Chitinase-Catalyzed Synthesis of Alternatingly *N*-Deacetylated Chitin: A Chitin−Chitosan Hybrid Polysaccharide

Akira Makino, Kazuhiro Kurosaki, Masashi Ohmae, and Shiro Kobayashi*,†

Department of Materials Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 615-8510, Japan

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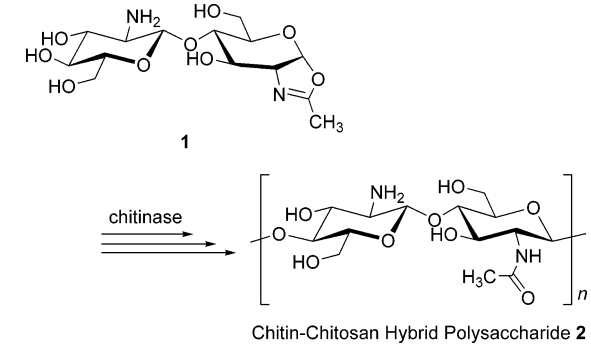
A chitin−chitosan hybrid polysaccharide (**2**) having a $\beta(1\rightarrow4)$-linked alternating structure of an *N*-acetyl-D-glucosamine (GlcNAc) unit and a D-glucosamine (GlcN) unit was synthesized via chitinase-catalyzed polymerization of an oxazoline derivative of a GlcN$\beta(1\rightarrow4)$GlcNAc monomer (**1**). Monomer **1** was designed as a transition-state analogue substrate (TSAS) monomer for chitinase catalysis, which belongs to the glycoside hydrolase family 18. Monomer **1** was effectively polymerized by the catalysis of enzymes from *Bacillus* sp., *Serratia marcescens* and *Streptomyces griseus*, under weak alkaline conditions, giving rise to a water-soluble hybrid polysaccharide (**2**) in good yields. Molecular weights of **2** reached 2020 with using chitinase from *Serratia marcescens*, which corresponds to 10−12 saccharide units.

Introduction

Chitin is a well-known natural polysaccharide consisting of $\beta(1\rightarrow4)$-linked *N*-acetyl-D-glucosamine (GlcNAc).[1] It is most abundant and distributed widely in invertebrate animals as a supporting material. Chitin has excellent characteristics of biodegradability and biocompatibility, particularly low immunogenicity. Therefore, it is frequently employed as a starting material in medicine, pharmaceutics, cosmetics, and food supplements, for instance, wound dressing sheets,[2] absorbable sutures,[3] carriers for prodrug[4] and drug delivery systems,[5] and humectants.[6] Chitosan has a similar structure to chitin, which is composed of $\beta(1\rightarrow4)$-linked D-glucosamine (GlcN). Due to the difference in the chemical structures between acetamido and amino functional groups, chitosan exhibits specific bioactivities such as antiviral[7] and antibacterial[8] activities, activation of complement[9] and macrophage,[10] promotion of plant growth,[11] and elicitation of phytoalexins.[12] Chemical modification of chitin and chitosan increases the efficiency of these activities[13] and expresses novel intriguing bioactivities;[14] sulfated chitin and its derivatives suppress metastasis of tumors.[15]

Such promising biomacromolecules of chitin and chitosan are synthesized in vivo by the catalysis of chitin synthase with uridine-5′-diphospho (UDP)-GlcNAc as a substrate for the chain elongation of the chitin molecule,[16] followed by *N*-deacetylation catalyzed by chitin deacetylase to generate chitosan.[17] However, chitin contains a GlcN unit in small amounts due to the incomplete action of the deacetylase,[18] causing structural diversities of these polysaccharides. Furthermore, both polysaccharides are insoluble in most organic solvents and water; chitin has rigid crystalline structures caused by the formation of multiple hydrogen bonds,[19] and chitosan is only soluble in weak acidic media like acetic acid. The poor solubility of these polysaccharides makes their chemical modification hard and limits their application to date. Accordingly, the modification requires normally severe reaction conditions, causing their degradation and unexpected side reactions.[20] Their structure−activity relationships have not been elucidated enough because of these structural diversities. Elucidation is therefore essential for the investigation of the biological activities of chitin, chitosan, and their derivatives at a molecular level.

Scheme 1. Chitinase-Catalyzed Synthesis of a Chitin−Chitosan Hybrid Polysaccharide



Chitin-Chitosan Hybrid Polysaccharide **2**

Enzymatic polymerization has been demonstrated for the synthesis of polysaccharides and oligosaccharides with well-defined structures.[21] For instance, alternatingly 6-*O*-methylated cellulose was prepared by the catalysis of cellulase with 6-*O*-methyl-β-cellobiosyl fluoride[22] and chondroitin 4-sulfate bearing sulfate groups at C4 in all *N*-acetylgalactosamine units by hyaluronidase with a 4-sulfated *N*-acetylchondroisine oxazoline derivative.[23] Particularly, synthetic chitin was produced by the chitinase-catalyzed polymerization of an *N*,*N*′-diacetylchitobiose (GlcNAc$\beta(1\rightarrow4)$GlcNAc) oxazoline as a transition-state analogue substrate (TSAS) monomer.[21,24] Synthetic chitin has a perfect repeating structure of $\beta(1\rightarrow4)$-linked GlcNAc, without the incorporation of GlcN units normally found in naturally occurring chitin.

The present paper describes chitinase-catalyzed polymerization of an *N*-acetylchitobiose oxazoline derivative (**1**), which has an amino group at the C2 position in its nonreducing terminal, for the synthesis of a chitin−chitosan hybrid polysaccharide (**2**) (Scheme 1). The polysaccharide **2** has an alternating structure of $\beta(1\rightarrow4)$-linked GlcNAc and GlcN with a well-defined structure.

* To whom correspondence should be addressed. E-mail: kobayash@kit.ac.jp. Tel/Fax: +81-75-724-7688.

† Present address: R&D Center for Bio-based Materials, Kyoto Institute of Technology, Kyoto 606-8585, Japan

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Experimental Section

Materials. Dichloromethane and 1,2-dichloroethane were used after distillation from P_2O_5. Molecular sieves AW300 purchased from Aldrich (Lot No. 04024CI) were grinded and then activated by heating over 100 °C under diminished pressure prior to use. Dowex 1-X4 was purchased from The Dow Chemical Co. Other chemicals were used without further purification. Chitinase from *Bacillus* sp. was purchased from Wako Pure Chemicals Inc. (Lot No. LDH7046). Chitinase from *Aeromonas hydorophila* was purchased from Seikagaku Corp. (Lot No. DN1187), and chitinases from *Serratia marcescens* and *Streptomyces griseus* were obtained from Sigma (Lot Nos. 45H4117 and 77H4055, respectively). All enzymes were used without further purification. Di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose, tetra-*N*-acetylchitotetraose, penta-*N*-acetylchitopentaose, and hexa-*N*-acetylchitohexaose were purchased from Seikagaku Corp. (Lot Nos. 9612020, 9809100, 9812110, 9906280, and 9812110, respectively) and were used as chitooligosaccharide standards for size exclusion chromatography (SEC) measurements. Dimer of GlcN, tetramer of GlcN, and hexamer of GlcN were purchased from Seikagaku Corp. (Lot Nos. 9710130, 0210100, and 0309160, respectively) and used as chitosanoligosaccharide standards for SEC measurements. Pullulan standards (M_w = 5900, 11800, 22800, 47300, and 112000, Lot No. 80301) were purchased from Showa Denko K. K.

