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Molecular Design and Synthesis of a Novel Substrate for Assaying Lysozyme Activity

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Abstract: A novel substrate {Galβ1,4GlcNAcβ1,4GlcNAc-β-*p*NP [Gal(GlcNAc)$_2$-β-*p*NP]} for assaying lysozyme activity has been designed using docking simulations and enzymatic synthesis via β-1,4-galactosyltransferase-mediated transglycosylation from UDP-Gal as the donor to (GlcNAc)$_2$-β-*p*NP as the acceptor. Hydrolysis of the synthesized Gal(GlcNAc)$_2$-β-*p*NP and related compounds using hen egg-white lysozyme (HEWL) demonstrated that the substrate was specifically cleaved to Gal(GlcNAc)$_2$ and *p*-nitrophenol (*p*NP). A combination of kinetic studies and docking simulation was further conducted to elucidate the mode of substrate binding. The results demonstrate that Gal(GlcNAc)$_2$-β-*p*NP selectively binds to a subsite of lysozyme to liberate the Gal(GlcNAc)$_2$ and *p*NP products. The work therefore describes a new colorimetric method for quantifying lysozyme on the basis of the determination of *p*NP liberated from the substrate.

Key words: lysozyme, enzyme assay, kinetics, chitin oligosaccharide derivatives, enzymatic synthesis

INTRODUCTION

Lysozyme (EC 3.2.1.17) classified as a member of glycoside hydrolase family 22 hydrolyzes bacterial cell wall polysaccharides resulting in bacterial lysis.[1)2)] The most common method for measuring the enzymatic activity of lysozyme is the cell turbidimetric assay using the cell wall of *Micrococcus lysodeikticus* as a substrate.[3)4)5)] However, the lytic action of lysozyme on *M. lysodeikticus* is not a suitable assay system for physical-organic chemical studies. As alternatives, several activity measuring methods using structurally defined chitin oligosaccharide derivatives linked to chromophores, such as *p*NP and 4-methylumbelliferyl groups, have also been reported.[6)7)8)] Although these substrates have the advantage of being able to easily and highly sensitively discriminate between the presence or absence of enzyme activity based on the rate of liberation of the chromophoric moiety, they are subject to multiple cleavages in the presence of lysozyme.[7)8)9)] Consequently, kinetic measurements using these substrates are laborious and cleavage often occurs at more than one point, making it necessary to dissect the overall rates into the rates for the individual processes. We have recently reported the synthesis of the substrate {4^4-*O*-β-D-galactosyl-β-tri-*N*-acetylchitotriosyl 2-acetamide-2,3-dideoxy-gluc-2-enopyranose [Gal(GlcNAc)$_3$DGN]} for lysozyme, which binds to the –3 to +2 subsites of HEWL and is hydrolyzed between Gal(GlcNAc)$_2$ and GlcNAc-DGN.[10)] As a result, an assay method for quantifying lysozyme was established which utilizes the Morgan-Elson reaction based on the generation of product DGN (2-acetamide-2,3-dideoxy-gluc-2-enopyranose), formed from Gal(GlcNAc)$_3$DGN by lysozyme through a conjugated reaction involving β-*N*-acetylhexosaminidase. Our objective in this study was to design a novel substrate for assaying lysozyme activity that liberates only a chromophore, such as *p*NP, thereby simplifying the assay system.

Here, we describe the design and synthesis of an end-modified β-D-galactosyl chitobiose derivative [Gal(GlcNAc)$_2$-β-*p*NP] with a chromophore starting from (GlcNAc)$_2$-β-*p*NP and using enzymatic modification. The mechanism of hydrolytic action and kinetics of HEWL with Gal(GlcNAc)$_2$-β-*p*NP were then analyzed. This study is the first report of a labelled chitin oligosaccharide derivative from which lysozyme liberates only a chromophore without multiple cleavages.

RESULTS AND DISCUSSION

Molecular design and enzymatic synthesis of Gal(GlcNAc)$_2$-β-pNP.

