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Enzymatic Synthesis of an Alternatingly 6-Fluorinated Chitin Derivative Catalyzed by Chitinase

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Alternatingly 6-fluorinated chitin derivative (**2**) was successfully synthesized by chitinase-catalyzed regio- and stereoselective ring-opening polyaddition of the *N,N'*-di-acetyl-6'-deoxy-6'-fluoro-chitobiose oxazoline derivative (**1**) as a transition state analogue substrate monomer. Compound **1** was synthesized via 23 step reactions by using a glucosamine hydrochloride as a starting material. **1** was catalyzed by the chitinase, giving rise to **2** as a white crystalline product in good yields. MALDI-TOF mass spectrum of the water-soluble part of **2** showed signals separated by 408 *m/z*, which corresponds to the molecular weight of the repeating unit of **2**. The X-ray diffractogram of the water-insoluble part of **2** indicated the characteristic peaks at 9.4, 19.2, 20.7 and 25.7 degrees. The values well agreed with those of synthetic chitin prepared through enzymatic polymerization. This result suggests that the product polysaccharide **2** has a crystalline structure similar to synthetic chitin. The optimal yield of **2** was obtained at pH 9.0 and at 30-40°C.

Key words: enzymatic polymerization, fluorinated chitin derivative, chitinase, transition state analogue

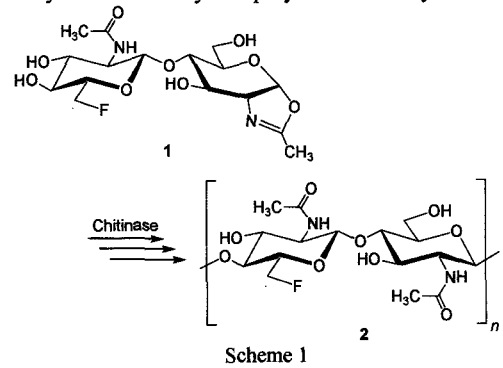
1. INTRODUCTION

Chitin is a linear homopolysaccharide consisting of *N*-acetyl-D-glucosamine (GlcNAc) connecting through β(1→4)-glycosidic linkages, widely found in nature such as in shells of insects and crabs, squid pens and fungi.[1] Chitin and the de-*N*-acetyleted derivative of chitosan are well-known as biocompatible and biodegradable substances.[2] In addition, they have many attractive bioactivities, for instance, antibacterial[3] and immuno-adjuvant[4] activities. Therefore, they are frequently used as materials for food supplements,[5] additives for cosmetics,[6] agriculture,[7] medical and pharmaceutical sciences.[8]

Fluorinated polysaccharide derivatives have been synthesized as fluorinated cellulose derivatives[9] and fluorinated chitin derivatives.[10] The former have been prepared by using partially acetylated cellulose derivatives with varying degree of substitution as starting materials followed by removal of the acetyl protecting groups. These compounds have characteristic features derived from the fluorine atom such as water and oil repellency, and permeability of gases.[11] The latter have been synthesized through direct fluorination of chitin polymer using diethylaminosulfur trifluoride (DAST) as a fluorinating agent. The degree of fluorination of the product ranged from 0.38 to 0.74 due to the reaction time. They also evaluated the cytotoxicity of the fluorinated chitin derivatives, which gave good cell viability in human and mouse fibroblasts cell cultures.

Enzymatic polymerization utilizing glycoside hydrolases as catalysts is an effective method to produce many kinds of natural and unnatural polysaccharides through a single step reaction,[12] for instance, synthetic cellulose by cellulase,[13] synthetic chitin by chitinase[14] and synthetic hyaluronan by hyaluronidase,[15] which belong to natural polysaccharides. Especially, synthetic chitin and synthetic hyaluronan have been generated by regio- and stereoselective ring-opening polyaddition of an *N,N'*-di-acetyl-chitobiose oxazoline derivative and an *N*-acetyl-hyalobiuronate oxazoline derivative, respectively as transition state analogue substrate monomers for the enzymes. In addition, synthesis of alternatingly 6-*O*-methyl-cellulose by cellulase[16] and cellulose-xylan hybrid polysaccharide by xylanase[17] have been achieved by this method, employing newly designed carbohydrate monomers of a 6-*O*-methyl-β-D-cellobiosyl fluoride and a β-D-xylopyranosyl-(1→4)-β-D-glucopyranosyl fluoride, respectively.

