Changes in Diad Sequence Distribution by Preferential Chain Scission during Thermal Hydrolysis of Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)

NOTE

Title:

Changes in Diad Sequence Distribution by Preferential Chain Scission during Thermal Hydrolysis of Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)

Running Head:

Diad Sequence Analysis of Thermal Hydrolyzed PHBHHx

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INTRODUCTION

Polyhydroxyalkanoates (PHAs) are microbial polyesters produced by many types of bacteria as an intracellular energy reserve material under the condition of substrate limitation as well as excessive carbon source.[1] The most commonly used poly((*R)*-3-hydroxybutyrate) (PHB) is the first member of PHA family to be discovered among more than 150 other monomer units which have been reported to date.[2,3] Poly((*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate) (PHBHHx) is a type of copolymer in PHA family that consists of randomly distributed (*R*)-3-hyroxybutyrate (HB) and (*R*)-3-hydroxyhexanoate (HHx) units.[4] This type of copolymer exhibits improved mechanical properties and processability compared to PHB and poly((*R*)-3-hydroxyvalerate) (PHBV).[5] PHBHHx copolymer is currently produced in large scale and was proven to be biocompatible in clinical studies using mouse fibroblasts cells as well as rabbit articular cartilage-derived chondrocytes.[6] PHBHHx is a highly favorable copolymer of PHB family not only due to its biodegradability, but also due to its flexible mechanical properties and good melt-processability.

Since PHBHHx has great potential uses in various fields, it is important to clarify its degradation properties, particularly thermal degradation and hydrolysis that induce serious deterioration of mechanical properties after melt-processing. Previously, degradation studies on PHA family were mainly concerned on enzymatic degradation,[7,8] thermal degradation,[9-10] hydrothermal degradation[11] as well as acid and alkaline hydrolysis,[12] where the resulted degradation products are very much dependent on the degradation mechanisms. It is well accepted that thermal degradation of PHB occurs through the random chain scission via β-elimination, resulting in final products with an unsaturated chain end. In the case of hydrothermal degradation of PHB, homogeneous and random degradation proceeded in a manner

of bulk erosion mechanism under high-temperature (180-300 °C) and high-pressure to produce water soluble monomers and oligomers.[11]

Previously, some studies on hydrolysis reaction of PHBHHx reported that hydrolysis occurred preferentially at amorphous phase than in crystalline phase.[13-15] However, there is lack information on the effect of HHx unit in the 3HB unit sequence on hydrothermal degradation of PHBHHx. In order to clarify the effect of HHx unit on the hydrothermal degradation, herewith super-heated steam (SHS) hydrolysis was introduced as an alternative method to hydrolyze PHBHHx. SHS is a type of unsaturated steam resulted from addition of heat to the saturated steam that enables the steam temperature to exceed its boiling point.[16] SHS is highly practical since it is operatable at atmospheric pressure,[17] and able to easily diffuse into PHBHHx homogeneously as well as specifically degrades the ester bonds without any dissolving out of into a liquid solvent of hydrolyzates that interrupts quantitative analysis of degradation behavior.[18]

In this study, effects of HHx unit on hydrothermal degradation of PHBHHx having 6% HHx unit content were investigated in comparison with PHB, particularly focusing on the changes in diad sequence distribution after the SHS hydrolysis. Diad sequence distribution for PHBHHx has been determined in previous studies,[13-14] however, to date there is lack of report on the changes in diad sequence distribution during degradation, *i.e.* effect of HHx unit in the sequence during hydrolysis. In order to clarify the subject, the changes in 1^{st}-order structure of PHBHHx during the SHS hydrolysis were monitored by precise structure analyses with size-exclusion chromatograph (SEC), proton (^{1}H) and carbon (^{13}C) NMR spectroscopies. Moreover, the reaction mechanism was validated by LUMO analysis of model compounds.