Measurements. NMR spectra were recorded with a Bruker DPX-400 spectrometer. All assignments were based on a correlation spectroscopy (COSY) experiment. High-resolution fast atom bombardment (HRFAB) mass spectra were obtained on a JEOL HX-110 spectrometer using 2,4-dinitrobenzyl alcohol or dithiothreitol−thioglycerol (1:1, v/v) as the matrix. Optical rotations were measured with a Jasco P-1010 polarimeter. Melting points were determined with a Yamato MP-21. The concentration of **1** in the reaction mixture was determined by high-performance liquid chromatography (HPLC) measurements using a Tosoh LC8020 system with a Shodex Asahipak NH2P-50 4E column (4.6 × 250 mm; eluent, distilled water−acetonitrile mixed solution (3:7, v/v); flow rate, 0.5 mL/min; 40 °C). An aliquot of the reaction mixture (3 μL) was sampled and injected to the column. Yields and molecular weights of **2** were determined by SEC measurements using a Tosoh GPC8020 system equipped with a Shodex Asahipak GS-320 HQ column (7.6 × 300 mm; eluent, 1% aqueous acetic acid containing 0.5 M sodium nitrate; flow rate, 1.0 mL/min; 40 °C). The molecular weight value was calibrated by using chitooligosaccharides, chitosanoligosaccharides, and pullulan standards. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed with a JEOL JMS-ELITE spectrometer using 2,5-dihydroxybenzoic acid as the matrix under positive ion mode.

3,4,6-Tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-α-D-glucopyranose (4). Compound **3**[25] (7.70 g, 17.4 mmol) was dissolved in tetrahydrofuran (THF, 60 mL) followed by the addition of benzylamine (2.10 mL, 19.1 mmol). After the reaction mixture was stirred at room temperature for 8 h under dry atmosphere, it was evaporated and the residue was diluted with chloroform and washed successively with 1 M aqueous HCl, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, filtrated through diatomaceous earth (Celite), and concentrated to dryness. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 2:1 then 1:1) to afford pure **4** (6.30 g, 15.7 mmol, 90%) as a white amorphous powder. ^{1}H NMR ($CDCl_3$) δ (ppm): 6.66 (1H, d, $J_{2,NH}$ = 9.04 Hz, N*H*), 5.36 (1H, dd, $J_{2,3}$ = 10.56 Hz, $J_{3,4}$ = 9.52 Hz, H-3), 5.36 (1H, t, $J_{1,2}$ = $J_{1,OH}$ = 3.50 Hz, H-1), 5.17 (1H, t, $J_{3,4}$ = $J_{4,5}$ = 9.56 Hz, H-4), 4.51 (1H, m, H-2), 4.27−4.22 (2H, m, H-5, H-6a), 4.15 (1H, d, $J_{6a,6b}$ = 10.52 Hz, H-6b), 2.11−2.03 (9H, s, $COCH_3$). These spectroscopic data were consistent with those previously reported.[26]

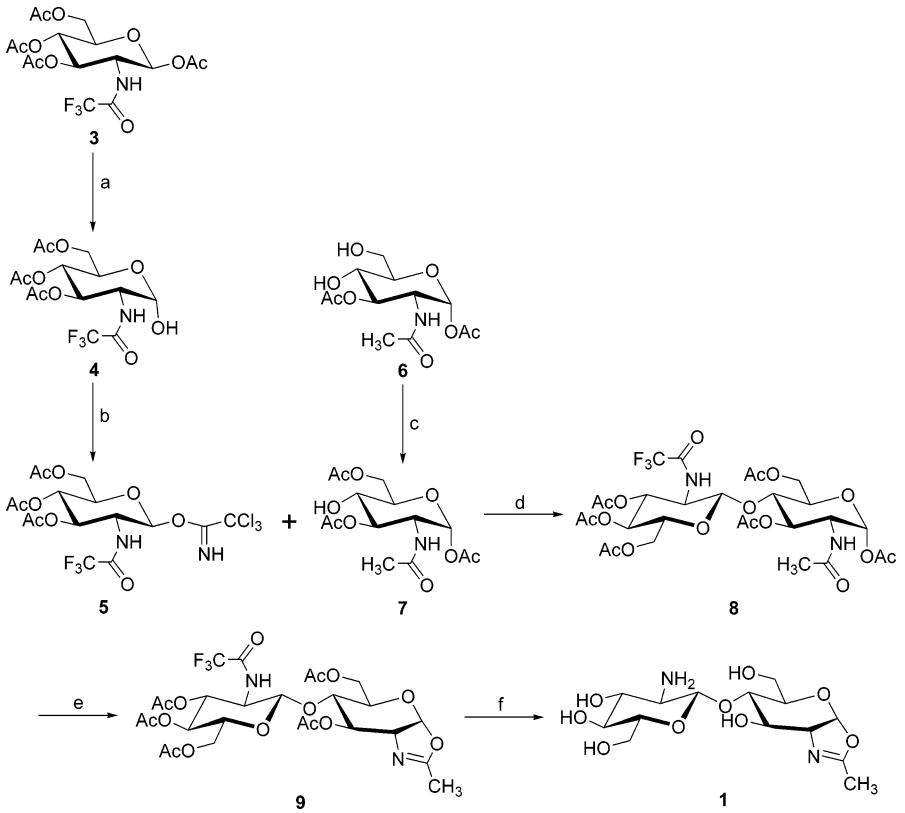
3,4,6-Tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranosyl Trichloroacetimidate (5). Trichloroacetonitrile (13.3 mL, 133 mmol) and 1,8-diazabicyclo-[5,4,0]-7-undecene (0.59 mL, 3.99 mmol) at 0 °C under dry atmosphere were added to a solution of compound **4** (5.33 g, 13.3 mmol) in dry dichloromethane (250 mL). After the reaction mixture was stirred at room temperature for 5 h, it was evaporated followed by purification by silica gel column chromatography (*n*-hexane/ethyl acetate 2:1 containing 0.3% triethylamine) to afford pure **5** (5.17 g, 3.97 mmol, 71%) as a yellowish amorphous powder. $[\alpha]^{25}_D$ +59° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ (ppm): 8.87 (1H, s, C=N*H*), 6.65 (1H, d, $J_{2,NH}$ = 8.53 Hz, NH), 6.45 (1H, d, $J_{1,2}$ = 3.52 Hz, H-1), 5.39 (1H, t, $J_{2,3}$ = $J_{3,4}$ = 12.04 Hz, H-3), 5.29 (1H, t, $J_{3,4}$ = $J_{4,5}$ = 9.56 Hz, H-4), 4.51 (1H, ddd, $J_{1,2}$ = 3.52 Hz, $J_{2,NH}$ = 8.56 Hz, $J_{2,3}$ = 12.04 Hz, H-2), 4.30 (1H, dd, $J_{5,6a}$ = 4.02 Hz, $J_{6a,6b}$ = 13.05 Hz, H-6a), 4.15−4.12 (2H, m, H-6b, H-5), 2.10−2.08 (9H, s, $COCH_3$). ^{13}C NMR ($CDCl_3$) δ (ppm): 171.66−169.11 (*C*OCH_3), 160.07 (*C*=N), 157.60 (q, *J* = 39.13 Hz, *C*OCF_3), 115.36 (q, *J* = 257.4 Hz, CO*C*F_3), 93.67 (C-1), 90.44 (CCl3), 70.44 (C-5), 70.32 (C-3), 66.82 (C-4), 61.23 (C-6), 52.59 (C-2), 20.66−20.43 (CO*C*H_3). HRMS-FAB: $[M + H]^+$ calcd for $C_{16}H_{19}O_9N_2F_3Cl_3$, 545.0108; found, 545.0108.

