doi:10.1246/cl.2012.689
Published on the web June 23, 2012

One-pot Chemoenzymatic Route to Chitoheptaose via Specific Transglycosylation of Chitopentaose–Oxazoline on Chitinase-template

Naoki Yoshida,[1] Tomonari Tanaka,[1,†] Masato Noguchi,[1] Atsushi Kobayashi,[1] Kumiko Ishikura,[1] Tatsuya Ikenuma,[1] Hiromu Seno,[1] Takeshi Watanabe,[2] Michinari Kohri,[1,††] and Shin-ichiro Shoda*[1]
[1]*Department of Biomolecular Engineering, Tohoku University, Aoba, Sendai, Miyagi 980-8579*
[2]*Department of Applied Biological Chemistry, Niigata University, Ikarashi, Niigata 950-2181*

(Received February 29, 2012; CL-120167; E-mail: shoda@poly.che.tohoku.ac.jp)

One-pot regio- and stereospecific synthesis of chitoheptaose ($(GlcNAc)_7$) has been achieved by using chitopentaose ($(GlcNAc)_5$) and chitobiose ($(GlcNAc)_2$) as starting materials. The key intermediate, 1,2-oxazoline derivative of $(GlcNAc)_5$, could be directly prepared in water and successfully transglycosylated to $(GlcNAc)_2$ catalyzed by a mutant chitinase with lower hydrolyzing activity.

Chitoheptaose ($(GlcNAc)_7$), the β-1,4-linked linear heptamer of *N*-acetyl-D-glucosamine (GlcNAc), shows higher elicitor activities, inducing the production of phytoalexin in suspension-cultured rice cells.[1] In spite of strong demand for chitoheptaose, its specific preparation has not been accomplished yet due to the difficulty in controlling degree of polymerization (DP). Chemical and enzymatic degradation of naturally occurring chitin afforded mixtures of chitooligomers having different DPs.[2,3] Enzymatic polycondensation techniques have also been attempted; however, the production of a monodisperse chitooligosaccharide was extremely difficult.[4] Here we report a new chemoenzymatic process for exclusive formation of $(GlcNAc)_7$ starting from commercially available chitopentaose ($(GlcNAc)_5$) and chitobiose ($(GlcNAc)_2$) via 1,2-oxazoline derivative of chitopentaose ($(GlcNAc)_5$-oxa) as synthetic intermediate.

The process was designed based on the retrosynthesis of $(GlcNAc)_7$ by cleaving the second glycosidic bond from the reducing end, which corresponds to the catalytic point of chitinase A1 from *Bacillus circulans* WL-12 (ChiA1), giving rise to $(GlcNAc)_5$-oxa (**1**) and $(GlcNAc)_2$ (**2**) (Figure 1). Based on these analyses, we postulated that a regio- and stereoselective synthesis of $(GlcNAc)_7$ would be possible if both of the substrates **1** and **2** were recognized by ChiA1 at the donor site (from subsite -5 to subsite -1) and the acceptor site (subsites $+1$ and $+2$) as glycosyl donor and acceptor, respectively.

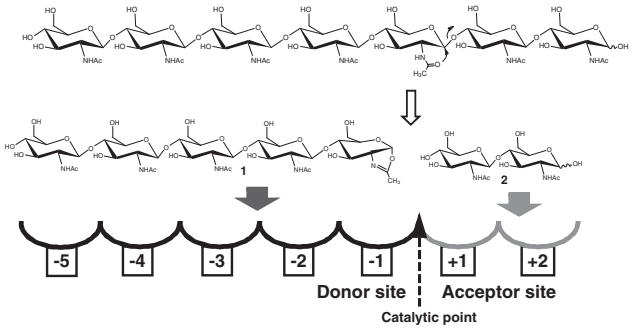
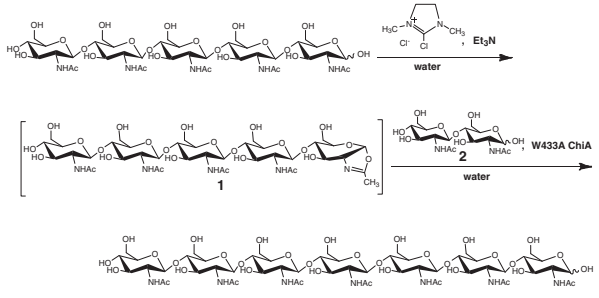


Figure 1. Retrosynthesis of $(GlcNAc)_7$ considering the catalytic point on the chitinase-template. The numbers -5 to $+2$ denote the subsites that recognize each GlcNAc unit of $(GlcNAc)_7$.

