

Enzymatic and Non-enzymatic Degradation of Poly (3-Hydroxybutyrate-co-3-Hydroxyvalerate) Copolyesters Produced by *Alcaligenes* sp. MT-16

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Poly(3-hydroxybutyrate-co-3-hydroxyvalerate), poly(3HB-co-3HV), copolyesters with a variety of 3HV contents (ranging from 17 to 60 mol%) were produced by *Alcaligenes* sp. MT-16 grown on a medium containing glucose and levulinic acid in various ratios, and the effects of hydrophilicity and crystallinity on the degradability of the copolyesters were evaluated. Measurements of thermo-mechanical properties and Fourier-transform infrared spectroscopy in the attenuated total reflectance revealed that the hydrophilicity and crystallinity of poly(3HB-co-3HV) copolyesters decreased as 3HV content in the copolyester increased. When the prepared copolyester film samples were non-enzymatically hydrolysed in 0.01 N NaOH solution, the weights of all samples were found to have undergone no changes over a period of 20 weeks. In contrast, the copolyester film samples were degraded by the action of extracellular polyhydroxybutyrate depolymerase from *Emericellopsis minima* W2. The overall rate of weight loss was higher in the films containing higher amounts of 3HV, suggesting that the enzymatic degradation of the copolyester is more dependent on the crystallinity of the copolyester than on its hydrophilicity. Our results suggest that the degradability characteristics of poly(3HB-co-3HV) copolyesters, as well as their thermo-mechanical properties, are greatly influenced by the 3HV content in the copolyesters.

Key words: *Alcaligenes* sp. MT-16, crystallinity, degradability, polyhydroxyalkanoate, poly (3HB-co-3HV) copolyesters

Polyhydroxyalkanoates (PHAs) comprise a class of naturally-occurring polyesters, which are synthesized by a wide variety of bacteria as a carbon and energy reserve material, usually under unbalanced growth conditions (Madison and Huisman, 1999). These polyesters have become the focus of widespread attention, as environmentally friendly polymers which can be used in a wide range of agricultural, marine, and medical applications. In particular, a copolyester consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), poly(3HB-co-3HV), has been receiving commercial interest as a promising candidate for the large-scale production of biodegradable and biocompatible thermoplastics, since this polymer exhibits a considerable range of thermo-mechanical properties which depend on its 3HV content (Doi, 1990).

Production of poly(3HB-co-3HV) is usually achieved by providing bacteria with a cosubstrate, such as propionate and valerate, along with a main carbon source. Although the 3HV fraction in the copolyester increases as the concentration of the cosubstrate in the culture medium

increases, the maximum content of 3HV in the copolyester is limited (generally less than 20 mol%) by the toxic effect of the cosubstrates at relatively low concentrations (Ramsay *et al.*, 1990). Copolyesters with a high 3HV content are not necessarily more useful, but alteration of the 3HV content in poly(3HB-co-3HV) is desirable from an industrial viewpoint, as it may offer an opportunity for the production of different thermoplastics, having various degrees of flexibility and toughness.

There is increasing interest regarding the degradation of PHAs in recent years, due to the biomedical industry's need for biodegradable polymer implants and controlled drug release systems (Zinn *et al.*, 2001). Moreover, actual understanding of the PHA degradation is very important for the eco-friendly management of polymer wastes (Jendrossek and Handrick, 2002; Kim and Rhee, 2003; Steinbüchel and Lutke-Eversloh, 2003). Even though PHAs are considered to be quite resistant to degradation in the animal body, both enzymatic and non-enzymatic processes can occur simultaneously under normal conditions. It is, therefore, important to understand both processes (Marois *et al.*, 1999). It is generally accepted that the rates of degradation are influenced by the characteristics of the

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polymer, such as chemical composition, structure, crystallinity, and molecular weight (Abe and Doi, 2002; Renstad *et al.*, 1999). The degradation of poly(3HB-co-3HV) copolyesters has been intensively investigated, but most of the available data are for copolyesters containing less than 25 mol% of 3HV. Reports regarding the biodegradation of poly(3HB-co-3HV) with high molar fractions of 3HV are relatively rare, as such polymers are not readily available. Therefore, the effect of 3HV content on the degradation of these copolyesters has not yet been well documented.

