC 3.2.1.165]. However, Hunt et al. (2008) postulated that this protein shows exo-β-D-glucosaminidase activity and cleaves the β-1,4-glycosidic linkage in a chitobiose [(GlcN)$_2$]-phosphate derivative made from (GlcN)$_2$ by the action of the phosphotransferase system.

© The Author 2010. Published by Oxford University Press. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com
(PTS) transporter. To better understand the chitin catabolism system, the activity of a GH9 protein found in Vibrionaceae should be determined.

Nonpathogenic *Photobacterium profundum* SS9 possesses a 4.1 Mbp major circular chromosome, a 2.2 Mbp minor circular chromosome and an 80 kbp circular plasmid (Vezzi et al. 2005). This tripartite structure is common to other Vibrionaceae; various gene clusters are also conserved in the genome sequences (Vezzi et al. 2005). The chitin catabolic operon found in *V. cholerae* is also observed in the genome sequence of *P. profundum* as shown in Table I. The deduced amino acid sequences of PBPRA0520 and VC0615 proteins show a high degree of similarity (88%). Based on the position of the open reading frame in the gene cluster and the conserved amino acid sequences, we assumed that the PBPRA0520 protein from *P. profundum* participates in the same way with regard to the chitin catabolic mechanism as the proteins from other *Vibrio* and *Photobacterium* species. We also aimed to determine the enzymatic properties of the PBPRA0520 protein with respect to the chitin catabolism cascade in marine bacteria.

In this study, we found that the PBPRA0520 protein hydrolyzes the O-glycosidic bond of chitobiose rather than that of cellobiose (CG2). This result led to the characterization of the enzymatic properties of this protein in detail. Here we describe the reaction mechanism of a GH9 glycosidase based on its substrate specificities and kinetic properties.

### Results

**Characterization of basic enzymatic properties**

The recombinant PBPRA0520 protein was expressed in *Escherichia coli* BL21(DE3) and purified, yielding a 60 kDa protein on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The molecular weight of the PBPRA0520 protein was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis (Figure 1). Two spectra ($m/z$ 66,658.32 [M + H]$^+$ and $m/z$ 133,110.92 [2M + H]$^+$) of the PBPRA0520 protein were detected in the analysis, suggesting that the former peak was a monomer of the protein (calculated [M + H]$^+$: 66,565.85 Da) and the latter peak was the dimer of the same protein (calculated [2M + H]$^+$: 133,130.7 Da). The enzyme was purified 9-fold as shown in Table II. The N-terminal sequence was Met-Gln-Leu-Leu-Thr. Since this sequence corresponds to that of the start codon, it is unlikely that the enzyme contains a signal peptide.

To determine the enzymatic activity, the hydrolytic products were detected by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and high-

![Figure 1](https://academic.oup.com/glycob/article-abstract/21/4/503/1990312/504)
Hydrolysis of oligosaccharides

The \((\text{GlcN})_n\) \((n = 3–5)\) degradation products of the PBPRA0520 protein were analyzed by TLC and HPLC. Figure 3 shows the time-dependent TLC profiles of the products of \((\text{GlcN})_n\) hydrolysis by the enzyme. Spots corresponding to the substrates GlcN and \((\text{GlcN})_{n-1}\) were detected in the initial stage of the hydrolytic reaction, suggesting that the enzymatic reaction proceeds in an exo-type cleavage manner. The enzyme released PNP from Glc-PNP and GlcN-PNP, suggesting that the enzyme hydrolyzed the glycosidic linkage from the nonreducing end of the substrate. Next, the enzymatic reaction products were analyzed by HPLC. Figure 4 shows the time courses of \((\text{GlcN})_n\) \((n = 3–5)\) hydrolysis by the enzyme. These results suggest that the enzyme released GlcN and \((\text{GlcN})_{n-1}\) from \((\text{GlcN})_n\) in the initial stages of the reactions. Oligosaccharides larger than the initial substrates were not detected even at an extended incubation time, indicating that the enzyme had no transglycosylation activity. Hydrolysis of CGn showed similar results as \((\text{GlcN})_n\) (data not shown).