2-Acetamido-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl Acetate (7). Acetyl chloride (1.02 mL, 1.13 g, 14.4 mmol) diluted with THF (3.0 mL) under dry atmosphere was added dropwise to a solution of compound **6**[27] (4.00 g, 13.1 mmol) in dry pyridine (15 mL). After the mixture was stirred at room temperature for 30 min, it was extracted with chloroform and successively washed with 4% aqueous $KHSO_4$, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, filtrated through Celite, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (toluene/ethyl acetate 3:1, 2:1, and 1:4) to afford pure **7** (3.00 g, 8.64 mmol, 66%) as a white amorphous powder. ^{1}H NMR ($CDCl_3$): δ (ppm): 6.15 (1H, d, $J_{1,2}$ = 3.62 Hz, H-1), 5.68 (1H, d, $J_{2,NH}$ = 8.95 Hz, N*H*), 5.15 (1H, dd, $J_{3,4}$ = 9.52 Hz, $J_{2,3}$ = 10.78 Hz, H-3), 4.62 (1H, dd, $J_{5,6a}$ = 3.25 Hz, $J_{6a,6b}$ = 12.47 Hz, H-6a), 4.42−4.38 (1H, m, H-2), 4.19 (1H, dd, $J_{5,6b}$ = 2.25 Hz, $J_{6a,6b}$ = 12.40 Hz, H-6b), 3.87−3.84 (1H, m, H-5), 3.67−3.64 (1H, m, H-4), 2.20−1.96 (12H, m, CH_3-CO). These spectroscopic data were consistent with those previously reported.[28]

2-Acetamido-3,6-di-*O*-acetyl-4-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranosyl)-2-deoxy-α-D-glucopyranosyl Acetate (8). A mixture of compounds **5** (200 mg, 0.37 mmol) and **7** (106 mg, 0.31 mmol) in anhydrous dichloromethane (5.0 mL) was stirred in the presence of activated molecular sieves AW300 (600 mg) under argon atmosphere at room temperature for 1 h. After the mixture was cooled to −20 °C, trimethylsilyl triflate (Me_3SiOTf, 6.6 μL, 0.037 mmol) was added to the reaction mixture. After it was stirred for 2 h, the mixture was filtered through Celite, diluted with chloroform, and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over with $MgSO_4$, filtered through Celite, and concentrated under diminished pressure. The residue was purified by silica gel column chromatography (*n*-hexane/ethyl acetate 1:1 then 1:4) to afford pure **8** (108 mg, 0.11 mmol, 48%) as a white crystal. $[\alpha]^{25}_D$ +23° (*c* 1.0, $CHCl_3$). Mp: 271−272 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.41 (1H, d, $J_{2',NH'}$ = 9.54 Hz, NH′), 6.11 (1H, d, $J_{1,2}$ = 4.02 Hz, H-1), 5.57 (1H, d, $J_{2,NH}$ = 9.03 Hz, NH), 5.22 (1H, dd, $J_{3,4}$ = 9.04 Hz, $J_{3,2}$ = 11.04 Hz, H-3), 5.16−5.08 (2H, m, H-3′, H-4′), 4.57 (1H, d, $J_{1',2'}$ = 8.03 Hz, H-1′), 4.45−4.36 (3H, m, H-2, H-6a, H-6a′), 4.16−4.03 (3H, m, H-2′, H-6b, H-6b′), 3.85 (1H, d, $J_{4,5}$ = 9.54 Hz, H-5), 3.69 (1H, t, $J_{3,4}$ = $J_{4,5}$ = 9.53 Hz, H-4), 3.64 (1H, m, H-5′), 2.21−1.94 (21H, s, $COCH_3$). ^{13}C NMR ($CDCl_3$) δ (ppm): 172.16−169.32 (*C*OCH_3), 101.19 (C-1′), 90.75 (C-1), 76.01 (C-4), 72.56 (C-5′), 72.38 (C-3′), 71.12 (C-5), 70.77 (C-3), 68.07 (C-4′), 61.69 (C-6), 61.87 (C-6′), 54.79 (C-2′), 51.40 (C-2), 23.39−20.72 (CO*C*H_3). HRMS-FAB: $[M + H]^+$ calcd for $C_{28}H_{38}O_{17}N_2F_3$, 731.2122; found, 731.2126.

2-Methyl-4,5-dihydro-[3,6-di-*O*-acetyl-4-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-trifluoroacetamido-β-D-glucopyranosyl)-1,2-dideoxy-α-D-glucopyranoso][2,1-*d*]-1,3-oxazole (9). Me_3SiOTf (32 mg, 26 μL, 0.14 mmol) under argon atmosphere at 55 °C was added to a solution of compound **8** (100 mg, 0.14 mmol) in anhydrous 1,2-dichloroethane (8.0 mL). The reaction mixture was cooled to 0 °C after stirring for 5.5 h, followed by the addition of triethylamine (1.0 mL) under argon

Scheme 2



(a) $BnNH_2$/THF, 90%; (b) CCl_3CN, DBU/CH_2Cl_2, 71%; (c) AcCl/pyridine, 85%; (d) Me_3SiOTf, MS-AW300/CH_2Cl_2, 67%; (e) Me_3SiOTf/1,2-dichloroethane, 89%; (f) (i) NaOMe/MeOH, (ii) DOWEX 1-X4 (OH^- form)/MeOH, quant.