EXPRIMENTAL PROCEDURE

Characterization details of materials: PHBHHx having 6.0 % HHx unit content (poly(*R*)-3-hydroxybutyrate-*co*-6%-(*R*)-3-hydroxyhexanoate) and PHB that have been subjected to a purification process were described in Supplementary Information. Film preparation was as follows: About 5g of PHA sample was weighed and placed on top of metal mold (10×10 cm^2, 0.5mm thickness) with teflon sheet basement, followed by up-down close-up with Al plates. The sample was preheated on a hotplate at 145 and 170 °C for PHBHHx and PHB samples, respectively, before being pressed at 10 MPa for 3 min followed by 40-50 MPa for 5 min to form transparent films. The film was cut into sample strips (1×4 cm^2, 0.5mm thickness) and used in SHS treatment. The sample strips were treated under prescribed conditions at 130-190 °C for 600-200 min at regular intervals: 60, 40, 30 and 20 min for 130, 150, 170 and 190 °C, respectively. After the SHS treatment, the samples were kept in a desiccator at room temperature overnight until constant weights were obtained. Then, the sample strips were kept in an airtight bag at 4 °C prior to analysis.

Details of characterization of samples were also described in Supplementary Information: Molecular weight changes were analyzed with a size exclusion chromatograph (SEC). Proton (^1H) (500-MHz, $CDCl_3$) and carbon (^{13}C) NMR (125-MHz, $CDCl_3$) spectroscopy were applied to determine the HB and HHx units composition and diad sequence distribution. A semi-empirical molecular orbital calculation was done on a TOSHIBA Dynabook N51/25M, equipped with 2.16 GHz Intel Celeron CPU N28 processor with the use of a Hulinks Spartan ’08. Equilibrium geometries were optimized by the Hartree-Fock/3-21G* method, resulted in showing up of lowest-unoccupied molecular orbital (LUMO) map painted the absolute value of LUMO onto an electron density surface of each model compound.

RESULTS AND DISCUSSION

Structural and thermal properties of PHBHHx having 6.0 % HHx unit content and PHB as a reference were listed in Table SI-1 (Supplementary Information). Based on the characterization results, PHBHHx had two endothermic peaks at 128 and 143 °C with low crystallinity value of 26 % calculated from whole sample by differential scanning calorimetry (DSC) analysis, while PHB showed higher melting point and crystallinity values of 170 °C and 50 %, respectively. The SHS hydrolyses of PHBHHx and PHB samples were carried out at 130, 150, 170 and 190 °C. At these temperatures, PHBHHx was practically in a molten state. On the other hand, PHB maintained the crystalline structure at 130 and 150 °C. Changes in SEC profiles of SHS-treated PHBHHx and PHB were illustrated in Figure SI-1~8 (Supplementary Information). The SEC profile was shifted to lower molecular weight region maintaining unimodal distribution profile close to the most probable distribution (MPD) up to a critical point. The MPD indicates that the hydrothermal degradation under SHS treatment conditions proceeded in a homogeneous random degradation manner via the bulk-erosion mechanism.[18-19] Beyond the critical point, the SEC profile gradually changed to multimodal distribution, meaning the shift to a heterogeneous random degradation manner. This observation is however different from the hydrolysis under high temperatures (180-300 °C) and high pressures[11]. It may rather be similar to the manner accompanying isolation of crystalline phase as being observed on poly(L-lactic acid) hydrolysis[18]. At 170 and 190 °C, in spite of molten state, the critical point was clearly observed around M_w: 32500 (150 min) and 20000 Da (80 min) respectively) for PHB, and around M_w: 29000 (180 min) and 23500 Da (80 min) for PHBHHx, respectively. These suggest a limitation of dominant and homogeneous bulk-erosion mechanism and/or a residual effect of the

heterogeneous chain-ends distribution around crystalline phases due to the placement of samples in a static state at atmospheric pressure without any agitation during the SHS treatment.

Relationships between lnM_w and time of PHBHHx and PHB during the SHS treatment are shown in Figure 1a and 1b, respectively. In the plotting, M_w was adopted for the calculation of kinetic parameters in order to evaluate it more precisely as previously reported.[18] Obviously, ln M_w and reaction time are in linear relationships, meaning that the reaction proceeded in a manner of the auto-catalytic random degradation kinetics.[20] Reaction rate constant value k was calculated according to Nishida's method as listed in Table SI-2, and the Arrhenius plots using ln k and $1/T$ as were depicted in Figure 2. From Figure 2, a good linear relationship was obtained for PHBHHx, resulting in an activation energy value, E_a = 90.6 kJ•mol^{-1}, while the plot of PHB showed deviation from linearity at 130 °C, giving a relatively higher k value due to accelerating effects of concentrated chain-end groups around crystalline phases that act as catalysts and hydrophilic groups. At higher temperatures of 170 and 190 °C, both samples were in the molten state, therefore, the k value became closer to each other. The tentative E_a value of PHB hydrolysis in the temperature range of 150-190 °C was estimated as 91.4 kJ•mol^{-1}, which was very close to the E_a value of PHBHHx hydrolysis, reflecting the dominant reaction on HB-HB bonds.