Anomeric analysis of the hydrolytic products

Figure 6 shows time-dependent \(^1H\)-NMR spectra of \((\text{GlcN})_n\) hydrolysis by the PBPRA0520 protein. The spectra showed the characteristic downfield signals of 5.34 ppm (\(\alpha'1\)H), 4.85 ppm (\(\beta'1\)H) and 4.72 ppm (internal \(^1H\)) from C1\(\alpha\), C1\(\beta\) and other C1 anomeric protons, respectively. The signal of 4.67 ppm results from the residual solvent. The standard equilibrium ratio of C1\(\alpha\):C1\(\beta\) anomers of 2.4:1 was calculated from peak areas. With an increase in the reaction time, the relative peak area of C1\(\alpha\) increased and that of internal \(^1H\) decreased (Figure 6). The peak area of the signal corresponding to C1\(\beta\) protons of GlcN at 4.82 ppm increased after 30 min, resulting from mutarotation of GlcN. These results suggest that hydrolysis by the PBPRA0520 protein proceeds by anomeric inversion.

Kinetic properties

The apparent kinetic parameters of CGn, \((\text{GlcN})_n\), GlcN-PNP and GlcN-PNP are summarized in Table III. The typical Michaelis–Menten relationship was observed. For the CG5 reaction, the \([S]–v\) curve was linear, because of the low water solubility of the substrate. Thus, we determined only \(k_{cat}/K_m\) of CGn hydrolysis for comparison with other substrates. The highest \(k_{cat}\) and \(K_m\) values were obtained using \((\text{GlcN})_2\) and CG5 as the substrates. \(k_{cat}/K_m\) of CGn hydrolysis was similar to that of the substrates, whereas that of the \((\text{GlcN})_n\) hydrolysis increased with an increase in the degree of polymerization. \(k_{cat}/K_m\) of Glc-PNP was three times greater than that of GlcN-PNP hydrolysis.
According to the CAZy database, GH9 comprises an endo-type cellulase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91), a β-glucosidase (EC 3.2.1.21) and a glucan 1,4-β-D-glucosidase (EC 3.2.1.74) (Cantarel et al. 2009). In this study, we elucidated that the GH9 protein from *P. profundi*um SS9 was an exo-β-D-glucosaminidase (EC 3.2.1.165), rather than a glucan 1,4-β-D-glucosidase (EC 3.2.1.74). This is the first report of an exo-β-D-glucosaminidase in the GH9 family. The enzymatic properties are discussed in further detail by comparison with the substrate specificities of

---

**Discussion**

According to the CAZy database, GH9 comprises an endo-type cellulase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91), a β-glucosidase (EC 3.2.1.21) and a glucan 1,4-β-D-glucosidase (EC 3.2.1.74) (Cantarel et al. 2009). In
Glucose-releasing GHs such as β-glucosidase and glucan 1,4-β-D-glucosidase belonging to GH9 family, and other exo-β-D-glucosaminidases.

Glucan 1,4-β-D-glucosidases belonging to GH9

The GH9 proteins were conserved in the genomic sequences of *Vibrio* and *Photobacterium*. The GH9 protein [VC0615, BglA] derived from *V. cholerae* was reported as an exo-type GH that yields glucose from cellooligosaccharides (Park et al. 2002a). In addition, the authors reported that the BglA protein has no activity toward gentiobiose, laminaribiose and sophorose (Park et al. 2002a). This substrate specificity was in consistent with that of a glucan 1,4-β-D-glucosidase (EC 3.2.1.74) as defined by Enzyme Nomenclature (http://www.chem.qmul.ac.uk/iubmb/enzyme/). Recently, Qi et al. (2008) reported that the external glucan 1,4-β-D-glucosidase (Cel9D) belonging to GH9 was cloned from *Fibrobacter succinogenes*, and the substrate specificity was characterized using various polysaccharides and oligosaccharides. The enzyme hydrolyzed only the β-1,4-glycosidic bond in the saccharides similar to that reported for BglA. The authors claimed that BglA could be categorized as a glucan 1,4-β-D-glucosidase, based on the similarities in substrate specificity (Qi et al. 2008). The PBPRA0520 protein also showed hydrolytic activity only toward the β-1,4-glycosidic bond of cellooligosaccharides. In addition, the PBPRA0520 protein released PNP from Glc-PNP similar to that reported for BglA. These results suggest that the PBPRA0520 protein possesses glucan 1,4-β-D-glucosidase activity.