atmosphere. After the evaporation of the mixture under reduced pressure, the residue was immediately subjected to silica gel column chromatography (*n*-hexane/ethyl acetate 1:1−1:4 containing 0.3% triethylamine) to afford pure **9** (82 mg, 0.12 mmol, 89%) as a white amorphous powder. $[\alpha]^{25}_D$ −8.3° (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ (ppm): 7.14 (1H, d, $J_{2',NH'}$ = 9.03 Hz, NH′), 5.92 (1H, d, $J_{1,2}$ = 7.53 Hz, H-1), 5.65 (1H, d, $J_{2,3}$ = 2.53 Hz, H-3), 5.20−5.11 (2H, m, H-3′, H-4′), 4.62 (1H, d, $J_{1',2'}$ = 8.54 Hz, H-1′), 4.52 (1H, dd, $J_{5,6a}$ = 3.01 Hz, $J_{6a,6b}$ = 12.05 Hz, H-6a), 4.29 (1H, dd, $J_{5',6a'}$ = 4.51 Hz, $J_{6a',6b'}$ = 12.54 Hz, H-6a′), 4.19−4.11 (3H, m, H-2, H-6b′), 4.00 (1H, dd, $J_{5,6b}$ = 2.01 Hz, $J_{6a,6b}$ = 12.55 Hz, H-6b), 3.79 (1H, m, H-5′), 3.46 (1H, d, $J_{4,5}$ = 9.54 Hz, H-4), 3.38 (1H, m, H-5), 2.14−2.12 (18H, m, $COCH_3$, CH_3 of oxazoline). ^{13}C NMR ($CDCl_3$) δ (ppm): 171.58−166.94 ($COCH_3$, CH_3C═N of oxazoline), 102.16 (C-1′), 99.15 (C-1), 77.64 (C-4), 72.62 (C-5′), 72.19 (C-3′), 70.42 (C-3), 68.00 (C-4′), 67.79 (C-5), 64.69 (C-2), 62.93 (C-6), 61.81 (C-6′), 54.40 (C-2′), 21.54−13.95 ($COCH_3$, CH_3 of oxazoline). HRMS-FAB: $[M + H]^+$ calcd for $C_{26}H_{33}O_{15}N_2F_3$, 671.1911; found, 671.1911.

2-Methyl-4,5-dihydro-[4-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-1,2-dideoxy-α-D-glucopyranoso][2,1-*d*]-1,3-oxazole (1). Sodium methoxide (28% methanol solution, 12 mg, 0.06 mmol) under dry atmosphere was added dropwise to a solution of compound **9** (82 mg, 0.12 mmol) in anhydrous methanol (3.0 mL). After the mixture was stirred for 30 min at room temperature, ion-exchange resin (Dowex 1-X4, OH^- form; 20 mg) was added to the mixture to remove the trifluoroacetyl group. The reaction mixture was stirred for 30 min at room temperature under dry atmosphere, filtered through cotton, and then concentrated to dryness under diminished pressure to afford pure **1** (28 mg, 0.69 mmol, quant) as a white amorphous powder. ^{1}H NMR (D_2O) δ (ppm): 6.05 (1H, d, $J_{1,2}$ = 7.53 Hz, H-1), 4.51 (1H, d, $J_{1',2'}$ = 8.03 Hz, H-1′), 4.41(1H, d, $J_{2,3}$ = 1.51 Hz, H-3), 4.17 (1H, m, H-2), 3.93 (1H, d, $J_{3',4'}$ = 12.55 Hz, H-3′), 5.20−5.11 (2H, m, H-3′, H-4′), 4.62 (1H, d, $J_{1',2'}$ = 8.54 Hz, H-1′), 4.52 (1H, dd, $J_{5,6a}$ = 3.01 Hz, $J_{6a,6b}$ = 12.05 Hz, H-6a), 4.29 (1H, dd, $J_{5',6a'}$ = 4.51 Hz, $J_{6a',6b'}$ = 12.54 Hz, H-6a′), 4.19−4.11 (3H, m, H-2, H-6b′), 4.00 (1H, dd, $J_{5,6b}$ = 2.01 Hz, $J_{6a,6b}$ = 12.55 Hz, H-6b), 3.79 (1H, m, H-5′), 3.46 (1H, d, $J_{4,5}$ = 9.54 Hz, H-4), 3.38 (1H, m, H-5), 2.14−2.12 (18H, m, $COCH_3$, CH_3 of oxazoline). ^{13}C NMR ($CDCl_3$) δ (ppm): 168.01 (CH_3C═N of oxazoline), 105.00 (C-1′), 100.22 (C-1), 79.42 (C-4), 77.21 (C-5′), 76.32 (C-3′), 71.82 (C-5), 70.76 (C-4′), 69.18 (C-3), 66.16 (C-2), 62.75 (C-6), 61.48 (C-6′), 57.15 (C-2′), 13.26 (CH_3 of oxazoline). HRMS-FAB: $[M + H]^+$ calcd for $C_{14}H_{24}O_9N_2$, 365.1560; found, 365.1560.

Typical Procedure for Enzymatic Polymerization of 1. Chitinase Sm (1.84 mg) dissolved in a phosphate buffer (50 mM, pH 9.0, 50 μL) was added to a solution of monomer **1** (36.4 mg, 0.10 mmol) in a phosphate buffer (50 mM, pH 9.0, 950 μL), and the mixture was kept standing at 30 °C. The reaction was monitored by means of HPLC with a Shodex Asahipak NH2P-50 4E column eluting with distilled water−acetonitrile mixed solution (3:7, v/v, flow rate of 0.50 mL/min) at 40 °C. After 26 h, the mixture was heated at 90 °C for 10 min to inactivate the enzyme and subjected to Sephadex G-10 column chromatography eluting with distilled water to give **2** (21.2 mg, 58% as an isolated yield).

Results and Discussion

Synthesis of Monomer 1. A newly designed TSAS monomer (**1**) was synthesized according to the reactions illustrated in Scheme 2. Compound **3** bearing a 2-trifluoroacetamido group was synthesized from D-glucosamine hydrochloride via three steps.[25] The anomeric *O*-acetyl group of **3** was selectively removed by benzylamine to give compound **4**. The resulting 1-OH group of **4** was reacted with trichloroacetonitrile to provide the glycosyl donor **5**. Compound **6** was synthesized from *N*-acetyl-D-glucosamine via three steps,[27] and then the 6-hydroxy group of **6** was selectively *O*-acetylated to afford the glycosyl acceptor **7**. Glycosylation of **7** with **5** was carried out using trimethylsilyl triflate (Me_3SiOTf) as a promoter to give the disaccharide derivative **8** in a 67% yield. Compound **8** was

