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BIODEGRADATION OF POLY(TETRAMETHYLENE SUCCINATE-CO-TETRAMETHYLENE ADIPATE) AND POLY(TETRAMETHYLENE SUCCINATE) THROUGH WATER-SOLUBLE PRODUCTS

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Abstract—Poly(tetramethylene succinate-co-tetramethylene adipate) (PBSA) and poly(tetramethylenesuccinate) (PBS) were hydrolyzed experimentally into water-soluble oligomers and monomers by *Chromobacterium* extracellular lipase. The oligomers were identified by high-performance liquid chromatography–mass spectrometry and ^{1}H-nuclear magnetic resonance, which indicated that a total of 28 oligomer species were liberated from PBSA, and that 13 of them were identical to the hydrolysates from PBS. Moreover, 20 of the species were polyester-based compounds of monomer units, and the other 8 species were small amounts of diurethane compounds. Bis(hydroxybutyl) succinate (BSB) and bis(hydroxybutyl) hexamethylene dicarbamate (BHB) were the typical oligomers and were chemically synthesized. Biodegradability of BSB and BHB was examined for 28 d in the activated sludge, and analysis of the results of this study indicated that the final conversion rate of constituent carbon to carbon dioxide was estimated at 80 mol% for BSB and 10 mol% for BHB. The remaining amount of carbon in the undegraded BHB was 20 mol%. In the presence of BSB, the biodegradability of BHB was increased by about 1.5 times. The suggestion was made that BSB induced a growth of microorganisms and helped BHB degradation. This is consistent with the observation that the biodegradation of BHB in native soil for 60 d reached $>$ 60%.

Keywords—Poly(tetramethylene succinate-co-tetramethylene adipate) Poly(tetramethylene succinate) Enzymatic hydrolysis Oligomers Biodegradability

INTRODUCTION

The environmental impact of non-biodegradable waste polymer materials has become steadily more acute. These materials find their was into the municipal solid waste stream. In contrast, biodegradable polyesters are expected to reduce the amount of waste. However, water-soluble intermediates of these polyesters are likely to be formed during degradation, and these intermediates might have environmental impacts. Evaluating the desirability of using biodegradable polyesters necessitates identifying and quantifying the intermediates formed during biodegradation, and determining the half-lives of these intermediates. We previously showed that some water-soluble oligomers could be readily obtained from poly(tetramethylene succinate-co-tetramethylene adipate) (PBSA) by enzymatic hydrolysis [1]. These oligomers were considered to be the intermediate products liberated from the polymer by extracellular enzymes. This experimental technique seems applicable to other polyesters as well.

In this study, we scaled up the system to provide enough sensitivity to survey all the hydrolysates from degradation of PBSA and poly(tetramethylene succinate) (PBS), and evaluated the half-lives of the obtained oligomers. We also focused on two typical oligomers, and showed that the conversion rates of the constituent carbons in the polymer to carbon dioxide, biomass, and dissolved metabolites depend on chemical structure and the environmental conditions of the microorganisms.

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MATERIALS AND METHODS

Chemicals

The PBSA and PBS used in this study were supplied by Showa Highpolymer (Tokyo, Japan). They are commercially available biodegradable plastics (PBSA, Bionolle 3020; PBS, Bionolle 1020), consisting of 1,4-butanediol (B), succinic acid (S), and adipic acid (A) with a composition ratio of 50:40:10 as B:S:A for PBSA and 50:50 as B:S for PBS. The PBSA and PBS were increased in molecular weight by chain-expansion with the addition of a slight amount of hexamethylene diisocyanate (H) as coupling agent. The resultant weight-averaged molecular weight (mol wt) was approximately 100,000 for both. They were powdered by freezer mill and sifted to 125 μm to 250 μm before the hydrolysis experiment. Bis(hydroxybutyl) succinate (BSB) was prepared through chemical synthesis based on a polycondensation reaction of B with S at a composition ratio of 50:50 as B:S, and was extracted with acetone. The extract contained BSB at 50 mol%, B at 24 mol%, BSBSB at 22 mol%, and BSBSBSB at 4 mol%. Bis(hydroxybutyl) hexamethylene dicarbamate (BHB) was also chemically synthesized by reacting H and excess amount of 4-hydroxybutyl methacrylate in anhydrous CH_3COOCH_3, followed by reduction of the methacrylic acid with NaOH in MeOH. The resulting BHB was extracted with acetone and had a purity of 98 mol%.

