Fungal Degradation of Polycaprolactones

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Synopsis

Three high molecular weight polycaprolactones ($\overline{M}_w$ = 35,000, 18,600, and 7,130) were utilized as the sole carbon source by five of six fungi tested by the American Standards for Testing and Materials (ASTM) agar plate method. The fungi were *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *Chaetomium globosum*, *Pencillium funiculosum*, and a *Fusarium sp.* Quantitative analysis of degradation was performed using gel permeation chromatography (GPC). GPC analysis demonstrated differences between the activities of organisms which appeared similar by the ASTM method, and showed that, while all molecular weight species within each polymer were hydrolyzed, in several cases low molecular weight end products were not assimilated. Depending on the organism, the dominant factor determining degradability was either polymer molecular weight or degree of crystallinity.

INTRODUCTION

Polycaprolactones (PCL), synthetic high molecular weight polyesters synthesized by Union Carbide Corp., have been shown to be biodegradable in both soil burial tests and fungal growth studies.[1] Fields and co-workers[2] analyzed the activity of *Pullularia pullulans* (renamed *Aureobasidium pullulans*[3]) toward PCLs of differing molecular weights and found negligible degradation of molecules above 15,000 MW by measuring the weight loss of cast films. This suggested that the degradation of mixed molecular weight polymers is restricted to the smaller components of the mixture and that the larger components are resistant. However, another study[4] found that a *Penicillum* species provided with a high molecular weight PCL ($\overline{M}_w$ = 25,000) reduced the organic carbon content of the medium to near zero in 15 days. It appears that the effect of molecular weight on degradability is not well understood and may vary with fungal species.

The present study was undertaken to examine the effect of molecular weight and crystallinity on PLC degradation by six fungi. Gel permeation chromatography (GPC) was used to quantitate the change in the molecular weight distribution of polymers following biodegradation. Using this technique, both selective degradation of species within the polymer mixture and the production of residual low molecular weight products could be observed. Differential scanning calorimetry (DSC) was used to evaluate the degree of crystallinity of

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PCL films prepared by different procedures. In addition, this study compares analyses with growth studies using procedures outlined by the American Society for Testing and Materials[5] with the GPC determination of residual polymeric materials. The ASTM procedure represents a common standardized method used for screening for the growth of fungi on synthetic, nonwater soluble substrates.

MATERIALS AND METHODS

Organisms and Media

The following fungi were used: *Penicillium funiculosum* (ATCC 11797), *Aspergillus niger* (ATCC 9642), *Aspergillus flavus* (ATCC 9643), *Chaetomium globosum* (ATCC 6205), a member of the *Aspergillus fumigatus* group (*Aspergillus fischeri*), an enrichment culture isolate, and a *Fusarium sp.* isolated in the laboratory. The fungi were stored as spore suspensions washed from Sabouraud's Dextrose Agar (Difco) with 0.1% (v/v) Triton X-100 in distilled water and standard volumes of each were used as the inocula for growth studies. The medium used consisted of a basal salts solution (BMS) containing, per liter of glass distilled water: K_2HPO_4, 0.7 g, and KH_2PO_4, 0.7 g, sterilized separately from $MgSO_4 \cdot 7H_2O$, 0.7 g; NH_4Cl, 1.0 g; $NaNO_3$, 1.0 g; and 1.0 mL of a trace salts solution, which had per milliliter: NaCl, 0.005 g; $FeSO_4 \cdot 7H_2O$, 0.002 g; $ZnSO_4 \cdot H_2O$, 0.007 g. The pH was adjusted to 6.5 with 10% (w/v) NaOH. For solid media, BMS was supplemented with 10.0 g of Noble Agar (Difco) per liter.

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. The PCL designated LPS-60 by the manufacturer contains a phthalic acid residue covalently linked to the chain ends but otherwise is structurally identical to PCL-700 and PCL-300. No low molecular weight contaminants were seen by chromatographic analysis of the polymers using THF, chloroform, or dichloromethane as solvents. Infrared spectroscopy of PCL-700 resulted in a spectrum identical to the published standard.[6] All polymers showed broad molecular weight distributions. Polydispersities (M_w/M_n) ranged from 1.892 to 1.978.

