Effects of Higher-Order Structure of Poly(3-hydroxybutyrate) on Its Biodegradation. I. Effects of Heat Treatment on Microbial Degradation

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SYNOPSIS

To clarify how the biodegradation of plastic is affected by physical properties based on the plastic's higher-order structure, the microbial degradability of heat-treated poly(3-hydroxybutyrate) (PHB) samples was studied. Cast-film samples and fibrous samples prepared with heat treatment were degraded by an isolated degrading bacterium: strain SC-17. Consequently, it was found that the higher-order structure properties such as crystallinity and modulus of elasticity of PHB acted as suppression factors. SEM observations of the degraded PHB films showed unique degradation patterns such as spherical and tubular holes on the surfaces and cells of SC-17 in process of degrading the PHB surface. These patterns are thought to result from colonization of SC-17 on the surfaces. From the results, it was suggested that the surface of the PHB was important as a growing field for the degrading bacterium and that the higher-order structure properties of the PHB affected not only the degradation reaction but also the colonization of the degrading bacterium on the surface.

INTRODUCTION

A storage polymer found in a range of bacterial groups, poly(3-hydroxybutyrate) (PHB) is accumulated as granules within the cytoplasm. It shows promise as a raw material for a biodegradable thermoplastic.

There appear to be many important factors that affect the biodegradability of plastic materials. These include, e.g., factors related to the first-order structure (chemical composition, molecular weight, molecular weight distribution), factors related to the higher-order structure (melting temperature, glass transition temperature, crystallinity, crystal structure, modulus of elasticity), and factors related to surface conditions (surface area, hydrophilic and hydrophobic properties). These factors are interrelated. For example, alteration of crystal structure often causes changes in surface morphology as well as in physical properties. It is thought that these changes influence the biodegradability of plastic and that results obtained without taking these changes into account provide a view of only one small facet of the highly complete picture of plastic biodegradability. Although many reports relating to the biodegradation of PHB and copolyesters have been published,[1-12] these have dealt mainly with the degradation mechanism by PHB depolymerases and the effects of copolyester chemical composition. It has therefore now become necessary to clarify the relationship between each factor in the higher-order structure of PHB and PHB biodegradability.

Various information relating to the higher-order structure of PHB and copolyesters is available. Merrick et al. reported that several treatments with acetone, HCl, or trichloroacetic acid, etc., rendered the native PHB granules completely resistant to hydrolysis by intracellular depolymerase.[1,2] Barnard and Sanders studied the mobility of PHB native granules by ^{13}C-NMR spectroscopy and found that the PHB molecules in the native granules were in a mobile state, not in a highly crystalline solid

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state.[13] Kawaguchi and Doi investigated the structure of native PHB granules of *Alcaligenes eutrophus* by X-ray diffraction analysis and reported that crystallization of native granules was caused by the removal of lipid components by various treatments.[14] Holland et al. examined the effect of a specimen preparation technique on hydrolytic degradation and showed that various forms of PHB and copolyesters had differing stabilities to hydrolytic attack.[9] Although enzymatic degradation of PHB-based blends has recently been the subject of frequent investigation,[11,12] it should be noted that such research involves complex interrelated factors.

To clarify the effects of physical properties based on the higher-order structure of PHB, we studied the microbial degradability of PHB using heat-treated samples under conditions that restrained the influence of surface morphology. Further, we investigated the microbiological action of the bacterium on the PHB sample surface by observation of degrading samples by scanning electron microscopy (SEM). Consequently, we found that the higher-order structure properties such as crystallinity and the modulus of elasticity of PHB acted as suppression factors and that the unique degradation patterns on the PHB sample surface probably were formed by colonization of the bacterium.

EXPERIMENTAL

Materials

PHB [BX GV9(EE) additive-free, technical grade granules] from *Alcaligenes* sp. was obtained from ICI (Japan) Limited. This was dissolved in hot chloroform and purified by precipitation with hexane. The weight-average molecular weight of the PHB was estimated to be about 20×10^4 from intrinsic viscosity data according to Marchessault et al.'s method.[15]

Films

PHB films were prepared by a conventional solvent-casting technique from solutions of the reprecipitated fibrous PHB in chloroform.

Heat Treatment

Film samples (initial film dimensions: 16 mm × 12 mm × 100 μ) were prepared under heat-treatment conditions of ① 30°C/36 h, ② 90°C/36 h, ③ 90°C/20 h + 150°C/16 h, and ④ 150°C/20 h. Reprecipitated fibrous samples were similarly prepared.

Wide-angle X-ray diffraction measurements of the PHB samples were made on a Rigaku Rint-1200 system. CuKα radiation (λ = 0.154056 nm) was used as the source. The X-ray diffraction patterns of the PHB samples were recorded in the range of 2θ = 6–40° at a scan speed of 2°/min. The percentage of crystallinity was calculated from diffracted intensity data according to Tsunoda et al.'s method.[16]

Dynamic viscoelastic measurements of the PHB samples were carried out for the films using a direct reading dynamic viscoelastometer (SEIKO Model SDM-5500 system).