Hydrolysis of PBSA or PBS by extracellular lipase

As hydrolyzing enzyme, Lipase Type XII (*Chromobacterium viscosum*, Sigma, St. Louis, MO, USA) was used with

further purification by dialysis against deionized water. The PBSA (7.5 g) powder was hydrolyzed in 750 ml of deionized water by 50 U/ml of the enzyme, maintaining the optimal pH of 7.5 by the addition of NaOH and maintaining the temperature optimum of 35°C for the enzyme. The PBS (8.0 g) powder was similarly hydrolyzed in 800 ml of deionized water. After 20 h of hydrolysis, the residual unreacted polymers were excluded by membrane filter with 0.2-μm pore size (Millipore Omnipore Membrane, Millipore, Bedford, MA, USA). The enzyme was subsequently excluded by ultrafiltration (Minitan, Millipore). The solution was finally freeze-dried and the obtained water-soluble oligomers were analyzed by high-performance liquid chromatography–mass spectrometry (HPLC-MS).

HPLC-MS and size exclusion chromatography-mass spectrometry (SEC-MS)

For the evaluation of water-soluble products in the hydrolysis experiments of PBSA and PBS, HPLC-MS measurement was carried out according to Ando et al. [1] with the mass spectrometer (JMS-SX102A, JEOL, Tokyo, Japan) connected to a high-performance liquid chromatograph equipped with frit-fast atom bombardment interface. Separation was performed at 40°C using a Shodex OHpak F-511A (Showa Denko K.K., Tokyo, Japan) column. Gradient elution was performed at a flow rate of 1.0 ml/min using two mobile phases containing 0.1% of trifluoroacetic acid (trifluoroacetic acid:acetonitrile and trifluoroacetic acid:water:acetonitrile). In the first step, the mobile phase consisted of 5% acetonitrile for 5 min, followed by a linear gradient which ran from 5 to 80% acetonitrile at 50 min. A 1.0% glycerol solution was added at 0.4 ml/min after the column as a matrix for fast atom bombardment ionization. For each component, the mass spectrum was measured and the chemical structure was determined by the pseudomolecular ion peak (MH^+). The amount of each product was quantified by the peak area of the pseudomolecular ion peak. The amount of each hydrolysate was quantified as disuccinic acid ester of 1,4-buthandiol (SBS) equivalent, and the mount of each monomer was quantified using itself as the standard.

For the evaluation of BSB and BHB in the biodegradation experiments, SEC-MS measurement was used. The SEC was performed at 40°C using a Shodex OHpak SB-8025HQ (Showa Denko K.K.) column and the mobile phase consisted of water: acetonitrile:acetic acid at a ratio of 60:40:0.05. The eluted materials were detected by a mass spectrometer (JMS-SX102A, JEOL, Tokyo, Japan) connected to the size exclusion chromatograph. The amount of residual BSB or BHB was determined by the pseudomolecular ion peak (MH^+), using itself as the quantitative standard.

^{1}H-Nuclear magnetic resonance

The ^{1}H-nuclear magnetic resonance was performed using a Bruker AMX-400 (Billerica, MA, USA) by dissolving the acquired oligomers into dimethylsulfoxide^{-d6}. The resonance spectrum was completely assigned to the chemical structure without unknown impurities below 1.0% (wt/wt).

Fourier transform infrared spectroscopy and chemical element analysis

Fourier transform infrared spectroscopy was performed with Perkin Elmer 1650 FTIR (Perkin-Elmer, Norwalk, CT, USA). Chemical element analysis was performed with a Yanagimoto MT-3 (Yanagimoto, Kyoto, Japan) and LECO CHNS-932 (LECO, St. Joseph, MI, USA). These techniques assisted the HPLC-MS analysis in determining the chemical structure.

