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Effect of low molecular weight additives on enzymatic degradation of poly(3-hydroxybutyrate)

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Abstract

The effects of low molecular weight additives on various properties of bacterial poly(3-hydroxybutyrate) (PHB) have been examined by utilizing dodecanol, lauric acid, tributyrin and trilaurin as additives. The analyses of glass transition temperature and cold crystallization temperature of PHB in the PHB/additive mixtures showed that these additives are miscible with PHB and act as plasticizers, though the miscibility is found in the limited mixtures with low additive content. The enzymatic degradation of the melt-crystallized films of the PHB/additive mixtures was investigated in aqueous solution of PHB depolymerase purified from *Alcaligenes faecalis* T1. The mixtures showed degradability different from pure PHB. A fairly small amount (1 wt%) of additive acts as an accelerator for the enzymatic degradation of PHB while a larger amount (9 wt%) of additive acts as a retardant. All the additives examined in this study showed similar trend. The retardation effect of additives observed for the PHB mixtures containing 9 wt% additive is ascribed to the segregation of the additive on the film surface. The additives on the surface prevent an attack of the enzymes on the PHB molecules. The higher molecular mobility in the amorphous phase and the thinner lamella are possible factors that accelerate the degradation of PHB containing 1 wt% additives. This result shows that the enzymatic degradability of PHB is controllable by adding low molecular-weight additives.

Keywords: Bacterial polyester; Poly(3-hydroxybutyrate); Enzymatic degradation

1. Introduction

A wide variety of microorganisms synthesize poly(3-hydroxybutyrate) (PHB) as an intracellular storage material [1–3]. PHB is a semicrystalline thermoplastic with environmental degradability. Due to this degradability, PHB has attracted much industrial attention.

In order to use PHB as an industrial material in a variety of fields, it is desirable to develop PHB-based materials with a wide diversity of properties. Biosynthesis of the copolyesters of 3-hydroxybutyrate (HB) with other hydroxyalkanoates, such as 3-hydroxyvalerate [3], 4-hydroxybutyrate [4], 5-hydroxyvalerate [5] and 3-hydroxypropionate [6], is one method to modify PHB properties. PHB-based copolymers have a wide range of properties depending on the chemical structure of the comonomer units as well as the comonomer composition [7–9]. Another method to modulate various properties of PHB is blending of PHB with other biodegradable polymers. Miscibility and some properties were reported for several blends including PHB/poly(vinyl alcohol) [10,11], PHB/poly(3-hydroxypropionate) [12], PHB/poly(lactic acid) [13], PHB/chemically synthesized poly((*R,S*)-3-hydroxybutyrate) [14], PHB/poly(butylene succinate-*co*-butylene adipate) [15] and PHB/poly(butylene succinate-*co*-ϵ-caprolactone) [15].

Biodegradability of PHB and PHB-based copolyesters has been evaluated in various environments such as in soil, sludge, river water and seawater [1]. Details of the degradation mechanism were elucidated through the examination of the enzymatic degradation of PHB and PHB-based copolyesters. Extracellular PHB depolymerases have been isolated from *Alcaligenes faecalis* T1 [16–19], *Pseudomonas lemoignei* [17,18], *Pseudomonas pikettii* [18] and *Comamonas testosteroni* [18]. These depolymerases hydrolyze the polyesters to water-soluble oligomers, which can be metabolized to water and carbon dioxide by bacteria. The rate of enzymatic degradation decreases with an increase in the crystallinity [19] and crystal size [20,21]. The degradability of PHB-based copolyesters also depends on the chemical structure of the comonomer units and the comonomer composition [16–19,22,23].

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Table 1
Chemical structure, molecular weight, melting temperature and solubility parameter of additives

	Chemical structure	Molecular weight	T_m (°C)	Solubility parameter ($J^{1/2}$ $cm^{-3/2}$)
Dodecanol	$CH_3(CH_2)_{10}CH_2OH$	186.3	23.4	16.6
Lauric acid	$CH_3(CH_2)_{10}COOH$	200.3	44.0	15.9
Tributyrin	$(C_3H_7COO)_3C_3H_5$	302.4	−75.0	17.8
Trilaurin	$(C_{11}H_{23}COO)_3C_3H_5$	639.0	14.0, 34.0, 43.9	16.4

The enzymatic degradation of some polymer blends containing PHB has also been investigated. We can intuitively understand the degradability of the blends of PHB with poly(3-hydroxypropionate) [12] and poly((*R*,*S*)-lactic acid) [13]. Linear relation between the degradation rate and the blend composition has been observed for these blends. On the other hand, the degradability of some other blend systems are more difficult to understand. A maximum was found in the degradation rate vs. composition curve for the PHB blends with poly((*R*,*S*)-3-hydroxybutyrate) [14], poly(butylene succinate-*co*-butylene adipate) [15] and poly(butylene succinate-*co*-ϵ-caprolactone) [15]. The addition of these second components accelerates the degradation of PHB and/or the second components. This observation prompted us to investigate the effect of other second components on the enzymatic degradability of PHB.