HEWL is known to have six subsites for sugar residue binding which correspond to hexa-*N*-acetyl-chitohexaose and these are termed –4 to +2.[11)12)13)] The enzyme cleaves the glycosidic linkage between the sugar residues at subsites –1 and +1 with the aid of Glu-35 and Asp-52, which are acidic amino acid residues.[14)15)] Nanjo *et al*. have reported that when lysozyme acts on (GlcNAc)$_3$-β-*p*NP, multiple

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Abbreviations: Gal(GlcNAc)$_2$-β-*p*NP, Galβ1,4GlcNAcβ1,4GlcNAc-β-*p*NP; HEWL, hen egg-white lysozyme; *p*NP, *p*-nitrophenol; DGN, 2-acetamide-2,3-dideoxy-gluc-2-enopyranose; β4GalTI, β-1,4-galactosyltransferase I; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

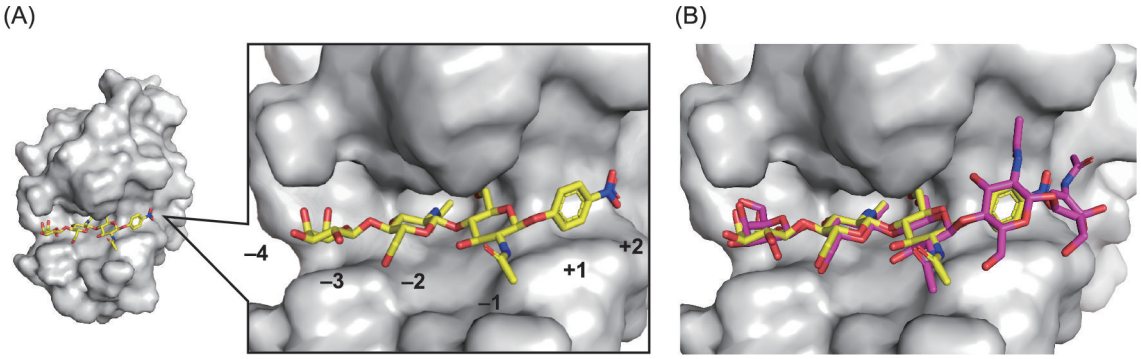

Fig. 1. (A) Position of Gal(GlcNAc)$_2$-β-*p*NP within the HEWL binding site, (B) The structure of Gal(GlcNAc)$_3$DGN in the HEWL binding site is shown in pink, and the structure of Gal(GlcNAc)$_2$-β-*p*NP is shown in yellow.

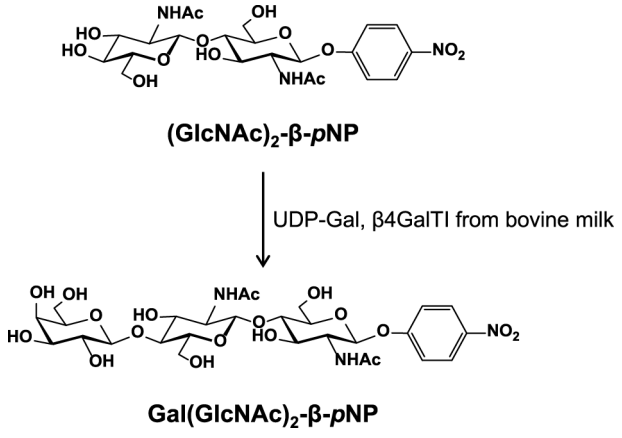

Fig. 2. Synthesis of Gal(GlcNAc)$_2$-β-*p*NP using β4GalTI.

cleavage reactions occur and a small amount of *p*NP is released.[7] In addition, we have recently reported that an end-modified galactosyl chitotetraose derivative [Gal(GlcNAc)$_3$DGN] binds to the –3 to +2 subsites of lysozyme in a structurally selective manner.[10] In the interaction of HEWL and chitin oligosaccharide, the C1,3-diaxial C-H hydrogens of the –3 GlcNAc residue have a face-to-face stacking interaction with the indole side chain of Trp-62, but the 2-*N*-acetyl group of the –3 GlcNAc residue has little involvement in such stacking interactions. Therefore, the Gal residues of Gal(GlcNAc)$_3$DGN with the C1,3-diaxial C-H hydrogens and without the 2-*N*-acetyl group preferentially binds to the –3 subsites. Based on these findings, we hypothesized that the novel substrate "Gal(GlcNAc)$_2$-β-*p*NP" would selectively bind to the –3 to +1 subsite of lysozyme and release only *p*NP. Initially, to evaluate the binding of Gal(GlcNAc)$_2$-β-*p*NP to the HEWL binding site, we performed docking simulations.[16] Furthermore, we compared the interactions in the Gal(GlcNAc)$_2$-β-*p*NP-HEWL complex with those in the Gal(GlcNAc)$_3$DGN-HEWL complex.[10] Figure 1A shows the docking of Gal(GlcNAc)$_2$-β-*p*NP to lysozyme, and Fig. 1B shows a superimposition of the Gal(GlcNAc)$_2$-β-*p*NP and Gal(GlcNAc)$_3$DGN substrates bound to lysozyme. We confirmed that docked Gal(GlcNAc)$_2$-β-*p*NP-HEWL has a similar binding mode to that of Gal(GlcNAc)$_3$DGN-HEWL in the –3 to +1 subsites. The results therefore demonstrate that Gal(GlcNAc)$_2$-β-*p*NP has the potential to be a novel substrate for measuring lysozyme activity.