Kinetic analyses of Glc-PNP and CG2 provided information on substrate-binding affinities around the cleavage site. $k_{cat}/K_m$.
of CG\textsubscript{2} hydrolysis by the PBPR\textsubscript{A0520} protein was higher than that of Glc-PNP hydrolysis, indicating that the +1 subsite was important for the recognition of the sugar moiety (+ and − indicate reducing and nonreducing ends of the subsite based on the proposed nomenclature) (Davies et al. 1997).

According to the kinetic analysis of Bgl\textsubscript{A}, the $k_{\text{cat}}/K_{m}$ of Glc-PNP hydrolysis was greater than that of CG\textsubscript{2} hydrolysis, suggesting that the binding affinity of the −1 subsite was higher than that of the +1 subsite (Park et al. 2002a). Conversely, Cel\textsubscript{9D} preferred to release glucose from CG\textsubscript{3} rather than from CG\textsubscript{2}. Furthermore, kinetic analysis of CG\textsubscript{n} hydrolysis by Cel\textsubscript{9D} indicated that the +2 subsite had the highest binding free energy among the subsites (Qi et al. 2008). These results suggest that the intracellular PBPR\textsubscript{A0520} protein and Bgl\textsubscript{A} specialize in the hydrolysis of saccharides rather than that of the external Cel\textsubscript{9D}.

**Exo-β-d-glucosaminidase activity**

In the Enzyme Nomenclature, exo-β-d-glucosaminidase activity is defined as the hydrolysis of chitosan or (GlcN)\textsubscript{n} to remove successive d-glucosamine (GlcN) residues from nonreducing termini (http://www.chem.qmul.ac.uk/iubmb/enzyme/). The deduced amino acid sequences of exo-β-d-glucosaminidases derived from *Thermococcus kodakaraensis* KOD1, *Amycolatopsis orientalis*, *Aspergillus species*, *Pyrococcus horikoshii*, and *Trichoderma reesei* PC-3–7 have been reported (Tanaka et al. 2003; Côté et al. 2006; Ike et al. 2006; Liu et al. 2006; Li et al. 2009). The enzyme from *T. kodakaraensis* KOD1 consisted of GH35 and GH42 domains. *A. orientalis* and *T. reesei* PC-3–7 exo-β-d-glucosaminidases were classified into GH2. The three-dimensional structures of these exo-β-d-glucosaminidases were found to have a (β/α)\textsubscript{8}-barrel structure similar to that of enzymes belonging to GH families 2, 35 and 42. In fact, the three-dimensional structure of *A. orientalis* was determined as a (β/α)\textsubscript{8}-barrel structure by X-ray crystal diffraction analysis (van Bueren et al. 2009). Enzymes belonging to GH families 2, 35 and 42 are categorized as retaining enzymes in the CAZy database, suggesting that the exo-β-d-glucosaminidases catalyze with retention of anomeric configuration during hydrolysis (Côté et al. 2006).

