Effect of Fluorine Substituent on the Chitinase-catalyzed Polymerization of Sugar Oxazoline Derivatives

Akira Makino,[1] Junji Sakamoto,[1] Masashi Ohmae,[1] and Shiro Kobayashi*[1,2]
[1]*Department of Materials Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 615-8510*
[2]*R&D Center for Bio-based Materials, Kyoto Institute of Technology, Kyoto 606-8585*

(Received October 24, 2005; CL-051339; E-mail: kobayash@kit.ac.jp)

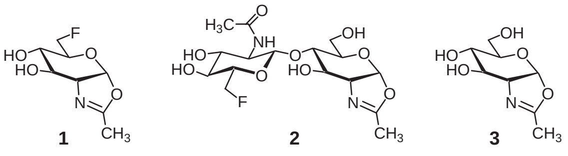
6-Deoxy-6-fluoro sugar oxazoline derivatives were synthesized and evaluated their polymerizabilities with the catalysis of chitinase. The polymerizability of an *N*-acetyl-6-deoxy-6-fluoroglucosamine oxazoline was lower than that of non-fluorinated *N*-acetylglucosamine oxazoline, only providing the ring-opened hydrolyzate. The disaccharide oxazoline derivative of *N*,*N*′-diacetyl-6′-deoxy-6′-fluorochitobiose exhibited lower polymerizability with the enzyme compared to that of the non-fluorinated *N*,*N*′-diacetylchitobiose oxazoline, providing the corresponding fluorinated chitin derivative in good yields within 4 h. These results suggest that the fluorine atom-incorporation reduced the reactivity of the oxazolines.

Fluorine atom is frequently introduced to natural products to investigate a number of biological events.[1] Particularly in the study of carbohydrate-associated enzymes, due to similar size of fluorine atom to oxygen atom (covalent radius: F = 0.64 Å, O = 0.66 Å),[2] fluorine-incorporated carbohydrates are used as synthetic substrates for enzymatic reactions.[3] Furthermore, the characteristic of fluorine atom (strong electronegativity) is quite useful to reveal biological activities by comparing with those of non-fluorinated, natural substrates.

We have demonstrated various chitinase-catalyzed polymerizations of the sugar oxazolines.[4–8] However, importance of the hydroxy groups on the sugar oxazoline units has not been well discussed in chitinase-catalyzed reactions. This study first demonstrates the effect of fluorine substituent in the sugar oxazolines on chitinase-catalyzed polymerizations.

Two kinds of 6-deoxyfluorinated sugar oxazoline derivatives (**1** and **2**) were designed. The monosaccharide derivative **1**[9] was synthesized via 6 step reactions starting from *N*-acetylglucosamine (GlcNAc).[10] The disaccharide derivative **2**[11] was prepared from GlcNAc via 24 step reactions.[12]

Compound **1** was subjected to enzymatic polymerization in a phosphate buffer catalyzed by chitinase from *Bacillus* sp., and the catalytic behavior was examined, comparing with a reference reaction of a non-fluorinated GlcNAc oxazoline derivative **3** (Scheme 1, Figure 1).[13] Without enzyme, compound **1** was decomposed to give the hydrolyzed product in a little slower rate compared to that of **3**. With chitinase, consumption rate of **1** was accelerated but significantly lower than that of **3**. This enzyme having (−2)(−1)(+1)(+2)(+3)(+4)-type subsite in the active site[14] produces mainly chitobiose, a disaccharide, as a smallest hydrolysis product.



Scheme 1.

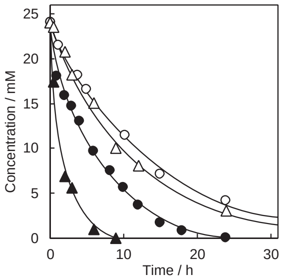
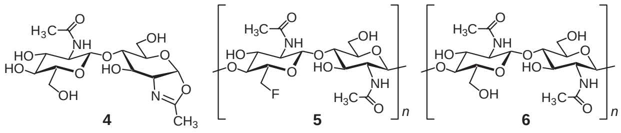


Figure 1. Reaction time-courses of **1** (circles) and **3** (triangles) with chitinase from *Bacillus* sp. (black symbols) and without enzyme (white symbols) at pH 7.7.

