Synthesis of Artificial Chitin: Irreversible Catalytic Behavior of a Glycosyl Hydrolase through a Transition State Analogue Substrate

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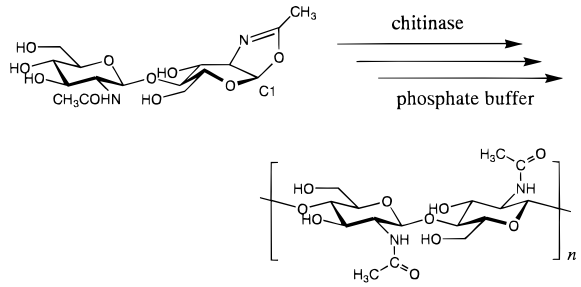
Chitin, a mucopolysaccharide from invertebrates, has recently been of great interest in numerous scientific and application fields as a multifunctional substance:[1] activator for immune systems,[2] inhibitor of metastases of tumor cells,[3] antibacterial substance,[4] wound-healing materials,[5] additives for cosmetics,[6] drug carriers,[7] health foods,[8] biodegradable polymers,[9] chelating polymers,[10] etc. Perfect artificial chitin is expected to advance these fields through superior gels,[11] enzyme immobilization,[12] and labeling.

The use of a glycosyl hydrolase as catalyst for selective synthesis of complicated polysaccharides has become a vivid topic in glycotechnology.[13] One of examples is the first successful *in vitro* synthesis of cellulose via a nonbiosynthetic pathway by utilizing cellulase as catalyst.[14] However, the limitation of many hydrolase-catalyzed reactions has long been pointed out by synthetic chemists because the yield of the reactions is normally low.[13] This is apparently attributed to the reversibility of the reaction catalyzed by the enzyme which promotes not only glycosylation of the substrate but also hydrolysis of the resulting product. The hydrolysis can not be avoided because the product is inherently a substrate for the hydrolysis enzyme. Therefore, the conformation of starting glycosyl substrates like glycosides,[15] phenyl glycoside derivatives,[16] and glycosyl fluorides[14,17−21] have to be very close to that of the product for the reaction to occur.

Scheme 1



Here, we report an extremely useful abiogenic method for chitin production where a combined use of a distorted substrate monomer and a hydrolysis enzyme allows the reaction to proceed only in the direction of polymerization at appropriate pH values. The reaction is designed on the hypothesis that the usage of a distorted glycosyl substrate close to a transition state structure lowers the free energy of activation, making the glycosylation possible even at the pH values where the hydrolysis enzyme does not cause the hydrolysis of the product. The ring-opening polyaddition of a chitobiose oxazoline derivative, a new monomer having a distorted structure with α configuration at C1, was exclusively promoted by chitinase, a hydrolysis enzyme of chitin, giving rise to high molecular weight chitin ($M_v > 4 \times 10^4$) (Scheme 1). As to the monomer size, we chose a chitobiose (disaccharide) derivative on the assumption that it would be preferentially recognized by the catalytic site of the enzyme over the *N*-acetylglucosamine (monosaccharide) derivative.

The new substrate monomer was easily prepared starting from chitobiose via three steps: acetylation of all hydroxy groups, formation of oxazoline ring by the action of trimethylsilyl trifrate,[22] and removal of acetyl groups by sodium methoxide. To a solution of the monomer (64 mg) in 0.01 M phosphate buffer (pH 10.6, 0.8 mL), an aqueous solution (0.2 mL) of chitinase (*Bacillus* sp., 1 wt % for the substrate) was added, and the mixture was incubated at 25 °C for 50 h. As the reaction proceeded, the initially homogeneous solution gradually became cloudy and finally the reaction system solidified with a white precipitate of the product polysaccharide. Tetrahydrofuran was added, and the mixture was heated at 90 °C for 5 min to deactivate the enzyme completely. The product was isolated by filtration and dried in vacuo (quantitative yield). The resulting polysaccharide was soluble in formic acid and a mixture of *N*,*N*-dimethylacetamide/*N*-methyl-2-pyrrolidone/lithium chloride but insoluble in water, methanol, and *N*,*N*-dimethylformamide.

The CP/MAS ^{13}C NMR spectrum of the product showed a signal at δ 105.0 ppm due to the C1 of the *N*-acetylglucosamine unit (Figure 1). The other signals at δ 84.4, 76.9, 74.3, 61.9, and 56.2 ppm correspond to the carbons C4, C5, C3, C6, and C2, respectively. Signals of the carbonyl carbon and the methyl carbon were observed at δ 175.0 and 23.8 ppm, respectively. No signals derived from the $\beta(1 \rightarrow 6)$[23] and $\beta(1 \rightarrow 3)$[24]

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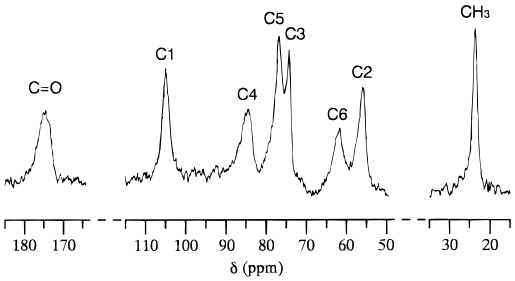

Figure 1. CP/MAS solid ^{13}C NMR (100 MHz) spectrum of artificial chitin.