Biodegradation

The biodegradation test was conducted according to the Ministry of International Trade and Industry (Toyko, Japan) test [2]. The test substance (30 mg) and 9 mg as mixed liquor suspended solid of activated sludge were added to 300 ml of mineral medium and stirred at 25°C for 28 d, during which time the biochemical oxygen demand (BOD) was measured continuously using a BOD meter (Coulometer, Ohkura Electric, Tokyo, Japan). The specific BOD demand of the test material was calculated as the difference between oxygen consumption in the test flasks and the blanks divided by the concentration of the test material at time *t*. The percentage biodegradation was calculated as the ratio of the specific BOD demand to the theoretical oxygen demand. Aniline was used as a positive control.

Soil burial test

The BHB (10 mg) was well mixed with 13 g of native soil containing 3 g of mineral water, and placed in a BOD reactor at 25°C for 50 d. The soils used for the biodegradation were a mixture of volcanic ash soil (Andosol) and red-yellow soil obtained from the surface of a cultivated field in Ibaraki Prefecture (Japan). The soils were sieved (<2 mm) and stored at 4°C before use. The biodegradability was calculated as mentioned above.

Determination of a carbon balance

The determination of a total carbon balance was carried out at the end of biodegradation. The total carbon balance was based on the summation of the amount of carbon derived from the following measurements: the carbon evolved as carbon dioxide, the carbon produced as new biomass, the carbon transformed into water-soluble organic metabolites, the carbon determined as dissolved organic carbon (DOC), and the carbon remaining in the undegraded polymer material. The carbon sum was compared with the amount of organic carbon in the test material introduced into the test system. The BOD evolved was used to determine the amount of carbon evolved as carbon dioxide. The culture medium was filtered using silver membrane filters of 0.45-μm pore size and 25-mm diameter (Millipore Silver Membrane AG4502500). The filter cake was dried in vacuum at 105°C for 3 h before weighing. The amount of carbon in the cake was determined by CHNS analysis using a LECO CHNS-932 (LECO). The amount of carbon in the residual materials was determined by SEC-MS. The filtrate was examined to determine the amount of DOC and dissolved inorganic carbon using a Shimazu TOC-5000A (Shimazu, Tokyo, Japan).

RESULTS AND DISCUSSION

HPLC-MS analysis of water-soluble products

The enzymatic hydrolysis of PBSA produces a mixture of monomers and oligomers as water-soluble products. The qualitative analysis of each product was made mainly by using its pseudomolecular ion peak. Some of the same molecular weight oligomers consisting of different monomer units were distinguished by both their mass spectra and values of retention time, whereas some products could not be distinguished, for example ABSB and SBAB, which have the same monomer composition and molecular weight. These oligomers are rep-

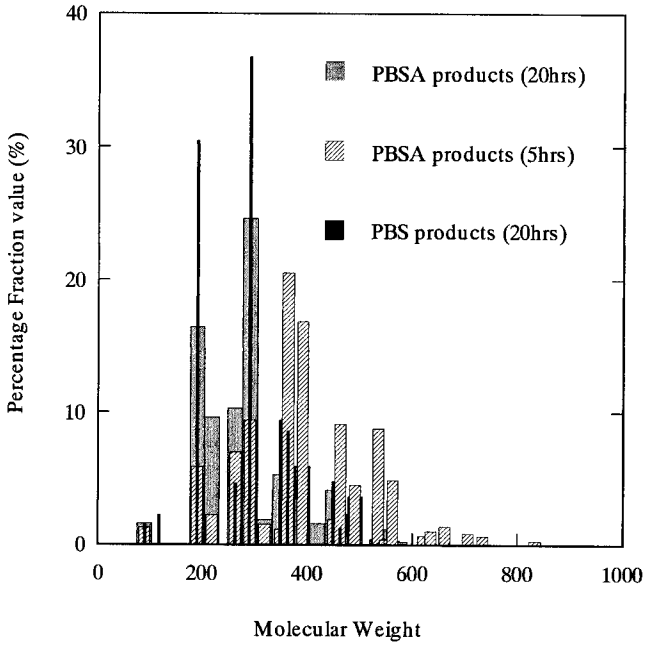


Fig. 1. Composition of the water-soluble products after 5 h and 20 h of enzymatic degradation of poly(tetramethylene succinate-co-tetramethylene adipate) (PBSA) powder and after 20 h of degradation of poly(tetramethylenesuccinate) (PBS) powder with *Chromobacterium* extracellular lipase at 35°C and pH 7.5. Each product is represented by the molecular weight corresponding to the structure in Table 1.

