Macromol. Symp. **2005**, *224,* 47-57

Structure, Properties and Biodegradation of Some Bacterial Copoly(hydroxyalkanoate)s

*Kazuki Ishida, Naoki Asakawa, Yoshio Inoue**

Department of Biomolecular Engineering, Tokyo Institute of Technology,
4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan
Fax: (+81) 45 924 5827; E-mail: yinoue@bio.titech.ac.jp

Summary: This article describes the relationships between not only comonomer-unit compositions but also their distributions and structures as well as properties for bacterial copoly(hydroxyalkanoate)s, including poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), poly(3-hydroxybutyrate-*co*-3 hydroxypropionate), poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate), poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-mercaptopropionate). It was found that as-produced copoly(hydroxy-alkanoate)s have broad comonomer-unit compositional distributions, which we can control to some degree. The structure and properties of copoly(hydroxy-alkanoate)s depend not only on the chemical structure and the comonomer-unit composition but also on the comonomer-unit compositional distribution.

Keywords: biodegradable; blends; fractionation of polymers; poly(hydroxyalkanoate)s; thermal properties

1. Introduction

Poly(3-hydroxybutyrate) [P(3HB)], a naturally occurring polyester produced by various microorganisms as an intracellular energy reserve, was discovered in 1925[1] and has attracted much attention from industrialists and academia due to its inherent biodegradability and biocompatibility.[2,3] Unfortunately, P(3HB) is rather brittle compared with common chemosynthesized plastics and thermally unstable at temperatures just slightly above its melting point.[4] Thus, so far, its application has been very limited. In order to overcome the inferior properties of P(3HB), a variety of poly(hydroxyalkanoate)s (PHAs) have been bacterially synthesized and their properties have been investigated.[5]

Recently, by using several bacterial strains such as *Ralstonia eutropha* and *Alcaligenes latus*, we have succeeded in preparing the following five kinds of 3HB-unit-containing copoly(hydroxyalkanoate)s with a wide range of comonomer-unit compositions; poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) [P(3HB-*co*-3HP)],[6-10] poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)],[11-14] poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)],[15,16] poly(3-hydroxybutyrate-*co*-3-hydroxyhexanonate)

DOI: 10.1002/masy.200550605

[P(3HB-*co*-3HHx)],[17,18] poly(3-hydroxybutyrate-*co*-3-mercaptopropionate) [P(3HB-*co*-3MP)][19] (Chemical structures shown in Figure 1).

Through solvent/non-solvent fractionation techniques, the as-produced copoly(hydroxyalkanoate)s mentioned above have been compositionally fractionated into a series of fractions with narrow compositional distributions. According to the fractionations, it was found that the as-produced samples were mixtures (blends) of random copolyesters and had broad and complicated comonomer-unit compositional distributions. As a result, some of the as-produced copolyesters were homogeneous blends and the others were in-homogeneous ones. Namely, the solid-state structure and properties of bacterial copoly(hydroxyalkanoate)s were shown to depend not only on their chemical structures and comonomer-unit compositions but also on their comonomer-unit compositional distributions. Based on their fractions, the relationships between the comonomer-unit composition and the physical properties as well as the biodegradability have been studied for these copolyesters.

This review article describes the structures, properties, in particular the thermal properties, and biodegradability of bacterial copoly(hydroxyalkanoate)s with a narrow comonomer-unit compositional distribution.

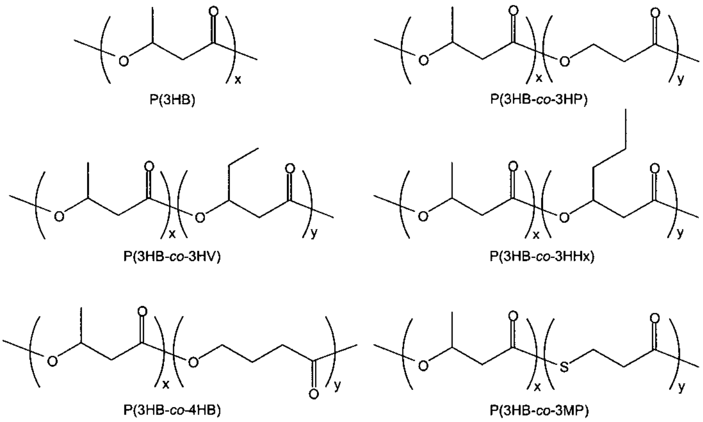

Figure 1. Chemical structures of bacterial poly(hydroxyalkanoate)s.