However, these monosaccharide monomers were recognized at the −1 subsite due to the oxazoline structure, which enhances the affinity to the −1 subsite. The initial rate of their concentration changes were estimated to be -5.0×10^{-7} $\mathrm{mol{\cdot}L^{-1}{\cdot}s^{-1}}$ (for **1** without enzyme), -6.4×10^{-7} $\mathrm{mol{\cdot}L^{-1}{\cdot}s^{-1}}$ (for **3** without enzyme), -1.8×10^{-6} $\mathrm{mol{\cdot}L^{-1}{\cdot}s^{-1}}$ (for **1** with chitinase), and -3.3×10^{-6} $\mathrm{mol{\cdot}L^{-1}{\cdot}s^{-1}}$ (for **3** with chitinase). Therefore, the fluorine-incorporation contributed a little to the stability of **1** for the hydrolysis, but it reduced more the recognizability of **1** to the −1 subsite of chitinase, leading to drastic reduction of the reactivity of **1**. Furthermore, no oligomeric products were produced during the enzymatic reaction of **1**, whereas **3** provided the corresponding chitooligosaccharides in good yields.[15] These results clearly indicate that **1** and/or its hydrolyzed ring-opened compounds could not be placed at the +1 subsite, which is essential for the glycosidic bond-formation by the catalytic center existing between the −1 subsite and the +1 subsite; in contrast, **3** and/or its hydrolyzed ring-opened compounds could be placed at the +1 subsite.

Polymerization behavior of **2** was examined in a phosphate buffer by comparing with that of a non-fluorinated *N*,*N*′-diacetylchitobiose oxazoline **4** (Figure 2).[16] Without enzyme, compound **2** was decomposed very slowly to give the hydrolyzed product in a similar rate to that of **4**, indicating that the fluorine atom incorporated at the C6′ hardly affected the stability of the oxazoline. With chitinase catalysis, consumption of **2** was remarkably accelerated but slower than that of **4**, which was consumed more rapidly. The initial rate of the concentration changes was estimated to be -2.2×10^{-7} $\mathrm{mol{\cdot}L^{-1}{\cdot}s^{-1}}$ (for **2** without enzyme), -2.5×10^{-7} $\mathrm{mol{\cdot}L^{-1}{\cdot}s^{-1}}$ (for **4** without enzyme), -7.1×10^{-6} $\mathrm{mol{\cdot}L^{-1}{\cdot}s^{-1}}$ (for **2** with chitinase), and -2.1×10^{-5} $\mathrm{mol{\cdot}L^{-1}{\cdot}s^{-1}}$ (for **4** with chitinase). Therefore, the fluorine-incorporation at the C6′ did not affect the stability of



Scheme 2.

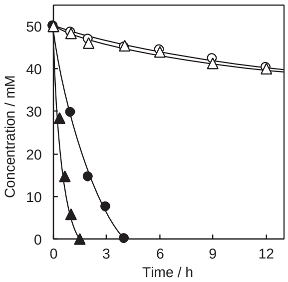


Figure 2. Reaction time-courses of **2** (circles) and **4** (triangles) with chitinase from *Bacillus* sp. (black symbols) and without enzyme (white symbols) at pH 9.0.

the oxazoline, but it reduced a little the recognizability of the sugar oxazoline to the active site of the enzyme, particularly to the −2 subsite. During the enzymatic reactions of **2** and **4**, white precipitates were formed in both reaction mixtures. IR spectrometry and size exclusion chromatography (SEC) confirmed that these precipitates were the corresponding alternating 6-deoxyfluorochitin derivative **5** and synthetic chitin **6**, respectively (Scheme 2).[17] Thus, compound **2** could serve as a transition state analogue substrate (TSAS) monomer[4,5] for the enzyme. This also indicates that TSAS monomer **2** and/or its hydrolyzed derivatives could act as a glycosyl acceptor placed at the +1–+2 subsites.

Notably, monomer **2** with lower initial consumption rate (-7.1×10^{-6} mol·L^{-1}·s^{-1}) provided polymer **5** in a higher yield of 81% compared to that of synthetic chitin **6** (65%) from **4** with higher initial consumption rate (-2.1×10^{-5} mol·L^{-1}·s^{-1}). It was reported that synthetic chitin was produced quantitatively from **4** under higher pH conditions (10.6), where hydrolysis activity of chitinase from *Bacillus* sp. was suppressed.[5] Therefore, the enzyme will maintain hydrolysis activity for monomer **4** and the growing polymer **6** at pH 9.0, resulting in reduction of the yield of **6**. In contrast, monomer **2** and product polymer **5** have 6-deoxyfluorine structures, which will make their enzymatic hydrolysis harder, leading to increased yield of **5** at pH 9.0.