PHB-degrading Bacterium

Farm soil, obtained from a taro field at Tsukuba, Japan, was incubated in a medium composed of 0.25% purified fibrous PHB, 10 ppm $FeSO_4 \cdot 7H_2O$, 200 ppm $MgSO_4 \cdot 7H_2O$, 1000 ppm $(NH_4)_2SO_4$, 20 ppm $CaCl_2 \cdot 2H_2O$, 100 ppm NaCl, 0.5 ppm $Na_2MoO_4 \cdot 2H_2O$, 0.5 ppm Na_2WO_4, 0.5 ppm $MnSO_4$, and 100 ppm yeast extract in 10.7 m*M* KH_2PO_4/K_2HPO_4 (pH 7.1) under vigorous aeration at 30°C for several days until the PHB was disintegrated and the turbidity of the medium developed. A pure culture was obtained by repeated application of subcultures on the same media and plating the cultured bacteria on the above medium containing 1.5% agar. An isolated strain (SC-17) was maintained on an agar slant at 5–10°C after incubation at 30°C for 72 h.

PHB Degradation Tests

A film sample (16 × 12 mm, about 20–30 mg) or fibrous sample (about 100 mg) was added to 100 mL of a test medium identical with the above culture medium except for the addition of the PHB. After the mixture of the PHB sample and the test medium contained in a 500 mL flask were autoclaved (at 120°C for 20 min), the bacterium was injected into it. The flask was aerated by stirring with a rotary shaker at 180 rpm and 30°C. After a prescribed incubation period, the pH of the medium was measured. Then, a small part of the medium (5 mL), which was filtered through a Millipore filter (0.22 μ pore size) and used for estimating the value of the water-soluble total organic carbon (TOC) with a Shimazu model TOC-500. The main portion of the medium was filtered through Toyo No. 2 filter paper and the remaining PHB sample was collected and washed with water. The filtrate and washings were mixed and cells were collected by filtration of the mixture with the Millipore filter. The collected PHB

sample and cells were weighed after drying at 105°C for 16 h and were confirmed by infrared spectra with a JASCO A-302 infrared spectrophotometer.

Scanning Electron Microscopy (SEM) Observations

PHB samples after the microbial degradation were observed under a JEOL model JSM-T220 with 15 kV acceleration.

RESULTS AND DISCUSSION

Changes in Higher-order Structure Properties of PHB Film by Heat Treatment

For altering the particular physical properties of the PHB film without changing others, cast films of the PHB homopolymer were heated in an oven. The changes in the physical properties of the PHB cast films are shown in Figure 1(a) and (b). Each of the measured properties, namely, crystallinity, half-value breadth of (020) plane, dynamic storage modulus E' at 30°C, and glass transition temperature (T_g) based on the peak temperature of the dynamic loss modulus E'', was obviously dependent upon the heat-treatment temperature. As the heat-treatment temperature increased, the crystallization advanced and E' and T_g rose correspondingly. However, SEM observation of the surfaces in the range of 150× to 5000× did not reveal any apparent morphological changes in the film samples [Figs. 5(a) and 6(a)] or in the fibrous samples either.

Effects of Heat Treatment of PHB on Microbial Degradation

Figure 2 shows the results of microbial degradation by the bacterium strain SC-17 of film samples ①, ②, and ③ and fibrous samples ①, ②, and ③, which were prepared with the heat treatment under the above conditions ①, ②, and ③, respectively. The fibrous samples were degraded rapidly, while the film samples were degraded gradually. The reason for this result is assumed to be that a larger surface area of the water-insoluble substrate increases the collision frequency in the enzymatic reaction and is advantageous for growth of the bacterium on the surface, as reported by Zobell[17] and Hattori et al.[18]

On the other hand, with regard to the effects of the heat treatment, it was found that the degradation rates of the film sample ③ and fibrous sample ③, which were the higher crystallized samples, were lower than those of the others. But, between samples

