Polycaprolactone Degradation by Mixed and Pure Cultures of Bacteria and a Yeast

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Synopsis

The degradability of three high molecular weight polycaprolactones ($\overline{M}_w$ = 35,000, 18,600, and 7,130) and one low molecular weight polycaprolactone diol ($\overline{M}_w$ = 2060) by mixed and pure cultures of microorganisms was assayed. A yeast, *Cryptococcus laurentii*, a gram-negative rod, *Acinetobacter calcoaceticus* var. *lwoffi*, and a gram-positive coryneform rod were used in the pure culture assays. The analysis of degradation by gel permation chromatography (GPC) allowed for quantitation independent of the growth of the organisms or the addition of supplementary growth factors. GPC analysis showed that the degradation effected by pure cultures was often enhanced when alternate carbon sources were present. This was not the case for mixed cultures. Mixed cultures completely metabolized polymer breakdown products while in some cases pure cultures did not.

INTRODUCTION

Polycaprolactones (PCL), high molecular weight polyesters, are degraded in soils[1] and by pure cultures of fungi.[1–4] Several fungi have been shown to be capable of the complete hydrolysis and metabolism of the PCL's.[3] There are many types of environments in which fungi would not be the dominant organismal type, particularly agricultural soils which are more neutral in pH relative to forest soils which are typically acid. Other types of microorganisms are rarely studied in the analysis of biodegradation and most techniques have evolved for fungal studies. Single cell microorganisms, bacteria and yeasts, do not lend themselves to growth assays of polymer metabolism since they do not spread to cover the surface of a polymer film, the criterion for the American Standards for Testing and Materials[5] (ASTM) procedure. In some cases, these organisms are unable to grow on polymer breakdown products alone in spite of their ability to cleave polymer chains.[6] This necessitates the addition of alternate growth substrates and an analysis of polymer degradation which is independent of the growth of the organism. Gel permeation chromatography (GPC) of residual polymeric materials was used here to determine the amount of polycaprolactone degraded under a variety of conditions. Bacteria obtained by enrichment techniques from environmental samples were studied, both in mixed and pure cultures. A yeast, *Cryptococcus laurentii*, was studied as a representative of

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that class of organisms. The effect of the provision of alternate growth substrates was also examined.

MATERIALS AND METHODS

Polymers

Polycaprolactone 700 (PCL-700), PCL-300 and LPS-60, with molecular weights of 35,000, 18,600, and 7,130 ($\overline{M}_w$), respectively, were obtained from Union Carbide Corp. (New York, N.Y.). LPS-60 contains a phthalic capping residue on the chain ends but otherwise is structurally identical to PCL-700 and PCL-300. A polycaprolactone diol, PCL-1250 ($\overline{M}_w$ = 2060) (Aldrich Chem. Corp., Milwaukee, Wisc.), was used in short term assays. From infrared spectroscopy and chromatographic analysis in tetrahydrafuran (THF), methylene chloride and chloroform the polymers were pure PCL and contained no low molecular weight contaminating materials.

Polymer Preparation for Biological Studies

In order to present the greatest surface area to biological attack, polymers were prepared in the form of films. All glassware was extensively washed, rinsed, and baked. Films were cast from chloroform (1.0 mL of 20 mg/mL solution) in 125-mL Erlenmeyer flasks containing a single layer of 4 mm glass beads or in sterile culture tubes (25 × 125 mm). After evaporation of the chloroform, high molecular weight polymers were heated in flasks or tubes for 4 h at 70°C and then cooled slowly. PCL-1250 tubes were rotated under a fume hood for 2 days to ensure total solvent evaporation.

For the qualitative evaluation of degradation, polymer top agar plates were prepared. PCL-1250 (1.5 mg/mL) was autoclaved in a basal salts (BMS)-agar solution,[3] blended in a Sorvall Omnimixer while still hot, and 5 mL dispensed on the surface of a clear agar plate (20 mL BMS). This created a turbid suspension of polymer which remained milky white after solidification. The degradative activity of microorganisms was detected as a clearing of the suspension around the colony.

Culture Medium

A liquid basal salts medium (BMS)[3] was used in all studies. Carbon sources tested as supplements to polymer included casamino acids (vitamin-free Difco, Detroit, Mich.) and succinate, both at 0.4% (w/v).