resented as adipate at a terminal, for convenience. Figure 1 shows the composition of water-soluble products during the enzymatic degradation of PBSA powder. The products range from 90 to 834 in molecular weight, corresponding to S and ABSBSBSBS, respectively. The main products after 5 h of the enzymatic degradation were oligomers of SBSBSB (mol wt 534), SBSBS (mol wt 462), ABSB (mol wt 390), and SBSB (mol wt 362). On the other hand, the main products after 20 h of the enzymatic degradation were oligomers of ABSB, SBSB, SBS (mol wt 290), and SB (mol wt 190). Ando et al. [1] showed that oligomers of one or two ester linkages were released in nearly equal ratios in the presence of three different extracellular lipases and were stable. The suggestion has been made that oligomers of one or two ester linkages would have low binding affinities for the lipases. The fraction of large oligomers containing more than three ester linkages in the products significantly diminished from 5 to 20 h during enzymatic degradation. Therefore, oligomers of one, two, or three ester linkages could be considered as the dominant products. Table 1 summarizes all the products from PBSA. In these products, SBHBSBSB (mol wt 792), SBHBSB (mol wt 620), ABHBS (mol wt 576), SBHBS (mol wt 548), ABHB (mol wt 476), SBHB (mol wt 448), and BHB (mol wt 348) differ structurally from the other polyester-based oligomers with respect to the diurethane segment. The diurethane segment comes from hexamethylene diisocyanate (H), which functions as a coupling agent to increase molecular weight [3]. The resulting PBSA behaves as a biodegradable plastic. These diurethane products also underwent enzymatic degradation and finally degraded to SBHB or BHB after 20 h. Consequently, we obtained 28 species of water-soluble products from PBSA and the small oligomers such as SB, AB, BSB, SBS, ABS, BHB, SBSB, ABSB, and SBHB were the major products at the end of enzyme degradation. We also degraded PBS in the same way and obtained the small oligomers that were SB, BSB, SBS, BHB, and SBSB; identical to those from PBSA.

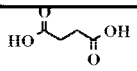
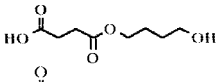
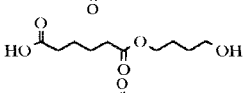
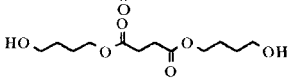
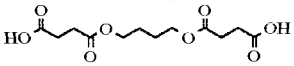
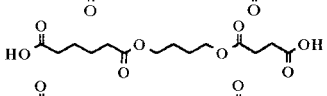
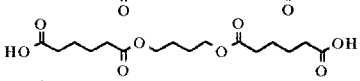
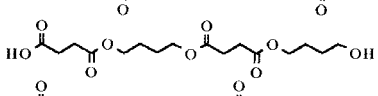
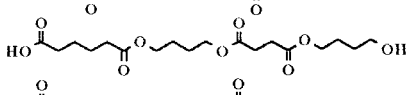
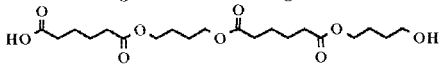
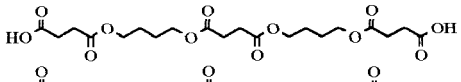
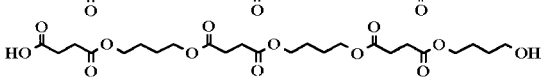
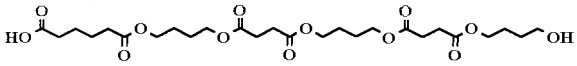
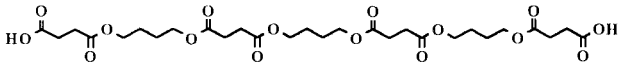
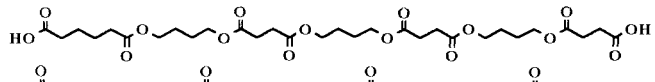
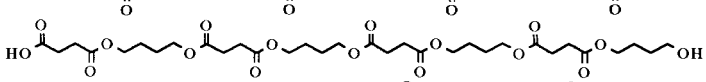
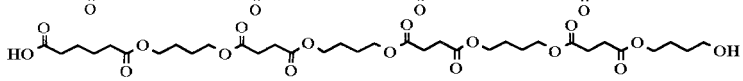
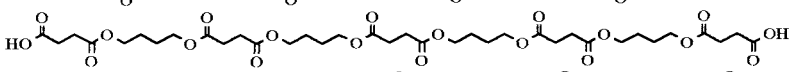
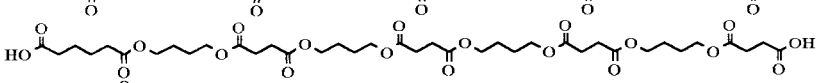
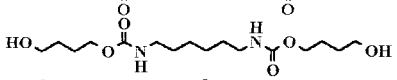
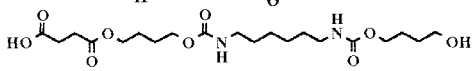
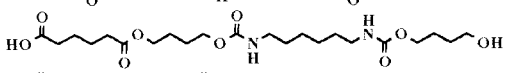
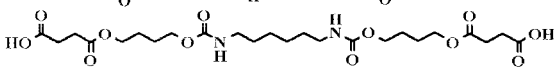
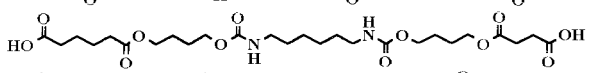
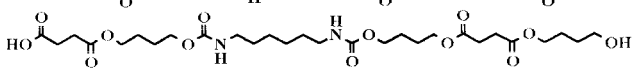
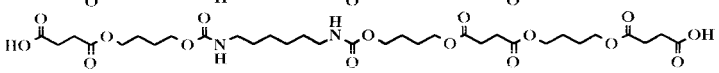
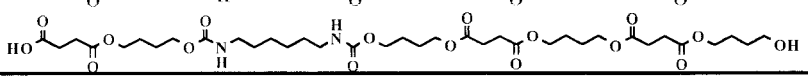
Specificity in biodegradation