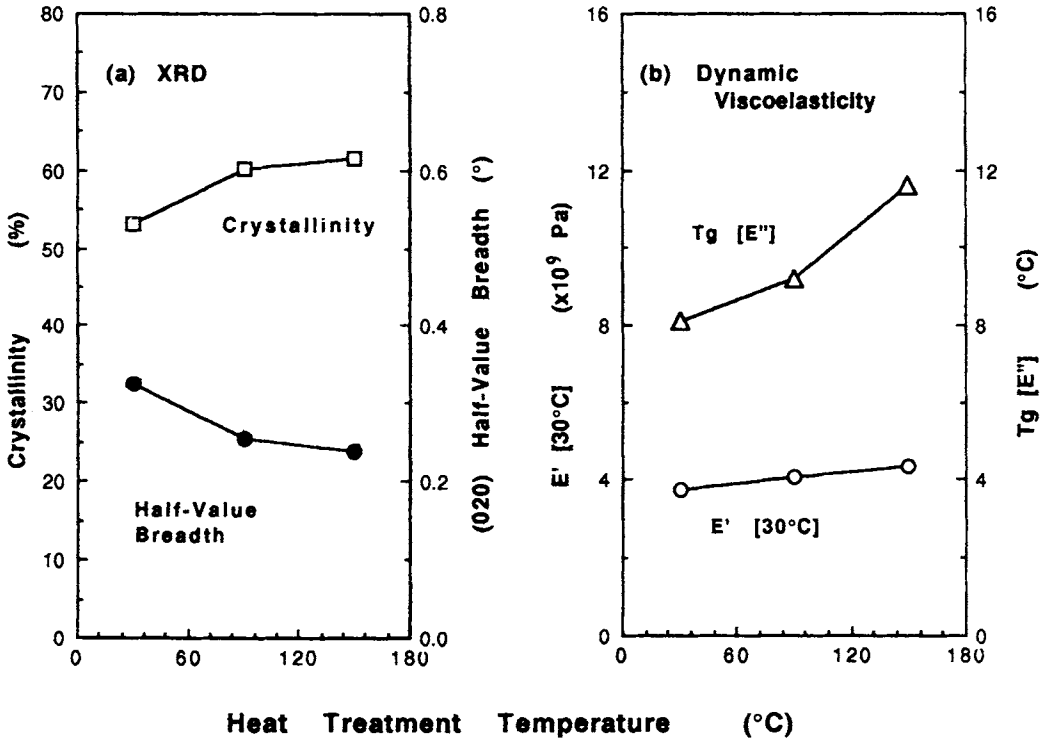

Figure 1 Changes in higher-order structure properties of PHB cast film by heat treatment.

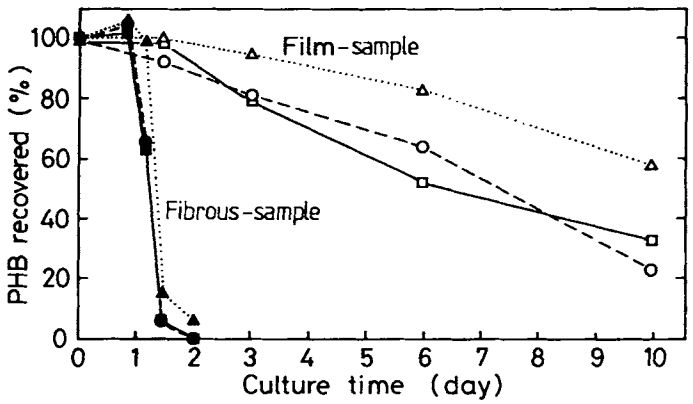

Figure 2 Comparison of progressive weight loss of film and fibrous samples heat-treated under three conditions: (□) film ①, (■) fibrous ①, 30°C/36 h; (○) film ②, (●) fibrous ②, 90°C/36 h; (△) film ③, (▲) fibrous ③, 90°C/20 h + 150°C/16 h.

① and ②, the expected difference in degradability was not observed. Taking into account the fast crystallization rate of PHB reported by Kunioka et al.,[19] it is considered that this lack of a difference results from the recrystallization during autoclave sterilization (20 min/120°C). Actually, the crystallinity values of film samples ① and ② after sterilization were nearly equal at about 60%. Therefore, the results in Figures 1 and 2 indicate that the microbial degradation is depressed with the development of crystallinity, crystal size, modulus of elasticity, and T_g in the PHB.

The above results suggest preferential degradation in the amorphous region of PHB, which is the flexible part. If the degradation of PHB proceeds in its amorphous region, this raises the question of whether the crystal part may remain without degrading completely. The answer was found in the following investigation.

Time Course of Microbial Degradation of Heat-treated PHB Film

The time course of degradation of film sample ④ by strain SC-17 is shown in Figure 3. The sample was prepared with heat treatment at 150°C for 20 h and had a high crystallinity value of about 64%. The degradation proceeded gradually, but after 34 days, the sample completely disappeared, whereas the weight loss of the control film without inoculation