Cultures and Isolates Used for Degradation Studies

Sediment samples (5 g) from the Willimantic River and Swan lake, a small pond on the campus of the University of Connecticut, were initially cultured at room temperature in BMS (100 mL, pH 7.3) containing 5.0% (v/v) hexanoic acid as the carbon source. After 1 week, 0.5 mL of each sediment enrichment was transferred to PCL-700 and PCL-300 coated Erlenmeyer flasks containing BMS (10 mL, pH 7.3). Individual polymer enrichment cultures were maintained

through four biweekly transfers followed by five monthly transfers, all incubated at room temperature without shaking. Gel permeation chromatographic (GPC) analysis of residual polymer was performed at the completion of each incubation in order to monitor the progress of each enrichment culture. Because early transfers showed little activity in 2 weeks, incubations were lengthened to 1 month. Yeast extract at 0.1% (w/v) was added as a supplement to a parallel set of enrichment cultures carried for 2 months. The enrichment cultures were transferred a total of ten times during the 8-month experiment.

Two separate isolations to obtain individual microorganisms capable of PCL degradation were performed. Samples from the enrichment flasks were plated on polymer-top agar plates which contained PCL-1250 as the sole carbon source, or PCL-1250 supplemented with one of the following: yeast extract, vitamin-free casamino acids, caproate, succinate, glycogen, raffinose, acetate, or pyruvate (all at 0.4% w/v). A total of 56 zone clearing colonies were picked, streaked to trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.), and gram stained. Of the 56, 17 appeared morphologically different and were further characterized. Of these 17, 16 were gram-positive coryneform rods. Isolate 107 was one of the most active enrichment isolates and is used here as a representative of the group. Biotin and thiamin, growth requirements for this isolate, were supplied to all media at 0.001 μg/mL and 0.5 μg/mL, respectively. *Cryptococcus laurentii*, a yeast, was isolated on a PCL-1250 top agar plate. *Acinetobacter calcoaceticus* var. *lwoffi* was obtained from a short-term PCL-700 enrichment of Willimantic River sediment by Douglas Marchuk in this laboratory. Pure cultures of the three isolates were tested for their ability to degrade all four PCL's in the presence of casamino acids, succinate (both at 0.4% w/v), or no addition. Sterile BMS (5.0 mL, pH 6.8) was added to sterile polymer coated tubes and inoculated with 0.1 mL of late logarithmic phase casamino acids grown cultures. Incubations were at 25°C for 1 week in the case of PCL-1250 and 1 month for the high molecular weight polymers. Experiments were stopped by lyophilization of the culture and samples were prepared for GPC as described below.

Strain Maintenance

Organisms freshly grown in trypticase soy broth were stored at −20°C in 50% (v/v) glycerol. Working cultures were transferred weekly on trypticase soy agar, and incubated at 25°C.

Gel Permeation Chromatograph (GPC)

GPC was performed using two series of columns: (1) three 60 × 0.75 cm columns; two 3×10^3 Å Styragel followed by one 500 Å Styragel (Waters Assoc., Milford, MA) for the analysis of the high molecular weight PCLs; (2) two 60 × 0.75 cm columns, both 500 Å Styragel for the analysis of experiments utilizing PCL-1250. Using polystyrene standards (Waters Assoc., Milford, MA) the upper exclusion limit of the first set was 70,000 $\overline{M}_w$ and of the latter set was 18,000 $\overline{M}_w$. Hexanoic acid (MW 116) eluted at the V_t of both sets. The GPC system has been described.[3]

The GPC system consisted of a piston pump (minipump, Milton Roy Co., Hollywood, FL), a Rheodyne injector, Model 7125 (Rheodyne, Berkeley, CA),

the columns, an R-4 Differential Refractometer (Waters Assoc., Milford, MA), and a Speedomax H recorder (Leeds and Northrup, Philadelphia, PA), with tetrahydrofuran (THF) as the eluant.

Lyophilized samples of high molecular weight polymers were extracted with 5.0 mL of THF for 1 h at room temperature followed by 1 h at 45–50°C. PCL-1250 samples were extracted with 2.5 mL THF for 2 h at 37°C. All were filtered through 0.5 μm Millipore FH filters and 100 μL sample volumes chromatographed. The molecular weight distribution after degradation was recorded and the area of the residual polymer peak eluting at or near the V_0 of the column systems compared to undegraded control samples treated in an identical manner. Uninoculated control polymers were quantitatively extractable and unaffected in distribution by experimental conditions. The reproducibility of data obtained after extraction, filtration, and injection of PCL 700 was tested and analyzed as previously described.[3] Glucose (0.4% w/v) grown cultures were treated identically to experimental cultures and produced no THF soluble products. Casamino acids (0.4%) grown cultures frequently produced low molecular weight THF soluble products. In these cases, products resulting from polymer hydrolysis were reported only when the experimental V_t peak was larger than the V_t peak from casamino acid-grown controls. No growth condition resulted in the extraction of material from organisms, which interfered with the quantitation of the polymer peak.