The significantly higher value of PHB at 130 °C, at which temperature PHB remained crystalline phase intact, is mainly due to the narrower amorphous phase, in which carboxyl end groups are concentrated. The concentrated carboxyl chain end groups may catalyze the hydrolysis of surrounding ester groups, resulting in the higher hydrolysis rate.

[Figure 1]

[Figure 2]

In order to elucidate effects of HHx unit on the SHS degradation of PHBHHx, changes in diad sequence distribution were calculated from integration values of the carbonyl resonance peaks for diad sequences: HB*HB, HB*HHx, and HHx*HB at 169.100, 169.253, and 169.284 ppm, respectively, on ^{13}C NMR spectra (Figure 3). Each resonance peak was simulated using the Lorentzian function to estimate the peak area, resulted in a list as shown in Table SI-3. The calculated diad sequence distribution values of original PHBHHx were compared with values calculated from the Bernoulian statistics applicable to a statistically random copolymerization.

In the Bernoulian model, mole fraction F_{i*j} of diad sequence i*j can be expressed as $F_{i*j} = F_iF_j$ with the mole fraction F_i and F_j of *i* and *j* units, reapectively. The calculated diad fractions: $F_{HB*HB,}$ $F_{HB*HHx,}$ F_{HHx*HB} and $F_{HHx*HHx}$ of original PHBHHx are 88.36, 5.64, 5.64, and 0.36 % respectively, which are in good agreement with the observed values: 88.6, 5.8, 5.6, and ~0 % for F_{HB*HB}, F_{HB*HHx}, F_{HHx*HB}, and $F_{HHx*HHx}$, respectively. A possible peak assignable to diad sequence fraction $F_{HHx*HHx}$ was hard to be detected on ^{13}C NMR spectrum due to very small portion of HHx*HHx unit sequence. Thus, it was confirmed that the sequence distribution of HB and HHx units in PHBHHx sample was statistically random.[11]

As shown in Figure 3 and Table SI-3, relative peak intensity of the diad sequence: HHx*HB gradually decreased, while the intensities of other diad sequences: HB*HB and HB*HHx increased contrastively. The changes in relative peak intensities of the diad sequences are clearly illustrated in Figure 4. Although the untreated PHBHHx sample has even relative intensity values of 5.8 and 5.6 % for HB*HHx and HHx*HB sequences, respectively, after the SHS treatment at 170 ℃ for 300 min, the relative intensity value of HHx*HB sequence

decreased to 3.9 %, while the value of HB*HHx sequence increased to 6.9 %. After the treatment at 190 ℃ for 200 min, although the spectrum broadened because of the extreme decrease in molecular weight (Table SI-3), the relative intensity values of HHx*HB and HB*HHx sequences were tentatively simulated using the Lorentzian function, and calculated to become 3.6 and 7.1 %, respectively. Moreover, the relative intensity value of HB*HB sequence was also slightly increased from 88.6 to 89.3 %. It is interesting to note that the HHx*HB sequence was preferentially degraded and contrastively the chain scission of HB*HHx sequences was suppressed during the SHS treatment (Scheme 1). These specific reaction properties were considered to be contributed by higher steric propyl group in HHx unit than methyl group in HB unit, *i.e.*, promoting effect due to concentration of HHx units in amorphous regions and suppressive effect due to steric hindrance and hydrophobicity of propyl group.

[Figure 3]

[Figure 4]

[Scheme 1]

