Enzyme-Catalyzed Polymerization Mechanism of Aliphatic Polyester Investigated by ^1H NMR and MALDI-TOF Mass Spectrum

Takahiko NAKAOKI,[†] Mari DANNO, and Kiyoshi KUROKAWA*

Department of Materials Chemistry, Faculty of Science and Technology, Ryukoku University,
Seta, Otsu, Shiga 520–2194, Japan

*Department of Anatomy, Shiga University of Medical Science, Seta, Otsu,
Shiga 520–2192, Japan

(Received May 19, 2003; Accepted July 31, 2003)

ABSTRACT: Enzyme-catalyzed polymerization mechanism of adipic acid and hexanediol was investigated by proton nuclear magnetic resonance (^1H NMR) and MALDI-TOF mass spectra. When the stoichiometric substrates were used for the reaction, ^1H NMR measurements showed that the hydroxyl terminated product was preferentially formed at the early stage, then with reaction time it changed to carboxyl terminated product. This process was also confirmed by MALDI-TOF mass spectra. First the chain propagation follows the step-growth mechanism accompanying the sequential addition of a diacid-diol unit. In this case, an enzyme-substrate complex is attacked by hydroxyl group of substrates, and as a result the hydroxyl terminated product was preferentially formed. And at the later stage of the reaction, the transesterification plays an important role to form the carboxyl terminated product. When an excess diacid was used, the end group structure showed the same behavior as the stoichiometric condition, *i.e.*, the predominant end group is consisted of the hydroxyl group at the initial stage then it rapidly changed to the carboxyl group although the time to change to carboxyl terminated product became shorter. In case of the condition of an excess diol, the reaction was avoided at the early stage because no enzyme forms a complex with diacid.

KEY WORDS Enzyme / Polycondensation / End Structure / Polyester / Nuclear Magnetic Resonance (NMR) / MALDI-TOF Mass Spectrum /

Enzyme-catalyzed polymerization in organic media has been extensively investigated during the last decade. Generally lipases are known to catalyze esterification and transesterification. Enzymatic ring opening polymerization such as lactones has been well-established by many researches. In 1993, Kobayashi *et al.* succeeded to polymerize different lactones using some lipases.¹ The advantage of this polymerization is that a leaving group is not produced and high molecular weight product is obtained. Lots of investigations on different ring size of lactones, solvent effect, and an enzymatic activity of lipases immobilized on different matrix have been carried out.^{2–16} In case of condensation of diol and diacid with aliphatic chain, the high molecular weight polyester can be obtained by removing a leaving group. Klivanov *et al.* have explored the enzymatic catalyzed asymmetric polymerization of aliphatic diol and diacid.¹⁷ They observed the formation of hydroxyl terminated oligomers, as an excess diol was used. Morrow *et al.* recognized the importance of stoichiometry and studied polycondensation using equimolar quantities of diol and diacid.¹⁸ They succeeded to prepare polyester with a several thousands of molecular weight. They also showed that the early stage of polymerization followed relatively rapid formation of hydroxyl terminated polyester and slower

formation of carboxyl terminated one. Enzymatic synthesis of aliphatic polyester in solvent-free system was studied by Uyama *et al.*, and the dependence of methylene length of monomers on polymerization was reported.^{19,20} Binns *et al.* investigated the polycondensation in stoichiometric adipic acid and butane-1,4-diol system.^{21,22} They proposed a step-growth mechanism following the sequential addition of a unit of diacid-diol. It was shown that the predominant product is hydroxyl terminated oligomers.

In this paper, the enzymatic polymerization process of adipic acid and hexamethylene diol was investigated by ^1H NMR and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopies. The reaction was conducted in a Dean-Stark apparatus. In order to avoid the deactivation of enzyme at high temperature, hexane with the boiling point of 69 °C was used as a reaction solvent. The purpose is to make clear the relationship between the end group structure and the reaction mechanism.