In conclusion, incorporation of a fluorine atom into the oxazoline derivative elevated the stability for hydrolysis of the oxazolines, but it decreased the recognizability of the oxazolines to chitinase. The effect of the fluorine substituent at the −1 subsite was investigated by using monomer **1**; however, in general, monosaccharide oxazoline was not well recognized by the enzyme. Further, chitinase-catalyzed reaction of **2** indicated the recognition of **2** at the −2 subsite and provided a white precipitate of an alternatingly 6-fluorinated chitin derivative **5**. Compound **1** did not induce a glycosylation with chitinase catalysis, which is due to its low recognizability with monosaccharide structure at the −1 subsite; the enzyme produces a chitobiose disaccharide as a minimum hydrolysis product. On the other hand, the disaccharide compound **2** produced the fluorinated chitin **5**. These results clearly indicate that to be a recognizable substrate for chitinase as TSAS monomer, the disaccharide structure is essential.

References and Notes

1 B. E. Smart, *Characteristics of C-F Systems*, in *Organofluorine Chemistry: Principles and Commercial Applications*, ed. by R. E. Banks, B. E. Smart, J. C. Tatlow, Plenum Press, New York, **1994**, p. 57.

2 S. Kobayashi, *J. Polym. Sci., Polym. Chem. Ed.* **2005**, *43*, 693.

3 M. C. T. Hartman, J. K. Coward, *J. Am. Chem. Soc.* **2002**, *124*, 10036.

4 a) S. Kobayashi, H. Uyama, S. Kimura, *Chem. Rev.* **2001**, *101*, 3793. b) S. Kobayashi, H. Uyama, M. Ohmae, *Bull. Chem. Soc. Jpn.* **2001**, *74*, 613. c) S. Kobayashi, S.-I. Shoda, H. Uyama, *Adv. Polym. Sci.* **1995**, *121*, 1. d) S. Kobayashi, J. Sakamoto, S. Kimura, *Prog. Polym. Sci.* **2001**, *26*, 1525.

5 S. Kobayashi, T. Kiyosada, S.-I. Shoda, *J. Am. Chem. Soc.* **1996**, *118*, 13113.

6 J. Sakamoto, S. Kobayashi, *Chem. Lett.* **2004**, *33*, 698.

7 H. Ochiai, M. Ohmae, S. Kobayashi, *Chem. Lett.* **2004**, *33*, 694.

8 H. Ochiai, M. Ohmae, S. Kobayashi, *Carbohydr. Res.* **2004**, *339*, 2769.

9 2-Methyl-4,5-dihydro-[1,2,6-trideoxy-6-fluoro-α-D-glucopyranoso][2,1-*d*]-1,3-oxazole (**1**). ^{1}H NMR (D_2O): δ 6.05 (1H, d, $J_{1,2} = 7.52$ Hz, H-1), 4.60 (1H, ddd, $J_{5,6a} = 2.00$ Hz, $J_{6a,6b} = 10.52$ Hz, $J_{6a,F} = 47.18$ Hz, H-6a), 4.56 (1H, ddd, $J_{5,6b} = 4.52$ Hz, $J_{6a,6b} = 10.52$ Hz, $J_{6b,F} = 47.18$ Hz, H-6b), 4.10 (1H, m, H-2), 3.95 (1H, t, $J_{2,3} = J_{3,4} = 3.26$ Hz, H-3), 3.67 (1H, dd, $J_{3,4} = 3.26$ Hz, $J_{4,5} = 9.30$ Hz, H-4), 3.52 (1H, dddd, $J_{5,6a} = 2.00$ Hz, $J_{5,6b} = 4.52$ Hz, $J_{4,5} = 9.04$ Hz, $J_{5,F} = 24.61$ Hz, H-5), 2.00 (3H, s, CH_3 of oxazoline); ^{19}F NMR (D_2O): δ −231.90 (dt, $J_{5,F} = 26.32$ Hz, $J_{6a,F} = J_{6b,F} = 48.88$ Hz, F-6); HRMS (FAB) *m/z*: calcd for $C_8H_{12}FNO_4$ $[M + H]^+$ 206.0829, found 206.0830.