We have previously reported the isolation of a mutant strain, *Alcaligenes* sp. MT-16, which accumulated poly(3HB-co-3HV) with significant amounts of 3HV units, using glucose as a sole carbon source (Choi *et al.*, 2003). The molar fractions of 3HV in the copolyester increased drastically, up to 77 mol%, when the organism was cultivated in a glucose medium supplemented with a cosubstrate, such as levulinic acid. In this study, we prepared poly(3HB-co-3HV) copolyesters with a variety of 3HV contents, ranging from 17 to 60 mol%, by growing the organism in glucose medium supplemented with different concentrations of levulinic acid, and evaluated both the enzymatic and non-enzymatic degradation of solution-cast films of these polyesters. Poly(3-hydroxybutyrate) (PHB) film was used as a control in the evaluation of the degradability of poly(3HB-co-3HV) copolyesters prepared during this study.

Materials and Methods

Preparation of PHAs

PHB was isolated from *Wautersia* (formerly *Ralstonia*) *eutropha* KHB-8862, as previously described (Chung *et al.*, 2001). Poly(3HB-co-3HV) copolyesters with varying 3HV contents were produced by growing *Alcaligenes* sp. MT-16 on basal medium (Yoon *et al.*, 1995) containing 20 g/l of glucose and levulinic acid in various ratios. Batch fermentations were conducted in a 5 L jar fermentor, with a working volume of 3 L. The medium was inoculated with a 10% (v/v) inoculum of an overnight culture in the same medium, except that it contained 10 g/l glucose and 4 g/l $(\text{NH}_4)_2\text{SO}_4$. Temperature and pH were automatically controlled at 37°C, and 7.0±0.1, respectively. The air flow rate was 1.0 vvm, and the agitation speed was 360 rpm. After 24 h of cultivation, the cells were harvested by centrifugation, followed by lyophilization. The polyesters were extracted from the lyophilized cells with hot chloroform using a Soxhlet apparatus, and were then purified by precipitation with methanol (Kang *et al.*, 2001). PHA films were prepared by solvent-casting techniques from chloroform solutions of polyester (0.2 g/ml), using glass dishes for casting surfaces. The solvent-cast films were aged for 2 weeks at room temperature, in order to achieve equilibrium crystallinity prior to analysis. Samples of the cast films measuring 1.0×1.0 cm were cut with a scalpel,

displaying a mean thickness of 0.10±0.02 mm, and a mean weight of 80.7±13.2 mg.

Degradation of PHA films

Non-enzymatic degradation of the PHA films was carried out at 37°C in small bottles containing 0.01 N NaOH (pH 11) solution. Following incubation for a given time, the cast films were periodically removed, washed with deionized water, and then dried in a vacuum at -50°C for 2 days, during which time the weight remained constant and was therefore used to determine mass loss (Marois *et al.*, 1999).

Enzymatic degradation of the PHA films was carried out at 37°C in a 0.1 M phosphate buffer (pH 7.5). The films were placed in small test tubes containing 5 ml of this buffer. The reactions were started by the addition of 10 µl of aqueous solution of PHB depolymerase (2 µg), purified from *Emericellopsis minima* W2 (Kim *et al.*, 2002b). The reaction solutions were incubated at 37°C with agitation. The films were periodically removed, washed with deionized water, and dried to constant weight. Weight loss was then calculated, as the percentage of weight decrease to original film weight.