The key mechanism of ester hydrolysis is nucleophilic acyl substitution, in which ester carbonyl carbon is the target of nucleophile. Given that the LUMO designates space available for a pair of electrons, a LUMO map indicates the location, at where nucleophile attack would be likely to occur. Three model compounds: Me-O-(*R*)-3HB-(*R*)-3HB-Me, Me-O-(*R*)-3HB-(*R*)-3HHx-Me, and Me-O-(*R)*-3HHx-(*R*)-3HB-Me were applied in order to calculate the LUMO value to estimate the hydrolyzability of ester carbonyl carbon on the nucleophile attack. The LUMO maps for model compounds, on which the absolute value of LUMO was painted onto an

electron density surfaces, were shown in Figure SI-9~11 (Supplementary Information). In the figures, near blue color indicates high concentration of the LUMO, showing the highest concentration over the ester carbonyl carbons. The obtained LUMO values over the carbonyl carbons are listed in Table SI-4 (Supplementary Information). It was found that the absolute LUMO value of HHx*HB carbonyl carbon was the highest (115.08 kJ•mol^{-1}), followed by HB*HB (112.95 kJ•mol^{-1}) and HB*HHx (109.58 kJ•mol^{-1}) carbonyl carbons. The results are in agreement with the result of changes in diad sequence distribution shown in Table SI-3 and Figure 4. Based on these results, the HHx*HB carbonyl carbon in PHBHHx had the highest absolute LUMO value and the acid-catalyzed hydrolysis[18] was accelerated on the HHx*HB sequence during the SHS treatment.

CONCLUSION

In order to clarify effects of co-monomer unit on hydrothermal degradation of microbial polyester, the degradation specificity of PHBHHx on the hydrolysis was investigated minutely by using SHS. As a result, it was estimated that the degradation of PHBHHx proceeded via auto-catalytic random degradation kinetics having the activation energy value E_a = 90.6 kJ•mol^{-1} in a temperature range of 130-190 °C. From the changes in diad sequence distribution, the HHx*HB sequence was preferentially hydrolyzed and contrastively the hydrolysis of neighbored HB*HHx sequence was suppressed. This degradation specificity was successfully supported by the LUMO analysis of model compounds. These specific reaction properties were considered to be contributed by the higher steric propyl group in HHx unit. Additional experiments using other copolymers having different percentage of HHx unit contents are currently in progress and will be reported in near future.

ACKNOWLEDGEMENTS

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Figure legends

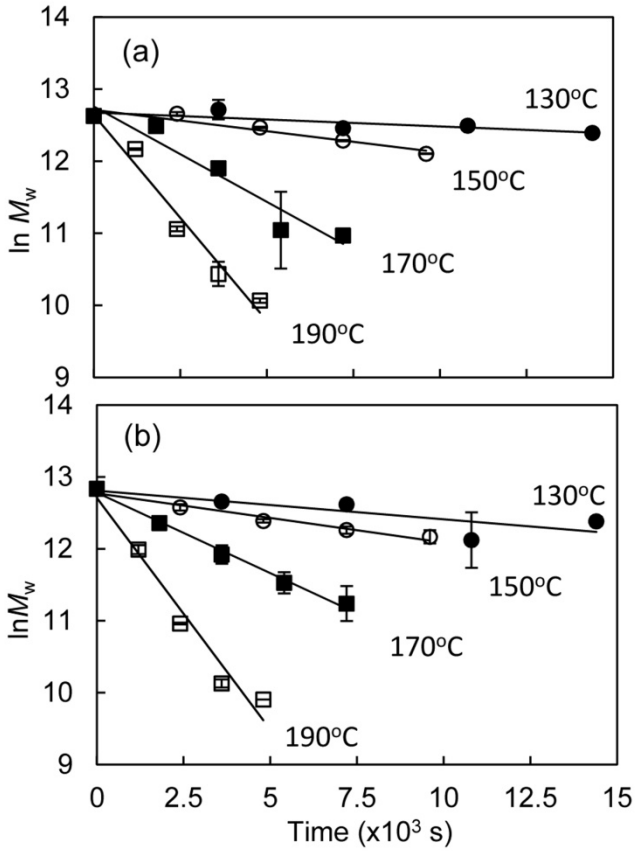
Figure 1. Plots of ln M_w vs. time for (a) PHBHHx and (b) PHB during SHS treatments in a temperature range of 130-190 °C. Bars show standard deviation.

Figure 2. Arrhenius plots for SHS degradation of PHBHHx and PHB.

Figure 3. Carbonyl resonances of diad sequences on ^{13}C NMR spectra of PHBHHx treated by SHS under different conditions.

Figure 4. Changes in diad sequence distribution of SHS treated PHBHHx.

Scheme 1. Schematic diagram of specific hydrolysis hydrolysis properties of PHBHHx.