Scheme 3. Two Kinds of Reactions Could Possibly Occur Using Monomer **1**

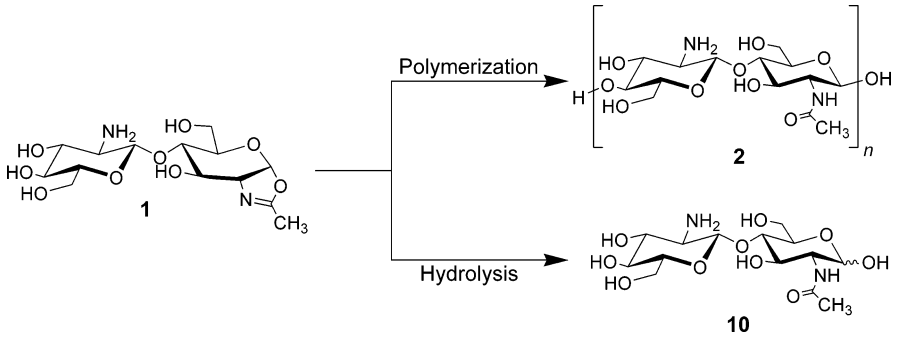


Table 1. Enzymatic Polymerization of **1** Catalyzed by Chitinase from Various Origins

	polymerization[a]		polymer **2**		
entry	chitinase	time/h[b]	yield/%[c]	M_n[d]	M_w[d]
1	Bs	0.7	64	1450	1550
2	Sg	0.7	34	1420	1870
3	Sm	16	62	1490	2310
4	Ah	120	0		

[a] In a phosphate buffer (50 mM, pH 8.5) at 30 °C with 10 wt % of enzyme for **1**. [b] Indicating the time for complete consumption of **1**. [c] Determined by HPLC measurements containing products with molecular weight higher than that of tetrasaccharide. [d] Determined by SEC calibrated with chitooligosaccharides, chitosanoligosaccharides, and pullulan standards.

treated with Me_3SiOTf to convert the corresponding oxazoline derivative **9** in an 89% yield. Finally, all of the *O*-acetyl protecting groups of **9** were removed with sodium methoxide in methanol followed by the removal of the trifluoroacetyl group using DOWEX 1-X4 (OH^- form) to give the target monomer **1** quantitatively.

Enzymatic Polymerization of 1 Catalyzed by Chitinase from Various Origins. It is to be pointed out that two kinds of reactions could possibly occur by using monomer **1**: polymerization of **1** to the product polymer **2** and hydrolysis of **1** via the oxazoline ring opening, leading to the formation of *N*-acetylchitobiose (**10**) (Scheme 3).

Chitinases belonging to the glycoside hydrolase family 18[24,29] have the potential to catalyze the above two reactions. Some chitinases from different origins are commercially available: *Bacillus* sp. (abbreviated as chitinase Bs), *Streptomyces griseus* (chitinase Sg), *Serratia marcescens* (chitinase Sm), and *Aeromonas* hydrophila (chitinase Ah). Figure 1 indicates the consumption rate of monomer **1** during the polymerization catalyzed by these four enzymes.

The concentration change of **1** was monitored by HPLC. With chitinases Bs and Sg, monomer **1** was completely consumed within 0.7 h (parts a and b of Figure 1). Chitinase Sm also accelerated the consumption of **1**, which disappeared within 16 h (Figure 1c). In contrast, it took 120 h for the complete consumption of **1** with chitinase Ah (Figure 1d). Monomer **1** was gradually decomposed without enzyme to give the ring-opened hydrolyzed product **10**. It remained in 97% at 0.7 h, 79% at 16 h, 36% at 120 h, and 13% at 240 h. These results indicate that all enzymes catalyze the oxazoline ring-opening reaction of **1**.

The reaction product was analyzed by HPLC and SEC measurements (Table 1). Chitinase Bs provided polymer **2** having a molecular weight (M_n) of 1450 in a 64% yield via the ring-opening polyaddition of monomer **1** under total control of regioselectivity and stereochemistry (entry 1). Chitinase Sg also catalyzed the polymerization of **1** (entry 2), affording **2** with an M_n of 1420 in a lower yield of 34% within the same reaction time of 0.7 h as that in entry 1. Chitinase Sm produced **2** with an M_n of 1490 in a similar yield of 62% to that in entry 1 within a longer reaction time of 16 h (entry 3). However, monomer **1** was not polymerized by the catalysis of chitinase Ah at all (entry 4); the hydrolysate **10** was detected as the sole product in the reaction mixture. These M_n values of **2** correspond to the octasaccharide, reaching the maximum saccharide units of 14 (see below). Thus, chitinases Bs and Sm were effective for the production of a chitin−chitisan hybrid polysaccharide **2** in terms of the yield and M_n value. Furthermore, it is to be pointed out that these reactions proceeded homogeneously, indicating that the product polymer **2** is soluble in aqueous media.

Structural Identification of Chitin−Chitosan Hybrid Polysaccharide 2. The mixtures after the reactions with chitinases Bs, Sm, and Sg were analyzed by MALDI-TOF MS. In all the spectra, the peaks with the same distance of *m*/*z* 364 were observed, which corresponds to the molecular weight value of monomer **1** as well as the repeating disaccharide of **2** (Figure 2).

Figure 3a shows the ^1H NMR spectrum of **2** obtained after purification by SEC through a Sephadex G-10 column. Two signals at δ 4.88 and 4.58 were derived from the anomeric protons of the internal GlcN and GlcNAc units, respectively. The coupling constants of these signals were 8.04 and 6.52 Hz, indicating that these saccharide units were connected through a β-glycosidic linkage. Furthermore, the ratio of the integration of the H-2 peak of the GlcN unit to the methyl peak of the *N*-acetyl group in the GlcNAc unit was 1:3. The ^{13}C NMR spectrum of **2** exhibited specific signals at δ 76.94 and δ 76.93 (Figure 3b), which correspond to the C4 of GlcNAc and GlcN, respectively. It is generally accepted that the carbon atom connecting to the glycosidic oxygen is characteristically shifted downfield (to a higher δ value) compared to other carbon atoms in the same pyranose unit.[30] Therefore, both the C4 carbons of GlcNAc and GlcN are adjacent to the glycosidic oxygen, indicating that polymer **2** has only a (1→4)-glycosidic linkage. The results from MALDI-TOF MS and NMR measurements clearly confirmed the structure of **2**, which is a chitin−chitosan hybrid polysaccharide having an alternating structure of β(1→4)-linked GlcN and GlcNAc.

Furthermore, it is to be pointed out that no peaks derived from polysaccharides having an odd number of saccharide chains were observed in the spectra. This indicates that the enzymes do not hydrolyze the (1→4)-β-glucosaminide linkages in **2** during the polymerization reaction.

Polymerization of 1 Catalyzed by Chitinase Bs under Various Conditions. To optimize the reaction conditions using chitinases Bs and Sm, which produced **2** in good yields, reaction parameters of pH, amount of enzyme, reaction temperature, and initial concentration of **1** were systematically examined.