Analytical procedures

Dry cell weight was measured by drying the harvested cells to a constant weight at 105°C. PHA content and its composition were determined by gas chromatography (GC) using Hewlett Packard (USA) gas chromatograph model 5890 Series II equipped with a HP-1 capillary column (30 m long × 0.25 mm in diameter), as described elsewhere (Park *et al.*, 1997), using poly(3HB-co-3HV) standards of known 3HB and 3HV monomer content. The total PHA quantity was determined by taking the sum of the amounts of 3HB and 3HV monomers, as determined by GC. Differential scanning calorimetry (DSC) measurements were taken using a TA Instruments (USA) model DSC 2010, from -40 to 200°C, at a ramp rate of 10°C/min. The average molecular weights of the polyesters were determined with a gel permeation chromatography (GPC) system, equipped with a Waters 6000 solvent delivery system (USA), RI detector, and U6K injector as described previously (Kim *et al.*, 1999). Water contact angles were measured using the sessile drop method at room temperature using an optical bench-type contact angle goniometer (Model 100-0, Rame-Hart, USA), as described previously (Chung *et al.*, 2003). Fourier-transform infrared spectroscopy in the attenuated total reflectance (FTIR-ATR) spectra was obtained using a Digilab FTS 80 (Bio-Rad, USA) spectrophotometer equipped with a KRS 5 internal reflection element (incidence angle, 45°). Scanning electron microscopic images were obtained with a Philips (Netherlands) scanning electron microscope model XL 30S, as described previously (Chung *et al.*, 2003).

Results and Discussion

Biosynthesis and general properties of poly(3HB-co-3HV) copolyesters

Table 1 shows the monomer compositions and molecular weights of PHB and poly(3HB-co-3HV) copolyesters, produced by *W. eutropha* and *Alcaligenes* sp. MT-16, respectively. Levulinic acid is 4-ketopentanoic acid which can be utilized by some PHA-producing microorganisms, as a 3HV precursor (Chung *et al.*, 2001; Jang and Rogers, 1996). When the levulinic acid concentration was increased from 0 to 2.0 g/L in culture medium, the 3HV fraction in the copolyesters produced by *Alcaligenes* sp. MT-16 increased from 17 to 60 mol%. The number average molecular weights (M_w) of poly(3HB-co-3HV) copolyesters were estimated to be 145,000 - 249,000, a result considerably lower than those of poly(3HB-co-3HV) copolyesters (M_w , 254,000 - 395,000) produced by *W. eutrophus*, from a mixture of butyrate and valerate (Doi *et al.*, 1990).

The melting temperature (T_m), glass-transition temperature (T_g), and enthalpy of fusion (ΔH_m) for the copolyesters decreased gradually with increases in 3HV content (Table 2). The T_m values decreased from 175 to 76, as the 3HV content increased from 0 to 60 mol%. In a previous study, Doi (1990) reported that the minimum T_m value (around 75°C) of poly(3HB-co-3HV) copolyesters produced by *W. eutropha* is observed at approximately 40 mol% 3HV, where the crystal lattice transition from PHB

to poly(3-hydroxyvalerate) occurs. This difference in thermal properties is probably due to the discriminative distribution of monomer composition in the copolyesters produced by different microorganisms with different carbon sources (Choi *et al.*, 2004). The elongation at the break increased from 8 to 620% with increasing 3HV content from 0 to 60 mol%, indicating that the poly(3HB-co-3HV) copolyesters exhibit a wide range of material properties, varying from crystalline plastic to elastic rubber, depending on copolyester composition.

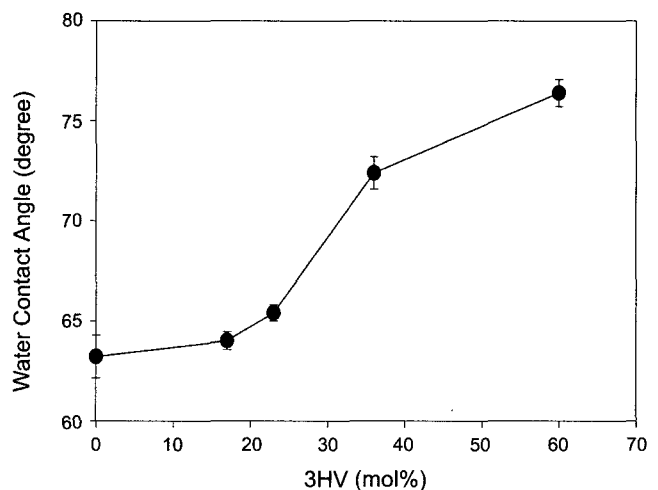


Fig. 1. Plot of 3HV content vs. water contact angle of poly(3HB-co-3HV) copolyesters. Error bars indicate the range of experimental readings obtained (sample number, $n=5$).