EXPERIMENTAL

Materials

Adipic acid and hexamethylene diol were purchased from Wako Co. Ltd., and used without further purification. The immobilized lipase from *Candida Antarctica*

[†]To whom correspondence should be addressed.

(Novozyme-435) was provided from Novozymes Japan Co. Ltd. Novozyme-435 was used after drying in a vacuum desiccator.

Measurements

^1H NMR spectra were recorded on Bruker DPX400 spectrometer. Each sample was dissolved in CDCl_3 , and measured at room temperature. Chemical shifts are referenced by tetramethylsilane (TMS). MALDI-TOF mass spectrum was measured by Voyager RP biospectrometry workstation equipped with a nitrogen laser (337 nm, 3 ns pulse width). The products were dissolved in tetrahydrofuran (THF, 1 mg mL^{-1}). $1\text{ }\mu\text{L}$ of the product solution was mixed with $10\text{ }\mu\text{L}$ of matrix solution of 2,5-dihydroxy benzoic acid (2,5-DHB) dissolved in THF (10 mg mL^{-1}). The mixture was put and dried on sample plate after coated by NaCl solution. The mass peaks observed in the spectrum are associated with the Na^+ complex, and the exact molecular mass was obtained by reducing 23 of Na from the observed one.

Polycondensation

A 5 mmol of adipic acid was added to a solution of 5 mmol of 1,6-hexanediol in 30 mL of hexane and Novozyme-435 (30 mg) was added. The solution was stirred at boiling point of hexane in a Dean-Stark apparatus with 0.5 g of molecular sieves. The reaction was terminated by adding 60 mL of cold chloroform and the enzyme and monomers were removed by filtration.

RESULTS AND DISCUSSION

Polycondensation with Equimolar Quantities of Diacid and Diol

In ^1H NMR spectrum, the methylene signals adjacent to hydroxyl and carboxyl end groups of polyester ($-\text{CH}_2-\text{OH}$ and $-\text{CH}_2-\text{COOH}$, respectively) give different chemical shifts from inner methylenes. These signals make it possible to estimate the integral ratio of hydroxyl and carboxyl terminated groups. The polycondensation process of adipic acid and hexamethylenediol was investigated as a function of reaction time. Since the methylene signal adjacent to carboxyl end group is overlapped with other peaks, an oxalyl chloride was added to the product solution in order to isolate this signal. Figure 1 shows typical ^1H NMR spectra of the product including oligomers before and after adding oxalyl chloride. A new peak due to carboxyl chloride $-\text{CH}_2-\text{COCl}$ was observed at 2.93 ppm. Similarly, the signal of hydroxyl terminated group shifted from 3.67 to 4.37 ppm. The integral intensity ratio for these signals provides the information

Figure 1. ^1H NMR spectra of the product from lipase catalyzed polycondensation of adipic acid with hexane diol for 6 h (a) before and (b) after adding an oxalyl chloride. The signals of methylene carbons adjacent to terminated groups $-\text{OH}$ and $-\text{COOH}$ shifted toward downfield.

Figure 2. Integral intensity ratio of hydroxyl and carboxyl terminated groups observed in ^1H NMR for equimolar adipic acid and hexane diol. The reactions were carried out more than twice and then the average values were calculated.

on molecular structure of end groups. In addition, the number average molecular weight (M_n) can be evaluated from the ratio of the peak intensity of methylene signal adjacent to inner ester groups ($-\text{CH}_2-\text{COO}-$) at 4.07 ppm and the total integral intensity of end groups. Figure 2 shows the intensity ratio of hydroxyl and carboxyl groups (OH/COOH) as a function of reaction time. Since the reaction was conducted under inhomogeneous condition, the estimated values accompanied with large fluctuation. Therefore the reactions were carried out more than twice, and the error bars were added in the figure. At the early stage of the reaction, the ratio of end group took about 5, indicating that pre-