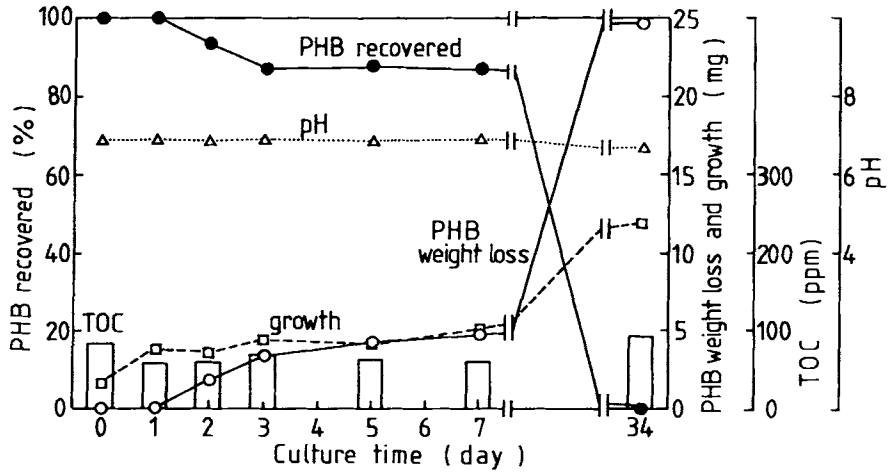

Figure 3 Time course of PHB film-sample ④ degradation by strain SC-17 at 30°C. Heat-treatment condition of sample ④ at 150°C for 20 h: (●) PHB recovered (%); (○) PHB weight loss (mg); (△) pH; (□) growth (dry cell weight) (mg); (bar) water-soluble TOC (ppm).

was 0.08% after 34 d. From this result, it was confirmed that both the crystal part and the amorphous part of the heat-treated PHB film was completely degraded by SC-17.

With decreasing sample ④ weight, the cell weight ("growth" in the figure) increased and finally reached 31.5% of the total initial weight of the sample and the yeast extract. The pH dropped slightly from 6.86 to 6.68. The water-soluble TOC decreased at first, maintained the lower level for some time, and finally increased above the initial value. From this TOC change pattern, it is presumed that the first stage was characterized by utilization of yeast extract by SC-17, the middle stage by the dominance of the metabolic rate of the decomposed compounds over the degradation rate by depolymerase, and the last stage by cell lysis or accumulation of metabolites, e.g., monomer, oligomer, and other organic acids.

For comparison, the time course of the fibrous sample degradation is shown in Figure 4. This sample was prepared without heat treatment. The degradation of fibrous PHB proceeded rapidly and was consequently completed in 40 h. The cell weight went up to 43.3% to the total initial weight. With the degradation, the pH dropped pronouncedly from 6.87 to 5.35. The water-soluble TOC accumulated in the medium from 20 to 30 h due to faster degradation than assimilation.

The results shown in Figures 3 and 4 suggest that the surface area and the higher-order structure of PHB affect the growth of the degrading microorganisms.

SEM Observations of PHB Films Degraded by Strain SC-17

To examine the PHB degradation mechanism and the growth of the degrading microorganisms, strain SC-17, on the surface of PHB, the degraded film samples were observed by SEM.

SEM observations of the degraded film samples ① and ③ are shown in Figures 5 and 6, in which time courses of the degradation patterns are illustrated. The initial surfaces of both samples were indistinguishable. As the degradation proceeded, the two samples showed similar degradation patterns by attack at each stage of degradation [compare Fig. 5(b) with Fig. 6(c) and Fig. 5(c) with Fig. 6(d)]. At a low percentage of degradation, small pits were observed on the surfaces of the PHB films. Then, with advancing degradation, the pits increased in size, combined, and changed into unique spherical holes measuring 50–200 μ in diameter. The spherical holes progressed into the inner portion of the films and some completely penetrated the film [Fig. 5(d)]. Figure 7 shows the relationship between spherical hole diameter and percentage of degradation. The results for two series of samples could be approximately represented by a single curve. From this result, it appears that the only difference between the two series of samples was a time lag in the growth of the holes.

Figure 8 shows the edge of degraded PHB film ③. The film was attacked from the lateral direction in some places, resulting in formation of tubular holes measuring about 50–100 μ in diameter.

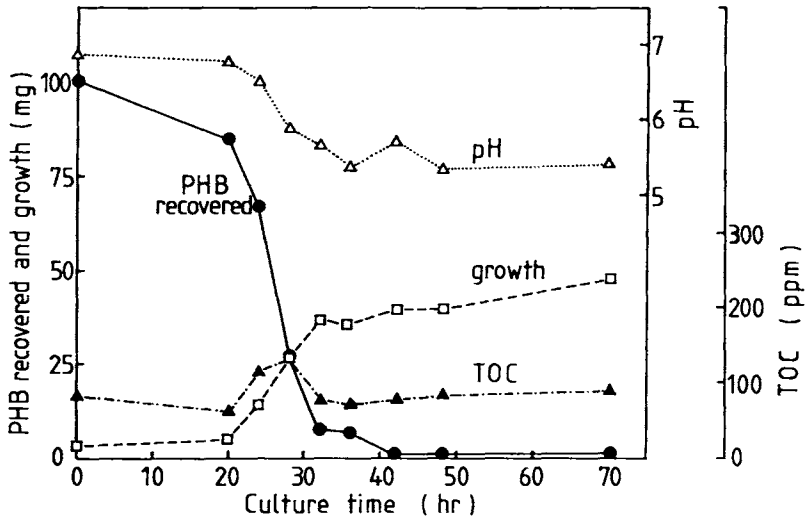

Figure 4 Time course of nonheated fibrous sample degradation by strain SC-17 at 30°C: (●) PHB recovered (mg); (△) pH; (□) growth (dry cells weight) (mg); (▲) water-soluble TOC (ppm).