Table 1. Monomeric composition and molecular weight of PHB and poly(3HB-co-3HV) copolyesters produced by *Wautersia eutropha* and *Alcaligenes* sp. MT-16, respectively

Sample code	Conc. of carbon source (g/l)		Polyester			
	Glucose	Levulinic acid	Composition (mol%)		Molecular weight	
			3HB	3HV	$M_n \times 10^{-3}$	PDI ^a
PHB	20	0	100	0	187	2.4
P(3HB-17-3HV)	20	0	83	17	145	3.5
P(3HB-23-3HV)	20	0.1	77	23	148	3.5
P(3HB-36-3HV)	20	1.0	64	36	249	2.5
P(3HB-60-3HV)	20	2.0	40	60	198	2.6

^aPolydispersity index (M_w/M_n).

Table 2. Thermal and mechanical properties of PHB and poly(3HB-co-3HV) copolyesters produced by *Wautersia eutropha* and *Alcaligenes* sp. MT-16, respectively

Sample	Thermal properties			Mechanical properties	
	T_m (°C)	T_g (°C)	ΔH_m (J/g)	Youngs modulus ^a (MPa)	Elongation to break ^a (%)
PHB	175	10.0	100.4	1023 ± 85	8 ± 0.9
P(3HB-17-3HV)	165	8.1	94.4	682 ± 56	32 ± 1.6
P(3HB-23-3HV)	138	5.1	54.3	545 ± 38	18 ± 1.9
P(3HB-36-3HV)	97	-2.4	43.2	362 ± 5	157 ± 13
P(3HB-60-3HV)	76	-4.3	33.4	213 ± 12	620 ± 28

^aAverage values ($n \geq 3$) and standard deviation.

Hydrophobicity and crystallinity of poly(3HB-co-3HV) copolyesters

The hydrophilicity of polymeric materials plays an important role in their degradability characteristics. The water contact angles of the polyesters, as determined via sessile

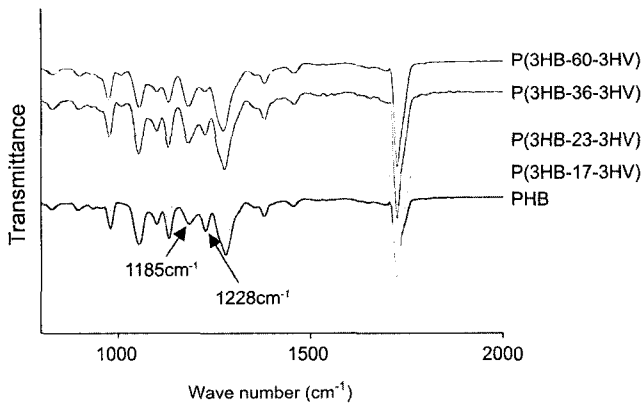


Fig. 2. FTIR-ATR spectra of PHB and poly(3HB-co-3HV) copolyesters.