Figure 3. MALDI-TOF mass spectra of the products polymerized for (a) 24 h and (b) 72 h with a DHB matrix. ○: $A(BA)_n$, ●: $B(AB)_n$, △: cyclic $(AB)_n$, and ▼: $(AB)_n$. The number of repetition units n was depicted in the figure.

ferred product is terminated by hydroxyl group. Binns *et al.* proposed the reaction mechanism for polycondensation of aliphatic diacid (abbreviated as A) and diol (abbreviated as B) in solvent free system.^{21,22} According to their result, AB unit plays a key role to make the chain grow. The enzyme-substrate complex (Enz-O-AB) is attacked by B, and then the oligomer consists of hydroxyl terminated products such as BAB, $B(AB)_2$, $B(AB)_3$, and so on. Finally they concluded that the preferred product is hydroxyl terminated one. The result that the hydroxyl terminated product was generated at the initial stage of the reaction as shown in Figure 2 consistent with their result. However with increasing reaction time, the hydroxyl terminated content was reduced. When the reaction time is 72 h, the ratio became less than 1, indicating that the carboxyl terminated products were preferentially formed. The analysis of ^1H NMR gives the average value of all products including various chain lengths with different terminated structures. In order to make clear the detail structure of end group, MALDI-TOF mass spectra were measured as shown in Figure 3. Figure 3a shows the mass spectrum of the product reacted for 6 h. The spectrum is characterized by regularly repeated peaks. These components distributed over wide range with high molecular weight, and were classified into four species of hydroxyl terminated and carboxylic terminated polyesters, a hydroxyl and carboxyl terminated polyester, and a cyclic polyester. These are represented by $B(AB)_n$, $(AB)_nA$, $(AB)_n$, and cyclic $(AB)_n$, respectively. The peak due to cyclic $(AB)_n$ provided

Figure 4. Number average molecular weight (M_n) calculated from ^1H NMR spectrum as a function of reaction time.

the strongest intensity. The content of cyclic products is estimated as 0.44 in all products. Since the nearest nucleophile is the hydroxyl terminated group of Enz-O- $(AB)_n$, the cyclic structures would be formed with ease. The peaks of $B(AB)_n$ displayed by close circles in Figure 3a have comparatively strong intensity and stronger than those of $A(BA)_n$. This leads to the result that the preferred product reacted for 6 h took the hydroxyl terminated structure. The spectrum for the product after 72 h polymerization was shown in Figure 3b. The peak distribution is almost the same as Figure 3a except the inverse intensity between $B(AB)_n$ and $(AB)_nA$. This indicates that with reaction time the terminated structure varied from hydroxyl terminated product to carboxyl one. This is in good agreement with the result of ^1H NMR analysis. This suggests that there exists the different mechanism between the early and later stages of the reaction. In Figure 4 shows M_n observed by ^1H NMR. First M_n increased with reaction time, but it decreased at 72 h. Perhaps the reaction would be dominantly the propagation process at the early stage, then the transesterification made the chain length smaller at the later stage.

Model Mechanism of Enzymatic Esterification

In general, it has been believed that ester hydrolases contain a Ser-His-Asp/Glu residues in a active site. This process was confirmed by Russell *et al.*; the enzyme catalyzes the reaction with the ester, since the ester monomer always provided less than diol during polymerization.^{23,24} The initiation step of the reaction is the acylation of hydroxyl group of serine with carboxyl group of substrates such as $A(BA)_n$ and $(AB)_n$ ($n = 1, 2, 3 \dots$), and Enz-O- $A(BA)_n$ or Enz-O-

Scheme 1. Initial stage. **Scheme 2.** Propagation step. **Scheme 3.** Cyclization. **Scheme 4.** Transesterification. **Scheme 5.** Termination.