Figure 2 shows the percentage biodegradation of BSB and BHB (products formed during PBSA biodegradation) during 28 d of degradation with activated sludge. Ando et al. [1] concluded that any oligomers containing hydroxyl terminals, such as BSB or BHB, were not detected at all. They speculated that this was because those oligomers were hydrolyzed quickly into other products by the action of lipases. However, we have detected these oligomers in the products. This confirms that our preparation technique was very useful for detecting a small amount of minor products. Comparison of the rates of biodegradation showed that the diurethane structure of BHB seemed very stable. The half-life for BSB was estimated as 3 d, but that for BHB was expected to be more than 50 d if the initial rate of biodegradation was maintained. Owen et al. [4] reported that the diurethane compound toluene-2,4-dicarbamic acid, diethyl ester was biodegraded to tolulene-2,4-diamine via the aromatic amine intermediates carbamic acid, (3-amino-4-methylphenyl)-, ethyl ester, and carbamic acid, (5-imino-2-methylphenyl)-, ethyl ester [4]. Analysis of the results of that study showed that the compounds of urethane groups were likely modified to the metabolic intermediates and accumulated in the culture before achievement of complete biodegradation. Therefore, we determined a total carbon balance to confirm the carbon evolved as carbon dioxide [5], the carbon produced as new biomass, and the carbon transformed into water-soluble organic metabolites for both BSB and BHB. The results are shown in Table 2. The biochemically oxidized amount of carbon (C_{BOD}; mg/L) in the test material introduced into the test system (carbon content [C_{MAT}; mg/L]) was calculated from the percentage biodegradation. The increase in biomass carbon in the test reactors containing test material (C_{BIO}; mg/L) was obtained from carbon analysis of the filter cakes. The increase in DOC during the incubation period (C_{DOC}; mg/L) was determined by DOC analysis. The amount of organic carbon in the residual test substance (C_{RES}; mg/L) was determined by SEC-MS analysis. In the case of BSB, C_{BOD}/C_{MAT}, C_{BIO}/C_{MAT}, C_{DOC}/C_{MAT}, and C_{RES}/C_{MAT} were approximately 0.80, 0.05, 0.02, and 0.00, respectively. In the case of BHB, those same relationships were approximately 0.10, 0.00, 0.90, and 0.20, respectively. This observation suggested that 70 mol% of BHB would be biochemically modified and accumulated in the culture medium as the water-soluble intermediate metabolites.

