Enzymatic Synthesis of Alternatingly 6-*O*-Carboxymethylated Chitotetraose by Selective Glycosidation with Chitinase Catalysis

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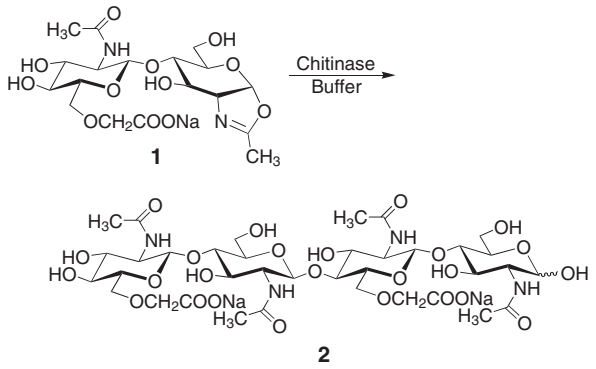
An alternatingly 6-*O*-carboxymethylated chitotetraose derivative was selectively synthesized via enzymatic glycosidation of a 6′-*O*-carboxymethylated chitobiose oxazoline derivative catalyzed by chitinase. The enzyme from *Streptomyces griseus* acted as an efficient catalyst for the reaction, giving rise to *N*,*N*′,*N*″,*N*‴-tetraacetyl-6′,6‴-di-*O*-carboxymethylchitotetraose in a 42% yield.

Chitin is a linear polysaccharide consisting of a $\beta(1 \rightarrow 4)$-linked *N*-acetyl-D-glucosamine (GlcNAc) repeating unit widely found as structural materials in invertebrates such as crabs, insects shells, and squid pens.[1] Biocompatibilities and bioactivities of chitin and its de-*N*-acetylated derivative of chitosan have been reported;[2] immuno-adjuvants, inhibitor of metastases of tumor cells, wound-healing materials, additives for cosmetics, drug carriers, biodegradable polymers, etc. In addition, anionic derivatives of chitin such as sulfonated,[3] phosphorylated,[4] and carboxymethylated (CM) chitins[5] show many attractive bioactivities, for instance, inhibition of virus infections,[6] promotion of blood coagulation,[7] and activation of macrophages.[8] Particularly the ability of CM-chitin to stimulate macrophages is strongly related to the degree of substitution and the position substituted in the GlcNAc unit.[8] The biological activities of CM-chitooligosaccharides, however, have not been well investigated because of the difficulty in preparation of structurally well-defined CM-chitooligosaccharide samples. Therefore, synthesis of such CM-chitooligosaccharides is crucial to elucidate the correlation between the CM group and the bioactivities at a molecular level.

Previously, we reported the regio-selective and stereo-controlled synthesis of chitin via ring-opening polyaddition of an *N*,*N*′-diacetylchitobiose oxazoline derivative catalyzed by chitinase from *Bacillus* sp.[9] The chitinase used is classified into glycoside hydrolase family 18 enzymes,[10] which cleave the $(1 \rightarrow 4)$-β-*N*-acetyl-D-glucosaminide linkage of chitin molecule through a substrate-assisted mechanism[9,11] involving an oxazolinium ion as transition state during the reaction. In this paper, we report a facile and efficient synthesis of *N*,*N*′,*N*″,*N*‴-tetraacetyl-6′,6‴-di-*O*-CM-chitotetraose (**2**) as a structurally well-defined CM-chitooligosaccharide by using *N*,*N*′-diacetyl-6′-*O*-CM-chitobiose oxazoline substrate (**1**) catalyzed by chitinase (Scheme 1).

Compound **1** was synthesized according to our recent report,[12] and subjected to the enzymatic reaction with chitinase from various origins (Table 1).[13] All the commercial enzymes employed here contain family 18 chitinases. The progress of the reaction was monitored by ^{1}H NMR spectroscopy with measuring the doublet signal ($\delta = 6.09$, $J = 5.52$ Hz) of H-1 of **1**.

Without enzyme, concentration of **1** very slowly decreased to 94% after 6 h, with giving the hydrolyzed product (**3**)[14] from



Scheme 1. Chitinase-catalyzed synthesis of regioselectively 6-*O*-carboxymethylated chitotetraose.