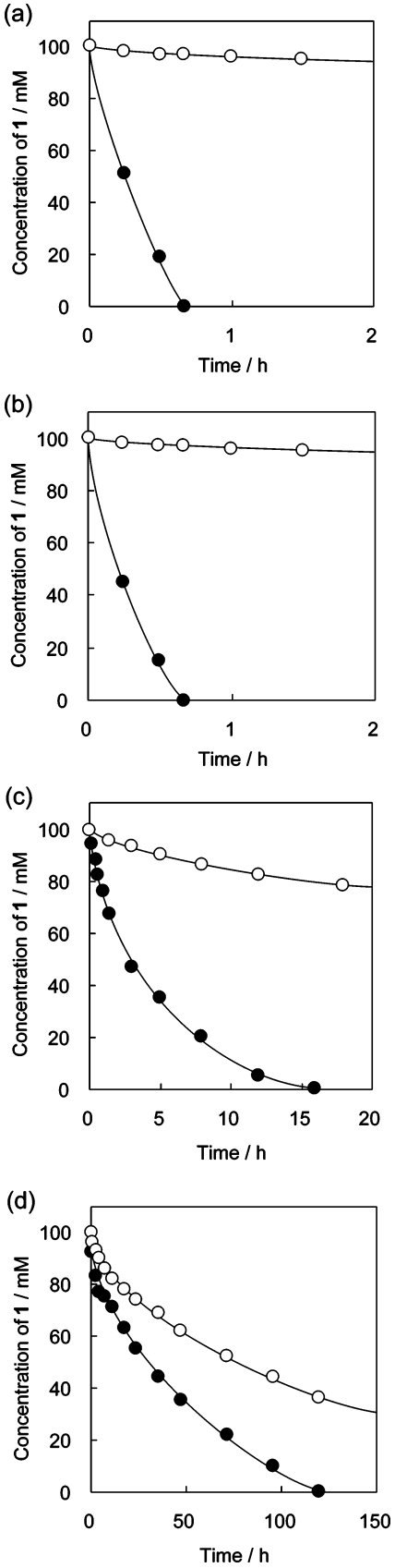


Figure 1. Consumption of monomer **1** vs time with chitinase (●) and without chitinase (○): (a) chitinase Bs, (b) chitinase Sg, (c) chitinase Sm, and (d) chitinase Ah. Reaction conditions: in a phosphate buffer, 50 mM, pH 8.5; amount of the enzyme, 10 wt % for **1**; reaction temperature, 30 °C; initial concentration of **1**, 100 mM.

First, the polymerization of **1** by Chitinase Bs was optimized. The pH conditions affected the yield of **2** and reaction time

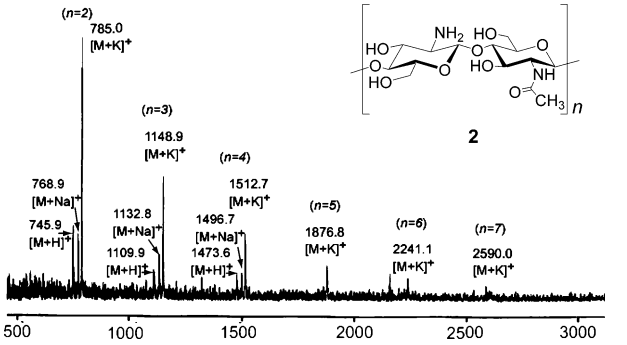


Figure 2. Typical MALDI-TOF MS spectrum of **2** obtained after reaction with chitinase Bs.

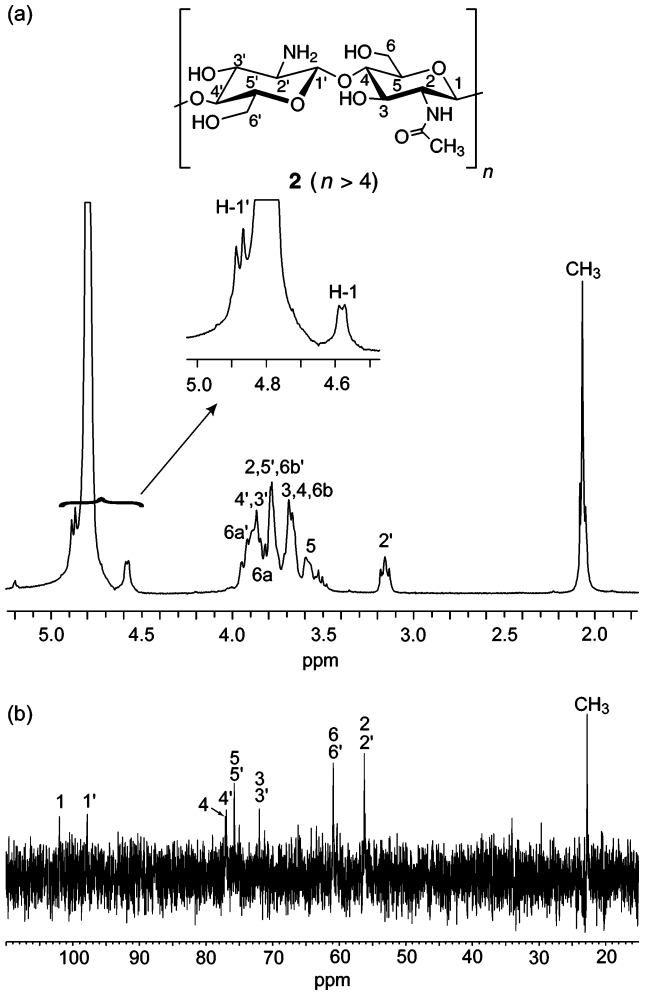


Figure 3. (a) ^{1}H and (b) ^{13}C NMR spectra of **2**. The inset in (a) is a magnified spectrum between δ 4.88 and 4.58.

(entry 1 in Table 1, entries 5−10). With an increase in pH value, the yield of **2** gradually became larger up to 64% at pH 8.5 (entry 1) then smaller to 43% at pH 10.0 (entry 9). The reaction of **1** with the enzyme was very fast at pH values ranging from 7.0 to 10.0 (entries 1, 5−9), which was finished within 0.2−1.7 h. However, it took more time, 18 h, for the complete consumption of **1** at pH 10.5 (entry 10), due to the reduced activity of the enzyme under weak alkaline conditions. The M_n value of **2** was narrowly distributed from 1250 to 1500, indicating that this reaction provided **2** mainly containing the corresponding octasaccharide (MW = 1475.41). Thus, monomer **1** was effectively polymerized by chitinase Bs at pH 8.5 (entry 1) in terms of the yield of **2**. Chitinase Bs has the pH optimum for hydrolysis at 7.8−8.6.[31] Monomer **1** has a GlcN unit as the

Table 2. Enzymatic Polymerization of **1** Catalyzed by Chitinase Bs under Various Reaction Conditions

	polymerization[a]					polymer **2**		
entry	pH	enzyme amount/wt %	temperature/°C	concentration of **1**/mM	time/h[b]	yield/%[c]	M_n[d]	M_w[d]
5	7.0	10	30	100	0.2	56	1250	1280
6	8.0	10	30	100	0.7	59	1400	1490
7	9.0	10	30	100	1.0	56	1450	1550
8	9.5	10	30	100	1.7	47	1420	1520
9	10.0	10	30	100	2.5	43	1440	1540
10	10.5	10	30	100	18	44	1500	1620
11	8.5	1	30	100	10	47	1380	1490
12	8.5	5	30	100	1.0	70	1450	1580
13	8.5	5	0	100	5.0	50	1600	1710
14	8.5	5	10	100	3.0	58	1600	1730
15	8.5	5	20	100	1.5	62	1450	1550
16	8.5	5	40	100	0.5	58	1360	1450
17	8.5	5	30	50	0.7	63	1430	1550
18	8.5	5	30	200	1.0	68	1440	1560

[a] In 50 mM phosphate buffer (entries 5−7, 11−18) and in 50 mM carbonate buffer (entries 8−10). [b] Indicating the time for the complete consumption of **1**. [c] Determined by HPLC measurements containing products with molecular weight higher than that of tetrasaccharide. [d] Determined by SEC calibrated with chitooligosaccharides, chitosanoligosaccharides, and pullulan standards.