Synergic effect in biodegradation

All of the water-soluble products from PBSA present after 20 h of enzymatic degradation were supplied into the activated sludge at the same time and biodegraded. The percentage biodegradation value of each product was calculated as the ratio of the specific BOD to the theoretical oxygen demand. The percentage residue products were determined by HPLC-MS analyses. The total percentage biodegradation value was continuously recorded and the percentages of residual products were measured after 1, 2, 3, and 16 d of cultivation and summed up as shown in Figure 3. Analysis of the results indicated that all the products were readily biodegradable and completely extinguished from the culture when the total biodegradability reached a constant value (80%). The apparent half-life for the aggregate is estimated to be 3 d, the same as

Table 1. Molecular weight (mol wt) and structure of all the water-soluble products from poly(tetramethylene succinate-co-tetramethylene adipate)

Oligomer[a]	mol wt	Structure
S	90	
B	118	
SB	190	
AB	218	
BSB	262	
SBS	290	
ABS	318	
ABA	346	
SBSB	362	
ABSB	390	
ABAB	418	
SBSBS	462	
SBSBSB	534	
ABSBSB	562	
SBSBSBS	634	
ABSBSBS	662	
SBSBSBSB	706	
ABSBSBSB	734	
SBSBSBSBS	806	
ABSBSBSBS	834	
BHB	348	
SBHB	448	
ABHB	476	
SBHBS	548	
ABHBS	576	
SBHBSB	620	
SBHBSBS	720	
SBHBSBSB	792	

[a] S = succinic acid; B = 1,4-butanediol; A = adipic acid; BHB = bis(hydroxybutyl) hexamethylene dicarbamate.

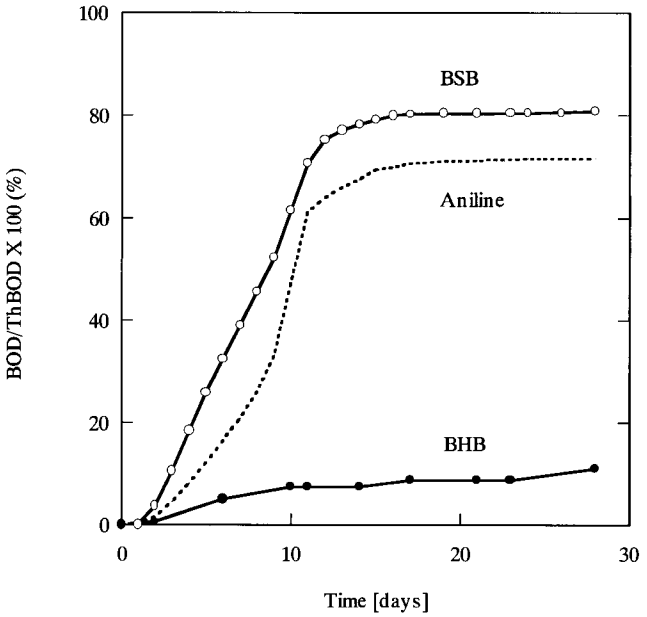


Fig. 2. Biodegradation curves of bis(hydroxybutyl) succinate (BSB) (○), bis(hydroxybutyl) hexamethylene dicarbamate (BHB) (●), and aniline as a positive reference (dotted line) in the activated sludge.

for single BSB. This observation indicates that the diurethane products such as BHB and SBHB were readily degraded in parallel with the other products. We then conducted the biodegradation test of BHB in the presence of BSB. The BSB was supposed to be able to induce a rapid growth of microorganisms in the culture and the increase in population was thought to possibly facilitate the degradation of BHB. The 24 mg of BHB and 6 mg of BSB (equivalent to mole ratio of BHB:BSB of 3:1) were supplied into the activated sludge and biodegraded for 28 d. The results are shown in Figure 4. The BHB and BSB had a biodegradability of 10% or 80%, respectively, when 30 mg of each substance was applied individually in the biodegradation test. If BHB and BSB were biodegraded independently, either would contribute to the percentage biodegradation value by 8% (10% × 24 mg/30 mg) and 16% (80% × 6 mg/30 mg), respectively. The total percentage biodegradation value was then expected to be 24%. However, the test revealed that the real measured value reached 31%. The BSB was assumed to be biodegraded rapidly and the rate of biodegradation was supposed to be limited. If so,