In a series of our studies on direct activation of the anomeric carbon atom,[5] we developed a facile method for synthesis of sugar oxazoline derivatives starting from the corresponding unprotected sugars[6] by the intramolecular dehydration reactions in water using 2-chloro-1,3-dimethylimidazolinium chloride (DMC)[7] as a dehydrating agent. According to this method, $(GlcNAc)_5$ can be converted to the corresponding $(GlcNAc)_5$-oxa (**1**) almost quantitatively without protecting the hydroxy groups.

These findings prompted us to develop a one-pot process toward $(GlcNAc)_7$ where both the synthesis of the glycosyl donor **1** and the subsequent transglycosylation can be performed in the same aqueous solution without isolating the synthetic intermediate **1** (Scheme 1). Since sugar oxazolines are known to behave as transition-state-analogue substrates,[8] we postulated that the reaction would proceed smoothly even if the template-protein possesses nearly no enzyme activity,[9] affording a monodisperse $(GlcNAc)_7$ without accompanying hydrolysis of the product. After screening various mutants of ChiA1, we found that the enzyme whose tryptophan residue 433 was changed to an alanine residue (W433A ChiA1) could behave as a template for the addition reaction of **2** to the oxazoline **1** without cleaving any *N*-acetylglucosaminide units in the substrates. The W433 residue is essential for hydrolysis of chitooligomers by distorting the conformation of the GlcNAc unit incorporated into the -1 subsite.[9b]

After investigating the reaction conditions with respect to reaction time, donor/acceptor ratio, DMC concentration, and enzyme concentration, we demonstrated the one-pot synthesis of $(GlcNAc)_7$ as follows.[12] A mixture of $(GlcNAc)_5$ (2.6 mg, 2.5 μmol) and triethylamine (7.8 μL, 56.3 μmol) in water (80 μL)



Scheme 1. One-pot synthesis of $(GlcNAc)_7$ by the addition of $(GlcNAc)_2$ (**2**) to $(GlcNAc)_5$-oxa (**1**) catalyzed by a mutant chitinase, W433A ChiA.

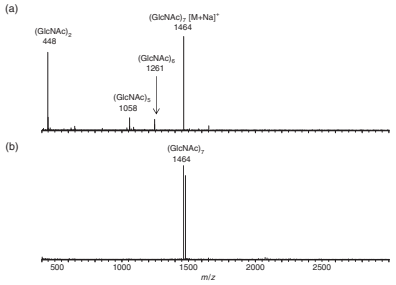


Figure 2. MALDI-TOF MS of the transglycosylated product. (a) Crude reaction mixture. (b) Product after washing with 50% aqueous methanol solution.

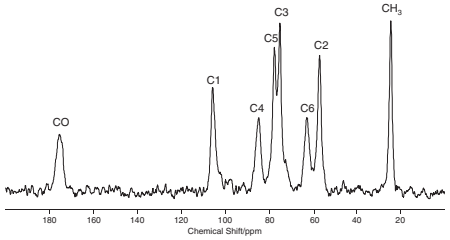


Figure 3. CP/MAS solid ^{13}C NMR (200 MHz) spectrum of $(GlcNAc)_7$ prepared by enzymatic addition reaction of $(GlcNAc)_2$ (**2**) to $(GlcNAc)_5$-oxa (**1**).

was treated with DMC (3.2 mg, 18.8 µmol) for 12 h at 0 °C followed by the addition of the glycosyl acceptor $(GlcNAc)_2$ (1.1 mg, 2.5 µmol) and W433A ChiA1 (10 µg), giving rise to the corresponding transglycosylated product (MALDI-TOF MS shown in Figure 2a). The resulting crude products were then washed with 50% aqueous methanol solution (1.0 mL) three times and dried in vacuo, giving rise to $(GlcNAc)_7$ (1.8 mg, 1.3 µmol, 52%) with higher purity (Figure 2b).

