ENZYMATIC RING-OPENING POLYADDITION FOR CHITIN SYNTHESIS : A CATIONIC MECHANISM IN BASIC SOLUTION?

Toshitsugu Kiyosada, Shin-ichiro Shoda

Department of Materials Chemistry, Graduate School of Engineering, Tohoku University, Sendai 980-77, Japan

Shiro Kobayashi*

Department of Materials Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 606-01, Japan

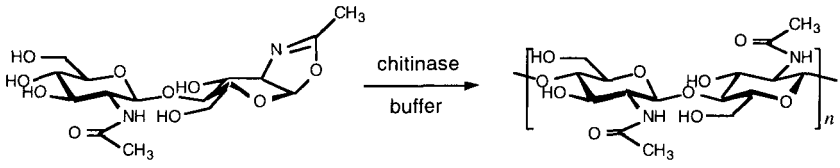
Abstract: The *in vitro* synthesis of chitin via a nonbiosynthetic path has been achieved for the first time by enzymatic ring-opening polyaddition of a chitobiose oxazoline monomer (**C2O**). Chitinase, a hydrolysis enzyme of chitin, recognized and polymerized the monomer regio- and stereoselectively. The structure of artificial chitin was confirmed by comparison with an authentic natural chitin sample with the use of CP/MAS ^{13}C NMR and IR spectroscopy. X-ray diffraction as well as NMR analysis showed its crystal structure of α-chitin. Characteristic features of the present polymerization are described.

INTRODUCTION

Chitin, which has a similar β(1→4) structure of cellulose, is the most abundant structural carbohydrate in animals. Most of chitin are biosynthesized by invertebrates. Chitin is an insoluble and non-swelling polysaccharide in any solvents. This is due to strong intermolecular hydrogen bonding originated largely from acetamide group. Recently, chitin and its derivatives attract much interest from such views as an inhibitor of metastases of tumor cells (Ref. 1), antibacterials (Ref. 2), wound-healing materials (Ref. 3), drug carriers (Ref. 4), biodegradable polymers (Ref. 5), etc. Until our recent work (Ref. 6), chitin synthesis had not been achieved, although *in vitro* synthesis of other polysaccharides, cellulose, amylose, and xylan, were achieved via enzymatic polycondensations using sugar fluorides as monomer and hydrolysis enzymes as catalyst (Ref. 7). We reported a completely novel approach for the synthesis of chitin by an enzymatic ring-opening polyaddition catalyzed by chitinase, not via a polycondensation, with the use of a novel oxazoline derivative monomer (**C2O**) from a disaccharide (Ref. 6). It was postulated that monomer **C2O** would be preferable as a substrate because it can be

 CCC 1022-1360/98/$ 17.50

recognized by the binding site of chitinase readily due to the structure resembling that of the transition state of the chitin hydrolysis by chitinase (Ref. 8). Thus, monomer **C2O** was polymerized very readily and is considered to be a "transition state analogue substrate".



C2O artificial chitin

RESULTS AND DISCUSSION

Polymerization of Chitobiose Oxazoline (**C2O**)

General procedures for polymerization of **C2O** were as follows. To a buffer solution (pH 8.0 - 12.9, 0.8 mL) containing 64 mg of **C2O** was added a chitinase (from *Bacillus* sp., 0.64 mg) buffer solution (0.2 mL) at 30 ℃. The mixture was kept at the same temperature for some hours. As the reaction proceeded, the initially homogeneous solution gradually became heterogeneous with a white precipitation. After the reaction, 1.0 mL of THF was added to the reaction mixture and heated at 90 ℃ for 5 minutes to inactivate chitinase. The mixture was poured into an excess methanol and water to remove the catalyst chitinase, chitooligosaccharides, and the oxazoline monomer, if remained. A part of the oxazoline derivative was converted to *N,N'*-diacetyl chitobiose by hydrolysis during work-up procedures. The resulting gel was collected by centrifugal separation and dried in vacuo to give white powdery materials that is a water insoluble part. Chitooligosaccharides of the water and methanol soluble part were dimer, tetramer and hexamer, which were determined by HPLC. The structure of the white materials has been characterized by IR, CP/MAS ^{13}C NMR spectroscopies (Ref. 9), X-ray diffraction (Ref. 10), and MALDI-TOF mass spectrum.