2. Fractionation of As-produced Copoly(hydroxyalkanoate)s and Their Comonomer-Unit Compositional Distribution

2.1. Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)s [P(3HB-co-4HB)s]. Ishida et al. have synthesized a series of bacterial P(3HB-*co*-4HB)s using *R. eutropha* and *A. latus* with a mixed carbon source of butyrate/4-hydroxybutyrate and that of sucrose/γ-butyrolactone, respectively, and carried out the fractionation of as-produced samples, using chloroform/*n*-heptane (or *n*-hexane) and chloroform/ethanol, into several fractions with different 4HB-unit contents.[15,16] The solubility of copolymers in a specific solvent is well-known to be affected by both the comonomer-unit composition and the molecular size. Two copolymers of different compositions and molecular sizes may have the same solubility in a specific solvent. However, it was found that some fractions weren't compositionally well-fractionated by only one kind of mixed solvent system, so these fractions were refractionated using another kind of mixed solvent system as mentioned above. It was found that there was a remarkable difference in the mode of comonomer-unit compositional distributions between as-produced P(3HB-*co*-4HB) samples produced by *R. eutropha* and *A. latus*. The former had bimodal distributions, that is, the as-produced samples from *R. eutropha* were mixtures of 3HB- and 4HB-rich P(3HB-*co*-4HB)s, while the latter had narrower and monomodal ones. The difference is attributable to their different metabolic systems. *R. eutropha* metabolizes both of the substrates in the mixed carbon source to acetyl-CoA via β oxidation, so the 3HB-units in the P(3HB-*co*-4HB)s are derived from both of the substrates while the 4HB-units are only from 4-hydroxybutyrate. On the other hand, *A. latus* may be unable to efficiently convert 4-hydroxybutyryl-CoA (produced by metabolizing γ-butyrolactone) to acetyl-CoA via β oxidation as suggested in the previous paper,[20] so the 3HB- and the 4HB-units are probably supplied respectively from sucrose and γ-butyrolactone; as a result, the comonomer-unit compositional distribution of P(3HB-*co*-4HB)s from *A. latus* became narrower and monomodal.

2.2. Poly(3-hydroxybutyrate-co-3-hydroxypropionate)s [P(3HB-co-3HP)s]. Cao et al.,[6,7] Arai et al.[8] and Wang et al.[9,10] have synthesized a series of bacterial P(3HB-*co*-3HP)s using *A. latus* with mixed carbon sources of 3-hydroxybutyrate/3-hydroxypropionate[6,7] and sucrose/3-hydroxypropionate,[8-10] and carried out the fractionation of the as-produced samples, using chloroform/*n*-heptane, into several fractions with different 3HP-unit contents. The 3HP-unit content of the fractionated

P(3HB-*co*-3HP)s tended to decrease with the increase of the *n*-heptane concentration in the mixed solvent. The width of the comonomer-unit compositional distribution was influenced by the pH value in the fermentation medium; the comonomer-unit compositional distribution became the narrowest at the pH value of 7.0 in the five controlled pH values of 6.0, 6.5, 7.0, 7.5 and 8.0.[9] Generally, it was found that the comonomer-unit compositional distributions for the bacterial P(3HB-*co*-3HP)s synthesized with the two kinds of mixed carbon sources were characteristically different from each other; those for P(3HB-*co*-3HP)s biosynthesized with 3-hydroxybutyrate/3-hydroxypropionate were rather broad and trapezoidal than monomodal, and those for P(3HB-*co*-3HP)s biosynthesized with sucrose/3-hydroxypropionate were narrower and monomodal.

