Biodegradable Polymers: Chymotrypsin Degradation of a Low Molecular Weight Poly(ester-Urea) Containing Phenylalanine

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Synopsis

A low molecular weight poly(ester urea), poly(L-phenylalanine/ethylene glycol/1,6-diisocyanatohexane), and a model diester diurea, dimethyldiphenylalaninehexamethylenediruea, were synthesized and found to be hydrolyzed by α-chymotrypsin solutions at pH 7.8. After ten-day exposure to 0.1 mg/ml enzyme solution at room temperature, 79.9% weight loss was observed for the model diester diurea (containing two ester linkages per molecule). A weight loss of 19.4% was observed for the poly(ester urea) of $\overline{M}_n$ 1930 (containing an average of eight ester linkages per molecule) after the same period of exposure. A poly(ester urea) of similar molecular weight, but derived from glycine instead of phenylalanine, was found to resist chymotrypsin hydrolysis.

INTRODUCTION

Many research efforts in synthetic polymer chemistry have been directed toward the synthesis of polymers resistant to biodegradation, and the production of such materials has been very impressive. In recent years, however, there is increasing interest in the design of biodegradable polymers for specialized applications such as controlled-release formulations of drugs, insecticide and pesticide carriers, as well as nontoxic surgical implant materials. (For recent reviews on biodegradable polymers and applications, see reference 1.)

An extensive testing program reported by Potts and co-workers[2] in 1973 on a broad spectrum of then available thermoplastics and thermosets showed that most materials resisted microbial degradation with the exception of aliphatic polyesters and polyester-based polyurethanes. It became apparent that new synthetic approaches were needed to enhance the biodegradabilities of polymers either through physical or chemical modification of existing materials or by development of new ones. For example, efforts have been directed toward the syntheses of photodegradable polymers giving fragments which might biodegrade,[3–5] mixing proteins or polysaccharides with synthetic polymers,[6] and crosslinking already degradable polymers such as cellulose[7] and gelatin[8] to increase physical strength.

For a material to be "biodegradable" by microorganisms, it must be enzyme degradable, and our approach has been to incorporate selected enzyme-susceptible bonds into polymer chains.[8,9] Because biochemical reactions are in general stereospecific, one can expect that the biodegradability of a synthetic polymer is greatly affected by its stereo configuration. α-Amino acids are readily

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available in optically pure forms and are thus ideal building blocks for stereospecific polymers. Bailey and co-workers, for example, have reported that polyamides derived from α-amino acids (glycine and serine) and ϵ-aminocaproic acid supported growth of soil microorganisms.[10] Another factor affecting biodegradability besides stereo configuration is the nature of any substituent adjacent to the susceptible linkage. One class of proteolytic enzymes is known to be effective in cleaving amide or ester bonds adjacent to aromatic side groups, and we now report studies on the ability of some of these enzymes to degrade isomeric polymers with and without a phenylalanine side group. Tabushi et al.[11] have independently found that chymotrypsin will hydrolyze a copolymer containing phenyllactic acid and lactic acid; the stereo configuration of their polymer was not specified.

EXPERIMENTAL

Melting points were taken on a Reichert hot-stage melting point apparatus and are uncorrected. Ultraviolet spectra were recorded on a Beckmann DB prism spectrophotometer while ninhydrin analyses were performed with the aid of a Beckmann DB-G grating spectrophotometer. Polymer molecular weights were determined using a Knauer vapor-phase osmometer with hexafluoroisopropanol (caution) as solvent. Elemental analyses were performed by the Baron Consulting Co. of Orange, Connecticut.

Reagents

L-Phenylalanine ($[\alpha]_D^{20} = -35.2°$) and 98% 1,6-diisocyanatohexane were purchased from Aldrich Chemical, Inc., Milwaukee, Wisconsin; D,L-phenylalanine and ethylene glycol, from Fischer Scientific Co., New York. The amino acids were used without further purification. The glycol and diisocyanate were vacuum distilled before use. Hexafluoroisopropanol was purchased from PCR Research Chemicals, Inc., Gainesville, Florida. Solvents were distilled prior to use. Ninhydrin (1,2,3-indantrione) was obtained from J. T. Baker Co., Phillipsburg, New Jersey, and anhydrous hydrindantin was supplied by Sigma Chemical Co.

The following enzymes were purchased from Sigma Chemical Co., St. Louis, Missouri: urease (from Jack beans); pepsin (from hog stomach mucosa); α-chymotrypsin (from bovine pancreas); and elastase (from hog pancreas). In addition, papain (from papaya latex) was obtained from Boehringer-Mannheim, New York, and acid protease (*Rhizopus chinensis*), from Miles Laboratories, Inc., Kankakee, Illinois.