Characterization of Product Chitin

The FT-IR spectrum of the white materials resembles that of natural α-chitin. Fig. 1 indicates CP/MAS ^{13}C NMR spectra of the product, natural α-chitin, and natural β-chitin, respectively. It is clear that the materials is very close to natural α-chitin in the ^{13}C NMR pattern. The signals at 105.0, 84.4, 76.9, 74.3, 61.9, and 56.2 ppm are due to the C1, C4, C5, C3, C6, and C2 carbon atoms, respectively. The signals of acetamide group are observed at 175.0 and 23.8 ppm as carbonyl and methyl carbon atoms, respectively. And, no signal due to the methylene carbon

atom (C6), which is linked as the β(1→6) glycosidic bond type, was observed around 70 ppm (Ref. 11). These results clearly show that the resulting products have a β(1→4) glycosidic linkage ; the product is thus to be called as "artificial chitin". Further, a X-ray diffraction pattern of the artificial chitin corresponds to natural α-chitin, but artificial chitin had higher crystallinity than native α-chitin as seen by sharp peaks at 2θ = 9.34 and 19.21 of artificial chitin (Fig. 2) (Ref. 10).

The molecular weight of the artificial chitin was determined by MALDI-TOF mass spectroscopy (Fig. 3). The chart indicates $Mw = 4.0 \times 10^4$ and $Mn = 2.9 \times 10^4$. The molecular weight was also determined by viscosity method. The intrinsic viscosity [η] of the artificial chitin in methanol / calcium chloride dihydrate solution was 3.2, which corresponds to the molecular weight (Mv) of 4.6×10^4, according to the Mark–Houwink–Sakurada equation ([η] = K•Mv^{α}, K = 2.54×10^{-2}, α = 0.45). (Ref. 12).

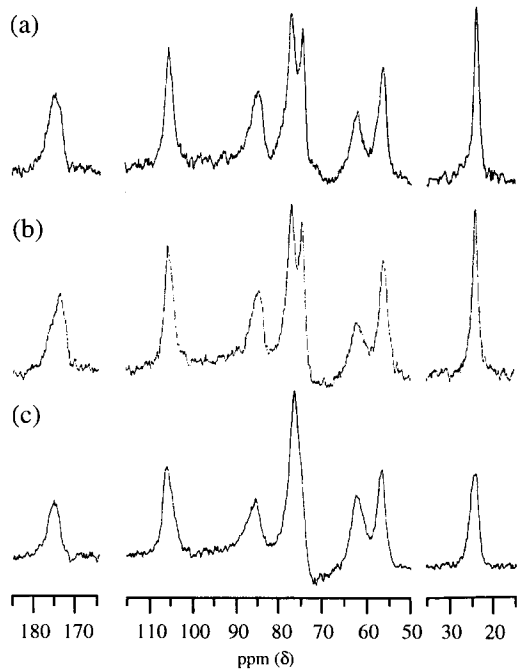


Fig 1. CP/MAS ^{13}C NMR (100MHz) spectra of a) the artificial chitin (the water insoluble part), b) natural α-chitin from queen crab, and c) natural β-chitin from squid pens

Treatment of artificial chitin with chitinase produced, of course, the hydrolysis products, mainly *N,N'*-diacetyl chitobiose.

All the above data fully support that the present artificial chitin is almost identical with natural α-chitin in higher structure.

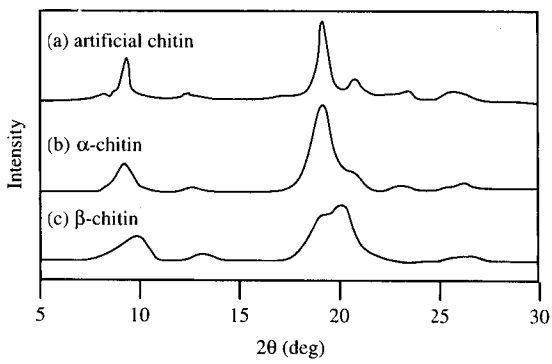


Fig 2. X-ray diffractograms of a) artificial chitin, b) natural α-chitin from queen crab, and c) natural β-chitin from squid pens

Characteristics of the Polymerization

Tab. 1 shows the effect of pH on the enzymatic polyaddition of **C2O** by chitinase. The reaction at pH 8.0 is a fast polymerization but gave low yield (38%) with lower molecular weight ($Mv = 0.9 \times 10^4$). At pH 9.0, the polymerization became slower but gave higher yield (62%) and higher molecular weight polymer ($Mv = 2.2 \times 10^4$). The reaction at pH 10.6 is the most effective polymerization system of **C2O**, with respect to yield and molecular weight (~100% and $Mv = 4.6 \times 10^4$, respectively), in spite of a slow polymerization. At a higher pH of 11.4, the polymerization became slower, giving rise to a lower molecular weight polymer. When the medium is more basic to pH 12.9, the polymerization did not take place. These phenomena are quite surprising, since the optimal pH value for hydrolysis of chitin by chitinase is 7.8 (Ref. 13). Chitinase catalyzed the polymerization irreversibly at a higher pH range under the complete suppression of its hydrolysis catalysis of the product chitin.