Table 3. Effects of Organic Cosolvent on Chitinase-Catalyzed Polymerization of **1**

	polymerization[a]		polymer **2**		
entry	organic cosolvent	time/h[b]	yield/%[c]	M_n[d]	M_w[d]
19	acetone	6.0	38	1100	1250
20	acetonitrile	6.0	58	1670	2190
21	dimethyl sulfoxide	6.0	61	1670	2010
22	ethanol	6.5	63	1710	2360
23	methanol	6.5	61	1720	2130
24	THF	6.0	58	1740	2370

[a] In phosphate buffer (50 mM, pH 8.5) containing 10% (v/v) organic solvent: reaction temperature, 10 °C; amount of chitinase Bs, 5 wt % for **1**. [b] Indicating the time for the complete consumption of **1**. [c] Determined by HPLC measurements containing products with molecular weight higher than that of tetrasaccharide. [d] Determined by SEC calibrated with chitooligosaccharides, chitosanoligosaccharides, and pullulan standards.

nonreducing end, which is not intact for the enzyme. The polymerization of **1** proceeded most effectively at pH 8.5, which is similar to the optimum pH for the hydrolysis reaction.

The amount of enzyme drastically influenced the yield of **2** and reaction time: 1 wt % of the enzyme for monomer **1** produced **2** in a lower yield of 42% within 10 h (entry 11), whereas the yield was improved to the maximum of 70% using 5 wt % of the enzyme (entry 12). Therefore, the following reactions were carried out with an enzyme amount of 5 wt %. It is to be noted that the polymerization of **1** proceeded at a temperature ranging widely from 0 to 40 °C, providing **2** in good yields (entries 12−16). Particularly, M_n of **2** reached the maximum of 1600 at 10 °C (entry 14). The initial concentration of **1** exerted little effects on the yield and M_n of **2** (entries 12, 16 and 17). Thus, chitinase Bs produced a chitin−chitosan hybrid saccharide **2** most effectively under the conditions at pH 8.5 and 10 °C with 5 wt % of the enzyme and 100 mM of **1**.

It is known that the addition of organic solvent to the reaction mixture improves the efficiency of enzymatic glycosylation.[32] For example, enzymatic polymerization to synthetic cellulose has been achieved successfully in an acetate buffer−acetonitrile mixture.[33] Therefore, we investigated the effect of an organic cosolvent on the production of the polysaccharide **2** (Table 3). In entry 19, acetone drastically reduced the yield and M_n of **2**. By the addition of acetonitrile (entry 20), dimethyl sulfoxide (entry 21), ethanol (entry 22), methanol (entry 23), and THF (entry 24), it took a longer time for the complete consumption of **1**, although the yield and M_n of **2** were slightly improved as compared with those in entry 14. Furthermore, the addition of 20% (v/v) of organic solvents caused the inactivation of the enzyme after 1−2 h, resulting in the production of **2** in much lower yields with a lower M_n value (data not shown). Thus, an aqueous buffer−organic cosolvent system was not suitable for this reaction.

Polymerization of 1 Catalyzed by Chitinase Sm under Various Conditions. Next, we investigated the chitinase Sm-catalyzed polymerization of **1** under varying reaction conditions of pH, enzyme amount, reaction temperature, and initial concentration (Table 4). At pH 7.0 (entry 25), polymerization proceeded quite fast; monomer **1** was completely consumed within 1.0 h, providing **2** in a low yield of 12% with a low M_n of 980 (mainly hexasaccharide), mainly producing the hydrolysis product **10**. The yield and M_n of **2** became higher with an increase in pH value (entry 3 in Table 1, entries 26−28); particularly at pH 9.0 (entry 27), polymer **2** was obtained in a good yield of 64% with an M_n of 1580. At pH 9.5 or higher (entries 28−30), the yield and M_n decreased gradually with an increase in the pH value. The reaction time varied from 1.0 to 96 h. The optimum pH of chitinase Sm for hydrolysis of chitin has been reported to be pH 5.0−6.0.[34] Furthermore, it has been reported recently that chitinase Sm also hydrolyzes chitosans having an *N*-acetyl group in 35−65%.[35] The hydrolysis rate of chitosan was accelerated by decreasing the amount of *N*-acetyl group. Monomer **1** having an amino group is, therefore, a good substrate for the enzyme, and the present polymerization proceeded most effectively at a higher pH of 9.0 than that for the hydrolysis due to suppression of the hydrolysis activity of the enzyme.[24] Thus, the following reactions were carried out at the fixed pH value of 9.0. In entry 31, 1 wt % of the enzyme provided polymer **2** in a lower yield of 51% with an M_n value of 1380 after a longer time of 120 h. With 5 wt % of the enzyme, the polymerization of **1** progressed very effectively, giving rise to polymer **2** within 24 h in the highest yield of 75% with the highest M_n of 2020 (M_w = 4280, mainly deca- and dodecasaccharides). These values exceeded those obtained using chitinase Bs. Thus, the amount of enzyme greatly influenced the yield and M_n of **2**. The reaction temperature was varied from 10 to 40 °C using 5 wt % of chitinase Sm (entries 32−35). At 10 °C (entry 33), it took 84 h for the complete consumption of

Table 4. Enzymatic Polymerization of **1** Catalyzed by Chitinase Sm under Various Reaction Conditions

	polymerization[a]					polymer (**2**)		
entry	pH	enzyme amount/wt %	temperature/°C	concentration of **1**/mM	time/h[b]	yield/%[c]	M_n[d]	M_w[d]
25	7.0	10	30	100	1.0	12	980	1000
26	8.0	10	30	100	11	43	1450	1990
27	9.0	10	30	100	18	64	1580	2610
28	9.5	10	30	100	18	60	1420	2000
29	10.0	10	30	100	24	57	1340	1810
30	10.5	10	30	100	96	47	1350	1800
31	9.0	1	30	100	120	51	1380	2300
32	9.0	5	30	100	24	75	2020	4280
33	9.0	5	10	100	84	50	1900	3520
34	9.0	5	20	100	42	71	1950	3840
35	9.0	5	40	100	21	68	1960	4060
36	9.0	5	30	50	21	70	1980	4000
37	9.0	5	30	200	28	73	2010	4180

[a] In 50 mM phosphate buffer (entries 25−27, 31−37) and in 50 mM carbonate buffer (entries 28−30). [b] Indicating the time for the complete consumption of **1**. [c] Determined by HPLC measurements containing products with molecular weight higher than that of tetrasaccharide. [d] Determined by SEC calibrated with chitooligosaccharides, chitosanoligosaccharides, and pullulan standards.