2.3. Poly(3-hydroxybutyrate-co-3-mercaptopropionate)s [P(3HB-co-3MP)s]. Tanaka et al. have synthesized a series of bacterial P(3HB-*co*-3MP) using *R. eutropha* with a mixed carbon source of sodium gluconate/3, 3'-thiodipropionate, and carried out the fractionation of the as-produced samples, using chloroform/*n*-hexane, into several fractions with different 3MP-unit contents.[19] An as-produced P(3HB-*co*-18 mol-% 3MP) sample was fractionated into 10 fractions with different 3MP-unit contents from 7 to 62 mol-%. As the concentration of *n*-hexane in the mixed solvent increased, the 3MP-unit content of the precipitated P(3HB-*co*-3MP) fraction gradually decreased in the first half of the fractionation procedure and the molecular weight of the following fractions gradually decreased, indicating that the P(3HB-*co*-3MP) copolymer was fractionated based on both the comonomer-unit content and the molecular weight.

3. Effect of Comonomer-Unit Composition and Its Distribution on Thermal Properties

3.1. Effect of Comonomer-Unit Composition on Thermal Properties. To clarify structure-property relationships for a series of P(3HB) copolyesters, that is, P(3HB-*co*-3HV), P(3HB-*co*-3HP), P(3HB-*co*-3HHx) and P(3HB-*co*-4HB), in Figure 2 the values of the melting temperature (T_m), the heat of fusion (ΔH_f) and the glass transition temperature (T_g) are plotted against the content of the second monomeric unit (3HV, 3HP, 3HHx and 4HB) for compositionally well-fractionated copolyester samples. Generally, as the content of the second monomeric unit increased, the T_m value for 3HB-rich P(3HB) copolyesters decreased, while that for the second monomeric unit-rich P(3HB) copolyesters increased, which is consistent with the relationship between the ΔH_f values and the contents of the second monomeric unit. This means that minimum crystallinity exists due to the disturbance of the crystalline lattice of the major component caused by the second repeating unit. On the other hand, the T_g value for all of these P(3HB) copolyesters decreased continuously with the increase of the second monomeric unit content,

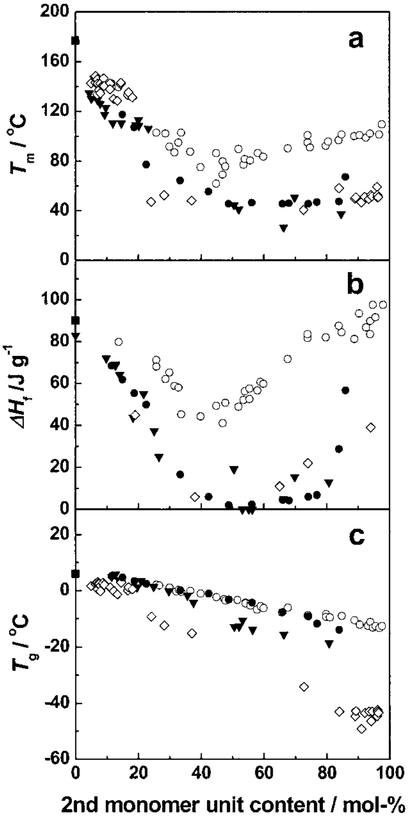

Figure 2. Plots of (a) melting temperature (T_m), (b) heat of fusion (ΔH_f) and glass transition temperature (T_g) versus content (mol-%) of second monomer (3HV, 3HP, 3HHx and 4HB) for comonomer-unit compositionally well-fractionated copolyesters; ■: P(3HB), ○: P(3HB-*co*-3HV), ●: P(3HB-*co*-3HP), ▼: P(3HB-*co*-3HHx), ◇: P(3HB-*co*-4HB) (cited from reference [18] and modified).

indicating the segmental mobility in the amorphous phase for all of these copolyesters increased with increasing the second repeating units, due to their longer side chains (3HV- and 3HHx-units), no side chain (3HP-unit) and longer backbone (4HB-unit) than the 3HB-unit.

3.2. Effect of Comonomer-Unit Compositional Distribution on Thermal Properties.

3.2.1. Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)s [P(3HB-co-4HB)s]. Figure 3a and 3b show the melting temperature (T_m) and the glass transition temperature (T_g) plotted against the 4HB-unit content, respectively, for the compositionally well-fractionated copolyester samples and homopolyesters P(3HB) and P(4HB).[21] Those values for as-produced unfractionated samples are also plotted against the average 4HB-unit contents.