Table 2. Carbon balance of bis(hydroxybutyl) hexamethylene dicarbamate (BHB) and bis(hydroxybutyl) succinate (BSB).[a] Percentage fraction values divided by C_{MAT} are shown in parentheses

Oligomer	C_{MAT} (mg/L)	C_{BOD} (mg/L)	C_{BIO} (mg/L)	C_{DOC} (mg/L)	C_{RES} (mg/L)
BSB	16.47 (100.0%)	13.31 (80.8%)	1.89 (11.5%)	0.42 (2.6%)	ND
BHB	16.56 (100.0%)	1.66 (10.0%)	ND	11.30 (68.2%)	3.31 (20.0%)

[a] C_{MAT} = carbon content in the test material introduced into the test system; C_{BOD} is the biochemically oxidized amount of carbon; C_{BIO} is the carbon content in the filter cake regarded as biomass carbon; C_{DOC} is the increase in dissolved organic carbon during incubation period; C_{RES} is the carbon content in the residual test material determined by size exclusion chromatography and mass spectroscopy; ND = not determined.

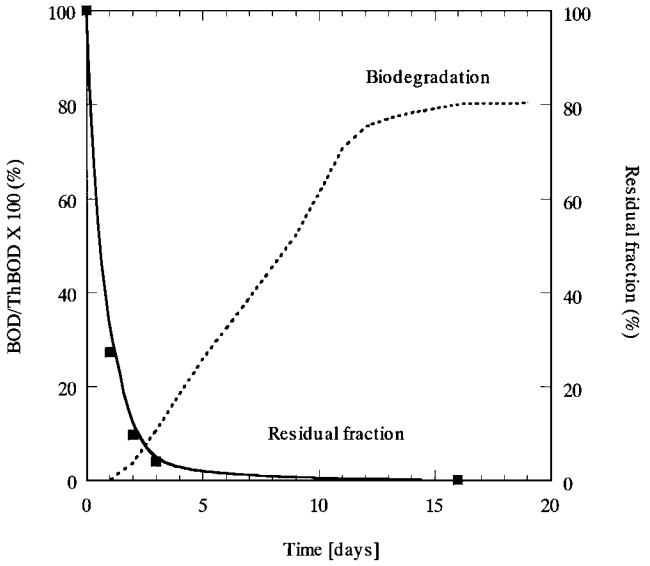


Fig. 3. Material balance in the biodegradation of all the water-soluble products prepared from poly(tetramethylene succinate-co-tetramethylene adipate). The percentage biodegradation was calculated from the amount of biochemical oxygen demand (BOD) and a biodegradation curve was obtained (dotted line). The total amount of residual products was measured by high-performance liquid chromatography–mass spectrometry after 1, 3, 5, and 16 d of cultivation (■) and the percentage residual fraction curve was obtained (solid line).

the percentage biodegradation of BHB in the presence of BSB would be expected to be 15% (31% − 16%), which is double that of BHB by itself (8%). The notion of synergism in biodegradation was reinforced by the soil burial test of BHB. Native soil is an inoculum rich in a variety of microorganisms. Figure 5 shows that the biodegradation of BHB attained a value