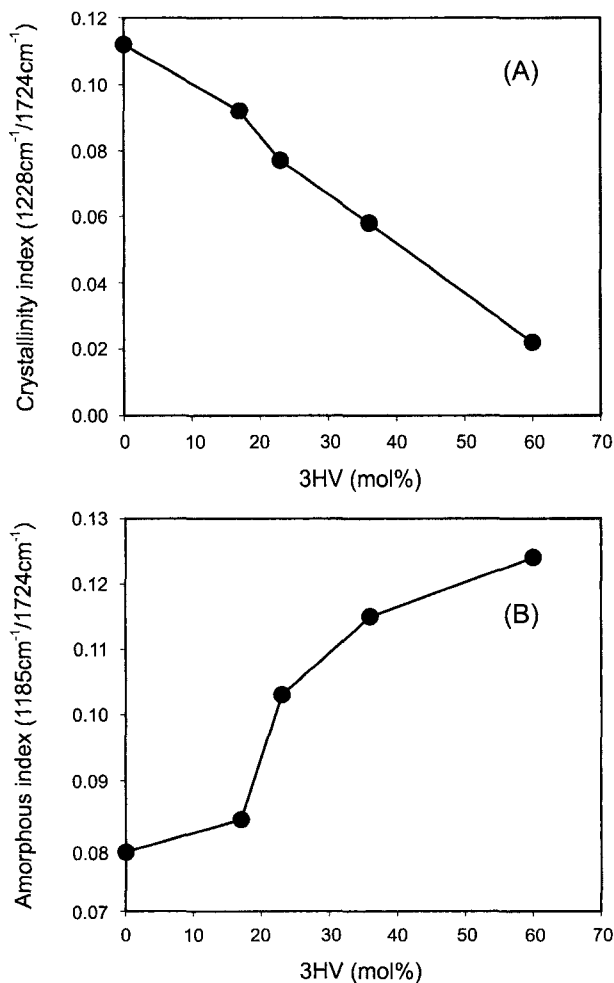


Fig. 3. Crystallinity index (A) and amorphous index (B) of poly(3HB-co-3HV) copolyesters determined by FTIR-ATR.

drop method, are shown in Fig. 1. All poly(3HB-co-3HV) copolyesters exhibited higher contact angles than did PHB, and the contact angles increased gradually with increasing 3HV content. It is widely accepted that increasing the side chain length of the constituents enhances the hydrophobicity of the polyester. Actually, our results indicated that the surfaces of the copolyesters become more hydrophobic as the 3HV content in the copolyesters increases.

Fig. 2 shows the typical FTIR-ATR spectra of the PHB and poly(3HB-co-3HV) copolyesters. Recent studies regarding the crystallinity of poly(3HB-co-3HV) by FTIR analysis of the copolyesters have revealed that the shapes and intensities of some IR bands are sensitive to the degree of crystallinity and, thus, some bands decrease in intensity, while others increase to different extents, as a response to greater crystallinity (Luo and Netravali, 2003; Galego *et al.*, 2000). Fig. 2 clearly indicates that the intensity of the band at 1185 cm⁻¹, the amorphous state-sensitive band, increased gradually with increased 3HV content. In contrast, the intensity of the band at 1228 cm⁻¹, the crystallinity-sensitive band, decreased with increased 3HV content. In order to determine the relative surface crystallinity of the polyesters, the crystallinity index (CI) and amorphous index (AI) were calculated from the spectra in Fig. 2, and plotted in Fig. 3. CI and AI were defined as the ratio of the intensities of the band at 1724 cm⁻¹, which is insensitive to the degree of crystallinity (Kansiz *et al.*, 2000), to the 1228 cm⁻¹ band, and to the 1185 cm⁻¹ band, respectively. As shown in Fig. 3, CI values decreased while AI values increased, as a response to increasing 3HV content, indicating that the copolyesters become more amorphous as the 3HV content of the copolyesters increases.

Non-enzymatic degradation of poly(3HB-co-3HV) copolyesters

Non-enzymatic hydrolysis in the degradation process of polymers may be important in the use of certain biological environments (*in vivo*) as implant materials. Marois *et al.* (1999) have suggested the importance of abiotic hydrolysis in the initiation of polyester degradation. In this study, however, the weight of all PHA films hydrolyzed non-enzymatically in 0.01N NaOH solution were unchanged over a period of 20 weeks, indicating that no significant polymer erosion had occurred, even at alkaline pH, during the incubation time. Similar results have been reported in another study (Doi, 1990), in which poly(3HB-co-8 mol% 3HV) was examined at various temperatures, for periods up to 200 days.