Table 1. Enzymatic synthesis of **2** from **1** catalyzed by chitinase from various origins[a]

Entry	[**1**] /M	Origin of Chitinase	pH	Time[b] /h	Yield of **2** /%[c]
1	0.05	*Bacillus* sp.	7.0	4.0	12[d]
2	0.05	*A. hydrophila*	7.0	80	10
3	0.05	*S. marcescence*	7.0	2.0	0
4	0.05	*S. griseus*	7.0	0.67	20
5	0.05	*S. griseus*	9.0	2.5	31
6	0.05	*S. griseus*	10.0	9.0	37
7	0.10	*S. griseus*	10.0	10.0	42
8	0.05	*S. griseus*	11.0	144	30

[a]In carbonate buffer (D_2O, 50 mM) at 30 °C. Amount of the enzyme: 10 wt % for **1**.
[b]Indicating the time for the complete consumption of **1**.
[c]Determined by HPLC measurements.
[d]Total yields of the oligosaccharide mixture.

1 in a 6% yield (data not shown). The present enzymatic reaction was accompanied by the formation of **3** as a hydrolyzed by-product in all cases.

The enzymatic reaction of **1** was performed at pH 7.0 and 30 °C with chitinase from *Bacillus* sp. (Wako Pure Chemicals Inc., Lot No. LDH7046, Entry 1), *Aeromonas hydrophila* (Seikagaku Co., Lot No. DN1187, Entry 2), *Serratia marcescence* (SIGMA, Lot No. 45H4117, Entry 3), and *Streptomyces griseus* (SIGMA, Lot No. 77H4055, Entry 4). The reaction with the enzyme from *Bacillus* sp. mainly gave **2** within 4 h (Entry 1); the product was a mixture of the oligosaccharides from disaccharides to hexasaccharides detected by HPLC. MALDI-TOF/MS analysis of the product showed formation of **2**, a tetrasaccharides with mono-*O*-CM group and hexasaccharides with di- and tri-*O*-CM groups. Furthermore, small amounts of pentasaccharides

and trisaccharides with di-*O*-CM group were also detected on the spectrum. These results suggest that three kinds of reactions occurred; glycosidation of **1** to generate the tetrasaccharide **2**, hydrolysis of **1** to give **3**, and transglycosylation to form oligosaccharide mixtures with rearranged structures from **2**. Chitinase from *A. hydrophila* catalyzed the reaction; however, it took 80 h for the complete consumption of **1**, affording **2** in a 10% yield (Entry 2). When chitinase from *S. marcescence* was used, **1** was rapidly consumed within 2 h (Entry 3). However, the disaccharide derivative **3** was a sole product. In contrast, **1** was consumed within 1 h after the addition of chitinase from *S. griseus*, selectively affording **2** in a 20% yield; the other compound being **3** in 80% (Entry 4). These results indicate that chitinase from *S. griseus* is the most effective and selective enzyme producing **2** at pH 7.0 and 30 °C.