$(\text{AB})_n$ is formed. This step was schematically shown in Scheme 1. And then, the oxygen atom of hydroxyl group of $(\text{AB})_n$ and B(AB)_n acts as nucleophile, and reacts with Enz-O-A(BA)_n or Enz-O-(AB)_n . This propagation process was depicted in Scheme 2. In case of Enz-O-(AB)_n , the hydroxyl group in the same molecule acts as nucleophile and the cyclic compound is formed (Scheme 3). Transesterification process is represented by Scheme 4. As can be seen in the scheme, this step allows the chain length reduce. The enzyme-substrate complex reacts furthermore as following Scheme 2. At last the polymerization is terminated by water as shown in Scheme 5.

The initial stage of the reaction has been proposed by Binns *et al.*^{21,22} First, the enzyme reacted with A, that is Enz-O-A was formed. This process corresponds to the Scheme 1(1) ($n = 0$). Then the hydroxyl group of B acts as nucleophile and AB is formed (Scheme 2(3) ($n = 0$ and $m = 0$)) A unit of AB will make an

Figure 5. Integral intensity ratio of hydroxyl and carboxyl terminated groups observed in ^1H NMR for the product polymerized under the condition of excess diacid. The reactions were carried out more than twice and then the average values were calculated.

enzyme-substrate complex, that is Enz-O-AB is formed (Scheme 1(2) $n = 0$). Then this complex is attacked by B or BA as nucleophiles following Scheme 2(5) ($n = 0$ and $m = 0$) or Scheme 2(6) ($n = 0$ and $m = 0$), respectively. At the initial stage, B exists much more than BA so that the preferred process is Scheme 2(5). And then B(AB)_m ($m = 1, 2, 3 \dots$) attacks Enz-O-AB (Scheme 2(5)) and B(AB)_m is formed by the addition of AB unit. These processes provide the hydroxyl terminated polyester, which is experimentally shown by ^1H NMR and MALDI-TOF mass spectra. With proceeding reaction, B will successively become a minor components and less than BA. The preferred process will be Scheme 2(6), instead of Scheme 2(5). In this case, $(\text{AB})_n$ ($n = 0, 1, 2 \dots$) forms the enzyme-substrate complex and Enz-O-(AB)_n is formed (Scheme 1(1)). Then following Schemes 2(5) and 2(6), B(AB)_n and $\text{B(AB)}_n\text{A}$ are formed. In any cases, the carboxyl terminated polyester would be formed. However, the experiments provided the fact that the OH/COOH ratio is less than 1, indicating that the major component is the polyester terminated by two carboxyl groups. Perhaps the transesterification would be important for this process. From Schemes 4(8) and 4(10), the product of Enz-O-A(BA)_n is expected. Then the carboxyl terminated polyester of $(\text{AB})_n\text{A}$ is formed through the Scheme 2(4). As can be seen in Scheme 4, the transesterification has another effect to make the molecular weight reduce. In fact, the experimental result supported this process, as shown in Figure 4.

Figure 6. MALDI-TOF mass spectra of the products polymerized for (a) 24 h and (b) 72 h with a DHB matrix.

Polycondensation under the Condition of Excess Diacid

Next we investigated the polycondensation mechanism under the condition of excess diacid, *i.e.*, the monomer ratio of A and B is 1.1 : 1. Figure 5 shows the intensity ratio of OH/COOH by ^1H NMR as a function of reaction time. When the reaction was conducted for 6 h, the OH/COOH ratio took about 2.5, corresponding that the preferred end group is the hydroxyl group. However it rapidly decreased with reaction time, then the preferred compound changed to carboxyl terminated product. As described in the above section, the AB unit is important for polymerization. After all diols are consumed to connect with diacids, further acylation between diacid and enzyme would be promoted. Then $(\text{AB})_n$ or $(\text{AB})_n\text{A}$ would be formed following Scheme 2(3) and 2(4). Figure 6a represents MALDI-TOF mass spectrum of the product reacted for 72 h. The main peaks of spectrum (a) consist of $\text{A}(\text{BA})_n$, corresponding that the preferred end group is carboxyl terminated group which is the same result as shown in ^1H NMR. So far, when the excess diacid was used, it has been reported that the product preferentially consists of the carboxyl terminated one. However in this investigation it was revealed that the initial stage of the polymerization provided the hydroxyl terminated product following the scheme shown in previous section and then changed to carboxyl terminated one. M_n estimated from ^1H NMR was shown in Figure 7. Judging from Figure 5, the propagation reaction finished within a few hours. Russell *et al.* reported that higher concentration of ester end group leads to a reactivity faster polymerization.^{23,24} Then M_n reached the highest value after