In this study, we examine the enzymatic degradability of PHB mixed with biodegradable low molecular-weight additives. Dodecanol, lauric acid, tributyrin and trilaurin are selected as additives. In addition to the effect of these additives on the degradability, we study the miscibility and the crystalline structures of the mixtures and discuss some factors that possibly play an important role in determining the biodegradability of PHB.

2. Experimental

2.1. Materials

PHB samples were bacterially synthesized by *Ralstonia eutropha* H16(ATCC17699) using a two-stage fermentation procedure [24,25]. This involves the first stages for bacterium growth and the second for polymer accumulation. Fructose was used as the sole carbon source in the second stage. PHB was extracted from the dried cells with hot chloroform and purified by repetitive reprecipitation into methanol and into hexane. The mixtures of PHB and low molecular weight additives were prepared by slowly casting from chloroform solution of PHB/additive mixture on glass plates at room temperature. Chemical structure, molecular weight and melting temperature of the additives are shown in Table 1.

The melt-crystallized films obtained by compression molding were used as the samples for the analysis. The cast mixtures were inserted between aluminum plates with an aluminum spacer (0.1 mm thickness) and were compression-molded at 195°C for 5 min under a pressure of 5 MPa, using a Toyoseiki Mini Test Press-10. The molten samples were then cooled to a selected crystallization temperature and kept for at least 4 weeks to reach equilibrium crystallinity prior to the analysis. In most cases, the samples were crystallized at room temperature, while the samples crystallized at 90°C were also prepared. The films washed in methanol were also prepared by immersing the melt-crystallized films in methanol for 2 h and then drying for 24 h under vacuum.

2.2. Enzymatic degradation

The extracellular PHB depolymerase was purified from *A. faecalis* T1 according to the method of Shirakura et al. [26] The enzymatic degradation of the polyesters was carried out for the melt-crystallized films at 37°C in 1 ml phosphate buffer (pH 7.4) containing 1 μg of PHB depolymerase. The initial dimensions of the films are 10 mm × 10 mm × 0.1 mm and their initial weights are 10–25 mg. The rate of enzymatic degradation was estimated through the measurement of weight loss of the films after exposing them to the enzyme solution for 5 h.

2.3. Analytical procedures

DSC measurements were conducted on a SEIKO EXSTAR6000 system equipped with a DSC 220U, using 1–3 mg of samples. Melting temperature, T_m, and crystallinity were determined by heating the melt-crystallized samples from room temperature to 200°C. Unless otherwise indicated, a heating rate of 20°C min^{-1} was used. The T_m value was determined from the maximum of the endothermic peak. Glass transition temperature, T_g, and cold crystallization temperature, T_{cc}, were determined by reheating the melt-quenched samples. The samples were melted in a DSC apparatus at 195°C for 5 min, quenched to −50°C and then reheated up to 200°C at a heating rate of 10°C min^{-1}. The T_g value was taken at the midpoint of the transition. The T_{cc} value was taken as the maximum of the exothermic peak.

Spherulite growth rates were measured by an Olympus BX 90 polarized microscope equipped with a Mettler FP82HT hot stage. Film samples were heated to 195°C, kept at this temperature for 1 min and then cooled to a selected crystallization temperature where they were kept

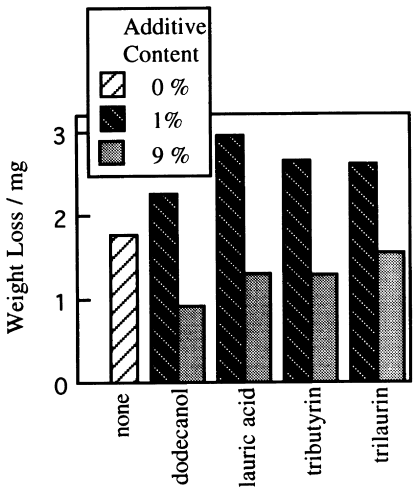

Fig. 1. Weight losses of PHB/additive mixtures crystallized at room temperature after 5 h of exposure to PHB depolymerase from *A. faecalis* T1. The data on pure PHB are also shown.

isothermally. Spherulite growth rates were taken as the slope of the linear plot of spherulite radius vs. time.