Next, the reactions of **1** with chitinase from *S. griseus* were performed under various pH to examine the optimal pH condition. The reaction at pH 9.0 proceeded smoothly, and **1** was completely consumed after 2.5 h, giving rise to **2** in a 31% yield (Entry 5). Compound **2** was formed within 9 h in a 37% yield at pH 10.0 (Entry 6), and the yield of **2** was improved to 42% by increasing the concentration of **1** (0.10 M; Entry 7). The reaction progressed very slowly at pH 11.0, providing **2** in a 30% yield after 144 h (Entry 8). In all the above reactions with *S. griseus*, compounds detected after the reaction were only two products, **2** and **3**. These observations show that chitinase from *S. griseus* catalyzes the glycosidation reaction of **1** at a pH ranging from 7.0 to 11.0. The optimal pH value is around 10.0, whereas that for the hydrolysis reaction is 5.5–7.0.[15] This trend is close to that in the synthesis of chitin via enzymatic polymerization catalyzed by chitinase from *Bacillus* sp. under weak alkaline conditions like pH 10.6, in which the reaction is the repetition of the regio-selective and stereo-controlled glycosidation of *N*,*N*′-diacetylchitobiose oxazoline derivative.[9] It is to be noted that the hydrolysis of chitin by chitinase catalysis involves a key-step of protonation at the glycosidic bond of chitin which needs at least pH 7 or lower, whereas the glycosidation of **1** or polymerization to chitin involves protonation at the oxazoline-ring which occurs even at a slightly basic conditions (pH 9–11).[9,16] On the other hand, chitinase from *S. griseus* catalyzed the glycosidation of **1** only once, resulting in the selective formation of the tetrasaccharide **2** in good yields. This implies that steric bulkiness and ionic moiety of CM group probably reduce the affinity of **1** and **2** at the active site of the enzyme. Once the glycosidation takes place, product **2** can no longer stay at and leaves from the active site, forming **2** with perfect selectivity. In addition, the chitinase from *S. griseus* may have a small positively numbered subsite, whereas the chitinase from *Bacillus* sp. has a $(-2)(-1)(+1)(+2)(+3)(+4)$-type subsite structure in the active site;[17] however, information about subsite mapping on chitinase from *S. griseus* has not been reported yet. The large positively numbered subsite in the chitinase active site from *Bacillus* sp. is probably responsible for the polymerization of chitin synthesis, where the glycosidation is repeated with chitinase catalysis between the oxazoline donor monomer at (-1) subsite and the acceptor at $(+)$ subsite.

In conclusion, the reaction of chitobiose oxazoline derivative with 6′-CM group **1** was effectively catalyzed by the chitinase from *S. griseus* at a pH ranging from 7.0 to 11.0, selectively affording **2** in good yields. The optimal pH value in this reaction was around 10.0, where **1** acted as a glycosyl donor as well as acceptor. Bioactivities of **2** are now under investigation.

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13 A typical procedure of the reaction: To a solution of **1** (16.2 mg, 33.3 μmol) in a carbonate buffer (333 μL, 50 mM, pH 10.0) was added chitinase from *S. griseus* (0.8 mg) at 30 °C. The mixture was kept standing at 30 °C for 10 h. After the reaction was completed, the enzyme was thermally inactivated at 80 °C for 20 min. Then the mixture was directly subjected to BioGel P−2 column chromatography eluting with distilled water to afford pure **2** (6.2 mg, 6.26 μmol, 19%). ^{1}H NMR (400 MHz, D_2O, methanol): δ 5.20 (0.55H, bs, H-1α), 4.69 (0.45H, bs, H-1β), 4.65 (1H, bd, $J = 7.56$ Hz, H-1″), 4.60 (2H, m, H-1′, H-1‴), 2.12–1.98 (m, 12H, $COCH_3$); ^{13}C NMR (100 MHz, D_2O, acetone): δ 178.71 and 178.35 (COONa), 175.20–175.11 (NHC=O), 102.18–101.67 (C-1′, C-1″, C-1‴), 95.46 (C-1β), 91.00 (C-1α), 22.90–22.54 (CH_3). HRMS-FAB (m/z): M^+ calcd for $C_{36}H_{56}N_4Na_2O_{25}$, 990.3029; found, $[M + H]^+$ 991.3150, $[M + Na]^+$ 1013.2925.
14 This is the oxazoline ring-opened compound through the nucleophilic attack of water molecule to the anomeric carbon atom of **1**, 2-acetamido-4-*O*-(2-acetamido-6-*O*-carboxymethyl-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranose (**3**). ^{1}H NMR (400 MHz, D_2O, acetone): δ 5.20 (0.57H, d, $J = 3.01$ Hz, H-1α), 4.72–4.70 (0.43H, m, H-1β), 4.61 (1H, d, $J = 8.54$ Hz, H-1′), 2.08–2.05 (6H, m, $COCH_3$); ^{13}C NMR (100 MHz, D_2O, acetone): δ 178.73 (COONa), 175.39–175.12 (NHC=O), 102.2–102.18 (C-1′α, C-1′β), 95.47 (C-1β), 91.02 (C-1α), 22.84–22.53 (CH_3). HRMS-FAB (m/z): M^+ calcd for $C_{18}H_{29}N_2NaO_{13}$, 504.1567; found $[M + Na]^+$ 527.1453.
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