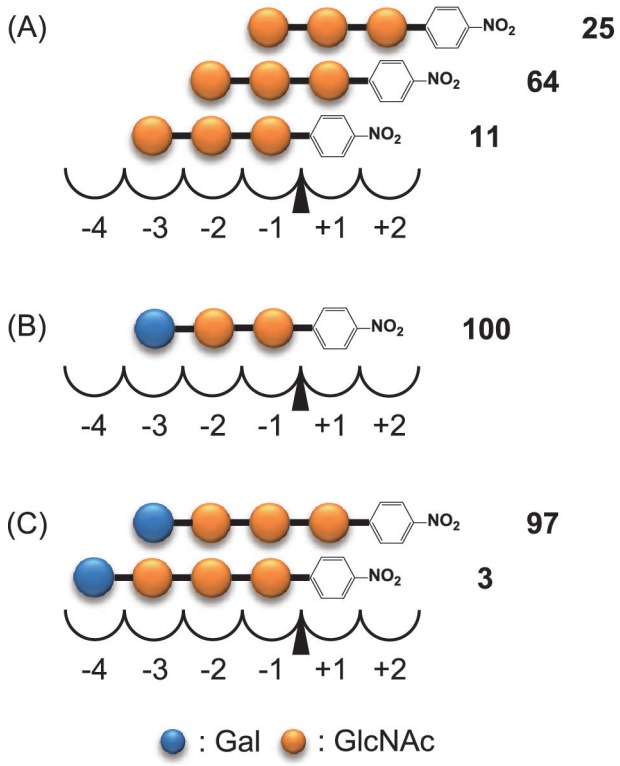

Fig. 3. The frequency of HEWL-catalyzed cleavage of glycosidic linkages of (A) (GlcNAc)$_3$-β-*p*NP, (B) Gal(GlcNAc)$_2$-β-*p*NP and (C) Gal(GlcNAc)$_3$-β-*p*NP.

The frequency of enzymatic attack on the glycosidic linkages of each substrate were estimated from the rates of product formation as shown in Fig. S3; (see Supplementary material). The numerical values represent the frequency of cleavage of the glycosidic linkages.

Subsequently, synthesis of Gal(GlcNAc)$_2$-β-*p*NP was performed based on the above design. The synthesis of Gal(GlcNAc)$_2$-β-*p*NP was achieved by galactosylation at the 4-position of the non-reducing terminal GlcNAc using (GlcNAc)$_2$-β-*p*NP as a acceptor, UDP-Gal as a donor and β-1,4-galactosyltransferase I (β4GalTI) from bovine milk as an enzyme source. As shown in Fig. 2, the reaction was regioselective and performed in one step. The time course of the enzymatic-transglycosylation reaction is shown in Fig. S1 (see Supplementary material), and was determined by high performance liquid chromatography (HPLC) quantification of the reaction products. The reaction mixture was easily purified by ODS column chromatography to

Table 1. Kinetic parameters of HEWL.

Substrates	K_m (mM)	V_{max} (mM/min)	k_{cat} (1/s)	k_{cat}/K_m (1/mM·s)	Relative ratio
$(GlcNAc)_3$-β-*p*NP	0.64	1.0×10^{-3}	9.6×10^{-5}	1.5×10^{-4}	1
$Gal(GlcNAc)_2$-β-*p*NP	0.30	2.7×10^{-4}	2.6×10^{-5}	8.4×10^{-5}	0.56

give $Gal(GlcNAc)_2$-β-*p*NP (yield of 50.9 % based on the amount of acceptor added). The structure of $Gal(GlcNAc)_2$-β-*p*NP was confirmed by ^1H and ^{13}C NMR analyses in D_2O solution (Figs. S2A and S2B; see Supplementary material) and also by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass analysis, as described in the experimental section. In addition, it is known that the substrate solubility in water is an important factor for enzyme activity measurement.[17] The solubility of the synthesized $Gal(GlcNAc)_2$-β-*p*NP in aqueous solution (> 18 mM) was greater than that of $(GlcNAc)_3$-β-*p*NP (> 8 mM), which has been shown to be a useful substrate for lysozyme assays.