Film surfaces were examined using a scanning electron microscope (Jeol JSM-5200). Films were sputter-coated with gold before examination.

3. Results and discussion

3.1. Enzymatic degradability of PHB/additive I

Enzymatic degradation of the PHB/additive mixtures crystallized at room temperature was investigated by using the extracellular PHB depolymerase purified from *A. faecalis* T1. The weight losses of the films of PHB/dodecanol, PHB/lauric acid, PHB/tributyrin and PHB/trilaurin after 5 h degradation by PHB depolymerase are shown in Fig. 1. It is clear that the additives alter the enzymatic degradability of the films considerably. The degradation of PHB containing 9 wt% additive is slower than pure PHB. The presence of a certain amount of additive retards the degradation of PHB. On the contrary, the degradation of PHB containing 1 wt% additive is faster than that of pure PHB. The presence of a fairly small amount of additive accelerates the degradation of PHB. Why does a very small amount of additive act as an accelerator while a larger amount of additive act as a retardant? Several factors, such as dispersion of additives, mobility of PHB [27], crystallinity [20] and crystalline size [21], are potentially responsible for the difference in the degradability of PHB. Here, each of these factors was examined.

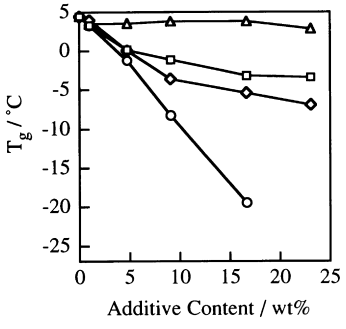

Fig. 2. Glass transition temperature (T_g) of PHB/dodecanol (□), PHB/lauric acid (◇), PHB/tributyrin (○) and PHB/trilaurin (△) mixtures observed as a function of additive content.

3.2. Plasticizing effects of additives on PHB

Figs. 2 and 3 show the variations in glass transition temperature, T_g, and cold crystallization temperature, T_{cc}, for the PHB/additive mixtures as a function of additive content. It was observed that the T_g and T_{cc} of the mixtures are lower than those of pure PHB. Therefore, the additives are miscible with PHB and improve the mobility of the molecules in the amorphous phase. The additives act as plasticizers.

Each of the additives, however, lowers T_g and T_{cc} to different extents. The T_g and T_{cc} of PHB/tributyrin continuously shift to lower temperature with an increase in the tributyrin content from 0 to 23 wt%, while those of PHB/dodecanol, PHB/lauric acid and PHB/trilaurin reach a plateau at additive content of 9, 9 and 1 wt%, respectively. Therefore, the miscibility of the mixtures with dodecanol, lauric acid and trilaurin with PHB is limited to low additive content. The additive content at which the T_g and the T_{cc} reach a plateau indicate the upper limit of the miscibility window. Therefore, the miscibility window of the additives with PHB is wider in the order of tributyrin $>$ dodecanol $\approx$ lauric acid $>$ trilaurin.

The solubility parameters, δ, of the additives and PHB were calculated according to the molar-attraction constants [28]. The δ values of the additives are listed in Table 1. The δ value of PHB is 19.0×10^4 $J^{1/2}$ $cm^{-3/2}$. Even if an additive is miscible with a semicrystalline polymer, the incorporation of the additive into the crystalline phase of the polymer hardly occurs. Thus, the solubility parameter of PHB was calculated based on the density of the amorphous phase. For PHB/tributyrin, difference in δ is not so large as to prevent mixing. On the other hand, the difference in δ for the other mixtures are relatively large, so that the mixtures are miscible only when the additive content is very low. This prediction is qualitatively consistent with the width of the miscibility window proposed earlier.

The growth of PHB spherulites from the melt of the mixtures was examined by polarized microscopy. Phase separation was observed for the melt of PHB/trilaurin at trilaurin content 5–23 wt%, while the homogeneous phase was observed for the others at additive content 0–23 wt%. This result is again consistent with the width of the miscibility window proposed earlier.

Fig. 4 shows the composition dependence of the growth rate, G, of PHB spherulites at 80°C for the mixtures. The growth rate of PHB/trilaurin is very close to that of pure

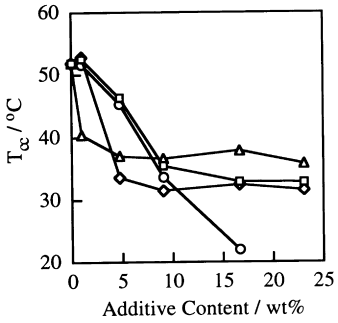

Fig. 3. Cold crystallization temperature (T_{cc}) of PHB/dodecanol (□), PHB/lauric acid (◇), PHB/tributyrin (○) and PHB/trilaurin (△) mixtures observed as a function of additive content.

