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Enzymatic Polymerization to an Alternating *N*-Phthaloyl Chitin Derivative Catalyzed by Chitinase

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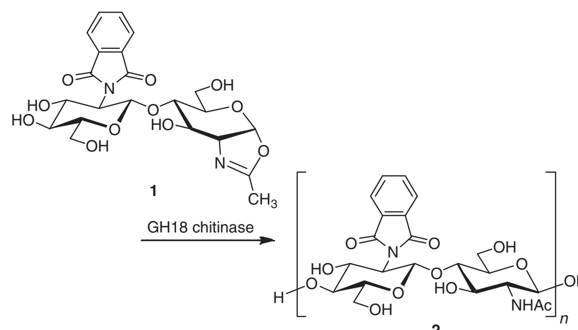
A chitin derivative with an alternating *N*-phthaloyl group was prepared via chitinase-catalyzed polymerization. A chitobiose oxazoline derivative with a *N*-phthaloyl group at the C2′ position was designed as a transition-state-analog substrate monomer for chitinase belonging to the glycoside hydrolase family 18. The monomer was successfully polymerized by the enzyme catalysis, providing the corresponding chitin derivative. This implies that the recognition at the C2 position of the substrate by the family 18 chitinase in the −2 and +1 subsites is quite loose.

Chitin is a well-known natural polysaccharide composed of $\beta(1 \rightarrow 4)$-linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc), which is one of the most abundant natural products produced by animals, bacteria, and fungi.[1] Chitin and its deacetylated form of chitosan have been extensively studied as biomaterials due to the nature of chitin and chitosan, such as biodegradability, biocompatibility, and immunological activities.[1,2] In order to produce high-performance biomaterials from chitin and chitosan, they are often modified chemically.[3] Particularly, their acylation and phthaloylation are frequently carried out to give solubility in organic solvents, which is essential for further modifications of chitin and chitosan.[3]

Chitinase-catalyzed polymerizations of sugar oxazoline derivatives have been performed to provide a wide spectrum of chitin derivatives.[4] In these polymerizations, the disaccharide oxazoline monomers bearing a variety of functional groups at the C2 position of the nonreducing end pyranose (the C2′) were successfully polymerized by chitinases belonging to the glycoside hydrolase family 18 (GH18);[5] synthetic chitin, cellulose–chitin hybrid, chitin–chitosan hybrid, and alternatingly *N*-sulfonated chitin derivative have been prepared from the monomer with NHAc, OH, NH_2, and $NHSO_3Na$ groups at C2′. The pyranosides with these functional groups are placed in at least the −2 and +1 subsites[6] of the enzyme, therefore these results strongly suggest that the GH18 chitinases have potential to recognize various C2-functionalized pyranosides at the −2 and +1 subsites and to exhibit catalysis for such substrates. In this study we demonstrate the GH18 chitinase-catalyzed polymerization of the sugar oxazoline monomer **1** with a phthalimido group at C2′ (Scheme 1), which is the most sterically bulky and hydrophobic substituent employed in a series of chitinase-catalyzed polymerizations.

Monomer **1** was prepared chemically; details are described in Supporting Information (SI),[12] briefly, glycosylation of readily accessible 2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose[7] with 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl trichloroacetimidate[8] was carried out to give the corresponding disaccharide derivative connecting through β-glycoside. The disaccharide was treated with trimethylsilyl trifluoromethanesulfonate to form an oxazoline ring, followed by removal of all of the *O*-acetyl groups by sodium methoxide in methanol to give **1**.



Scheme 1.

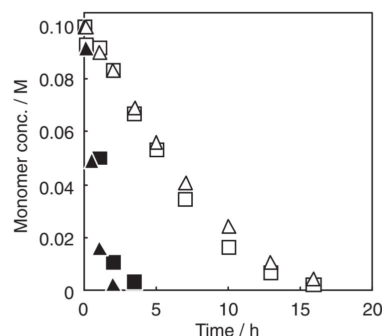


Figure 1. Reaction-time courses of **1** with chitinase from *Bacillus* sp. (▲, pH 7.0; ■, pH 8.0) and without enzyme (△, pH 7.0; □, pH 8.0): In a phosphate buffer (50 mM) at 30 °C; initial concentration of **1**, 0.1 mol L^{-1}, amount of enzyme, 10 wt % for **1**.

Monomer **1** was then subjected to enzymatic polymerization catalyzed by chitinase from *Bacillus* sp. The enzyme is a member of the GH18 chitinases and can polymerize various disaccharide oxazoline derivative monomers.[4] Thus, the enzyme was employed in this study.

Figure 1 shows the time dependence of concentration of **1** with chitinase from *Bacillus* sp. and without enzyme at pH 7.0 and 8.0.[9] In Figure 1, consumption of **1** was dramatically accelerated by the addition of the enzyme in both cases at pH 7.0 and 8.0; the monomer disappeared within 5 h, whereas without enzyme it took over 15 h for complete consumption.[10] This indicates that monomer **1** is recognized and catalyzed by chitinase from *Bacillus* sp., leading to the oxazoline ring-opening. Furthermore, formation of a white precipitate was observed in each reaction mixture containing the enzyme. The supernatant of the mixture at pH 7.0 was then subjected to MALDI-TOF-MS analysis (Figure 2). The peaks with equal distance of *m*/*z* 494, which correspond to molecular mass of the repeating disaccharide unit of **2**, were observed up to *m*/*z* 2491 ($n = 5$) in the spectrum. This clearly indicates the formation of