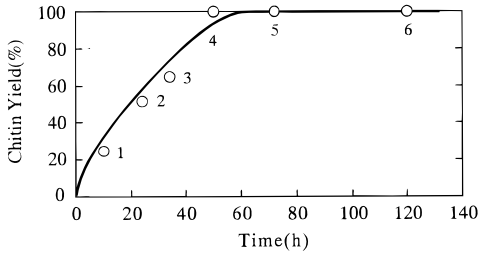

Figure 2. Relationships of chitin yields and molecular weight values versus reaction time. Polymerization performed at 25 °C in phosphate buffer (pH 10.6). Chitin was isolated by filtration followed by washing with methanol and then water. Molecular weight values (M_v) were the following: 1, 4.1×10^4; 2, 4.2×10^4; 3, 4.1×10^4; 4, 4.6×10^4; 5, 4.2×10^4; 6, 4.4×10^4.

glycosidic linkage were observed around δ 70 and 80 ppm, respectively. These results clearly indicate that the glycosidic bond formation occurred in a regio- and stereoselective manner between chitobiose units with the inversion of configuration at C1 during the polymerization, giving rise to the stereoregular polysaccharide having a $\beta(1 \rightarrow 4)$ linkage (*artificial chitin*).

The molecular weight of the isolated artificial chitin was measured in methanol containing calcium chloride dihydrate.[25] The intrinsic viscosity [η] of the above solution was 3.2 and the molecular weight (M_v) was determined to be 4.6×10^4 according to the Mark−Houwink−Sakurada equation, $[\eta] = KM^{\alpha}$ ($K = 2.54 \times 10^{-2}$, $\alpha = 0.45$).[25,26]

Figure 2 shows product yields and molecular weight values obtained as a function of reaction time at pH 10.6. The yield reached quantitative after 50 h. The yield and molecular weight did not decrease at longer reaction times, showing an unusual irreversible behavior of a glycosyl hydrolase. The polymerization by chitinase was efficiently catalyzed at this pH; whereas, the hydrolysis of the product chitin is completely suppressed, since chitinase has an optimal hydrolysis at pH 7.8.[27] All chitins obtained at six different reaction times had similar high molecular weights. The trend of yield (monomer conversion) versus molecular weight resembles that of the chain polymerization of vinyl monomers. To our knowledge, this is the first example that a polyaddition behaved similarly to a chain polymerization. According to the present method, the elongation of single glucan chain is possible in solution to produce a high molecular weight polymer before crystallization occurs as a result of polymer−polymer interaction via intermolecular hydrogen bonds.

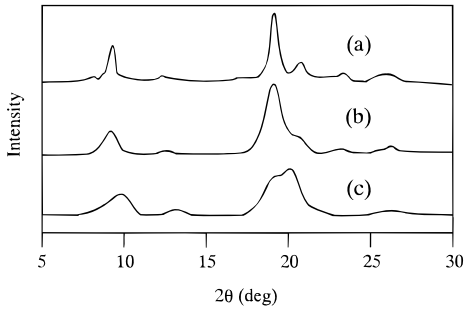

Figure 3. X-ray diffractograms of (a) artificial chitin, (b) natural α-chitin from queen crab, and (c) natural β-chitin from squid pens.

The X-ray diffraction measurement of the artificial chitin (Figure 3a) showed peaks at $2\theta = 9.34$ and 19.21, which are characteristic to α-chitin (Figure 3b)[28] and readily distinguishable from β-chitin (Figure 3c).[29] The sharp peaks of the artificial chitin indicated that the chitin had higher crystallinity than native α-chitin.

Since native chitin exists as a complex with proteins and inorganic materials, recovery of native chitins normally requires an acid or alkaline treatment. Consequently, the *N*-acetyl group in the *N*-acetylglucosamine unit becomes partially damaged, which results in imperfect chitins. This chitin must be reacetylated in order to obtain perfect chitins.[30] The present method allows the production of the perfectly *N*-acetylated chitins. Further, this method enables the synthesis of a modified chitin (e.g., labeled chitin), allowing biological studies to be made on chitin activity in living cells. It is also to be noted that the combined use of the transition state analogue monomer and the hydrolysis enzyme allows the reaction to proceed only in the direction of polymerization at appropriate pH values while suppressing hydrolysis of the product in aqueous media. The present methodology which involves the ring-opening polyaddition of a sugar oxazoline derivative as a glycosyl donor opens a new door for development of enzymatic glycosylation of 2-amino-2-deoxy sugars.

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