As shown in Figure 3a, the T_m values of all of the P(3HB-*co*-4HB)s are plotted in two separate temperature regions, i.e., 120–160 and 40–60 ℃. As for the fractions, the T_m value decreased with the increase of the 4HB-unit content up to ca. 40 mol-%, and then increased slightly, though the data for the fractions with intermediate 4HB-unit content wasn't obtained. When the 4HB-unit content exceeded ca. 20 mol-%, the T_m value was depressed significantly, suggesting significant thinning of the crystalline lamellar thickness. However, almost all of the unfractionated as-produced samples showed two T_ms in the higher and the lower regions, which is attributable to the fact that these as-produced samples are mixtures of random P(3HB-*co*-4HB)s with different 4HB-unit contents. The 4HB-unit content of these as-produced samples was actually an overall one consisting of at least two immiscible blends with low and high 4HB-unit contents.

As shown in Figure 3b, the T_g value of compositionally well-fractionated samples decreased continuously with the increase of the 4HB-unit content. Those values of as-produced samples produced by *A. latus* (average 4HB-unit contents were 21.7 and 31.2 mol-%) were exactly located on the straight line obtained for the fractionated samples in Figure 3b, suggesting that as-produced samples from *A. latus* are miscible blend systems. The T_g values of almost all of the as-produced samples produced by *R. eutropha* were also detected by DSC as single values. But most of these T_gs deviated extraordinarily from the straight line. T_gs of these unfractionated samples with the 4HB-unit contents of 20.9, 39.6, 50.9 and 69.8 mol-% were all detected at temperatures lower than −40 ℃. These T_gs were derived from the 4HB-rich phase in the immiscible blends of 3HB- and 4HB-rich copolyesters. T_gs derived from a 3HB-rich phase in these as-produced samples were not

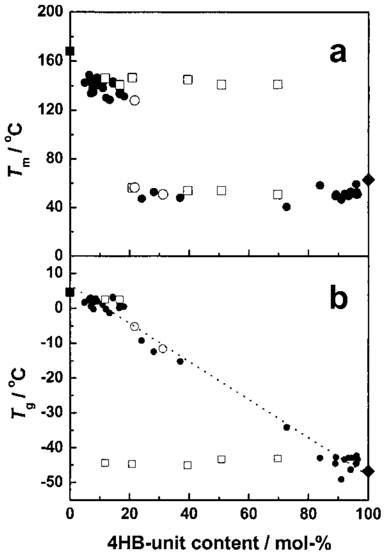

Figure 3. 4HB-unit content dependences of (a) T_m and (b) T_g for the unfractionated as-produced P(3HB-*co*-4HB)s from *R. eutropha* (□) and *A. latus* (○) and their fractions (●). The T_m and T_g values of P(3HB) (■) and P(4HB) (◆) are also plotted.

detected in the DSC thermograms because the glass transitions were hidden by the crystallization exotherms of the 4HB-rich components. An as-produced sample with the 4HB-unit content of 11.9 mol-% from *R. eutropha* showed two T_gs in the DSC thermogram, caused by two separated phases of the immiscible blend. Another as-produced sample with the 4HB-unit content of 16.7 mol-% from *R. eutropha* showed a single T_g located near the straight line, which was derived from the 3HB-rich phase in the immiscible blend, while the T_g value of the 4HB-rich phase in this unfractionated sample was not detected in the DSC thermogram because its content in the as-produced sample was very small.