Action of lysozyme on Gal(GlcNAc)$_2$-β-pNP.

(1) Reaction product and bond cleavage frequencies.

Initially, we examined the frequency of glycoside bond cleavage mediated by lysozyme using $Gal(GlcNAc)_2$-β-*p*NP and the reference substrate $(GlcNAc)_3$-β-*p*NP. The amount of each product formed at an early stage (within the first 15 % of total hydrolysis) from the initial substrate during incubation with lysozyme was analyzed by HPLC (Figs. S3A and S3B; see Supplementary material). Based on these data, the frequency of lysozyme-catalyzed cleavage of each glycosidic linkage in $Gal(GlcNAc)_2$-β-*p*NP and $(GlcNAc)_3$-β-*p*NP was determined based on the subsite structure of the enzyme (Fig. 3). Each substrate demonstrated a completely different binding pattern with respect to the –4 to +2 sugar-binding subsites of lysozyme. As reported previously, lysozyme hydrolysed $(GlcNAc)_3$-β-*p*NP at three cleavage positions; at the first (bond 1), second (bond 2), and third glycosidic bonds (bond 3) from the *p*NP residue in a ratio of 11:64:25, respectively (Fig. 3A). In contrast, the novel substrate $Gal(GlcNAc)_2$-β-*p*NP is cleaved exclusively at bond 1 to liberate $Gal(GlcNAc)_2$ and *p*NP (Figs. 3B and S4; see Supplementary material). This finding is in good agreement with the docking simulation data of $Gal(GlcNAc)_2$-β-*p*NP bound to HEWL (Fig. 1). The relative rates of hydrolysis of $Gal(GlcNAc)_2$-β-*p*NP compared with the reference compound $(GlcNAc)_3$-β-*p*NP (100) was 27 (Table 1). Therefore, although $Gal(GlcNAc)_2$-β-*p*NP has substituted the non-reducing end with galactose residues, it still acts as a substrate. In contrast to $Gal(GlcNAc)_2$-β-*p*NP, $Gal(GlcNAc)_3$-β-*p*NP synthesized as a comparison showed multiple cleavages at the first and second bonds (bonds 1 and 2) in a ratio of 3:97, respectively (Fig. 3C). These results indicate that terminal galactose residues of the *p*NP derivative of β-D-galactosyl chitin oligosaccharide can bind to the –4 and –3 subsites (and *p*NP is not bond to –1). Therefore, incubation of lysozyme with $Gal(GlcNAc)_2$-β-*p*NP liberates only the chromophore without multiple cleavages by enzyme.