Figure 7. Number average molecular weight calculated from ^1H NMR spectrum as a function of reaction time.

Figure 8. Integral intensity ratio of hydroxyl and carboxyl terminated groups observed in ^1H NMR for the product polymerized under the condition of excess diol. The reactions were carried out more than twice and then the average values were calculated.

24 h of reaction, and it slightly decreased due to transesterification.

Polycondensation under the Condition of Excess Diol

Finally, the polycondensation mechanism under the condition of excess diol, *i.e.*, the ratio A : B = 1 : 1.1 will be described in this section. Figure 8 shows OH/COOH ratio obtained by ^1H NMR depending on reaction time. The hydroxyl terminated content is much larger than carboxyl one. Figure 6b shows MALDI-TOF mass spectrum of the product polymerized for 72 h. The preferred product is the hydroxyl terminated $\text{B}(\text{AB})_n$. This

Figure 9. Number average molecular weight calculated from ^1H NMR spectrum as a function of reaction time.

Figure 10. Number average molecular weight as a function of monomer ratio of diacid and diol.

result is in good agreement with that of ^1H NMR. Since the formation of enzyme-diacid complex catalyzes the esterification, the polymerization would be hard to promote if there exists only a small amount of diacid A. As for the molecular weight shown in Figure 9, ^1H NMR provided the average value as low as 2000, and it kept almost constant independent of reaction time. The propagation reaction would finish within 6 h because of the lack of diacid. Russell *et al.* also observed that the excess diol provided the product with the low molecular weight. In order to make clear the effect of excess diol, the reaction was conducted for the different molecular ratio of diacid and diol, that is A : B = 1 : 1.05, 1 : 1.1, and 1 : 1.2. Figure 10 shows the M_n depending

on OH/COOH ratio. It was revealed that M_n decreased with increasing monomer content of diol. The more the diol is, the faster the free diacid was lost in the solution. The hydroxyl terminated product would restrict the propagation process.

CONCLUSIONS

Enzymatic polycondensation mechanism of adipic acid and hexandiol was investigated by ^1H NMR and MALDI-TOF mass spectra. The ratio of end group of OH and COOH for the polyester was estimated by ^1H NMR. When the equimolar substrates were used for the reaction, the hydroxyl terminated product preferentially yielded at the early stage, and then with increasing reaction time the preferred product changed to carboxyl terminated one. This process was followed by MALDI-TOF mass spectra. The products consist of four species of $(\text{AB})_n$, $\text{B}(\text{AB})_n$, $(\text{AB})_n\text{A}$ and cyclic $(\text{AB})_n$. First the hydroxyl terminated $\text{B}(\text{AB})_n$ was a preferred product, because $\text{B}(\text{AB})_n$ ($n = 0, 1, 2, 3 \dots$) attacked Enz-O-AB as a nucleophile. The early stage of the reaction can be interpreted by Binns's proposal.^{21,22} However at the later stage of the reaction, the carboxyl terminated product was preferred through transesterification. Even for the reaction conducted under the condition of excess diacid, the early stage took the hydroxyl terminated polyester and then it changed to the carboxyl terminated one at the later stage. This result shows that the initiation and propagation mechanism followed Schemes 1 and 2 with no relation to the amount of diacid. In case of the condition of excess diol, there is a fewer opportunity to form enzyme-substrate complex. As a result, most of the product consists of hydroxyl terminated polyester.