Herein, we report the chitinase-catalyzed synthesis of an alternatingly 6-fluorinated chitin derivative (**2**) by using an *N,N'*-di-acetyl-6'-deoxy-6'-fluoro-chitobiose oxazoline derivative (**1**) as a novel transition state analogue substrate monomer (Scheme 1). This type of chitin derivative is expected to be a new material with a well-defined structure for investigating the bioactivities of fluorinated chitin derivatives as well as for applying as a raw material for many fields of sciences such as medicine, pharmaceutics, enzymology, crystallography, carbohydrate chemistry and polymer chemistry.

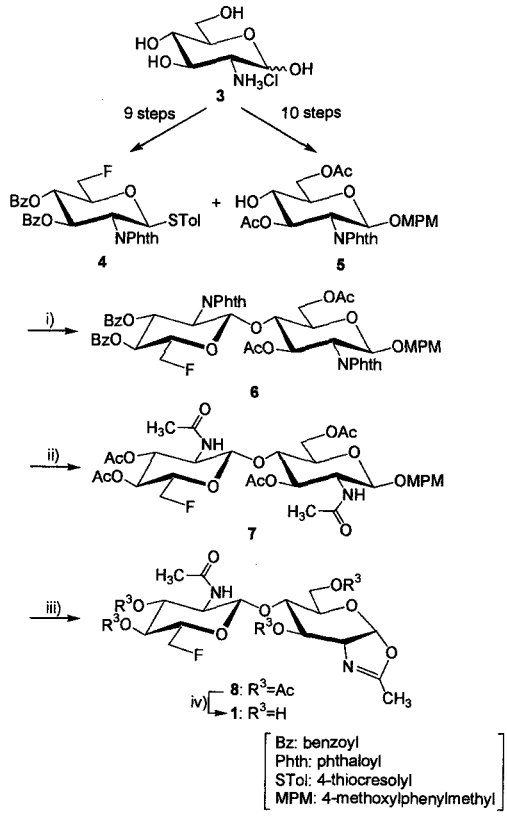


Scheme 1

2. RESULTS AND DISCUSSION

2.1 Monomer synthesis.

The disaccharide monomer **1** was prepared according to the reactions outlined in Scheme 2. The glycosyl donor (**4**) and acceptor (**5**) were prepared from glucosamine hydrochloride (**3**) via 9 and 10 step reactions, respectively. Compound **4** was glycosidated with **5** by using NIS-AgOTf as a promoter in the presence of activated powdery molecular sieves AW-300 to afford **6**. *O*-Acetyl and *O*-benzoyl groups of **6** were removed by sodium methoxide in methanol, and then *N*-phthaloyl groups were removed by hydrazine monohydrate in *N,N*-dimethylformamide followed by acetylation by acetic anhydride in pyridine to give **7**. The MPM group was hydrogenated by palladium hydroxide on activated carbon followed by acetylation to provide a peracetylated derivative with the 6'-fluorine group. Formation of an oxazoline ring was performed using TMSOTf in 1,2-dichloroethane to afford **8**. All *O*-acetyl groups of **8** were then removed by sodium methoxide to give **1**.



Scheme 2. i) NIS-AgOTf / CH_2Cl_2, -40°C then rt, 5h, 71%, ii) CH_3ONa / CH_3OH, rt, 0.5h, hydrazine monohydrate / DMF, 80°C, 15h, then acetic anhydride / pyridine, rt, 10h, 79% (3 steps), iii) H_2, $Pd(OH)_2$ / $CHCl_3$-CH_3OH, rt, 5h then acetic anhydride / pyridine, rt, 3h, TMSOTf / CH_2Cl_2, 50°C, 6h, 81% (3 steps), iv) CH_3ONa / CH_3OH, rt, 0.5h, quant.