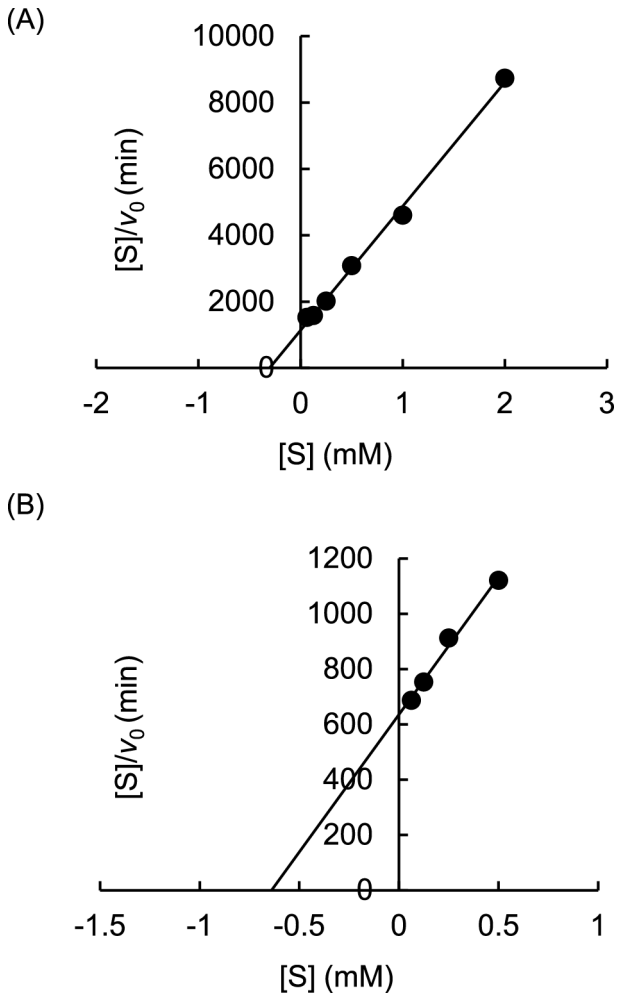

Fig. 4. Hanes-Woolf plots for the hydrolysis of (A) $Gal(GlcNAc)_2$-β-*p*NP and (B) $(GlcNAc)_3$-β-*p*NP as substrate for HEWL.

(2) Kinetic studies.

Next, the kinetic parameters for the lysozyme mediated hydrolysis of two substrates [$Gal(GlcNAc)_2$-β-*p*NP and $(GlcNAc)_3$-β-*p*NP] were determined (Fig. 4 and Table 1). The results show that the affinity for $Gal(GlcNAc)_2$-β-*p*NP (K_m = 0.30 mM) is 2.1-fold higher than that for $(GlcNAc)_3$-β-*p*NP (K_m = 0.64 mM). However, the k_{cat}/K_m value for $Gal(GlcNAc)_2$-β-*p*NP is 1.8-fold lower than that for $(GlcNAc)_3$-β-*p*NP (Table 1). Therefore, the catalytic efficiency for the novel substrate is slightly lower relative to the conventional *p*NP derivative. These results show that the terminal 4-*O*-substituted Gal residue of $Gal(GlcNAc)_2$-β-*p*NP does not significantly prohibit the hydrolysis reaction and can function as a substrate for activity measurement.

Lysozyme assay system using Gal(GlcNAc)$_2$-β-pNP.

Based on these results, an analytical procedure for lyso-

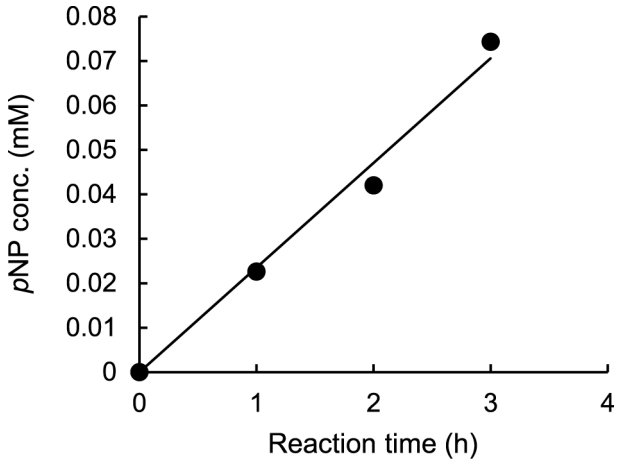

Fig. 5. A reaction mixture (20 μL) containing 1 mM Gal(GlcNAc)$_2$-β-*p*NP and 100 μg of HEWL in 20 mM sodium acetate buffer (pH 5.0) was incubated at 37 °C. The color intensity of *p*NP was measured as described in the text.

zyme activity using Gal(GlcNAc)$_2$-β-*p*NP as a substrate. The conditions for the lysozyme assay were established as follows. A reaction mixture (20 μL) containing 1 mM Gal(GlcNAc)$_2$-β-*p*NP and 100 μg of HEWL in 20 mM sodium acetate buffer (pH 5.0) was incubated for several hours at 37 ºC. After inactivation of the reaction by addition of 36 μL of 1 M Na_2CO_3, the liberated *p*NP was quantified spectrophotometrically at 405 nm. As the results, Gal(GlcNAc)$_2$-β-*p*NP gave a linear relationship between color intensity of the liberated *p*NP with time (Fig. 5). In addition, liberation of *p*NP was not observed without HEWL. One unit of activity was defined as the amount of enzyme required to liberate 1 μmol of *p*NP per min.

In conclusion, we have established a facile method for synthesis of a novel substrate useful for routine microgram assay of lysozyme. Of particular note is the establishment of lysozyme activity measurements that liberate only *p*NP from the oligosaccharide substrate which leads to simplification of the assay.