PHB at every composition, while the rate increases with an increase of additive content for the other mixtures. Fig. 5 shows the growth rate, G, of PHB spherulites for 91/9 PHB/lauric acid as a function of crystallization temperature. The growth rate of PHB/lauric acid is very close to that of pure PHB at temperature higher than 90°C, at which the growth rate of pure PHB reaches the maximum. On the other hand, the growth rate of this mixture is higher than that of pure PHB at temperature lower than 90°C. Similar growth rate shift is observed for PHB/dodecanol and PHB/tributyrin (data not shown). The mobility of molecules is a main factor that determines the spherulite growth rate at a temperature between T_g and the temperature of the maximum growth rate. Therefore, the growth rate shift also supports that dodecanol, lauric acid and tributyrin improve the mobility of PHB molecules.

3.3. Crystal structure

Crystal structures of the mixtures have been examined through the analysis of the melting behavior of the samples. Fig. 6 shows DSC thermograms of PHB and the mixtures of 77/23 PHB/additive crystallized at room temperature. The endothermic peaks at ≈170°C and 20–50°C correspond to the melting of PHB and additives, respectively. Only one melting peak of PHB crystals (at ≈170°C) was observed for

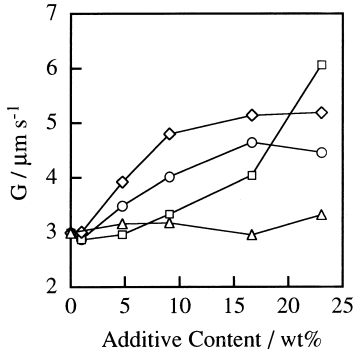

Fig. 4. Radial growth rate of PHB spherulites at 80°C in PHB/dodecanol (□), PHB/lauric acid (◇), PHB/tributyrin (○) and PHB/trilaurin (△) mixtures observed as a function of additive content.

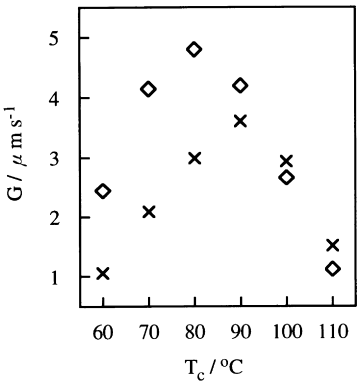

Fig. 5. Radial growth rate of PHB spherulites for PHB (×) and 91/9 PHB/lauric acid mixture (◇) observed as a function of crystallization temperature.

PHB, PHB/tributyrin and PHB/trilaurin, while two peaks were observed for PHB/dodecanol and PHB/lauric acid.

In order to examine the two-peak behavior, DSC thermograms for PHB/dodecanol and PHB/lauric acid were obtained at various heating rate, as illustrated in Fig. 7. Two-peak behavior is often observed in single-polymer systems in which melt/recrystallization process occurs during DSC heating run. In that case the relative peak area of the higher temperature peak increases with decreasing heating rate. Though the melt-crystallized PHB sample used in this study has one melting peak, PHB samples having other thermal history often show the two-peak melting behavior, which is usually ascribed to the melt/recrystallization process [29]. For PHB/dodecanol and PHB/lauric acid, however, the relative peak areas are independent of heating rate. Therefore, two PHB crystalline phases are formed in these mixtures.

The melting temperatures of dodecanol and lauric acid are higher than room temperature (see Table 1). The crystallization of PHB competes with that of the additives at room temperature. When the crystallization of PHB precedes that

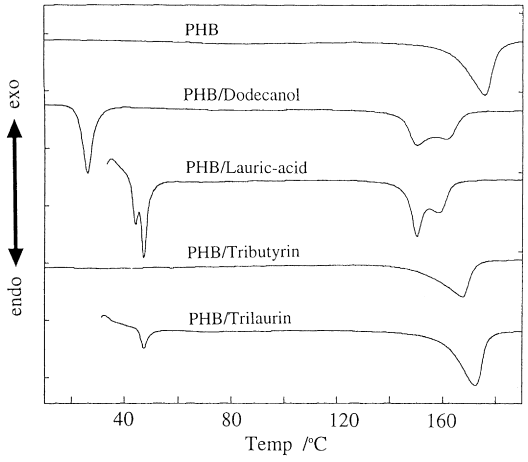

Fig. 6. DSC thermograms of PHB and of 77/23 PHB/additive mixtures melt-crystallized at room temperature.