10 A. Makino, M. Ohmae, S. Kobayashi, *Polym. Prepr. Jpn.* **2001**, *50*, 998.

11 2-Methyl-4,5-dihydro-[4-*O*-(2-acetamido-2,6-dideoxy-6-fluoro-β-D-glucopyranosyl)-1,2-dideoxy-α-glucopyranoso][2,1-*d*]-1,3-oxazole (**2**). ^{1}H NMR (D_2O): δ 6.09 (1H, d, $J_{1,2} = 7.04$ Hz, H-1), 4.81 (1H, s, H-6a′), 4.72 (1H, s, H-6b′), 4.62 (1H, d, $J_{1',2'} = 8.52$ Hz, H-1′), 4.42 (1H, s, H-3), 4.21 (1H, m, H-2), 3.73–3.30 (8H, m), 2.07 (3H, s, $COCH_3$), 2.06 (3H, s, CH_3 of oxazoline); ^{13}C NMR (D_2O): δ 174.75 ($\mathit{C}OCH_3$), 168.67 ($N{=}\mathit{C}(CH_3)O$), 103.51 (C-1′), 100.08 (C-1), 82.44 (d, $J_{6',F} = 168.25$ Hz, C-6′), 79.33 (C-4), 74.96 (d, $J_{5,F} = 19.56$ Hz, C-5′), 73.66 (C-3′), 71.22 (C-3), 69.05 (C-5), 68.89 (d, $J_{4',F} = 5.87$ Hz, C-4′), 65.54 (C-2), 62.10 (C-6), 56.09 (C-2′), 22.41 ($CO\mathit{C}H_3$), 13.21 (CH_3 of oxazoline); ^{19}F NMR (D_2O): δ −235.91 (dt, $J_{5',F'} = 25.19$ Hz, $J_{6a',F'} = J_{6b',F'} = 48.13$ Hz, F-6′); HRMS (FAB) *m/z*: calcd for $C_{16}H_{25}FN_2O_9$ $[M + H]^+$ 409.1622, found 409.1622.

12 A. Makino, J. Sakamoto, M. Ohmae, S. Kobayashi, *Polym. Prepr. Jpn.* **2003**, *52*, 1049.

13 A typical procedure of the reaction: To a solution of **1** (0.98 mg, 4.8 µmol) in a phosphate buffer (200 µL, 10 mM, pH 7.7) was added chitinase from *Bacillus* sp. (0.10 mg) at 30 °C. The mixture was kept standing at 30 °C for 24 h, then the enzyme was thermally inactivated at 90 °C for 5 min. The mixture was analyzed by size exclusion chromatography (SEC) with Shodex SUGAR KS-802 column eluting with distilled water at 80 °C.

14 C. Sasaki, A. Yokoyama, Y. Itoh, M. Hashimoto, T. Watanabe, T. Fukamizo, *J. Biochem.* **2002**, *131*, 557.

15 Yields of the oligosaccharides: total 35% (dimer 24%, trimer 10%, and tetramer 1%). See also: T. Kiyosada, Ph. D. Dissertation, Tohoku University (1998), Chap. 2.

16 A typical procedure of the reaction: To a solution of **2** (4.09 mg, 10 µmol) in a phosphate buffer (200 µL, 10 mM, pH 9.0) was added chitinase from *Bacillus* sp. (0.20 mg) at 40 °C. The mixture was kept standing at 40 °C for 4 h followed by thermal inactivation of the enzyme at 90 °C for 5 min. A white precipitate was separated by centrifugation as a water-insoluble part of **5** (2.21 mg, 54%), and the supernatant was analyzed by HPLC to determine the yield of a water-soluble part of **5** (27%).

17 IR spectroscopic data: 3480–3440 cm^{-1}, OH stretching; 3270–3260 cm^{-1}, NH stretching; 2960–2878 cm^{-1}, CH stretching; 1660–1650, C=O stretchning (amido I); 1560–1550 cm^{-1}, NH bending vibration (amido II). M_n of the solid **5** was determined to be 1380 (mainly 6 saccharide units) by SEC with Tosoh α-3000 column eluting with 0.6 mol·L^{-1} LiCl-NMP/DMAc (2:1, v/v) calibrated by chitooligosaccharides (M_n = 424, 628, 831, 1034, and 1237) and pullulan standards (M_n = 5900 and 11800). MALDI-TOF/MS analysis showed **5** connected up to 10 saccharide units.

Published on the web (Advance View) December 27, 2005; DOI 10.1246/cl.2006.160