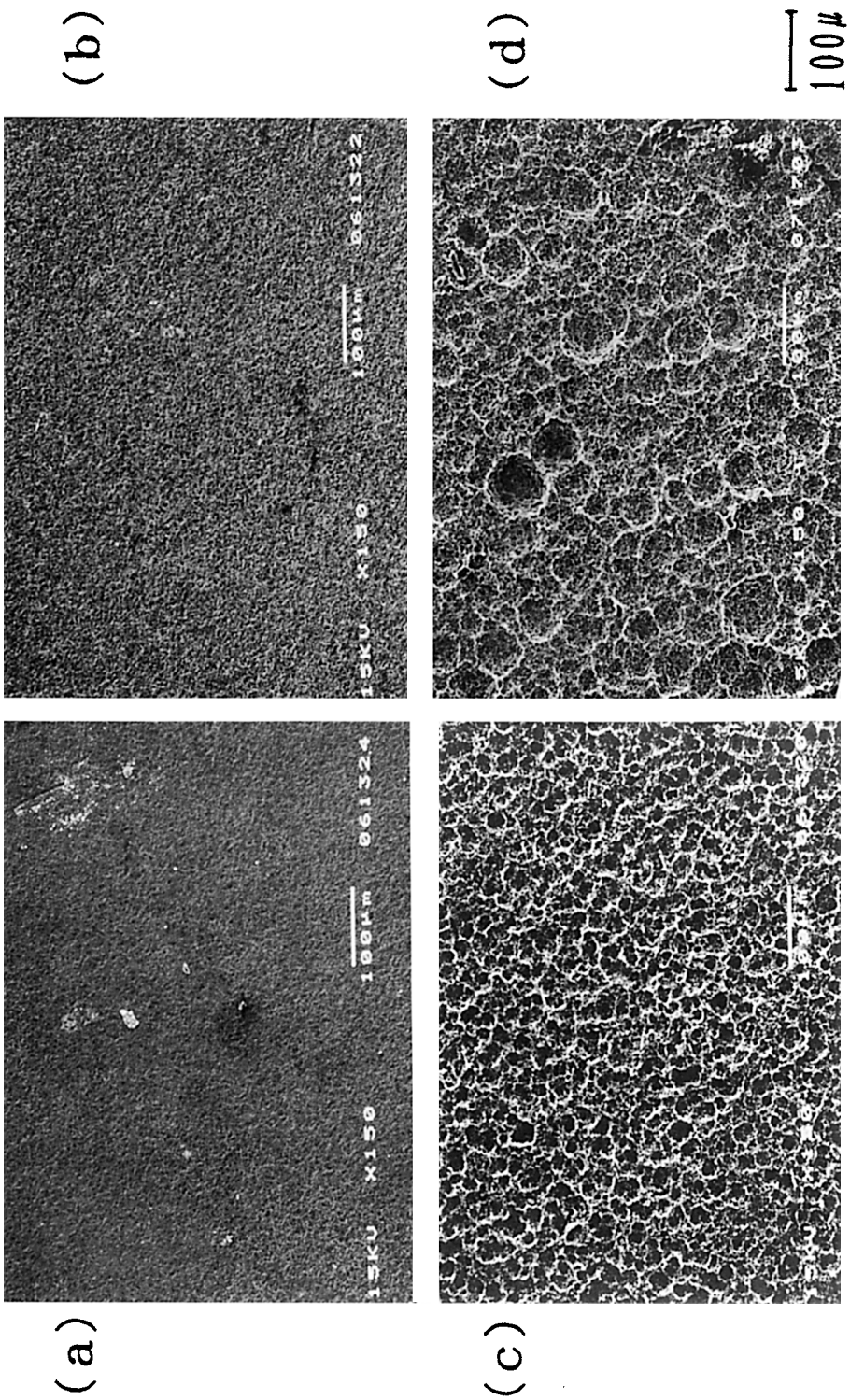

Figure 5 SEMs of surface of degraded PHB film sample ⑨. Culture time: (a) 0 h; (b) 72 h; (c) 143 h; (d) 239 h.