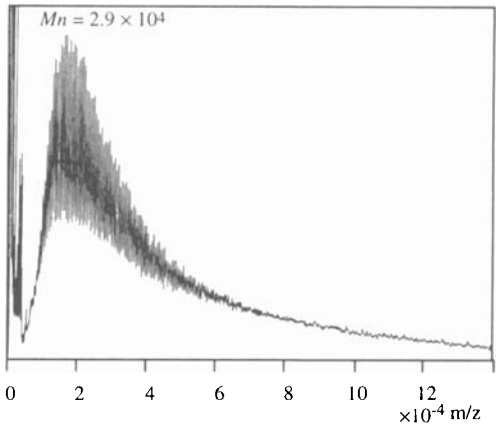


Fig 3. MALDI TOF-mass spectrum of artificial chitin

Tab. I. Effect of pH on the enzymatic polyaddition[a] of **C2O**

entry	pH	time(h)	yield of chitin(%)	$Mv^{b}(\times 10^{-4})$
1	8.0	2.5	38	0.9
2	9.0	20	62	2.2
3	10.6	50	quant.	4.6
4	11.4	150	87	<0.5
5	12.9	300	0	–

[a] Polymerization in phosphate buffer at 25℃, [**C2O**] = 0.2M, chitinase = 1wt%. [b] Determined by viscosity.

Fig. 4 shows profiles of polymerization time with respect to chitin yield and to molecular weight. Under the polymerization conditions of pH 10.6 at 25℃, the chitin yield became quantitative after 50 hr. Even at a longer reaction time the chitin yield did not decrease, indicating no hydrolysis of the product chitin. It is to be noted that the molecular weight was already high at a

low conversion of the monomer and its value was in a small range for all six runs. This phenomenon is very close to that of a radical chain polymerization of typical vinyl monomers, involving a slow initiation and a fast propagation. To our knowledge the present reaction is the first case in the polyaddition type polymerizations to show such a molecular weight-time relationship.

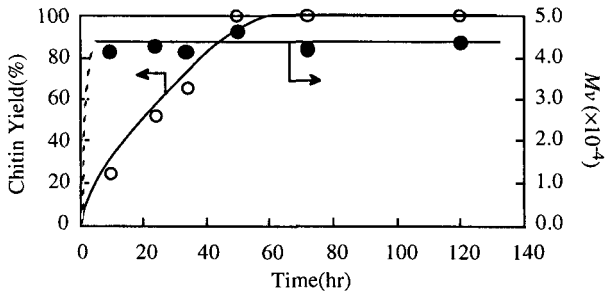
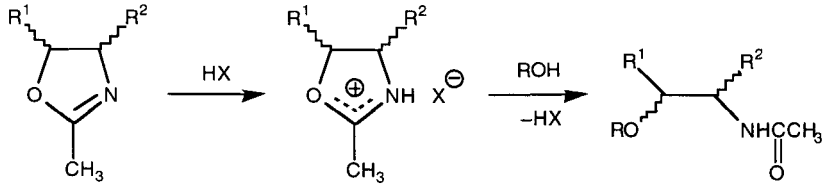


Fig 4. Relationships of chitin yields and molecular weight values versus reaction time. Polymerization was performed at 25°C in phosphate buffer (pH10.6)

Based on the above observations, the reaction involves protonation of the 2-methyl-oxazoline ring of **C2O** monomer even under basic reaction conditions according to a well-known reaction mode (Ref. 14) catalyzed by chitinase.



In the polymerization, HX probably corresponds to a carboxylic acid group of the active site in chitinase and ROH to 4'-hydroxy group of the monomer.

CONCLUSION

The first in vitro synthesis of chitin via a nonbiosynthetic path has successfully been achieved with use of a chitobiose oxazoline monomer (**C2O**) as a " transition state analogous substrate " for chitinase (from *Bacillus* sp.), a hydrolysis enzyme of chitin. The enzymatic polyaddition of **C2O** took place even in a weakly basic medium via a cationic reaction mode. It is also to be noted that the present method of using a " transition state analogous monomer " for a hydrolytic enzyme has opened a way to the future development of new methodology for synthesis of various polysaccharides including chitin and a variety of biologically important poly- and oligosaccharides.

ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Science and Culture, Japan (08102002).

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