EXPERIMENTAL

Materials. (GlcNAc)$_2$-β-*p*NP and (GlcNAc)$_3$-β-*p*NP were kindly provided by Yaizu Suisan Kagaku Industry Co., Ltd. (Shizuoka, Japan). β4GalTI from bovine milk was purchased from Sigma-Aldrich (St. Louis, MO, USA). UDP-Gal was a gift from Yamasa Co. (Chiba, Japan). HEWL, which was recrystallized six times, was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). All other reagents were of the highest quality commercially available and were used without further purification.

Analytical methods. The HPLC system consisted of a Unison UK-C18 (4.6 × 250 mm; Imtakt Corp., Kyoto, Japan), a JASCO Intelligent System Liquid Chromatograph (Jasco Corp., Tokyo, Japan), and detection at 300 nm. The bound material was eluted in 20 % methanol at a flow rate of 0.75 mL/min at 40 ºC. MALDI-TOF mass spectra were acquired using an AutoFlex spectrometer (Bruker Daltonics GmbH, Bremen, Germany) in positive reflection mode with 20 kV ion acceleration and without post acceleration. The spectra were recorded using a detector voltage of 1.65 kV and averaged from at least 300 individual laser shots. A solution of 65 mM 2,5-dihydroxybenzoic acid in 30 % ethanol was used as the matrix. Samples were dissolved in water and mixed with the matrix (1:4 v/v). Each mixture (1 μL) was spotted onto a stainless steel platform and allowed to crystallize at room temperature. The spectrometer was calibrated with peptide calibration standard II (Bruker Daltonics). 500 MHz ^{1}H NMR spectra and 125 MHz ^{13}C NMR spectra were recorded using a JEOL ECX-500 II spectrometer (Jeol Ltd., Tokyo, Japan). Chemical shifts were expressed in ppm relative to the methyl resonance of the external standard sodium 3-(trimethylsilyl)propionate. HEWL concentration was calculated from a standard curve by measuring the absorbance at 280 nm.

Docking simulations. Docking simulations were performed for evaluating the binding region of Gal(GlcNAc)$_2$-β-*p*NP against HEWL using the AutoDockVina 1.1.2 program.[11] The crystal structure of the HEWL (accession code: 4HP0) complex with ligand 4-*O*-β-tri-*N*-acetylchitotriosyl moranoline was obtained from PDB.[18] The ligand 4-*O*-β-tri-*N*-acetylchitotriosyl moranoline was removed using the PyMOL Molecular Graphic System 2.0.2 (www.pymol.org/) before initiating docking simulations. Ligand [Gal(GlcNAc)$_2$-β-*p*NP] was edited from the crystal structure of lysozyme in complex with Glcβ1,4Glcβ1,4Glc-β-*p*NP (accession code: 3VOI).[19]All 3D structures of ligands were obtained with Avogadro 1.2.0 and geometry optimization was performed by the MOPAC AM1 method[20] using MOPAC 2016 (http://OpenMOPAC.net). Water molecules were removed, and polar hydrogen atoms added to the HEWL and Gal(GlcNAc)$_2$-β-*p*NP using AutoDockTools-1.5.6rc3. A grid box with a size of 36(x) × 18(y) × 16(z) was centered on the active site of the enzyme. The exhaustiveness parameter was set to 50 (default 8). The docked structure of Gal(GlcNAc)$_2$-β-*p*NP with HEWL was displayed using the PyMOL program and compared with the docked structure of Gal(GlcNAc)$_3$DGN with HEWL.

Enzymatic synthesis of Gal(GlcNAc)$_2$-β-pNP using β4GalTI. (GlcNAc)$_2$-β-*p*NP (22 mg, 0.040 mmol) and UDP-Gal (610 mg, 0.082 mmol) were first dissolved in a solution that contained 4.08 mL of 50 mM MOPS buffer (pH 7.4) containing 8 mM $MnCl_2$ and 0.01 % (w/v) NaN_3, and then 200 mU (0.1 mL) of β4GalTI was added. The mixture was incubated for 24 h at 37 ºC, and the reaction terminated by boiling for 5 min. The supernatant was isolated by centrifugation (8,000 × G, 10 min) and then loaded onto an ODS column (1.5 × 30 cm) equilibrated with 25 % methanol at a flow rate of 1.5 mL/min and a fraction size of 20 mL. The absorbance of the eluate was monitored at 210, 260, and 300 nm. An aliquot from pooled fractions 37–40 was concentrated by evaporation and lyophilized. Gal(GlcNAc)$_2$-β-*p*NP was obtained in a yield of 50.9 % (14.4 mg) relative to the amount of acceptor. MALDI-TOF mass: *m/z* 730.357 $[M + Na]^+$ (calcd for $C_{28}H_{41}N_3NaO_{18}$, 730.228); ^{1}H NMR (D_2O, 500 MHz) δ: 8.14 (d, 2H, $J_{m,o}$ 9.0 Hz, *m*-Ph proton), 7.07 (d, 2H, $J_{o,m}$ 9.0 Hz, *o*-Ph proton), 5.21 (d, 1H, $J_{1,2}$ 8.5 Hz, H-1), 4.54 (d, 1H, $J_{1',2'}$ 8.0 Hz,