The GH9 enzymes hydrolyze the β-glucosidic bond by anomeric inversion according to the CAZy database. This study indicated that hydrolysis by the PBPR\textsubscript{A0520} protein occurred by anomeric inversion, as shown in Figure 6. To our knowledge, this is the first report of an inverting exo-β-d-glucosaminidase. The difference between the anomeric forms of the products indicated that the active site structure of the PBPR\textsubscript{A0520} protein was different from that of other retaining exo-β-d-glucosaminidases. The three-dimensional structures of the GH9 enzymes have been reported to have a (α/α)\textsubscript{6}-barrel fold (Sakon et al. 1997; Parsiegla et al. 2002; Mandelman et al. 2003; Schubot et al. 2004; Pereira et al. 2009). Recently, the three-dimensional structure of the VP2484 protein derived from *Vibrio parahaemolyticus* RIMD 22106633 was registered in the Protein Data Bank (http://www.wwpdb.org/). According to the three-dimensional structure (accession number: 3H7L), this GH9 protein has a (α/α)\textsubscript{6}-barrel fold. The amino acid sequence identity and similarity between VP2484 and PBPR\textsubscript{A0520} proteins was 63 and 77%, respectively, suggesting that the PBPR\textsubscript{A0520} protein has a similar three-dimensional structure. The three-dimensional structure of PBPR\textsubscript{A0520} protein was modeled using the 3D-jigsaw program based on the 3H7L structure (Bates et al. 2001). The modeled structure suggested that PBPR\textsubscript{A0520} protein consisted of a (α/α)\textsubscript{6}-barrel structure (see Supplementary data, Figure S1). The active site of the enzyme was deduced from mutational analysis of Cel\textsubscript{9D} based on the sequence alignment between these enzymes (amino acid identity: 37%) (Qi et al. 2008). The catalytic residues (D139, D143 and E555; PBPR\textsubscript{A0520} numbering) were conserved in the PBPR\textsubscript{A0520} at the amino acid level and were found in the cleft of the (α/α)\textsubscript{6}-barrel structure. C-terminal residues were located on the opposite side of the active site, suggesting that the His\textsubscript{n} tag did not affect the enzymatic activity.

The (GlcN)\textsubscript{n} hydrolytic pattern of the PBPR\textsubscript{A0520} protein was similar to that reported for other retaining exo-β-d-glucosaminidases. The enzyme produced GlcN and (GlcN)\textsubscript{n−1} from (GlcN)\textsubscript{n}, and released PNP from GlcN-PNP, suggesting that it hydrolyzes from the nonreducing end of chitoooligosaccharides. These properties were in consistent with the definition of the Enzyme Nomenclature. According to the kinetic analysis of exo-β-d-glucosaminidase from *T. kodakaraensis*, the highest $k_{\text{cat}}$ and $K_{m}$ were obtained using (GlcN)\textsubscript{n} as the substrate (Tanaka et al. 2003). This is in consistent with the observations made in our study regarding the kinetic analysis of (GlcN)\textsubscript{n} hydrolysis by the PBPR\textsubscript{A0520} protein. The $k_{\text{cat}}/K_{m}$ for (GlcN)\textsubscript{n} hydrolysis by the PBPR\textsubscript{A0520} protein was 250 times greater than that for GlcN-PNP hydrolysis. This suggested that the +1 subsite of the enzyme recognized the sugar moiety, resulting in the formation of a nonproductive complex between the enzyme and GlcN-PNP.

Based on the kinetic parameters of CG\textsubscript{n} and (GlcN)\textsubscript{n} hydrolysis by the enzyme, we concluded that the PBPR\textsubscript{A0520} protein is an exo-β-d-glucosaminidase rather than a glucan 1,4-β-d-glucosidase.

**Amino acid sequence comparison within the GH9 family**

Phylogenetic analysis was performed with amino acid sequences of GH9 proteins characterized by enzymatic activity (see Supplementary data, Figure S2). We categorized the GH9 enzymes into three groups, based on the relationship between their action patterns and amino acid sequences. They were separated into “endo-type GHS” [cellulase (EC 3.2.1.4) and lichenase (EC 3.2.1.73)], “disaccharide-releasing GHS” [cellobiohydrolase (EC 3.2.1.91)] or “monosaccharide-releasing GHS” [glucan 1,4-β-d-glucosidase (EC 3.2.1.74) and exo-β-d-glucosaminidase (EC 3.2.1.165)]. Furthermore, three-dimensional structural alignment was performed using the EXPRESSO (3DCoffee) program (Armougom et al. 2006). The result indicated that the α-helix structure (amino acid sequence number 151–158 in PBPR\textsubscript{A0520}) was conserved in monosaccharide-releasing GHS (see Supplementary data, Figure S3). According to alignment of Cel\textsubscript{9D}, VC0615 and cellulases belonging to GH9, this region was significantly different by comparison with endo-type cellulases (Qi et al. 2008). This α-helix was found to be located near the active site of the
Role of the GH9 protein in chitin catabolism in Vibrionaceae