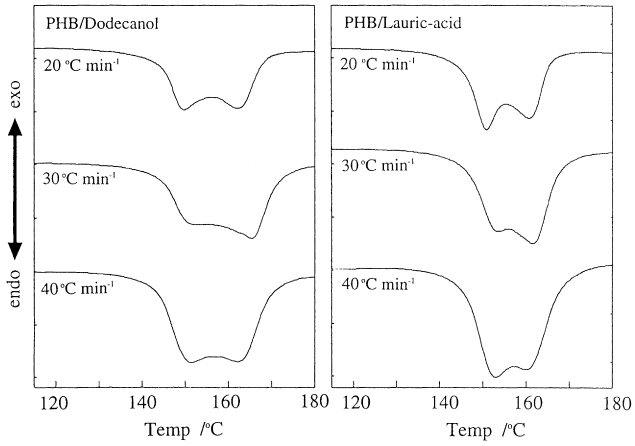

Fig. 7. DSC thermograms with various heating rates for 77/23 PHB/dodecanol and 77/23 PHB/lauric acid mixtures melt-crystallized at room temperature.

of the additive, the lamella of PHB similar to that of PHB in the pure state can be formed from the miscible melt. On the other hand, when the crystallization of the additives precedes that of PHB, the preformed crystals of the additives probably reduce the mobility of PHB molecules in melt, and as a result, retarding the lamella thickening of PHB. Thinner lamella has lower melting temperature. Therefore, the two melting peaks of PHB probably correspond to the melting of the PHB crystals formed from the miscible melt and those formed from the melt containing the additive crystals. Actually, when the mixtures are crystallized at temperature higher than the melting temperature of the additives, only one PHB crystalline phase is formed. Fig. 8 shows DSC thermograms of the mixtures of 77/23 PHB/additive crystallized at 90°C. Every sample exhibits only one melting peak of PHB crystal, indicating that only one PHB crystalline phase is formed at 90°C. Although the melting temperature of trilaurin is also higher than room temperature, the PHB/trilaurin mixture crystallized at

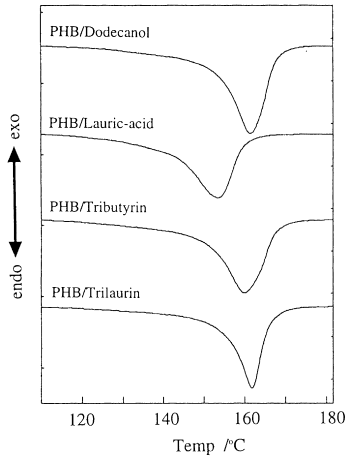

Fig. 8. DSC thermograms of 77/23 PHB/additive mixtures melt-crystallized at 90°C.

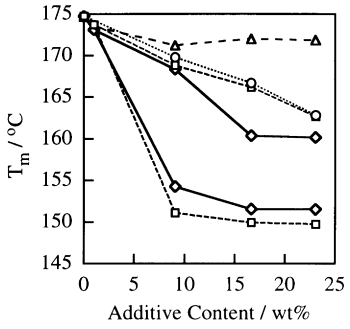

Fig. 9. Melting temperature (T_m) of PHB/additive mixtures melt-crystallized at room temperature as a function of additive content: (□) PHB/dodecanol; (◇) PHB/lauric acid; (○) PHB/tributyrin; (△) PHB/trilaurin. For PHB/dodecanol and PHB/lauric acid mixtures, two melting temperatures are plotted.

room temperature exhibits one-peak melting behavior (Fig. 6). This can be ascribed to the fact that PHB and trilaurin are miscible only when the trilaurin content is very low. The PHB/trilaurin mixture forms separate phases in the melt and the PHB crystals grow from almost pure PHB melt.

The apparent melting temperature, T_m, reflects the lamella thickness. Fig. 9 shows the composition dependence of the T_m of PHB crystals for the mixtures crystallized at room temperature. The mixtures have one or two T_m lower than that of pure PHB, suggesting that they have thinner lamella.

The degree of crystallinity of the samples was determined from the ratio $\Delta H_f^*/\Delta H_f^0$, where ΔH_f^0 and ΔH_f^* are the enthalpies of fusion of perfect PHB crystal ($=146\ J\ g^{-1}$) [30] and the apparent enthalpy of fusion observed for PHB/additive mixtures, respectively. The ΔH_f^* value was obtained from the area of the endothermic peak in the DSC thermograms. Fig. 10 shows the degree of crystallinity of the samples crystallized at room temperature. For all the samples, the crystallinity is 50–60% regardless of composition. The additives give little effect on the crystallinity.