3.2.2. Poly(3-hydroxybutyrate-co-3-hydroxypropionate)s [P(3HB-co-3HP)s]. Cao et al. have performed the DSC measurement for an as-produced P(3HB-*co*-30.6 mol-% 3HP) and its fractions with the 3HP-unit contents ranging from 8.5 to 60.5 mol-%.[6] The fractions with the 3HP-unit contents of 8.5, 12.8 and 15.8 mol-%, as well as the as-produced sample, displayed an endothermic peak due to the crystal reorganization at temperatures just higher than the T_m peak. In general, the T_m value decreased with increasing the 3HP-unit content. The as-produced sample showed a characteristically similar melting behavior to the fractions with 3HP-unit content of 8.5 and 12.8 mol-%, though weight ratios of these fractions were very small (5.4 and 7.0 wt.-% for the fractions with 3HP-unit contents of 8.5 and 12.8 mol-%, respectively), which is attributable to the larger melting endotherms due to higher crystallinities for the fractions having low 3HP-unit contents. As seen in this case, as-produced P(3HB-*co*-3HP)s can reveal only the feature of the melting behavior corresponding to that of the minor fraction with a 3HP-unit content markedly different from the average 3HP-unit content of the as-produced sample.

4. Effect of Comonomer-Unit Composition and Its Distribution on Biodegradability

The biodegradation of a series of compositionally well-fractionated P(3HB-*co*-3HP)s in the presence of PHA depolymerases purified from *R. pickettii* T1 and *Acidovorax sp.* TP4 has been investigated by Cao et al.[22] and Wang et al.,[23] respectively. It was found that the copolyesters forming the P(3HB)-type crystalline structures were degraded by *R. pickettii* T1 depolymerase at a relatively fast rate compared with those forming the P(3HP)-type crystalline structure, and that the amorphous copolyesters can hardly be degraded. For example, weight losses of melt-crystallized P(3HB)-type crystalline polyester films of P(3HB) and P(3HB-*co*-23.8 mol-% 3HP) were ca. 0.3 and 2.5 $\mathrm{mg \cdot cm^{-2} \cdot h^{-1}}$, respectively, and those of P(3HP)-type ones of P(3HB-*co*-89.6 mol-% 3HP) and P(3HP) were ca. 0.8 and 0.7 $\mathrm{mg \cdot cm^{-2} \cdot h^{-1}}$, respectively. On the other hand, amorphous P(3HB-*co*-60.1 mol-% 3HP) film was hardly degraded by the *R. pickettii* T1 depolymerase. The maximum degradation rate of the P(3HB) crystalline type copolyester was observed for the P(3HB-*co*-3HP) fraction with the 3HP-unit content of 20–30 mol-%. These results indicate that the enzymatic degradation rate of fractionated P(3HB-*co*-3HP) is affected not only by the crystallinity of the copolyester but also by the chemical structure of the monomeric units and the substrate specificity of the PHA depolymerase. The crystallinity, and hence the biodegradability of copolyesters, is affected by the comonomer-unit composition. In contrast to the *R. pickettii* T1 depolymerase, the *Acidovorax sp.* TP4 depolymerase hydrolyzed P(3HB-*co*-3HP) films with a lower degree of crystallinity at a higher rate, no matter what kind of crystalline structures were formed. The depolymerases purified from *R. pickettii* T1 and *Acidovorax sp.* TP4 are defined respectively as PHA depolymerase types I and II according to the position of the lipase box in the catalytic domain. The difference of the degradation results of amorphous P(3HB-*co*-3HP) films by *R. pickettii* T1 and *Acidovorax sp.* TP4 depolymerases may be accounted for by the fact that *Acidovorax sp.* TP4 depolymerase can adsorb on the surface of the amorphous polymer and then cleave it, while *R. pickettii* T1 depolymerase cannot adsorb on the amorphous surface. The behavior of environmental degradation in river water was

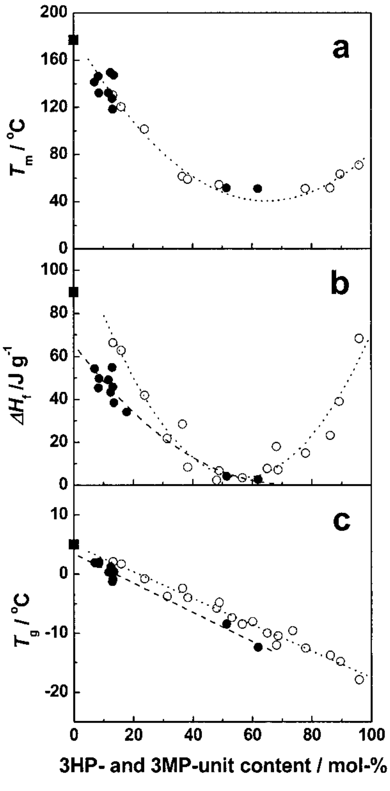

Figure 4. Plots of (a) melting temperature (T_m), (b) heat of fusion (ΔH_f) and (c) glass transition temperature (T_g) versus 3MP- and 3HP-unit contents (mol-%) for well fractionated copolyesters. ●: P(3HB-*co*-3MP); ○: P(3HB-*co*-3HP); ■: P(3HB) (cited from reference [19] and modified).