The chitin catabolic cascade in Vibrionaceae has been investigated in both biochemical and bioinformatics studies (Park et al. 2002b; Meibom et al. 2004; Hunt et al. 2008). The GH9 protein is conserved in a gene cluster regulated by the chitin sensor protein gene (ChiS) in Vibrio and Photobacterium species (Heidelberg et al. 2000; Chen et al. 2003; Kim et al. 2003; Makino et al. 2003; Meibom et al. 2004; Ruby et al. 2005). The GH9 protein (VC0615, BglA) found in V. cholerae has been reported to be an exo-type β,1,4-α-D-glucosidase that acts on celloligosaccharides (Park et al. 2002a). However, V. cholerae does not grow on cellobiose (Hunt et al. 2008). The authors speculated that the enzyme played a role in the hydrolysis of certain arylglycosides to release phenolic compounds (Park et al. 2002a). Microarray expression profiling analysis of V. cholerae has indicated that the cellobiose PTS transporter (VC1281–VC1286) exhibits (GlcN)2-specific transportation rather than cellobiose-specific transportation (Meibom et al. 2004). Bioinformatic analysis of the chitin pathway in V. cholerae has predicted that VC0615 (BglA) plays a role in the cleavage of the β,1,4-α-D-glucosaminidic bond in the (GlcN)2-phosphate derivative in the cytosol (Hunt et al. 2008). Our results indicate that the PBPPRA0520 protein hydrolyzed the β,1,4-α-D-glucosaminidic linkage in (GlcN)n from the nonreducing end. Considering the gene location and substrate specificity, conserved GH9 proteins in the chitin catabolic operon in Vibrionaceae may be key enzymes, which act to cleave the glycosidic linkage in the (GlcN)2-phosphate derivative, which is derived from (GlcN)2 by the action of the PTS transporter, in the cytoplasm.

Materials and methods

Materials

The P. profundum SS9 strain (BAA-1253™) was obtained from the American Type Culture Collection (Manassas, VA). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA), and the DNA polymerase from T. kodakaraensis KOD1 was obtained from Toyobo (Osaka, Japan). Cellooligosaccharides (CGn; n = 2–5), chitoooligosaccharides [(GlcN)n; n = 2–5] and laminarinbiose were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Gentiobiose and sophorose were purchased from Sigma-Aldrich (St Louis, MO). Xylobiose was obtained form Wako Pure Chemicals (Kyoto, Japan). Avicel® was purchased from Merck (Darmstadt, Germany). Glc-PNP and lactose were purchased from Nacalai Tesque (Kyoto, Japan). Dai-chitosan (100 (VL) (98% deacetylated chitosan) was kindly gifted by Dainichiseika Color & Chemicals Mfg. Co., Ltd. (Tokyo, Japan). All other reagents were of analytical grade and were obtained commercially.

DNA manipulation

Recombinant DNA techniques and agarose gel electrophoresis were performed as described by Sambrook et al. (1989). Plasmid DNA was isolated using a QIAprep Spin Plasmid Kit (Qiagen, Hilden, Germany). Digestion by restriction enzymes was performed in an appropriate buffer at concentrations of 1–10 U/µg of DNA for 0.5–16 h at 37°C. Completion of the reaction was confirmed by agarose gel electrophoresis.

Nucleotide sequence analysis

The nucleotide sequence was determined by the dideoxy-nucleotide chain termination method using an automated DNA sequencer (3100 Genetic Analyzer; Applied Biosystems, Foster City, CA) with a dRhodamine Terminator Kit (Perkin-Elmer, Fremont, CA). At least three independent clones of each PCR product were sequenced. Sequence data were analyzed using the GENETYX-WIN software, version 9.0 (Genetyx Software Development Co., Ltd., Tokyo, Japan).