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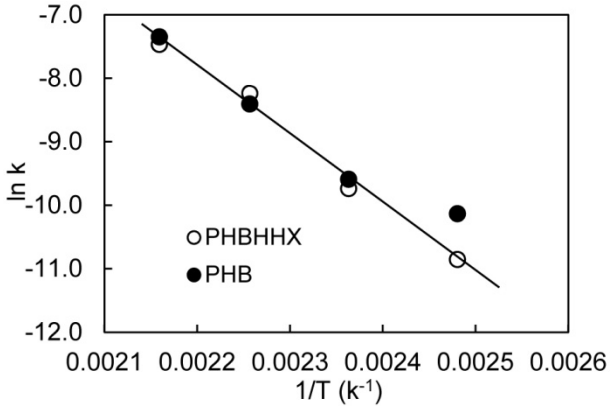
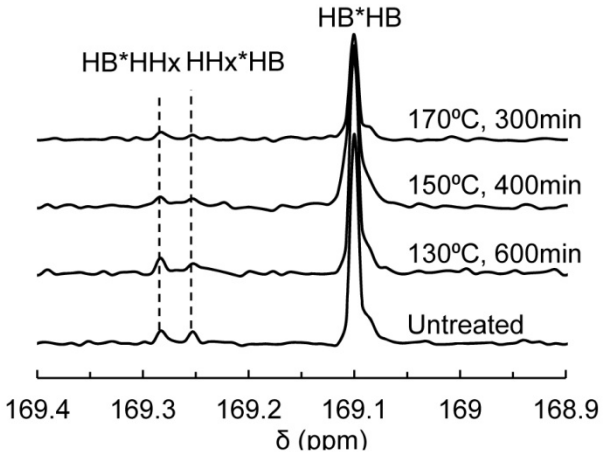


Figure 2. Arrhenius plots for SHS degradation of PHBHHx and PHB.



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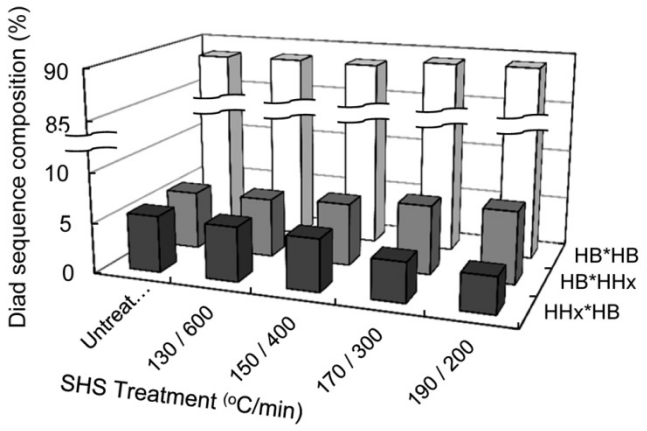

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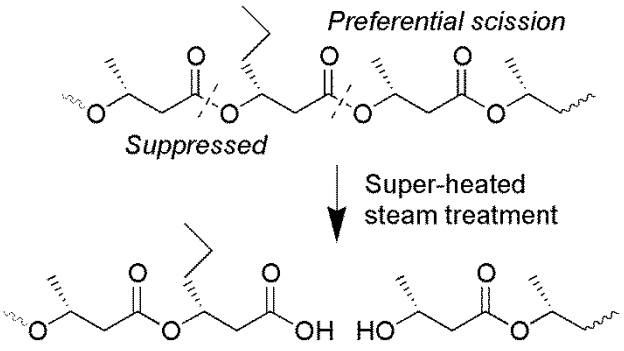

Scheme 1. Schematic diagram of specific hydrolysis hydrolysis properties of PHBHHx.

Graphical Abstract

Changes in Diad Sequence Distribution by Preferential Chain Scission during Thermal Hydrolysis of Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)

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Specific hydrolysis properties of microbial copolyester P(3HB-*co*-6%-3HHx) was found during super-heated steam treatment in a temperature range of 130-190 °C. From changes in diad sequences on ^{13}C NMR spectra, preferential and suppressive chain scission of HHx*HB and HB*HHx sequences, respectively, were determined. These specific reaction properties were considered to be contributed by higher steric propyl group in HHx unit than methyl group in HB unit, *i.e.*, promoting effect due to concentration of HHx units in amorphous regions and suppressive effect due to steric hindrance and hydrophobicity of propyl group. Further, it was validated by the LUMO analysis using a semi-empirical molecular orbital calculation method.