3.4. Segregation of additives on film surface

Fig. 11 shows the scanning electron micrographs of the

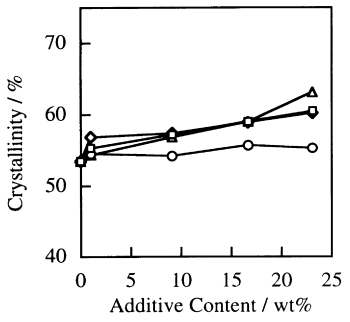

Fig. 10. Degree of crystallinity of PHB/additive mixtures melt-crystallized at room temperature as a function of additive content: (□) PHB/dodecanol; (◇) PHB/lauric acid; (○) PHB/tributyrin; (△) PHB/trilaurin.

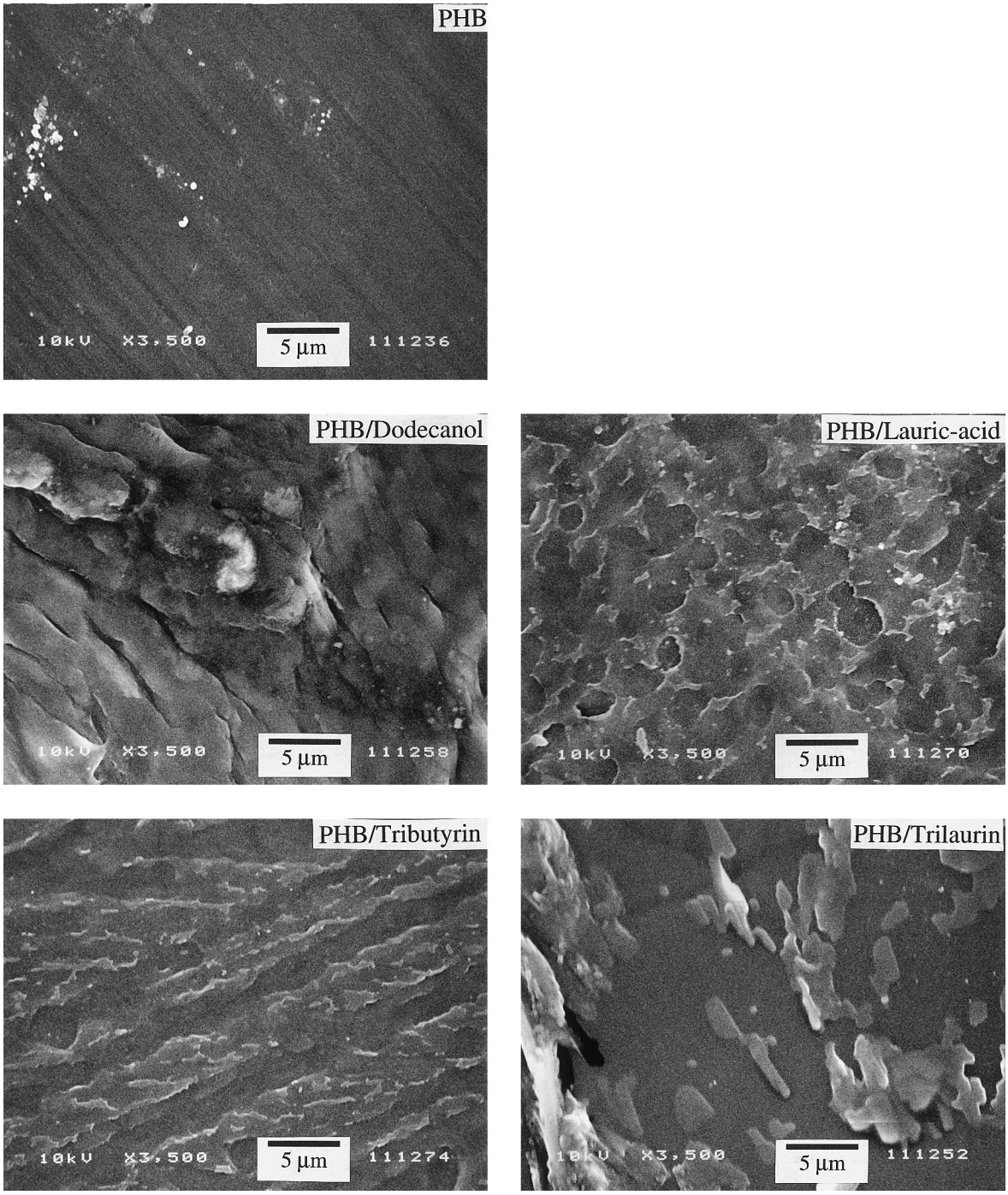

Fig. 11. Scanning electron micrographs of film surfaces of PHB and 91/9 PHB/additive mixture.

surfaces of PHB and the 91/9 PHB/additive mixtures. The surfaces of the 99/1 mixtures are indistinguishable from that of pure PHB because of low additive content. The surfaces of the 91/9 mixtures are rougher compared to that of pure PHB. This observation suggests that the segregation of the additives occurs at the surface area. When the additives segregate at the surface areas, they may disturb the contact of PHB depolymerase with PHB molecules.