Expression of the PBPPRA0520 protein in E. coli

The gene encoding PBPPRA0520 was amplified from the genomic DNA of P. profundum SS9 by PCR using the forward primer 5'-CATATGCAGCTGTGACTAATACGCTCTT GGT-3' (containing an NdeI site denoted by bold type) and the reverse primer 5'-CTCGAGACATGATTTCGCTCTT GAATTCG-3' (containing an XhoI site denoted by bold type). The amplified fragments were cloned into pCR®2.1-TOPO® (Invitrogen, San Diego, CA) and the DNA sequence was confirmed. The plasmid was then digested with NdeI and XhoI. The digest fragment was ligated into pET30b (Novagen, Madison, WI) at the corresponding sites, generating the plasmid pET30b-PBPPRA0520 encoding the PBPPRA0520 protein with the His6 sequence added to its C-terminal end. Next, the pET30b-PBPPRA0520 were transformed into E. coli BL21 (DE3), and the resulting transformants were incubated in Luria broth medium containing 0.05 mg/mL kanamycin at 37°C until the optical density at 600 nm reached 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to give a final concentration of 1 mM, and the cultures were incubated for 24 h at 25°C. The PBPPRA0520 protein expressed was extracted from the wet cells (1 g) in 3 mL of 50 mM sodium phosphate buffer (pH 8.0) by sonication.

Purification of the recombinant PBPPRA0520 protein

The cell-free extract was loaded onto a Ni-NTA agarose (Qiagen) column (1 × 3 cm), and the enzyme was eluted with a stepwise gradient of imidazole (step 1, 10 mM; step 2, 20 mM and step 3, 250 mM) in 50 mM sodium phosphate buffer (pH 8.0) containing 0.3 M NaCl. The purified enzyme was desalted using an Amicon Ultra-4 10k (Millipore, Billerica, MA) at the corresponding sites, generating the plasmid pET30b-PBPPRA0520 encoding the PBPPRA0520 protein with the His6 sequence added to its C-terminal end. Next, the pET30b-PBPPRA0520 were transformed into E. coli BL21 (DE3), and the resulting transformants were incubated in Luria broth medium containing 0.05 mg/mL kanamycin at 37°C until the optical density at 600 nm reached 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to give a final concentration of 1 mM, and the cultures were incubated for 24 h at 25°C. The PBPPRA0520 protein expressed was extracted from the wet cells (1 g) in 3 mL of 50 mM sodium phosphate buffer (pH 8.0) by sonication.
sequence of the purified recombinant PBPRA0520 protein was determined using a Procise 494 HT protein sequencing system (Applied Biosystems). MALDI-TOF MS spectra of the PBPRA0520 protein (Matrix: 3,5-dimethoxy-4-hydroxycinnamic acid) were recorded in a linear mode using a Voyager-DE STR (Applied Biosystems). Thioredoxin (m/z 11,674.48), apomyoglobin (m/z 16,952.56) and aldolase (m/z 39,212.28) were used for external calibration.

Enzyme assay

CG2–CG5 activity was determined by measuring the increase in glucose concentration during hydrolysis. The enzymatic reaction was routinely performed in 50 mM sodium phosphate buffer (pH 7.0) at 37°C. Periodically, a portion of the reaction mixture was boiled for 5 min to inactivate the enzyme, and the concentrations of glucose and CG2–CG5 were quantified by HPIC using a CarboPac PA1 column (2 × 250 mm, Dionex, Sunnyvale, CA) equipped with a pulsed amperometric detector (ICS-3000, Dionex). Chromatography was performed using a linear gradient of 0–100% 0.2 M sodium acetate–water (78:22, v/v) at a flow rate of 0.25 mL/min.

(GlcN)n (n = 2–5) hydrolysis was determined by measuring the increase in GlcN during saccharide hydrolysis. The enzymatic reaction was performed in 50 mM sodium phosphate buffer (pH 7.0) containing various concentrations of (GlcN)n at 37°C. At the appropriate reaction time, aliquots of the reaction mixture were withdrawn and mixed with 0.25 M NH3 solution. This solution was immediately boiled for 5 min to inactivate the enzyme. (GlcN)n (n = 2–5) hydrolysis was monitored by HPLC using a Shodex Asahipak NH2-50 4E column (4.6 × 250 mm, Showa Denko, Japan) and eluted with acetonitrile–water (78:22, v/v) at a flow rate of 1.0 mL/min at 30°C. The products were monitored using a charged aerosol detector (Corona CAD, Esa, MA).

Hydrolysis of Glc-PNP and GlcN-PNP was determined by measuring the increase in the rate of PNP released at 400 nm after addition of an equal volume of 1 M Na2CO3 to the reaction mixture.