On the other hand, morphological changes were observed on the surface of films during hydrolysis tests. The SEM photographs of the polyesters both before and after 20 weeks of non-enzymatic hydrolysis are shown in Fig. 4. As seen, the surface of the initial PHB film was

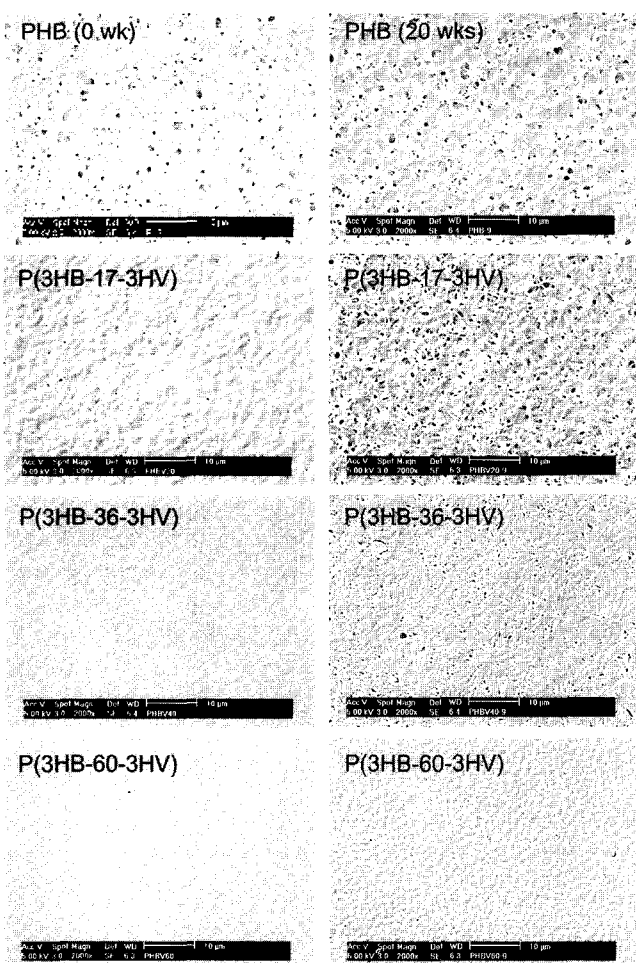


Fig. 4. SEM photographs of PHB and poly(3HB-*co*-3HV) copolyester films both before (0 wk, panels on the left) and after 20 weeks (panels on the right) of non-enzymatic hydrolysis in 0.01 N NaOH solution (scale bars, 10 μ m).

very rough, revealing an interwoven fibrillar structure with small holes. By contrast, the surfaces of the poly(3HB-*co*-3HV) copolyesters were smooth, and surface smoothness increased with increasing copolyester 3HV content. After 20 weeks of exposure to NaOH solution, the surfaces of all samples became rougher, along with an increased density of hole formation on their surfaces. From these results, it can be surmised that the non-enzymatic degradation of PHAs progresses on their surfaces before noticeable weight loss occurs. In addition, the lowest hole density on the surface of the poly(3HB-*co*-60 mol% 3HV) film, and the highest hole density on the surface of the PHB film, bolster the notion that the rate of non-enzymatic degradation decreases as 3HV content in the polyesters increases. This phenomenon might be attributable to the increase in the hydrophobicity of polyester with increased 3HV content.