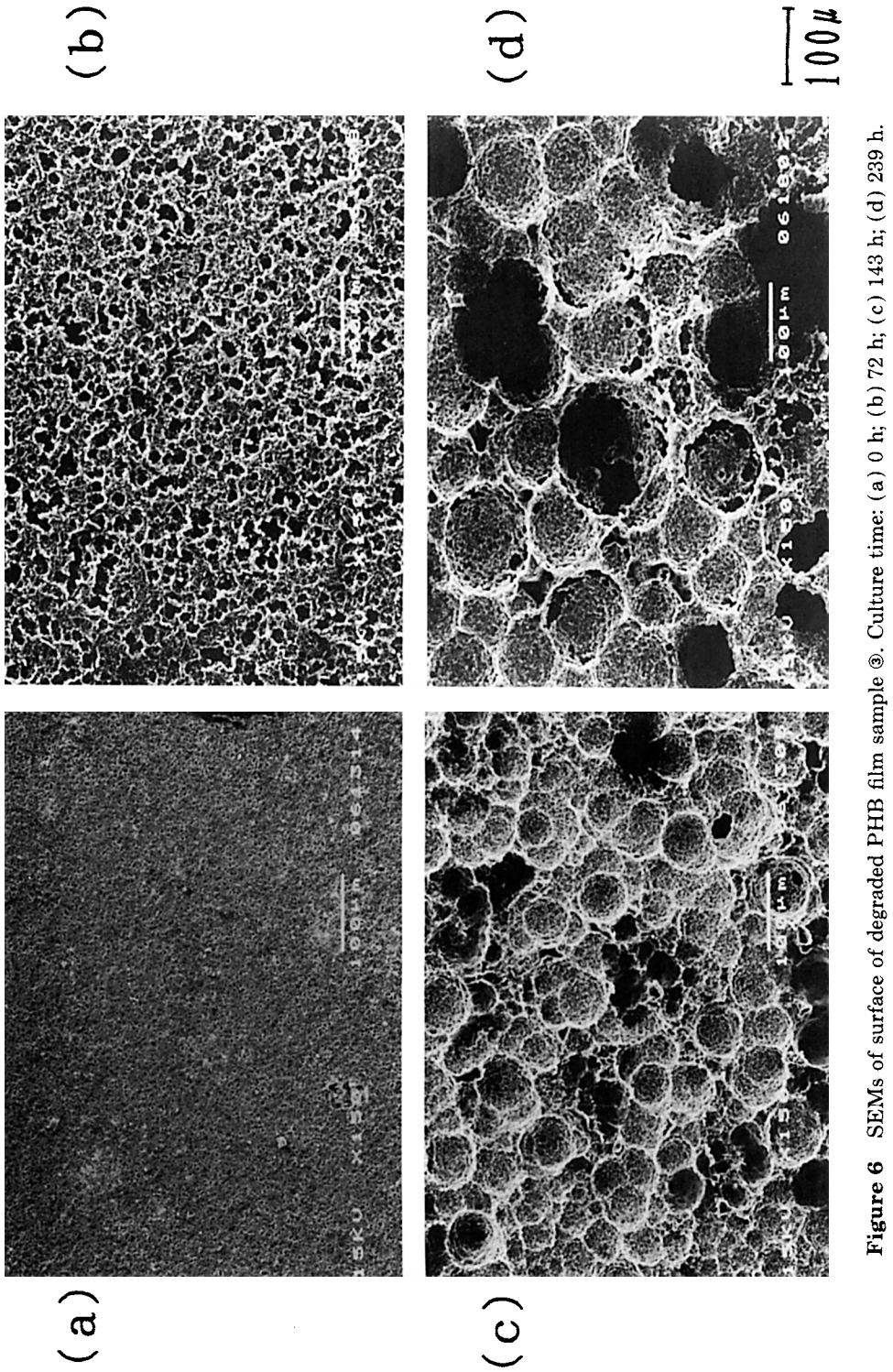

Figure 6 SEMs of surface of degraded PHB film sample ③. Culture time: (a) 0 h; (b) 72 h; (c) 143 h; (d) 239 h.

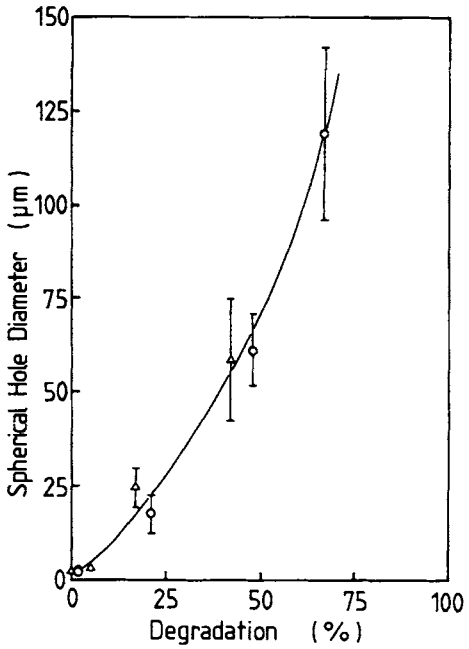

Figure 7 Relationship between degradation percentage and diameter of spherical holes on film surfaces of PHB film samples. Heat-treatment condition of film samples: (○) film sample ①, 30°C/36 h; (△) film sample ③, 90°C/20 h + 150°C/16 h. Bar = SD.