Effect of pH and temperature on enzymatic activity

Enzymatic activity was measured by hydrolysis of 1.3 mM GlcN-PNP at 37°C, while the pH of the reaction mixture was varied. The buffer systems used were sodium acetate (pH 4.2–5.7), sodium phosphate (pH 6.6–8.0) and 3-cyclohexylaminopropanesulfonic acid (pH 9.9–10.2). The final pH values of the reaction solutions were determined after adding the enzyme and the substrates. The optimum temperature for enzymatic activity was determined using 50 mM sodium phosphate buffer (pH 7.0).

Analysis of the products

The reaction products from various disaccharides except (GlcN)n were separated by TLC on a silica gel 60 F254 plate (5.0 × 7.5 cm; Merck) with a solvent system of acetonitrile–water (4:1, v/v). Sugars were detected by baking after dipping the plate in 5% sulfuric acid in methanol. For the analysis of the reaction mixture of (GlcN)n hydrolysis, the product was analyzed using a solvent system of 1-propanol–28% ammonia solution (2:1, v/v) (Côté et al. 2006). Chitooligosaccharides were detected by baking the plate after dipping in ninhydrin reagent. When necessary, the amounts of the products were quantified by HPIC and HPLC as described in the Enzyme assay. The amounts of reducing sugar liberated during polysaccharide hydrolysis by the enzyme were determined by the modified Schales procedure or the Somogyi–Nelson method using α-glucose or α-glucosamine as the standard (Nelson 1944; Somogyi 1952; Imoto and Yagishita 1971).

Analysis of the anomeric form of the products

The anomic form of the hydrolytic product from (GlcN)4 (12.4 mM) was determined using 1H-NMR spectrometer (ECX-400P; JEOL, Tokyo, Japan). The enzymatic reaction was performed in 10 mM sodium phosphate buffer (pH 7.0) at 26°C with an enzyme concentration of 0.5 μM. After addition of the enzyme solution, the NMR tube was immediately loaded into the NMR probe. Accumulation of 1H-NMR spectra started at the appropriate reaction time. The accumulation of data required 1.5 min.

Kinetic analysis

To determine the apparent kinetic parameters of cellooligosaccharides [CG2–CG5] and chitooligosaccharides [(GlcN)−(GlcN)n], these substrates were subjected to hydrolysis in 50 mM sodium phosphate buffer (pH 7.0) at 37°C. The initial hydrolytic rates were measured as the increase in α-glucose or α-glucosamine by HPIC and HPLC as described in the Enzyme assay. The kinetic parameters were calculated over the substrate concentration range of 0.2–3Km with the Michaelis–Menten equation by the curve-fitting method using Kaleidagraph™ ver. 3.51 (Synergy Software, Reading, PA).

Homology-modeled structure

The modeled three-dimensional structure of PBPRA0520 protein was constructed using the 3D-jigsaw program (Bates et al. 2001). The three-dimensional structure of VP2484 protein was used as a template.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Funding

This work was supported in part by a grant-in-aid for Young Scientists (B), Ministry of Education, Culture, Sports, Science, and Technology, Japan (22780095, to Y.H.) and in part by a grant from Elizabeth Arnold Fuji Foundation.

Acknowledgements

We are grateful to Dr. Nobuaki Ishida for his technical advice on NMR spectroscopy, Dr. Shinya Fushinobu for useful advice on structural analysis and Mr. Takuya Hiroi and Mr. Takashi Kanazawa for their technical assistance during the course of this study.
Conflict of interest statement
None declared.

Abbreviations
CAZy, Carbohydrate-Active EnZymes; CG2, cellobiose; CGn, cellooligosaccharide with n degrees of polymerization; GH, glycoside hydrolase; GlcN, α-glucosamine; (GlcN)n, chito-oligosaccharide with n degrees of polymerization; GlcNAc, N-acetyl-α-glucosamine; GlcN-PNP, 4-nitrophenyl-β-D-glucoside; Glc-N-acetyl-β-D-glucosaminide; Glc-PNP, 4-nitrophenyl-β-D-glucoside; HPIC, high-performance ion-exchange chromatography; HPLC, high-performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NMR, nuclear magnetic resonance; PNP, 4-nitrophenol; PTS, phosphotransferase system; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

References