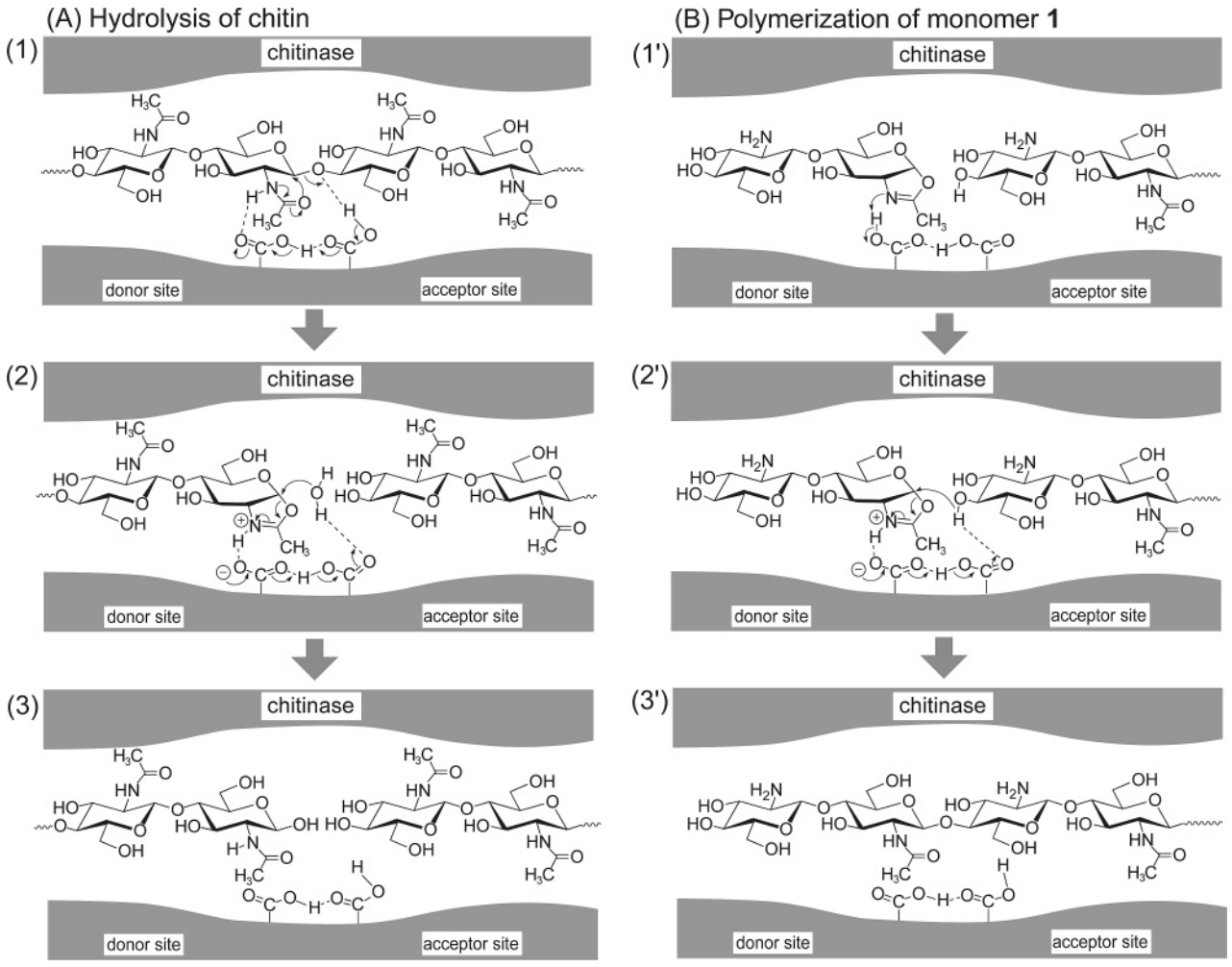


Figure 4. Postulated reaction mechanisms of chitinase catalysis.

1, affording **2** in a reduced yield of 50% with an M_n of 1900. At 20 °C or higher (entries 34 and 35), the polymerization proceeded slightly faster. Changes in the initial concentration of **1** did not affect the polymer yield and M_n so much (entries 36 and 37).

Reaction Mechanism of Chitinase. Figure 4 shows the postulated reaction mechanisms of chitinase in (A) hydrolysis of chitin and (B) polymerization of monomer **1**. In the hydrolysis, the glycosidic oxygen of the saccharide chain placed between the donor site and the acceptor site of chitinase is protonated by the carboxylic acid in the active center of the enzyme immediately after recognition as illustrated in stage 1. Then, the acetamido oxygen at the C2 position of the saccharide unit at the donor site attacks the neighboring C1 carbon to form the corresponding oxazolinium ion[29] stabilized by another carboxylate in the active center, leading to scission of the glycosidic linkage (stage 2). Nucleophilic attack by a water molecule from the β-side opens the ring of the oxazolinium to

accomplish the hydrolysis reaction, giving rise to the hydrolyzate having a β-configuration (stage 3). In the polymerization, the oxazoline monomer **1** is effectively recognized at the donor site of chitinase as a TSAS.[21a] The nitrogen atom in the oxazoline ring is immediately protonated by the carboxylic acid after the recognition to form the corresponding oxazolinium ion (stage 1′), which is stabilized there. Then, the hydroxy group at the C4 of another monomer or the growing chain end attacks the C1 of the oxazolinium from the β-side (stage 2′), resulting in the formation of a $\beta(1\rightarrow4)$ glycosidic linkage (stage 3′). Repetition of these reactions is a ring-opening polyaddition, leading to the formation of a chitin−chitosan hybrid polysaccharide under total control of regioselectivity and stereochemistry. The key-point is the structure of the transition state (or the intermediate), which is commonly involved in both stages 2 and 2′ as a protonated oxazolinium moiety. Monomer **1** is very close to the moiety, showing the importance of the concept of a TSAS monomer.[21]

Conclusion

This study demonstrated the ring-opening polyaddition of an *N*-acetylchitobiose oxazoline derivative (**1**) catalyzed by chitinases Bs and Sm. Monomer **1** was effectively polymerized by these enzymes catalysis, giving rise to a water-soluble chitin−chitosan hybrid polysaccharide (**2**) in good yields under total control of regioselectivity and stereochemistry. The M_n value of **2** reached 1670 and 2020 using chitinases Bs and Sm, respectively, which correspond to 8−10 ($n = 4-5$) and 10−12 ($n = 5-6$) saccharide units, respectively. A chitin−chitosan hybrid polysaccharide produced in this study has a well-defined structure, which is difficult to achieve via conventional chemical and biochemical methods. Therefore, this polysaccharide is expected to become an important material to open the door for the investigation of the biological activities of chitin, chitosan, and their derivatives at a molecular level.

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