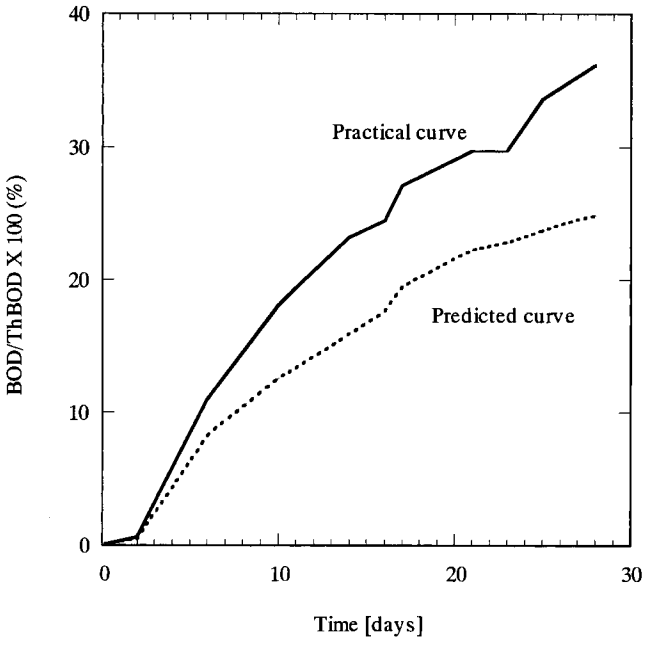


Fig. 4. Biodegradation curve of the blend of bis(hydroxybutyl) succinate (BSB) and bis(hydroxybutyl) hexamethylene dicarbamate (BHB) at a mole ratio of 1:3 as BSB:BHB (actual curve; solid line). The predicted biodegradation curve (predicted curve; dotted line) was calculated from the biodegradation curves of single BHB and that of single BSB assuming that BSB and BHB are independently biodegraded.

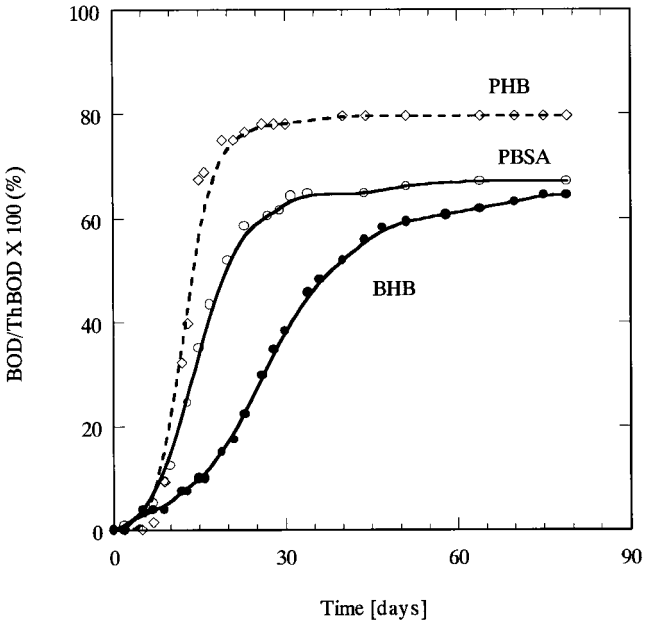


Fig. 5. Biodegradation curve of bis(hydroxybutyl) hexamethylene dicarbamate (BHB) (●), poly(tetramethylene succinate-co-tetramethylene adipate) (PBSA) (○), and poly(3-hydroxybutyrate) (PHB) as a positive reference (◊) in soil.

of 60% in 60 d in the soil burial test. The half-life of BHB in soil was estimated to be 30 d. Owen et al. [4] demonstrated that *Exophiala jeanselmei* is able to hydrolyze the urethane groups in tolylcarbamate compounds that resemble the urethane segments in tolulene diisocyanate–based polyurethanes [4]. This strain was isolated from soil and might be absent from activated sludge. On the other hand, Figure 5 also shows that the half-degradation time of PBSA in the same condition is about 15 d, which is comparable to that of poly(3-hydroxybutylate) as a positive reference. The difference in half-lives between BHB (30 d) and PBSA (15 d) suggests that the readily biodegradable substances such as BSB have an important role in promoting PBSA biodegradation.

Conclusion

The water-soluble products from enzymatic hydrolysis of PBSA or PBS are classified into polyester-based products and diurethane products. The formers have half-lives of about 3 d. On the other hand, the latter are biodegraded at a much slower rate. However, the polyester-based products likely enhance the activities of microorganisms in a degradation environment and thereby facilitate the biodegradation of the diurethane products. We showed that a few small products were preferentially digested, meaning that microorganisms could select them in proportion to the molecular sizes and utilize the polyester in preference to the polyurethane. These products would be metabolized in the cell and become oxidized. With regards to their metabolites, the small products such as BSB and BHB were important as the starting material in the metabolic pathway. The BHB seemed to undergo β-oxidation and be transformed. The significant feature of this work is its indication of the environmental friendliness of PBSA and PBS. This approach could be applied to other biodegradable polymers.

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