Culture Conditions

The protocol outlined by the American Society for Testing and Materials (ASTM) was followed for the solid surface growth studies.[5] A film of the polymer was cast on the surface of a BMS–agar plate by spreading 1.0 mL of 20 mg/mL PCL in chloroform over the surface of the plate and evaporating the chloroform. The centers of the plates were inoculated with 0.1 mL of spore suspensions, and the plates were incubated in a moist chamber at 25°C for 1 month. The amount of growth was scored on a scale of 1–4 as described in Table I.

For quantitative assays of degradation, films (1.0 mL of 20 mg/mL PCL in chloroform) were cast over the surface of a single layer of sterile 4 mm glass beads

TABLE I
Fungal Degradation of Polycaprolactones: ASTM[a] and GPC[b] Analyses

Organism	PCL-700 Growth ASTM	PCL-700 Growth GPC	PCL-300 Growth ASTM	PCL-300 Growth GPC	LPS-60 Growth ASTM	LPS-60 Growth GPC	Growth controls[a] Chloroform	Growth controls[a] Glucose
A. flavus	4	44	4	40	4	59[c]	1	4
	4	60	4	44	4	61		
A. niger	2	12	2	0	3	65[c]	1	4
	2	11	2	0	3	68[c]		
Fusarium sp.	4	88	4	79	4	77[c]	1	4
	4	86	4	89	4	81[c]		
Ch. globosum	4	45[c]	4	41[c]	4	63[c]	1	4
	4	64[c]	4	44[c]	4	54[c]		
P. funiculosum	4	33	4	35	4	65[c]	1	4
	3	37	3	38	4	58[c]		
A. fischeri	4	100	4	95	4	100[c]	2	4
	4	100	4	95	4	ND[d]		

[a] Numbers represent duplicate determinations using the ASTM rating scheme: % area of plate covered by mycelial growth: (1) 0–10; (2) 10–30; (3) 30–60; (4) 60–100.
[b] Duplicate determinations of % decrease in area of polymer peak.
[c] Production of low molecular weight material detected by chromatography.
[d] ND = not done.

in sterile 125 mL Erlenmeyer flasks and the solvent fully evaporated by heating at 70°C for 4 h. After cooling, 10.0 mL BMS and a 0.1 mL inoculum were added aseptically and the flasks incubated at 25°C for 1 month. Controls for stability of the polymer to nonbiological hydrolysis with incubation and viability of the inoculum were included. These experiments were terminated by lyophilization of the culture in the growth flask. Experiments were performed in duplicate.

Gel Permeation Chromatography (GPC)

High pressure liquid chromatography (HPLC) was performed using a series of three 60 × 0.75 cm columns: two 3×10^3 Å Styragel followed by one 500 Å Styragel (Waters Assoc., Milford, MA). The HPLC system consisted of a piston pump (Mini-pump, Milton Roy Co., Hollywood, FL.), the columns, a U6-K injector and R-4 Differential Refractometer (Waters Assoc., Milford, MA), and a Speedomax H recorder (Leeds and Northrup, Phila., PA), with THF as the eluant. The described column system is capable of resolving polystyrene standards of 70,000 to 2350 $\overline{M}_w$. Hexanoic acid (MW 116) elutes at the V_t of the columns.

Lyophilized samples were extracted with 5.0 mL of THF for 1 h at room temperature followed by 1 h at 45–50°C. Extracted samples were filtered through 0.5 μm Millipore FH filters and 100 μL sample volumes were chromatographed with a flow rate of 2 mL/min. Coating flasks with solvent solutions of polymer and subsequently removing all solvent soluble material ensured that all carbon sources available were delivered with solvent and accounted for by chromatography. The molecular weight distribution after degradation was recorded, and the area of the residual polymer peak compared to undegraded control samples treated in an identical manner. The reproducibility of data obtained after extraction, filtration, and injection of PCL-700 was tested, and

a 3.4% standard deviation was calculated for five samples for peak height or area (determined by weighing traced peaks). Standard dilutions were done to ensure a linear relationship between detector response and concentration within the range used in these experiments. This confirmed that small decreases in polymer peak height or area could be detected and ascribed to degradation. Controls were included which tested for the solubility of fungal material in THF. Glucose (0.4% w/v) grown fungal cultures were treated identically to experimental cultures and produced no THF soluble products. Polymers added to cultures just prior to harvest were quantitatively extractable from the mixture, resulting in peak areas identical to uninoculated controls. This eliminated the possibility of absorption of the polymers to mycelia and demonstrated the absence of any nonbiological alteration in peak shape or area.