In order to confirm this suggestion, we prepared the film samples washed in methanol. The films crystallized at room temperature were immersed in methanol for 2 h and then dried for 24 h under vacuum. The weight loss after washing were 8.1, 8.7, 8.8 and 1.1%, for the 91/9 mixtures of PHB/dodecanol, PHB/lauric acid, PHB/tributyrin and PHB/trilaurin, respectively. For PHB/dodecanol, PHB/lauric acid and PHB/tributyrin, the amount of weight loss is close to the total amount of the additive contained in the films before washing, indicating that most additives were

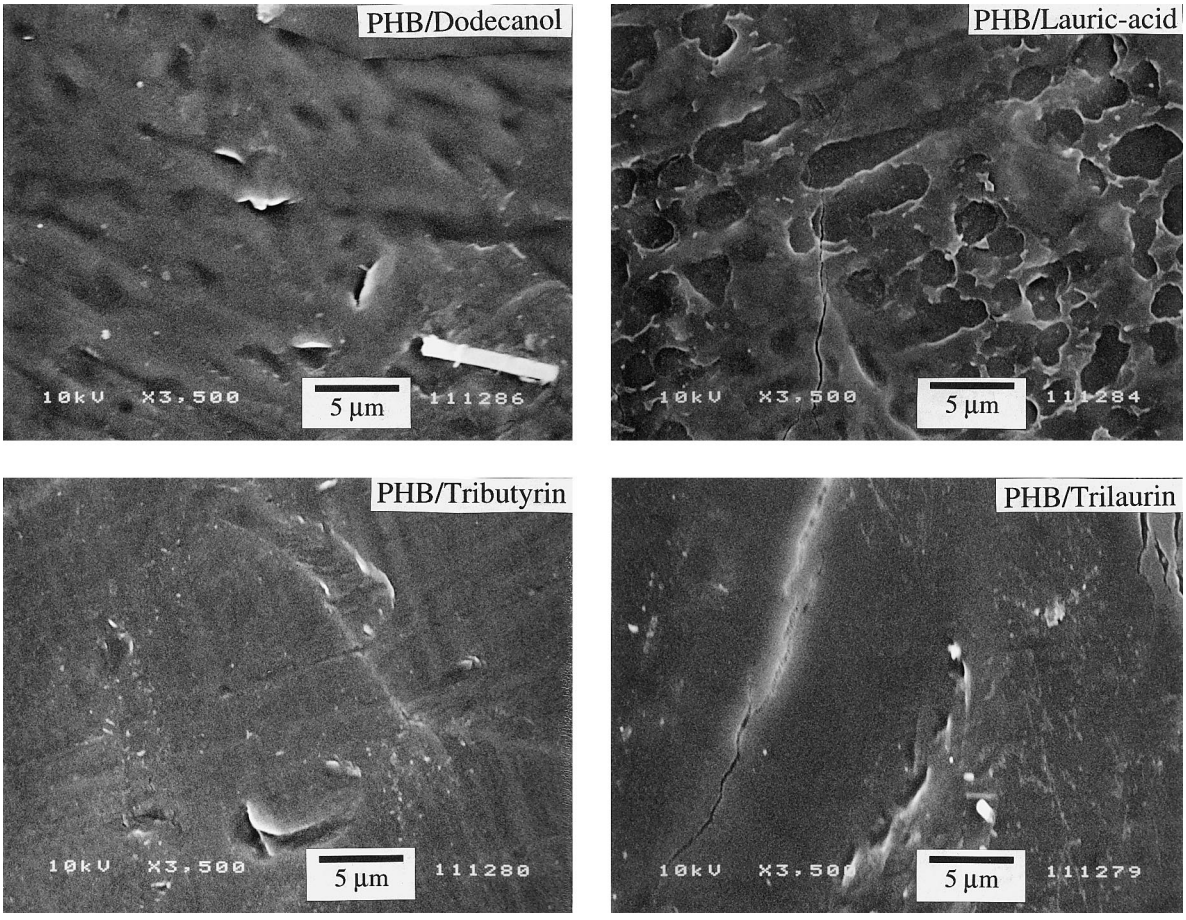

Fig. 12. Scanning electron micrographs of film surfaces of 91/9 PHB/additive mixtures after methanol washing.

removed from the mixtures upon washing. Small weight loss for PHB/trilaurin indicates that only the additive on the film surface is removed. In order to confirm whether or not this is a result of low solubility of trilaurin in methanol, the films of PHB/trilaurin were washed with hexane. The weight loss after washing was 0.7%. Thus, trilaurin in the film forms a domain structure, which is discontinuous to the film surface.