assessed for bacterial P(3HB), chemosynthesized P(3HP) and a series of compositionally well-fractionated P(3HB-*co*-3HP)s by the BOD method.[22] As a result, all of the samples except for P(3HP) were completely biodegraded in the experimental time, suggesting that only bacterially synthesized polyesters can be degraded in the river water and the presence of a natural 3HB-unit can induce the environmental degradation of copolyesters regardless of their different 3HB-unit contents.

5. Effects of Thio/Oxo Ester Linkages on Thermal Properties of Poly[3-hydroxybutyrate-*co*-3-(mercapto/hydroxy)propionate]s

Recently, P(3HB-*co*-3MP) has been bacterially synthesized, which is the first example on a bacterial PHA containing sulfur atoms in the backbone.[24-26] The properties of polymers containing sulfur atoms in the backbone linkage are expected to be different from those of polymers containing oxygen atoms in the corresponding linkage, for example, the melting point of poly(ethylene sulfide) (T_m = 216 ℃)[27] is significantly higher than that of its counterpart polymer with oxygen atoms at the corresponding positions, that is, poly(ethylene oxide) (T_m = 66~69 ℃).[28,29] It is reasonable to expect that physical properties of P(3HB-*co*-3MP) differ more or less from those of its counterpart, P(3HB-*co*-3HP).

Tanaka et al. have investigated the 3MP-unit content dependence of the thermal properties (T_m, ΔH_f and T_g) for compositionally well-fractionated P(3HB-*co*-3MP)s with 3MP-unit contents up to 62 mol-% as shown in Figure 4.[19] P(3HB-*co*-3MP)s exhibited marked similarity of thermal properties to those of P(3HB-*co*-3HP)s. However, the destructive effect of the 3MP-unit on the P(3HB) homopolymer-type crystalline lattice was found to be larger than that of the 3HP-unit, according to the 3HP/3MP-units content dependence of the ΔH_f values.

It has been reported that the melting point of bacterial poly(3-mercaptopropionate) [P(3MP)] synthesized by recombinant *E. coli* strains was 170 ℃,[30] which is much higher than that of chemosynthesized poly(3-hydroxypropionate) [P(3HP); T_m = 73 ℃[31]]. Thus, it is interesting to investigate the 3MP-unit content dependence of crystalline morphology of 3MP-unit rich P(3HB-*co*-3MP)s, although such polymers except for P(3MP) have so far not been biosynthesized.

6. Conclusion

This article describes the fractionation of bacterial as-produced copoly(hydroxyalkanoate)s and the properties, including the thermal properties and biodegradability, for as-produced samples and their fractions. From the results of the fractionation, it can be concluded that as-produced copoly(hydroxyalkanoate)s can be separated into several fractions with different comonomer-unit compositions, indicating they have broad comonomer-unit compositional distributions. The comonomer-unit compositional distribution proved to be controllable by changing bacterial strains, carbon substrates, pH in the fermentation medium, and so on. It was found in this study that the solid-state structure, physical properties and biodegradabilities depend not only on the chemical structure and the content of the monomeric units but also on the comonomer-unit compositional distribution.