RESULTS

Polycaprolactone (PCL) Degradation by Mixed Cultures

Lake and river sediment samples, transferred regularly for 6 months in BMS containing PCL-700 or PCL-300 as the sole carbon source, were monitored for their ability to degrade the polymers. Following each transfer, residual polymeric materials were solubilized from lyophilized cultures and the amount remaining determined by GPC. The percent reduction in the area of the high molecular weight peak eluting at or near the V_0 of the column system was the amount utilized by the culture or "percent degradation." The initial 2-week incubations showed no degradation. Incubations were lengthened to 4 weeks, and parallel cultures supplemented with yeast extract were included for organisms possibly depleted of growth factors. The results for the final 4 monthly transfers are shown in Table I. Willimantic River sediment cultured on PCL-700 (Willi 700) showed an increase to 100% degradation early in the transfer series. The same sediment cultured on PCL-300 (Willi 300) never achieved significant levels of degradation. Both Swan Lake sediments (Swan 700 and Swan 300) showed activities which progressed slowly. In the final culture, Swan 300 and Willi 300 showed no degradation activity. In low activity cultures a small part of the population may have been responsible for the degradation, and sampling may not have transferred sufficient numbers of the active organisms.

The addition of yeast extract did not stimulate activity, and in three cases actually repressed activity. Ten days into the first monthly incubation (7th subculture), viable cell counts on trypticase soy agar were done for each of the eight enrichments. At the level used (0.1% w/v), yeast extract did not produce an increase in cell numbers over the levels found in parallel cultures lacking yeast

TABLE I
Percent Degradation of Polycaprolactones by Enrichment Cultures[a]

Culture	Subculture number			
	7	8	9	10
Willi 700	61	100	100	100
+ YE	46	100	ND[b]	ND[b]
Willi 300	4	0	12	0
+ YE	8	0	ND[b]	ND[b]
Swan 700	9	2	13	16
+ YE	9	8	ND[b]	ND[b]
Swan 300	9	15	25	0
+ YE	3	0	ND[b]	ND[b]

[a] Determined from GPC using two 3×10^3 Å Styragel columns and one 500 Å Styragel column in series; experimental peak areas were compared to uninoculated polymer control areas to determine the percent peak reduction.

[b] ND = not done.

extract. All flasks contained between 6 and 60×10^7 viable cells/mL of culture. Because of the lack of enhancement of degradation, the supplementation with yeast extract was discontinued.

Analysis of elution profiles showed that all species of the high molecular weight polymers were degraded. No low molecular weight residues were observed, indicating the complete incorporation of breakdown products by cells.

Polycaprolactone Degradation by Pure Cultures

The abilities of two bacterial isolates and a yeast, *Cryptococcus laurentii*, to degrade high molecular weight PCL's in 1 month were evaluated by GPC. The fact that 16 of the original 17 enrichment isolates were detected by their ability to produce a zone on top-agar plates containing nutrient sources in addition to polymer, suggested that alternate sources may enhance or be required for hydrolytic activity. For comparison, the low molecular weight PCL-1250 was included; values were obtained for this PCL after 1-week incubations.

Evaluation of the chromatograms revealed that none of the isolates selectively degraded low molecular weight species of the high molecular weight polymers. The peak of degraded polymer was evenly reduced in size for all species. Low molecular weight materials were detected in several cases. Residual materials were produced from the phthalate capped polymer, LPS-60, under all three conditions by all isolates where significant degradation was seen. Low molecular weight materials from the other polymers appeared to be dependent on the growth condition. The degradation of PCL-1250 was frequently accompanied by the production of low molecular weight residues, possibly due to the shorter (1 week) incubation time. The presence of casamino acids appeared to produce residues greater in area than any seen in controls grown on casamino acids alone. This was seen in only one case with succinate.

Cr. laurentii (Table II) significantly degraded all the polymers. The effect of increasing polymer molecular weight was a general reduction in activity, most clearly seen with no addition or in the presence of succinate. Degradation of all the polymers was greatly enhanced by casamino acids, but not by succinate.

TABLE II
Cryptococcus laurentii: Effect of Supplementary Substrates on the Extent of Polycaprolactone Degradation

Polymer (0.4%)	% Degradation, added substrate (0.4%)		
	None	Casamino acids	Succinate
1250	33[a]	71[a]	56
LPS-60	28[a]	39[a]	24[a]
300	7	48[a]	0
700	11	29	6

[a] Reduction of high molecular weight peak accompanied by production of low molecular weight material.