2.3 Enzymatic polymerization of **1**.

Figure 1 shows the reaction-time courses of **1** by the enzyme in phosphate buffer (10 mM, pH 10.5) at 30°C. Concentration of **1** decreased faster by the addition of chitinase than without addition of the enzyme. This result indicates that **1** can serve as a good substrate for the enzyme. After the reaction was completed, a white precipitate was formed in the reaction mixture in case of enzyme addition. In the reaction without enzyme, a hydrolyzed product with oxazoline ring-opened structure derived from **1**, 2-acetamido-2,6-di-deoxy-6-fluoro-β-D-glucopyranpsyl-(1→4)-2-acetamido-2-deoxy-D-glucopy ranose was produced.

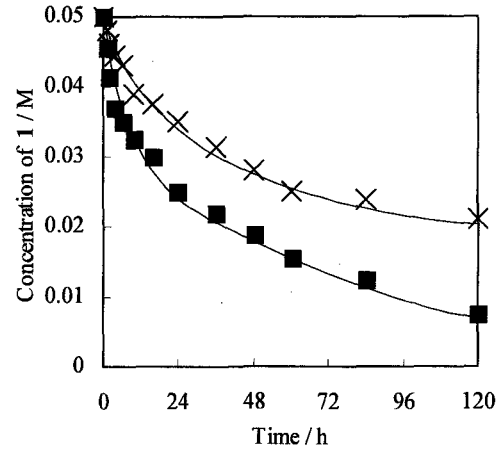


Fig. 1 Reaction-time courses of **1** with chitinase (■) and without enzyme (×).

The precipitate was separated by centrifugation and rinsed with distilled water. The supernatant and washings were combined, and then analyzed by HPLC and MALDI-TOF/MS. The precipitate was subjected to elemental analysis and X-ray diffraction analysis. Figure 2 illustrates the MALDI-TOF mass spectrum of the water-soluble part of the product. All signals observed were separated by 408 *m/z*, the value of which corresponds to the molecular mass of the repeating disaccharide unit of **2**.

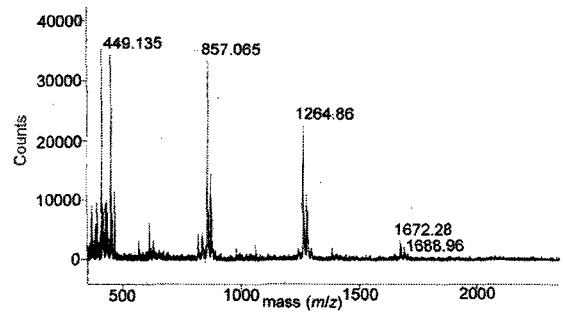


Fig. 2 MALDI-TOF mass spectrum of the water-soluble part of **2**.

Elemental analyses of the precipitate proved the inclusion of the fluorine atom. The fluorine content agreed with the theoretical value. These results indicated that the product was the fluorinated chitin derivative **2**. The X-ray diffraction analysis of **2** showed a spectrum with characteristic peaks at 9.4, 19.2, 20.7 and 25.7 degrees, which strongly resembled that of synthetic chitin with an α-chitin crystalline structure prepared through enzymatic polymerization (Fig. 3). This result suggests that the water-insoluble product of **2** has a crystalline structure very similar to that of synthetic chitin.

Table I. Enzymatic polymerization of **1** by chitinase from *Bacillus* sp.[a]

Entry	[1] / M	pH	Temperature / °C	Enzyme amount / wt% for **1**	Time[b] / h	Yield / %	
						Insoluble part[c]	Soluble part[d] (n<6)
1	0.05	7.0	30	1.0	12	10	23
2	0.05	8.0	30	1.0	24	40	26
3	0.05	9.0	30	1.0	24	40	27
4	0.05	10.0	30	1.0	36	30	29
5	0.05	11.0	30	1.0	168	0	0
6	0.05	9.0	40	1.0	18	40	30
7	0.05	9.0	50	1.0	18	0	19

[a]In phosphate buffer (10 mM).
[b]Indicating the time for complete consumption of 1.
[c]Isolated yield.
[d]Determined by HPLC.