[1] M. Lemoigne, *Ann. Inst. Pasteur* **1925**, *10*, 135.
[2] Y. Doi, "*Microbial polyester: Microorganisms and poly(3-hydroxyalkanoates)*", VCH Publishers, New York, **1990**, p. 33.
[3] Y. Inoue, N. Yoshie, *Prog. Polym. Sci.* **1992**, 17, 571.
[4] F.-D. Kopinke, M. Remmler, K. Mackenzie, *Polym. Degrad. Stab.* **1996**, *52*, 25.
[5] A. Steinbüchel, H. E. Valentin, *FEMS Microbiol. Lett.* **1995**, 128, 219.
[6] A. Cao, M. Ichikawa, K. Kasuya, N. Yoshie, N. Asakawa, Y. Inoue, Y. Doi, H. Abe, *Polym. J.* **1996**, *28*, 1096.
[7] A. Cao, K. Kasuya, H. Abe, Y. Doi, Y. Inoue, *Polymer* **1998**, *39*, 4801.
[8] Y. Arai, A. Cao, N. Yoshie, Y. Inoue, *Polym. Int.* **1999**, *48*, 1219.
[9] Y. Wang, M. Ichikawa, A. Cao, N. Yoshie, Y. Inoue, *Macromol. Chem. Phys.* **1999**, *200*, 1047.
[10] Y. Wang, Y. Inoue, *Int. J. Biol. Macromol.* **2001**, *28*, 235.
[11] N. Kamiya, Y. Yamamoto, Y. Inoue, R. Chujo, Y. Doi, *Macromolecules* **1989**, *22*, 1676.
[12] N. Yoshie, H. Menju, H. Sato, Y. Inoue, *Macromolecules* **1995**, *28*, 6516.
[13] Y. Wang, S. Yamada, N. Asakawa, T. Yamane, N. Yoshie, Y. Inoue, *Biomacromolecules* **2001**, *2*, 1315.
[14] S. Yamada, Y. Wang, N. Asakawa, N. Yoshie, Y. Inoue, *Macromolecules* **2001**, *34*, 4659.
[15] K. Ishida, Y. Wang, Y. Inoue, *Biomacromolecules* **2001**, *2*, 1285.
[16] K. Ishida, Y. Inoue, *Biomacromolecules* **2004**, *5*, 1135.
[17] T. Watanabe, Y. He, T. Fukuchi, Y. Inoue, *Macromol. Biosci.* **2001**, *1*, 75.
[18] L. D. Feng, T. Watanabe, Y. Wang, T. Kichise, T. Fukuchi, G. Q. Chen, Y. Doi, Y. Inoue, *Biomacromolecules*, **2002**, *3*, 1071.
[19] S. Tanaka, L. D. Feng, Y. Inoue, *Polym. J.* **2004**, *36*, 570.
[20] Y. Saito, S. Nakamura, M. Hiramitsu, Y. Doi, *Polym. Int.* **1996**, *39*, 169.
[21] K. Ishida, unpublished data.
[22] A. Cao, Y. Arai, N. Yoshie, K. Kasuya, Y. Doi, Y. Inoue, *Polymer* **1999**, *40*, 6821.
[23] Y. Wang, Y. Inagawa, T. Saito, K. Kasuya, Y. Doi, Y. Inoue, *Biomacromolecules* **2002**, *3*, 828.
[24] T. Lütke-Eversloh, K. Bergander, H. Luftmann, A. Steinbüchel, *Microbiology* **2001**, *147*, 11.
[25] T. Lütke-Eversloh, J. Kawada, R. H. Marchessault, A. Steinbüchel, *Biomacromolecules* **2002**, *3*, 159.
[26] T. Lütke-Eversloh, A. Steinbüchel, FEMS *Microbiol. Lett.* **2003**, *221*, 191.
[27] K. Tani, H. Tadokoro, *Macromolecules* **1974**, *7*, 507.
[28] Y. Takahashi, H. Tadokoro, *Macromolecules* **1973**, *6*, 672.
[29] Y. He, N. Asakawa, Y. Inoue, *Polym. Int.* **2000**, *49*, 609.
[30] T. Lütke-Eversloh, A. Fischer, U. Remminghorst, J. Kawada, R. H. Marchessault, A. Bogershausen, M. Lalwei, H. Eckert, R. Reichelt, S.-J. Liu, A. Steinbüchel, *Nature Materials* **2002**, *1*, 236.
[31] A. Cao, N. Asakawa, N. Yoshie, Y. Inoue, *Polym. J.* **1998**, *30*, 743.