Acknowledgment. This investigation was financially supported by Riko Kikin at Ryukoku University. The authors thank Mr. Noboru Urushiyama for the assistance in acquiring the MALDI-TOF mass spectra.

REFERENCES

1. H. Uyama and S. Kobayashi, *Chem. Lett.*, 1149 (1993).
2. D. Knani, A. L. Gutman, and D. Kohn, *J. Polym. Sci., Part A: Polym. Chem.*, **31**, 1221 (1993).
3. R. T. MacDonald, S. K. Puapura, Y. Y. Svirkin, R. A. Gross, D. L. Kaplan, J. Akkara, G. Swift, and S. Wolk, *Macromolecules*, **28**, 73 (1995).
4. Y. Y. Svirkin, J. Xu, R. A. Gross, D. L. Kaplan, and G. Swift, *Macromolecules*, **29**, 4591 (1996).
5. L. A. Henderson, Y. Y. Svirkin, R. A. Gross, D. L. Kaplan, and G. Swift, *Macromolecules*, **29**, 7759 (1996).
6. F. Dong and R. A. Gross, *Int. J. Biol. Macromol.*, **25**, 153

- (1999).
7. A. Kumar and R. A. Gross, *Biomacromolecules*, **1**, 133 (2000).
8. A. Kumar and R. A. Gross, *J. Am. Chem. Soc.*, **122**, 11767 (2000).
9. A. Kumar, B. Kalra, A. Dekhterman, and R. A. Gross, *Macromolecules*, **33**, 6303 (2000).
10. H. Uyama, K. Takeya, N. Hoshi, and S. Kobayashi, *Macromolecules*, **28**, 7046 (1995).
11. H. Uyama, K. Takeya, and S. Kobayashi, *Bull. Chem. Soc. Jpn*, **68**, 56 (1995).
12. H. Uyama, H. Kikuchi, K. Takeya, N. Hoshi, and S. Kobayashi, *Acta Polym.*, **47**, 357 (1996).
13. S. Namekawa, H. Uyama, and S. Kobayashi, *Polym. J.*, **30**, 269 (1998).
14. S. Kobayashi, H. Uyama, S. Namekawa, and H. Hayakawa, *Macromolecules*, **31**, 5655 (1998).
15. H. Uyama, Y. Shigeru, and S. Kobayashi, *Polym. J.*, **31**, 380 (1999).
16. T. Nakaoki, Y. Mei, L. Miller, A. Kumar, B. Kalra, O. Kirk, M. Cristensen, and R. A. Gross, to be submitted.
17. A. L. Margolin, J. Y. Creene, and A. M. Klibanov, *Tetrahedron Lett.*, **28**, 1607 (1987).
18. J. S. Wallace and C. J. Morrow, *J. Polym. Sci., Part A: Polym. Chem.*, **27**, 3271 (1989).
19. H. Uyama, K. Inada, and S. Kobayashi, *Chem. Lett.*, 1285 (1998).
20. H. Uyama, K. Inada, and S. Kobayashi, *Polym. J.*, **32**, 440 (2000).
21. F. Binns, P. Harffey, S. M. Roberts, and A. Taylor, *J. Polym. Sci., Part A: Polym. Chem.*, **36**, 2069 (1998).
22. F. Binns, P. Harffey, M. Stanley, S. M. Roberts, and A. Taylor, *J. Chem. Soc., Perkin Trans. I*, 2671 (1999).
23. A. K. Chaudhary, E. J. Beckman, and A. J. Russell, *Biotechnol. Bioeng.*, **55**, 227 (1997).
24. A. K. Chaudhary, E. J. Beckman, and A. J. Russell, *AIChE Journal*, **44**, 753 (1998).