H-1′), 4.37 (d, 1H, $J_{1'',2''}$ 8.0 Hz, H-1′′), 3.97 (dd, 1H, $J_{1,2}$ 8.5, $J_{2,3}$ 10.5 Hz, H-2), 3.91-3.55 (16H, H-3, H-4, H-5, H-6a, H-6b, H-2′, H-3′, H-4′, H-5′, H-6′a, H-6′b, H-3′′, H-4′′, H-5′′, H-6′′a, H-6′′b), 3.44 (dd, 1H, $J_{1'',2''}$ 8.0, $J_{2'',3''}$ 10 Hz, H-2′′), 1.98 (s, 3H, CH_3CO-′), 1.92 (s, 3H, CH_3CO-). ^{13}C NMR (D_2O, 125 MHz) δ: 174.7 ($CH_3\underline{C}ONH$-′), 174.5 ($CH_3\underline{C}ONH$-), 161.7 (*p*-Ph carbon), 142.7 (Ph carbon attached to the phenolic oxygen), 126.2 (*m*-Ph carbon × 2), 116.5 (*o*-Ph carbon × 2), 103.0 (C-1′′), 101.5 (C-1'), 98.5 (C-1), 79.0 (C-4), 78.3 (C-4′), 75.4 (C-5′′), 75.0 and 74.9 (C-5, C-5′), 72.6 (C-3′′), 72.2 and 72.1 (C-3, C-3′), 71.0 (C-2′′), 68.6 (C-4′′), 61.1 (C-6′′), 60.0 and 59.9 (C-6, C-6′), 55.2 (C-2′), 54.8 (C-2), 22.2 ($\underline{C}H_3CONH$-′), 22.1 ($\underline{C}H_3CONH$-).

Cleavage frequencies and kinetic studies. Incubations with chitin oligosaccharide derivatives as substrates (0.0625–2 mM) were performed at 37 ºC in 200 µL of 20 mM sodium acetate buffer (pH 5.0). Reactions were initiated by the addition of HEWL (35 nmol). Samples (20 µL) were taken at 1 h intervals (0, 0.5, 1, 2, 3, 4, 5 and 6 h) during the incubation, and inactivated by addition of 180 µL of H_2O and boiling. The amount of product formation was determined by HPLC. The quantity of product was calculated from the peak areas on the chromatogram. The amount of each product increased linearly with time during the initial stage (i.e. within the first 15 % of hydrolysis) of the reaction. On the basis of these data, the frequency of lysozyme-catalyzed cleavage of glycosidic linkages was determined. By measuring the amount of products as described above, the initial velocities (υ) were also obtained directly from the initial slopes of the time-course plots of the reaction. Various substrate concentrations (0.0625–2 mM) were used in these experiments. The parameters of Michaelis-Menten type kinetics were evaluated by Hanes-Woolf plots ([S]/υ *versus* [S]) and the least-squares method.

Colorimetric lysozyme assay system using Gal(GlcNAc)$_2$-β-pNP. A reaction mixture (20 µL) containing 1 mM Gal(GlcNAc)$_2$-β-*p*NP and 100 µg of HEWL in 20 mM sodium acetate buffer (pH 5.0) was incubated at 37 ºC. A quarter of the reaction mixture was removed at 1 h intervals and then immediately transferred to a microplate containing 36 µL of 1 M Na_2CO_3 to stop the reaction. The amount of *p*NP liberated was determined by measuring the absorbance at 405 nm using a microplate reader (Corona Grating Microplate Reader SH-9000 Lab, Corona Electric Co., Ltd., Ibaraki, Japan).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

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