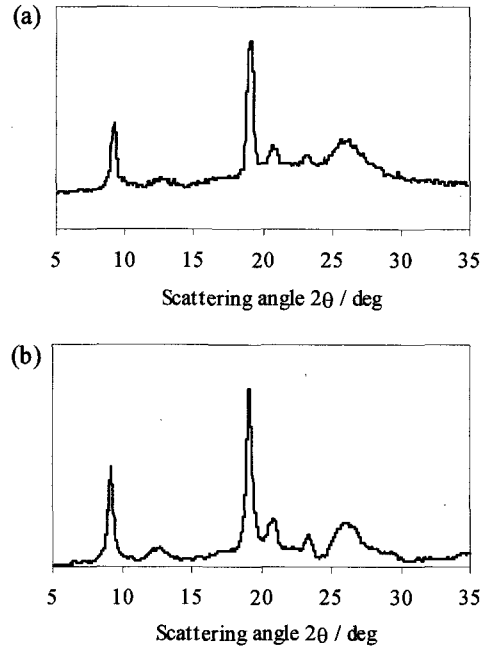


Fig. 3 X-Ray diffractograms of the water-insoluble part of **2** (a) and synthetic chitin (b).

2.4 Polymerization reactions of **1** under various conditions.

In order to optimize the reaction, polymerization of **1** was performed under various reaction conditions (Table I). In entries 1 to 5, the pH value was varied from 7.0 to 11.0. The total yield of the water-insoluble and water-soluble parts became larger with increasing pH value, however, it became smaller at pH 10.0. In the reaction at pH 11.0, compound **2** was not produced. The maximum yield was obtained at pH 9.0 within 24 h (entry 3, total 67%). In entries 6 and 7, the reaction temperature was varied. The yield of the water-soluble part was slightly improved to 30% at 40°C within shorter reaction time (18 h), whereas the yield of the water-insoluble part was the same as that in entry 3. The reaction at 50°C did not provide the water-insoluble part and the water-soluble part in low yield. These results indicate that the optimal temperature is 30-40°C for the reaction.

3. CONCLUSION

An alternatingly 6-fluorinated chitin derivative (**2**) was successfully synthesized via enzymatic polymerization catalyzed by chitinase from *Bacillus* sp. with an *N,N'*-di-acetyl-6'-fluoro-chitobiose oxazoline derivative (**1**) as a novel transition state analogue substrate monomer. The polymerization reaction proceeded smoothly under weak alkaline conditions, giving rise to **2** as a white crystalline product in good yields. The optimal yield was obtained at pH 9.0 and at 30-40°C. The result of the X-ray diffraction analysis suggests that the product polysaccharide **2** has a similar crystal structure to that of synthetic chitin. Further investigations about the structure of **2** as well as its biological functions are now under progress.

4. EXPERIMENTAL

4.1 Measurements.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-400 spectrometer for confirmation of the monomer structure. HPLC measurements were performed with a Tosoh LC-8020 system equipped with a ChemcoBond ODS-W (Chemco; eluent: distilled water/acetonitrile=98/2 (v/v); flow rate: 1.00 mL/min; 30°C) or a Sugar KS-802 (Shodex; eluent: distilled water; flow rate: 0.50 mL/min; 80°C). MALDI-TOF mass spectrum was recorded on a JEOL JMS-Elite using 2,5-dihydroxybenzoic acid as a matrix. X-Ray diffractograms were obtained by X-ray diffraction analyzer, Rigaku RINT 1400 using CuK α-ray beam (1.541 Å).

4.2 A typical polymerization procedure.

Monomer **1** (2.04 mg, 0.005 mmol) was dissolved in phosphate buffer (10 mM, 80 μL), and the pH was finely adjusted to 9.0 by using hydrogen chloride. To the solution 20 μL of the enzyme solution (10 mM phosphate buffer containing 0.0204 mg of chitinase) was added. The reaction mixture was kept standing at 30°C for 24 h. The concentration of **1** was confirmed by HPLC measurement. After the complete consumption of **1** was confirmed, the enzyme was thermally denatured at 90°C for 10 min to terminate the reaction. The mixture

was then centrifuged to separate the water-insoluble part from the reaction mixture. The separated precipitate was rinsed with 100 μL of distilled water (3 times) and dried under diminished pressure to give a pure compound of **2** (0.8 mg, 40%) as a white solid.

5. ACKNOWLEDGMENT

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