Ac. calcoaceticus had the lowest activity towards the high molecular weight polymers (Table III). Of the organisms tested, its ability to degrade PCL's seemed the most affected by the molecular weight of the polymer. *Ac. calcoaceticus* activity towards PCL-1250 was high and enhanced by casamino acids but not by succinate. Succinate did enhance activity towards LPS-60.

Of the organisms tested, isolate 107 (Table IV) had the highest activity towards high molecular weight PCL's in the presence of casamino acids or succinate. This isolate did not degrade the lower molecular weight LPS-60 and PCL-300 to a greater extent than PCL-700. The amount of LPS-60 degradation was much less than expected, based on the amount of PCL-700 degradation. This isolate differed from all other isolates in this regard.

DISCUSSION

Most synthetic high molecular weight polymers resist microbial attack. Of the many types of polymers studied, the polyesters appear to support fungal[1–4] and bacterial[6] growth best.

Mixed cultures of organisms obtained by the enrichment of sediment samples with PCL substrates, bacterial pure cultures isolated from those enrichments, and a yeast, *Cr. laurentii* degraded high molecular weight PCL's. Chromatographic analysis of residual polymeric materials following degradation showed that even the highest molecular weight species of PCL-700 were degraded. The mixed culture, Willi 700, *Cr. laurentii*, and isolate 107 were the most active

TABLE III
Acinetobacter calcoaceticus: Effect of Supplementary Substrates on the Extent of Polycaprolactone Degradation

Polymer (0.4%)	% Degradation, added substrate (0.4%)		
	None	Casamino acids	Succinate
1250	42[a]	53[a]	38
LPS-60	0	0	47[a]
300	0	0	0
700	3	5	0

[a] Reduction of high molecular weight peak accompanied by production of low molecular weight material.

TABLE IV
Isolate 107: Effect of Supplementary Substrates on the Extent of Polycaprolactone Degradation

Polymer (0.4%)	% Degradation, added substrate (0.4%)		
	None	Casamino acids	Succinate
1250	26[a]	21[a]	26[a]
LPS-60	8[a]	28[a]	10[a]
300	0	39	44
700	18	81	75

[a] Reduction of high molecular weight peak accompanied by production of low molecular weight material.

towards PCL-700. Values of 100%, 29%, and 81% degradation, respectively, were obtained for these cultures. Complete utilization of breakdown products was observed with these organisms. In the absence of alternate carbon sources, *Cr. laurentii* and isolate 107 showed significant activity towards the high molecular weight PCL's but degraded PCL-700 more than PCL-300. Because these polymers are chemically identical an inhibitory role of crystallinity is possible as has been suggested for the fungal degradation of PCL's.[3,4] The sample preparation methods used here resulted in films of PCL-300 which are 15% more crystalline than PCL-700 films.[3] This may negate the potential for increased degradability of PCL-300 due to its lower molecular weight. This possibility is supported by the work of Cook et al.[4] which demonstrated preferential utilization of amorphous areas, followed by the removal of crystalline polymer. Similar results were seen with the GPC analysis of fungal degradation of PCL's.[3]

The activity of isolate 107 towards LPS-60 under all conditions was inhibited 10–65% relative to its activity towards PCL-700 and PCL-300. This could be due to a structural difference in the polymer caused by the presence of the phthalate molecule. It is also possible that the depolymerization produced a compound which inhibited further enzymatic or cellular functions of isolate 107. The degradation of LPS-60, the only polymer containing the phthalate cap structure, produced residual materials in all of the assays where it was degraded. It has not been proven, but the evidence strongly suggests that the residual material is related to the presence of the phthalate cap. Aromatic hydrocarbons similar to phthalate are degraded by some organisms[7] so the possibility exists that if a modified form of phthalate was formed by LPS-60 hydrolysis, it would be degraded by other organisms in the environment.

The bacterial isolates and *Cr. laurentii* effect less degradation of the high molecular weight PCL's than several species of fungi studied by the same method.[3] The fungi and the Willi 700 mixed culture required no substrate supplementation in order to obtain high levels of degradation as was the case for the bacteria and yeast pure cultures. In general, the presence of casamino acids resulted in a marked enhancement of activity, whereas succinate in most cases did not. Casamino acids are generally considered to be noncatabolite repressive growth substrates, whereas the effect of succinate is variable depending on the organism.

In view of the wide range of activities observed, the utilization of several test organisms and culture conditions is clearly advantageous when an accurate de-

termination of the biodegradability of a compound is desired. Evidence has been presented here which confirms the biodegradability of polycaprolactones by bacteria, a yeast, and a mixed culture. Analytical techniques were used which allow for the quantitation of degradation and the analysis of the fate on breakdown products independent of growth conditions.

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