Differential Scanning Calorimetry (DSC)

DSC was performed on polymer films deposited on glass beads as in liquid growth studies to determine the percent of the sample which was crystalline. Film (4.0–5.0 mg) was scraped from the glass beads and sides of the flasks and placed in the sample pan of a DuPont 900 Thermoanalyzer (E. I. DuPont Co., Wilmington, DE). The ΔH_M was determined with a heating range of 20°C/min, and the percent crystallinity calculated using the formula

$$\% \text{ crystallinity} = \frac{\Delta H_M \text{ sample}}{32.4 \text{ cal/g}} \times 100$$

assuming that the ΔH_M of a completely amorphous sample is zero. Khambutter et al.[7] determined the ΔH_M for 100% crystalline PCL to be 32.4 cal/g.

RESULTS

ASTM Growth Studies

Based on the standards set by the ASTM procedure, the evaluation of percent mycelial coverage of a 100-mm agar plate showed that all of the six fungi tested exhibited some growth on each of the three tested polymers when compared to chloroform treated control plates (Table I). In most cases a rating of 4, representing a coverage of 60–100% of the surface, was given. The least active organism was *A. niger*, which achieved its highest rating of 3 on the lowest molecular weight polymer. For the other organisms, the ASTM system did not readily discriminate between relative activities of the organisms or activities of a single organism towards the different molecular weight polymers. The polymers appeared equally degradable by this method.

GPC Analysis: Quantitative

Comparison of the area under the high molecular weight peaks of GPC chromatograms of control polymers and polymers subjected to the action of the fungi allows the determination of degradation by the observation of a decreased area in the chromatograms of the treated samples. Such a comparison showed that all three polymers were significantly degraded within 1 month and that the tested

organisms demonstrated a range of activities toward them (Table I). The lowest molecular weight PCL, LPS-60 ($\overline{M}_w$ = 7,130), was consistently more extensively degraded than PCL-700 ($\overline{M}_w$ = 35,000) or PCL-300 ($\overline{M}_w$ = 18,600). The relationship between degradability and molecular weight for the two higher molecular weight polymers was less clear. In fact, where there were significant differences, PCL-700 was hydrolyzed to a greater extent than PCL-300, suggesting the involvement of a structural factor other than molecular weight that affected the amount of degradation. By the average of the two independent determinations, four of the six test organisms degraded PCL-700 more than PCL-300, while the *Fusarium sp.* and *P. funiculosum* showed less than a 4.0% difference between activities on the two polymers. *A. niger* was unique among the tested organisms in having low activity towards both high molecular weight polymers, but an activity on LPS-60 comparable to that of the other organisms. *A. fischeri* represented the other extreme, with high hydrolytic capacity towards all three polymers. The range of activities towards LPS-60 was narrower than that for the other polymers. The phthalic acid cap structure may affect molecular orientation to some extent, which in turn may affect degradability. The hydrolysis of LPS-60 was always accompanied by the production of low molecular weight material ($\overline{M}_w$ = 480) which eluted near the V_t of the column.

GPC Analysis: Qualitative

The column system used in these studies was selected to optimize the separation of the original polymer from breakdown products. Figures 1 and 2 are chromatograms of control samples of PCL-700 and LPS-60 with chromatograms of experimental samples. The polymers eluted at or near the upper exclusion limit of the column system (V_0) while any residual degraded materials of low molecular weights eluted at the lower exclusion limit (V_t). The molecular weight distributions of PCL-300 (not shown) and LPS-60 were nearly Gaussian. PCL-700, chromatographed using a larger pore size Styragel matrix (3×10^4 Å), also showed a Gaussian molecular weight distribution, although it eluted in a void volume peak using the column series reported here. All three polymers were