A similar phenomenon has often been reported regarding investigation of native starch degradation by various amylases,[20,21] but with regard to the degradation of PHB, very few data are available. Doi et al. reported SEM observations of PHB cast films after enzymatic degradation by extracellular PHB depolymerase from *Alcaligenes faecalis* T1,[10] and although they indicated that the enzymatic degradation occurred in the surface layer, they did not suggest the above phenomenon.

Figure 9 shows enlarged views of a spherical hole in Figure 5(c). The hole appears to be filled up with the cells of SC-17 in the process of degrading the PHB film surface. The cells were straight rods, 1 × 2–3 μ in size. The morphology was about the same as we observed under phase-contrast microscopy. The photograph suggests that the cells adhere to the surface.

From the above observations, it is considered that the spherical holes occur when the degrading microorganisms adhere to the film surface and then propagate and degrade the surrounding material. This is probably the first photographic observation of the state of PHB in the process of degradation.

Figure 10 shows the changes in the size of the spherical holes. The diameters of the holes enlarged linearly with culture time. This linear enlargement is similar to the linear colony growth on an agar plate, as reported by Pirt.[22] This fact supports the hypothesis that the unique degradation patterns are the result of the colonization of SC-17 on the PHB surface. Furthermore, Figure 10 shows two differences between film samples ① and ③. Specifically, the beginning time and rate of hole enlargement were slower in the higher crystallized sample ③. This indicates that the heat treatment of PHB affects the growth of strain SC-17 on the PHB surface in addition to the degradation rate.

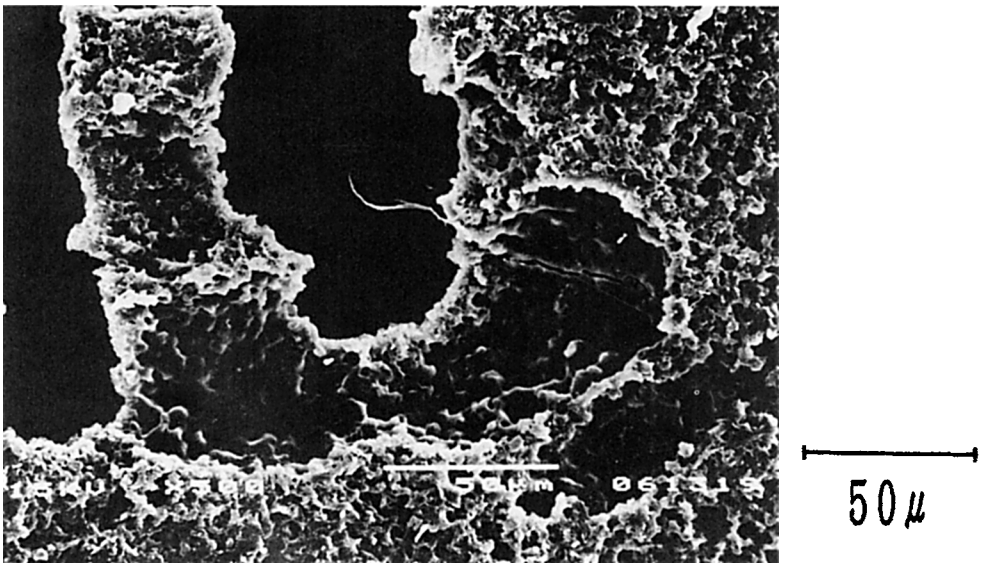

Figure 8 SEM of edge of degraded PHB film sample ③ after culture for 239 h.