Fig. 12 shows the scanning electron micrographs of the surface of the films after washing. The smooth surface like pure PHB film appears after washing for the films of PHB/dodecanol, PHB/tributyrin and PHB/trilaurin mixtures. Therefore, the roughness of the surface observed for the films before washing is ascribed to the additives for these mixtures. Although almost all lauric acid is removed by washing, the roughness on the film surface remains. The addition of lauric acid modifies the morphology of PHB.

3.5. Enzymatic degradability of PHB/additive II

The observation of the film surface of the mixtures indicates that the lower rate of degradation for the 91/9 mixtures (Fig. 1) can be ascribed to the segregation of the additive on the film surface. The additives on the surface prevent the attack of enzyme on the PHB molecules. For the 99/1 mixtures, the segregation of the additive is not observed because of very low additive content. The higher rate of degradation for the 99/1 mixtures is probably attributed to the structure of the film inside.

There is strong evidence that PHB depolymerases initially hydrolyze accessible chains in the amorphous phase and subsequently erode the chains in the exposed crystalline lamellae [20,21,31,32]. Further, Scandola [27] has suggested that the mobility of the molecules in the amorphous phase also affects the biodegradability. All the additives examined here improve the mobility of PHB molecules in the amorphous phase. The higher mobility should be a possible factor that causes the higher degradability of PHB containing 1 wt% additive.

Another factor that affects the degradability is crystalline structure. Kumagai and Doi [20] have reported that the enzymatic degradability of PHB decreases with an increase in crystallinity. All the samples investigated in this study, however, have similar crystallinity. The change in degradability cannot be ascribed to the difference in crystallinity. Abe et al. [22] showed that the degradation rate decreases with an increase in lamella thickness. The mixtures of PHB/additive have T_m lower than the T_m of pure PHB, indicating

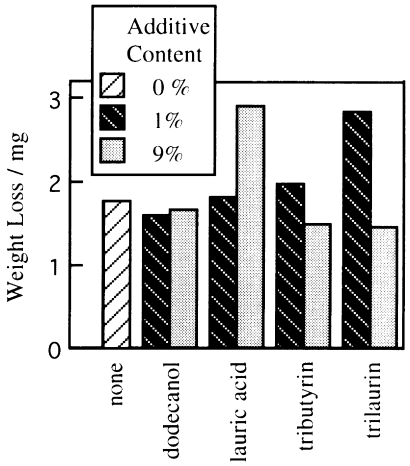

Fig. 13. Weight losses of methanol-washed PHB/additive mixtures after 5 h of exposure to PHB depolymerase from *A. faecalis* T1. The data on pure PHB before washing are also shown.

that the lamellae of the mixtures are thinner than those of pure PHB. Therefore, the thinner lamella is another possible factor that accelerates the degradation of PHB containing 1 wt% additive.

The enzymatic degradability of the methanol-washed films is shown in Fig. 13. As shown in Section 3.4, dodecanol, lauric acid and tributyrin were almost fully removed from the films of PHB/additive mixtures by methanol washing. Thus, molecular mobility in the amorphous phase of these mixtures after methanol washing must be similar to that of pure PHB. Further, DSC thermograms of these mixtures after washing were indistinguishable from that of pure PHB shown in Fig. 6 (data not shown). The lamella structure similar to pure PHB has been re-formed in these samples. Therefore, it is a matter of course that the degradability of these methanol-washed mixtures except PHB/trilaurin and 91/9 PHB/lauric acid is similar to that of pure PHB. The higher degradability of washed 91/9 PHB/lauric acid can be ascribed to the roughness on the film surface remaining even after washing. The degradability of the films of PHB/trilaurin, from which the additive only on the film surface region is removed by washing, was invariant upon washing and still higher than that of pure PHB. Therefore, the additive on the film inside affects the degradability.

We showed that the rate of enzymatic degradation of PHB can be controlled by the addition of certain kinds of low molecular-weight compounds. Research for low molecular-weight compounds appropriate for controlling the degradation rate of PHB-based copolymers is now in progress.

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