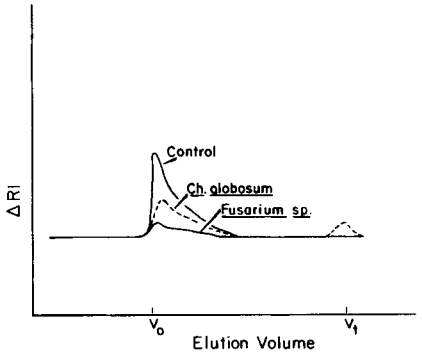

Fig. 1. Gel permeation chromatogram of PCL-700 control and reduced high molecular weight peak and low molecular weight materials produced by *Ch. globosum*. Also shown is the reduced high molecular weight peak for the *Fusarium sp.* The material chromatographed was extracted from growth flasks and eluted with THF.

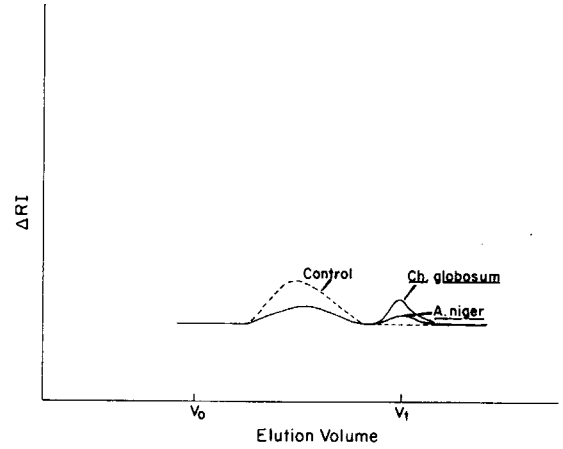

Fig. 2. Gel permeation chromatogram of LPS-60 control and products of degradation by *Ch. globosum* and *A. niger*. Both organisms degraded LPS-60 to the same extent, but differing amounts of low molecular weight material are evident. The material chromatographed was extracted from growth flasks and eluted with THF.

shown to have broad molecular weight distributions. Values for polydispersities ranged from 1.892 to 1.978. Figure 2 illustrates the low molecular weight product produced by *Ch. globosum* and *A. niger* from LPS-60 hydrolysis. The elution pattern shown was typical of that for all organisms growing on LPS-60. *Ch. globosum* alone incompletely utilized material from PCL-700 (Fig. 1) and PCL-300. Because the identity of these low molecular weight materials is unknown and the response of the differential refractometer is related to the physical and chemical properties of a compound as well as its concentration, these peaks could not be quantitated. Fungal cultures grown on glucose produced no THF extractable material. These chromatograms and those of all other samples tested showed that all species within the high molecular weight peaks were reduced in concentration. The polycaprolactones did not contain any contaminating materials detectable by GPC.

DSC Analysis

The degradation data suggest that, although PCL-300 is lower in molecular weight than PCL-700, it is not more degradable. PCL-300, with a percent crystallinity of 75.5, was 15% more crystalline than PCL-700 (60.5% crystalline), and LPS-60 had an intermediate crystallinity (71.2%). The lower molecular weight of PCL-300 (relative to PCL-700) may facilitate greater crystallization in the casting process and subsequent incubation, resulting in lowered degradation. Several different methods of polymer preparation were used to try to alter polymer crystallinity. It has found that the PCLs all reach an equilibrium crystallinity, represented by the numbers given, after several days incubation at room temperature.

DISCUSSION

Previous studies showed that polycaprolactones are degraded within a year when buried in the soil[1] and that the degradation may[2] or may not[4] be very de-

pendent on molecular weight. In the present study the three polycaprolactones tested were shown by two methods to be degradable. Molecular weight was a factor in the extent of degradation within a month for some organisms, and crystallinity also appeared to play a significant role.