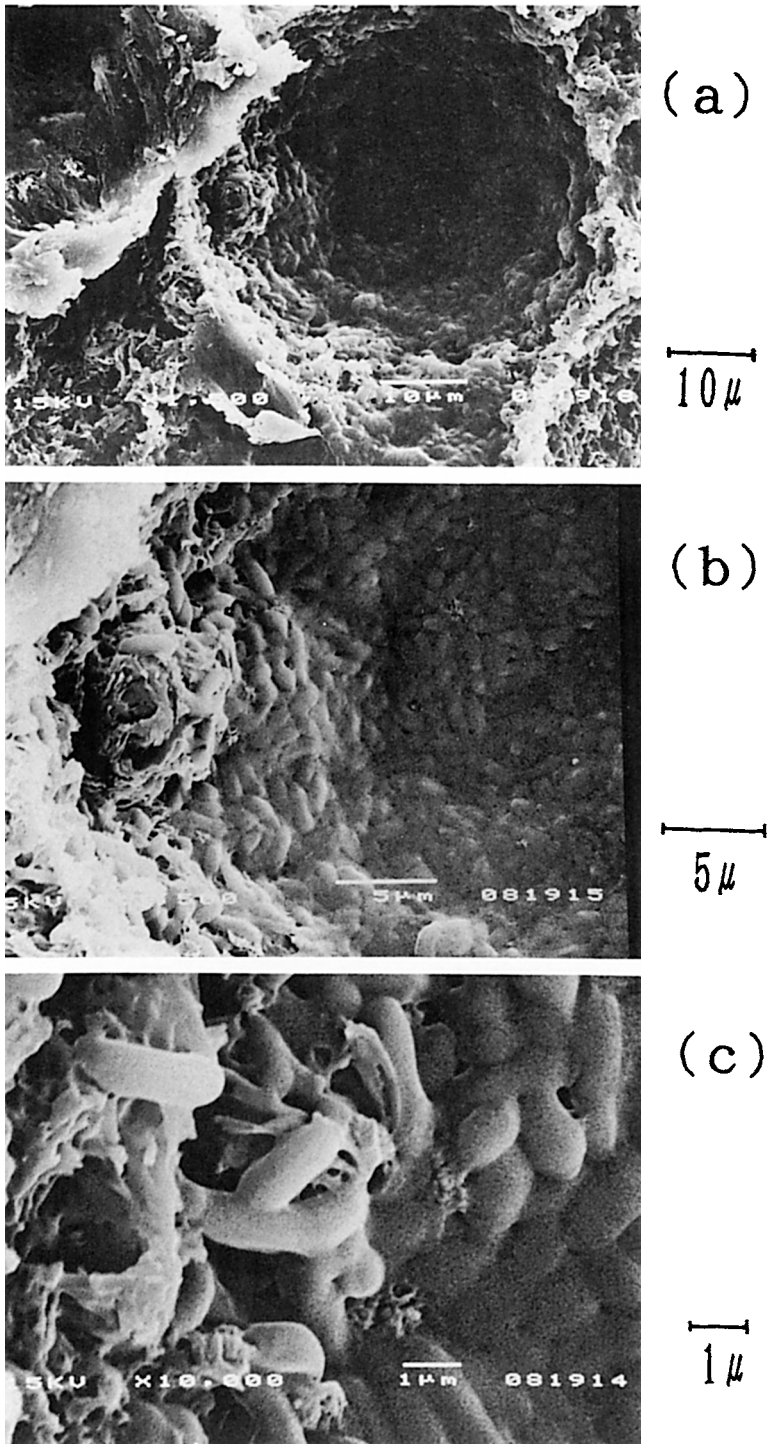

Figure 9 SEMs of PHB degrading bacteria in the process of degrading PHB film sample ① after culture for 143 h: (a) 1500×; (b) 3500×; (c) 10,000×.

The most important information that can be derived from the above results is that the microbial degradation by strain SC-17 proceeds without distinction between the crystal and amorphous phases of PHB and produces the unique degradation patterns on the surfaces, while it is, nevertheless, obviously affected by the higher-order structure properties.

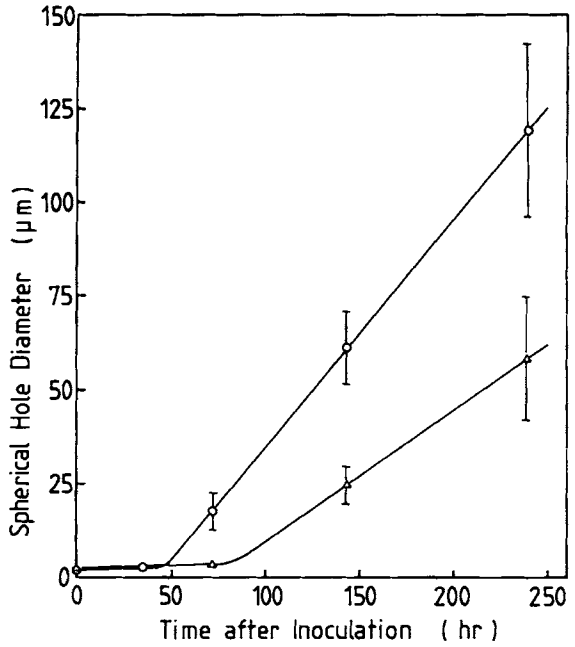

Figure 10 Time-course change in diameter of spherical holes on degraded PHB film surfaces. Heat-treatment condition of film samples: (○) film sample ①, 30°C/36 h; (△) film sample ③, 90°C/20 h + 150°C/16 h. Bar = SD.

CONCLUSION

This investigation took up the two effects on microbial degradation of PHB: the physical properties based on higher-order structure and the growth of the PHB degrading bacteria on the PHB surface. It was suggested that the surface of the PHB is important as a growing field for the degrading bacteria and that the higher-order structure properties of the PHB affected not only the degradation reaction but also the colonization of the degrading bacteria on the surface. To confirm the above conclusions, we will investigate the enzymatic degradation by PHB depolymerase from strain SC-17.

For understanding the relationship between the biodegradation and the higher-order structure of PHB, the role of each factor in the higher-order structure has to be more fully clarified. Unfortunately, the present work does not allow us to discuss the individual property factors in detail. However, work on elucidating the effect of each factor on microbial degradation is now in progress in our laboratory.

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