The ^{1}H NMR spectrum of the product showed signals around 4.44 ppm due to the six anomeric protons of internal units.[12] The CP/MAS ^{13}C NMR spectrum of the product showed a signal at 105.7 ppm due to the C1 of the *N*-acetylglucosamine unit (Figure 3).[12] The other signals at 84.7, 77.6, 75.0, 62.7, and 56.9 ppm correspond to the carbons C4, C5, C3, C6, and C2, respectively. No signals derived from the $\beta(1 \rightarrow 6)$[10] and $\beta(1 \rightarrow 3)$[11] glycosidic linkage were observed around 70 and 80 ppm, respectively. These results clearly indicate that the glycosidic bond formation between chitopentaose unit and chitobiose unit occurred in a regio- and stereoselective manner with the inversion of configuration at C1, giving rise to the stereoregular oligosaccharide having a $\beta(1 \rightarrow 4)$ linkage.

This is a completely protection-free process for construction of chitoheptaose where both glycosyl donor preparation and the subsequent enzymatic transglycosylation can be performed in aqueous media without using any organic solvents. The combined use of a transition state analogue substrate and a deactivated enzyme allows an efficient synthesis of $(GlcNAc)_7$ while suppressing hydrolysis of the product.

We thank Dr. Shinichiro Yoshida of Research and Analytical Center for Giant Molecules, Tohoku University for measuring ^{1}H and CP/MAS ^{13}C NMR spectrum. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Industrial Technology Research Grant Program in 2006 from NEDO of Japan and an International Center of Research & Education for Molecular Complex Chemistry in 2007 from TOHOKU UNIVERSITY Global COE Program.