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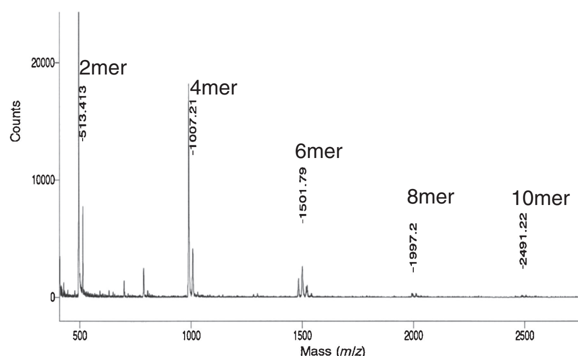

Figure 2. A MALDI-TOF mass spectrum of water-soluble part of **2**. All of the peaks were observed as $[M + H]^+$ form.

Table 1. Polymerization of **1** by chitinase from *Bacillus* sp.[a]

Entry	Enzyme amount[b] /wt %	Temp /°C	[**1**] /mol L^{-1}	Time[c] /h	Yield of **2** (insoluble)[d] /%
1	1	30	0.2	7.0	14 (5)
2	5	30	0.2	3.5	53 (20)
3	10	30	0.2	2.5	87 (40)
4	20	30	0.2	1.5	40 (30)
5	10	20	0.2	3.5	82 (20)
6	10	40	0.2	2.5	69 (20)
7	10	30	0.05	1.5	41 (0)
8	10	30	0.1	2.5	80 (40)

[a]In a phosphate buffer (50 mM) at pH 7.0. [b]Added against the amount of **1**. [c]Indicating the time for complete consumption of **1**. [d]Total yields of water-soluble and -insoluble parts of **2**. The former was determined by HPLC measurements (tetra- and hexasaccharides) and the latter was an isolated yield. In parentheses is shown an isolated yield of the water-insoluble part.

2 by chitinase-catalyzed polymerization of **1**. ^{13}C NMR of the hexasaccharide of **2** further supported the success of the polymerization (details are described in SI[12]). Notably, the polymerization at pH 9.0 also progressed, however, the cyclic imido group of **1** was hydrolyzed to the corresponding phthalamate after addition of buffer due to instability of the phthalimido group under basic conditions, and then the decomposed oxazoline monomer and **1** were "copolymerized," affording a complex mixture. Thus, the following polymerization reactions were carried out at pH 7.0 in order to avoid the structural damage to the product polymer.

The polymerization reaction was optimized at pH 7.0 in terms of enzyme amount, reaction temperature, and monomer concentration (Table 1). In Entries 1–4, amount of the enzyme drastically affected yield of **2**; the yield became higher with increase of the enzyme amount and reached 87% in Entry 3. Further, the excess enzyme caused decrease of the yield (Entry 4), particularly in that of the water-soluble part, which suggests occurrence of hydrolysis of water-soluble oligomers of **2** by the enzyme. Reaction temperature also exerted influence on the yields; those of the water-insoluble portion decreased to 20% at 20 and 40 °C (Entries 5 and 6). Notably, the water-insoluble portion of **2** was not obtained in 0.05 mol L^{-1} of **1** (Entry 7), whereas it recovered drastically to 40% (around 80% in total yields) in 0.1 and 0.2 mol L^{-1} of **1** (Entries 3 and 8). Thus, the best result in terms of total yields was obtained via polymerization at 30 °C in 0.2 mol L^{-1} of **1** with 10 wt % enzyme (Entry 3).

In conclusion, an alternating *N*-phthaloyl chitin derivative was successfully synthesized via chitinase-catalyzed polymerization. To date, a variety of chitin derivatives bearing an alternating C2-substituent have been produced in a series of polymerizations of C2′-substituted disaccharide oxazoline derivative monomers catalyzed by GH18 chitinases.[4] The C2′-substituents introduced to the monomers are acetamido (for synthetic chitin), hydroxy (for cellulose–chitin hybrid), amino (for chitin–chitosan hybrid), and sulfoamino (for alternatingly *N*-sulfonated chitin) groups, which are hydrophilic, and are neutral, cationic, and anionic, respectively. In contrast, monomer **1** has a hydrophobic phthalimido group as the C2′-substituent, however, the enzyme recognized and polymerized it very effectively. Furthermore, the phthalimido group is sterically most bulky among the C2′-substituents of the disaccharide monomers employed for chitinase-catalyzed polymerizations. Therefore, GH18 chitinases are capable of polymerizing disaccharide monomers with a variety of C2′-substituents. It should be pointed out that all of the monomers providing the corresponding polysaccharides via chitinase-catalyzed polymerizations have the C2′-substituents in the equatorial orientation. In contrast, the disaccharide oxazoline monomer of Manβ(1 → 4)GlcNAc-oxazoline (Man: D-mannopyranose), which has an axially oriented hydroxy group at the C2′, cannot serve as a glycosyl donor for GH18 chitinase catalysis at all.[11] Therefore, an equatorially oriented C2′-substituent is essential for successful monomer design for chitinase-catalyzed polymerization. Thus, these provide new insights into the substrate specificities as well as the catalysis mechanism of GH18 chitinases. In the near future we will report synthesis of high-performance chitin derivatives bearing other functional groups at the C2 position.

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