Enzymatic degradation of poly(3HB-*co*-3HV) copolyesters
Extracellular PHB depolymerases consist of a catalytic

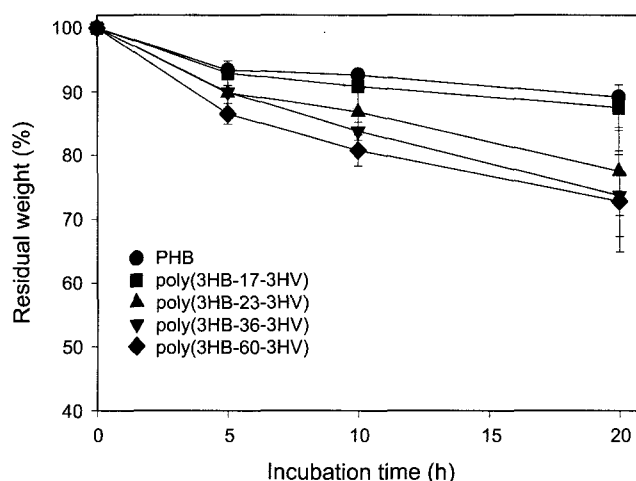


Fig. 5. Enzymatic degradation profiles of PHB and poly(3HB-*co*-3HV) copolyester films treated with PHB depolymerase from *Emericellops minima* W2.

domain in the N-terminus and a substrate-binding domain in the C-terminus (Jendrossek and Handrick, 2002). The enzymes first adsorb onto the surfaces of polyester films by the function of their binding domains, and then catalyze the hydrolysis of polymer chains by the function of their catalytic domains. It has been suggested that the biodegradability characteristics of polyesters comprised of hydroxyalkanoates can be significantly affected by initially small microstructural differences (Kim *et al.*, 2002a). Fig. 5 depicts the enzymatic degradation profiles of PHB and poly(3HB-*co*-3HV) films treated with PHB depolymerase from *E. minima* W2, as a function of degradation time. The enzymatic degradation rates of the poly(3HB-*co*-3HV) films were higher than those of PHB films, and the weight loss of the copolyesters increased in a linear fashion up to 10-25% at 20 h. In particular, the overall rate of weight loss was higher for the films containing higher 3HV concentrations, in spite of their reduced hydrophilicity.

The most important factors influencing the enzymatic degradation rates of PHA films are crystallinity, molecular weight, and accessibility of the polymer surface (Brandl *et al.*, 1995; Jendrossek and Handrick, 2002). Doi and his colleagues (1990) have reported that the PHA film degradation rates by various microbial depolymerases decreases with increasing crystallinity, and that the rate of enzymatic degradation is largely independent of molecular weight. Similar results were also seen in other recent studies (Molitoris *et al.*, 1996; Quinteros *et al.*, 1999; Yoshie *et al.*, 1999). Our current results also reveal that the enzymatic degradation rate of the poly(3HB-*co*-3HV) copolyesters is more dependent on the crystallinity of the copolyesters than on their hydrophilicity.

Depending on the depolymerase, the hydrolysis products of PHA can be either monomers, monomers and

Table 3. Monomer contents in water-soluble products after enzymatic degradation of poly(3HB-co-3HV) copolyesters^a

Sample	3HB (mol%)	3HV (mol%)
Poly(3HB-23-3HV)	63.0	37.0
Poly(3HB-36-3HV)	55.0	46.0
Poly(3HB-60-3HV)	36.2	73.8

^aThe enzymatic degradation of the copolyesters was carried out on solution-cast films at 37°C for 20 h in 5 ml phosphate buffer (pH 7.5) containing 2 µg of PHB depolymerase from *Emericellopsis minima* W2.

dimers, or a mixture of oligomers (Jendrossek and Handrick, 2002). In a previous paper, it was reported that monomers and dimers comprise the majority of hydrolysis products induced by the PHB depolymerase from *E. minima* W2 (Kim *et al.*, 2002b). After 20 h of PHB depolymerase treatment of poly(3HB-co-3HV) copolyesters, the 3HV content in the water-soluble products produced by enzymatic hydrolysis was determined by GC. As shown in Table 3, the 3HV content in the degradation products was somewhat higher than in the original poly(3HB-co-3HV) copolyester samples before degradation, suggesting that the ester bonds between 3HV and the 3HB units are hydrolyzed faster by the action of the PHB depolymerase than are those between 3HB and the 3HB units. This result is consistent with the finding that the rate of enzymatic degradation is higher for poly(3HB-co-3HV) films containing higher concentrations of 3HV.

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