References and Notes

† Present address: Department of Biobased Materials Science, Kyoto Institute of Technology, Matsugasaki, Kyoto 606-8585
†† Present address: Division of Applied Chemistry and Biotechnology, Chiba University, Yayoi-cho, Chiba 263-8522
1 a) A. Yamada, N. Shibuya, O. Kodama, T. Akatsuka, *Biosci., Biotechnol., Biochem.* **1993**, *57*, 405. b) T. Yamaguchi, Y. Maehara, O. Kodama, M. Okada, M. Matsumura, N. Shibuya, *J. Plant Physiol.* **2002**, *159*, 1147.
2 a) J. A. Rupley, *Biochim. Biophys. Acta, Spec. Sect. Mucoproteins Mucopolysaccharides* **1964**, *83*, 245. b) S. Hirano, S. Tsuneyasu, Y. Konodo, *Agric. Biol. Chem.* **1981**, *45*, 1335.
3 a) A. Ohtakara, M. Mitsutomi, in Proceedings of the 2nd International Conference on Chitin and Chitosan, ed. by S. Hirano, S. Tokura, The Japan Society of Chitin and Chitosan, **1982**, pp. 117–121. b) W.-J. Jung, A. Souleimanov, R.-D. Park, D. L. Smith, *Carbohydr. Polym.* **2007**, *67*, 256.
4 a) A. Masaki, T. Fukamizo, A. Otakara, T. Torikata, K. Hayashi, T. Imoto, *J. Biochem.* **1981**, *90*, 527. b) T. Usui, Y. Hayashi, F. Nanjo, K. Sakai, Y. Ishido, *Biochim. Biophys. Acta, Gen. Subj.* **1987**, *923*, 302. c) T. Usui, H. Matsui, K. Isobe, *Carbohydr. Res.* **1990**, *203*, 65. d) K. Akiyama, K. Kawazu, A. Kobayashi, *Carbohydr. Res.* **1995**, *279*, 151. e) T. Hattori, Y. Sakabe, M. Ogata, K. Michishita, H. Dohra, H. Kawagishi, K. Totani, M. Nikaido, T. Nakamura, H. Koshino, T. Usui, *Carbohydr. Res.* **2012**, *347*, 16.
5 a) J.-i. Kadokawa, M. Mito, S. Takahashi, M. Noguchi, S.-i. Shoda, *Heterocycles* **2004**, *63*, 1531. b) T. Tanaka, M. Noguchi, A. Kobayashi, S.-i. Shoda, *Chem. Commun.* **2008**, 2016. c) T. Tanaka, W. C. Huang, M. Noguchi, A. Kobayashi, S.-i. Shoda, *Tetrahedron Lett.* **2009**, *50*, 2154. d) T. Tanaka, T. Matsumoto, M. Noguchi, A. Kobayashi, S.-i. Shoda, *Chem. Lett.* **2009**, *38*, 458. e) T. Tanaka, H. Nagai, M. Noguchi, A. Kobayashi, S.-i. Shoda, *Chem. Commun.* **2009**, 3378. f) T. Tanaka, M. Noguchi, K. Watanabe, T. Misawa, M. Ishihara, A. Kobayashi, S.-i. Shoda, *Org. Biomol. Chem.* **2010**, *8*, 5126. g) N. Yoshida, M. Noguchi, T. Tanaka, T. Matsumoto, N. Aida, M. Ishihara, A. Kobayashi, S.-i. Shoda, *Chem.—Asian J.* **2011**, *6*, 1876.
6 M. Noguchi, T. Tanaka, H. Gyakushi, A. Kobayashi, S.-i. Shoda, *J. Org. Chem.* **2009**, *74*, 2210.
7 H. B. Koenig, S. Wilfried, H. D. Cologne, K. G. Metzger, Ger. Offen. DE 2104579, **1972**; H. B. Koenig, S. Wilfried, H. D. Cologne, K. G. Metzger, *Chem. Abstr.* **1972**, *77*, 140048.
8 For example: a) S. Kobayashi, T. Kiyosada, S.-i. Shoda, *J. Am. Chem. Soc.* **1996**, *118*, 13113. b) S. Kobayashi, H. Morii, R. Itoh, S. Kimura, M. Ohmae, *J. Am. Chem. Soc.* **2001**, *123*, 11825. c) S.-i. Shoda, M. Fujita, C. Lohavisavapanichi, Y. Misawa, K. Ushizaki, Y. Tawata, M. Kuriyama, M. Kohri, H. Kuwata, T. Watanabe, *Helv. Chim. Acta* **2002**, *85*, 3919. d) S. Kobayashi, S.-i. Fujikawa, M. Ohmae, *J. Am. Chem. Soc.* **2003**, *125*, 14357. e) S.-i. Shoda, M. Kohri, A. Kobayashi, in *Biocatalysis: Chemistry and Biology*, ed. by A. Trincone, Research Signpost, Kerala, **2005**, pp. 83–98. f) S. Kobayashi, M. Ohmae, H. Ochiai, S.-i. Fujikawa, *Chem.—Eur. J.* **2006**, *12*, 5962. g) M. Kohri, A. Kobayashi, M. Noguchi, S.-i. Kawaida, T. Watanabe, S.-i. Shoda, *Holzforschung* **2006**, *60*, 485.
9 a) T. Watanabe, K. Suzuki, W. Oyanagi, K. Ohnishi, H. Tanaka, *J. Biol. Chem.* **1990**, *265*, 15659. b) T. Watanabe, Y. Ariga, U. Sato, T. Toratani, M. Hashimoto, N. Nikaidou, Y. Kezuka, T. Nonaka, J. Sugiyama, *Biochem. J.* **2003**, *376*, 237.
10 J. Defaye, A. Gadelle, C. Pedersen, *Carbohydr. Res.* **1989**, *186*, 177.
11 G. M. Lipkind, A. S. Shashkov, Y. A. Knirel, E. V. Vinogradov, N. K. Kochetkov, *Carbohydr. Res.* **1988**, *175*, 59.
12 Supporting Information is available electronically on the CSJ-Journal Web site, http//www.csj.jp/journals/chem-lett/index.html.