By the ASTM method, all of the polymers allowed for the growth of five of the six fungi, with little discrimination between the organisms or the polymers. The exception to this was *A. niger*, which showed poorer growth on PCL-700 and PCL-300. This lack of discrimination is particularly evident for *A. niger* and *P. funiculosum* when the ASTM results are compared with those from chromatographic analyses. *A. niger* had the lowest activity by both methods, but GPC detected slight activity against PCL-700 and none against PCL-300, while growth results were the same for both polymers. *P. funiculosum*, with the next lowest GPC values, received growth ratings comparable to the high activity fungi. For *P. funiculosum* and the remaining four fungi, mycelial growth that warranted an ASTM rating of 4 is associated with hydrolysis values ranging from 35% to 100%. Therefore, GPC represents a much more reliable and quantitative method for the analysis of polymer degradation.

Five of the six fungi tested showed significant degradation of the higher molecular weight polymers by GPC analysis. Additionally, chromatographic analysis indicated that all size species within the polymer mixtures were hydrolyzed, in contrast to the preferential hydrolysis of low molecular weight components reported for *Pullularia pullulans*.[2] PCL-300 is significantly lower in molecular weight than PCL-700, but no organism tested here had greater activity against it. In four cases PCL-300 was less extensively degraded than PCL-700. The potential enhancement due to lower molecular weight may have been negated by an increase in crystallinity. The DSC data support the hypothesis that crystallinity is an important factor in the degradability of these polymers. LPS-60 has an intermediate crystallinity and less than half the molecular weight of PCL-700 but is not proportionally more degradable by most of the fungi. Attempts to vary the crystallinity of the PCLs were not successful due to the tendency of polymers with low T_g values (less than 25°C) to reorganize to an equilibrium crystallinity at room temperature. Scanning electron microscopy has shown that the amorphous region of PCL films are degraded more readily than crystalline areas.[8] The reduced order within amorphous regions may facilitate enzyme diffusion to available linkages.

Low molecular weight material was produced by *Ch. globosum* from each of the polymers. This could have been due to slow utilization relative to the rate of polymer hydrolysis or to the accumulation of nonutilizable end products. However, the hydrolysis products are probably assimilated since *Ch. globosum* grew well on the polymers. The low molecular weight material produced in the degradation of LPS-60 by all the fungi apparently represents nonutilizable material.

Post-degradation chromatographic analysis of polymers has clear advantages over traditionally used growth, weight loss, and tensile strength loss assays. The evaluation of growth is only semiquantitative, and any trace contaminant can produce ambiguous results. Alterations in tensile strength and weight can be caused by the partial hydrolysis or even the nonenzymatic hydrolysis of the polymer followed by the diffusion of smaller components into the medium. These components may or may not be utilized by the test organism, and in either case are not detected by the method of analysis. Weight loss studies can be

further complicated by the adherence of cellular material to the polymer matrix. GPC allows for the direct determination of the effects of an organism has on a polymer, independent of the ability of that organism to grow on the polymer. In this study several fungi were shown to hydrolyze polymeric material and to remove breakdown products from the medium.

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References

1. J. E. Potts, R. A. Clendinning, W. B. Ackart, and W. D. Niegisch, *Am. Chem. Soc., Polym. Chem. Prepr.*, **13,** 629 (1972).

2. R. D. Fields, F. Rodriquez, and R. K. Finn, *J. Appl. Polym. Sci.*, **18,** 3571 (1974).

3. M. M. Ellis, *Dematiaceous Hyphomycetes*, Commonwealth Mycological Institute, Kew, Surrey, England, 1971, p. 515.

4. Y. Tokiwa, T. Ando, and T. Suzuki, *J. Ferment. Technol.*, **54,** 603 (1976).

5. American Society for Testing and Materials, ASTM G21-70, *1976 Annual Book of Standards*, American Society for Testing Materials, Philadelphia, Pa., 1976, pp. 781–785.

6. C. J. Pouchert, Ed., *The Aldrich Library of Infrared Spectra*, 2nd ed., Aldrich Chemical Co., Milwaukee, Wisc., 1975, p. 1352.

7. F. B. Khambutter, F. Warner, T. Russell, and R. S. Stein, *J. Polymer. Sci. Polym. Phys. Ed.*, **14,** 1391 (1976).

8. W. J. Cook, J. A. Cameron, J. P. Bell, and S. J. Huang, *J. Polym. Sci., Polym. Lett. Ed.*, **19,** 159 (1981).

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