Syntheses

L-Phenylalanine Methyl Ester Hydrochloride.[13] To 48.9 ml (1.21 mole) absolute methanol chilled to 0°C and stirred magnetically was added 8.70 ml (0.121 mole) thionyl chloride. The addition was continued over a period of 1 hr and the temperature maintained near 0°C. L-Phenylalanine (20.0 g, 0.121 mole) was then added and the mixture refluxed for 6 hr and cooled. Removal of solvent in vacuo and recrystallization of the resulting solid twice from absolute ethanol–ether gave 20.6 g (78.9%) L-phenylalanine methyl ester hydrochloride as colorless needles, mp 162–165°C (lit. 159–160°C).[14]

L,L-Diester Diurea Model Compound (1). L-Phenylalanine methyl ester (4.37 g, 0.0244 mole) was obtained as a pale-yellow oil after neutralization in an aqueous sodium bicarbonate solution of its hydrochloride salt (5.83 g, 0.0270 mole) and repeated extraction with anhydrous ether. The combined ethereal extracts were dried over anhydrous magnesium sulfate and filtered before removal of the ether in vacuo.

The ester was dissolved in 50 ml dry toluene and placed in a three-necked flask equipped with a reflux condenser, drying tube, dropping funnel, and nitrogen inlet. Hexamethylene diisocyanate (98%), 2.10 g, 0.0125 mole, was dissolved in 10 ml toluene and placed in the dropping funnel. The reaction vessel was heated in an oil bath to 60°C, and the diisocyanate solution was added dropwise over a period of 10 min. Almost immediately, the reaction mixture became waxy, and uniform mixing was difficult. Gentle heating was continued for 2 hr.

The mixture was cooled, filtered by suction, and washed with generous portions of *n*-hexane. The white solid was dried overnight in vacuo to leave 5.85 g (89.2%) of the crude product. Recrystallization from ethanol–water gave 5.59 g (95.5% recovery) of white crystals, mp 163–164°C.

ANAL. Calcd for $C_{28}H_{36}N_4O_6$: C, 63.86; H, 7.27; N, 10.64. Found: C, 64.11; H, 7.28; N, 10.46.

D,L-Diester Diurea Model Compound (1). D,L-Phenylalanine methyl ester hydrochloride (6.00 g, 0.0278 mole) was dissolved in 20 ml distilled water. Sodium bicarbonate was added until an evolution of CO_2 was no longer observed. The aqueous solution was extracted with six 25-ml portions of anhydrous ether. The combined ethereal extracts were dried over anhydrous magnesium sulfate, filtered, and the ether removed in vacuo. The yield of D,L-amino acid ester was 4.67 g.

The ester was dissolved in 50 ml dry toluene and placed in a three-necked flask fitted with a reflux condensor, drying tube, dropping funnel, nitrogen inlet, and magnetic stirring bar. The solution was heated to 60°C. A solution of 2.22 g (0.0132 mole) 98% 1,6-diisocyanatohexane in 10 ml toluene was added dropwise to the ester solution over a 2-min interval. Heating was continued for 2½ hr.

The resulting white solid was filtered by suction washed with *n*-hexane, and dried at 60°C overnight. The yield of solid was 6.59 g (95.4%). Recrystallization from ethanol–water gave 5.96 g (90.4%) white crystals, mp 114–116°C.

ANAL. Calcd for $C_{28}H_{36}H_4O_6$: C, 63.86; H, 7.27, N, 10.64. Found: C, 63.72; H, 7.36; N, 10.39.

L,L-Phenylalanine Ethylene Glycol Diester *p*-Toluenesulfonate. Into a 500-ml flask was placed 36.7 g (0.223 mole) L-phenylalanine, 44.2 g (0.232 mole) *p*-toluenesulfonic acid monohydrate, 6.1 ml (0.11 mole) entylene glycol, and 300 ml reagent benzene. The flask was fitted with a reflux condensor, drying tube, Dean-Stark tube, and magnetic stirring bar. The suspension was refluxed for 26 hr. Removal of the benzene in vacuo gave 76.4 g of the unpurified mixture. Recrystallization from water gave 4.16 g (54.5%) colorless crystalline rods, mp 227–230°C.

ANAL. Calcd for $C_{34}H_{40}N_2O_{10}S_2$: C, 58.34; H, 5.75; N, 4.00; S, 9.15. Found: C, 58.34; H, 5.85; N, 4.17; S, 9.01.

Poly(L,L-phenylalanine/Ethylene Glycol/1,6-Diisocyanatohexane) (2). A solution of 8.00 g (0.0114 mole) L,L-phenylalanine ethylene glycol diester *p*-toluenesulfonate in 15 ml distilled water was prepared with heating on a hot plate. While still warm, 1.92 g (0.0228 mole) sodium bicarbonate was added slowly. A vigorous evolution of carbon dioxide was observed. The aqueous solution was extracted with seven 25-ml portions of reagnet-grade chloroform. The combined chloroform extracts were dried over anhydrous magnesium sulfate, filtered, and the chloroform removed in vacuo. The yield of diamine as a colorless oil was 3.65 g (0.0103 mole).

The oil was dissolved in 70 ml dry toluene and placed in a 200-ml three-necked flask fitted with a dropping funnel, nitrogen inlet, and $CaSO_4$ drying tube. A solution of 1.76 g (0.0105 mole) 98% 1,6-diisocyanatohexane in 20 ml dry toluene was prepared and placed in the dropping funnel. The diisocyanate solution was added dropwise to the diamine solution at room temperature over a period of 10 min. A white precipitate was observed within 20 min. Stirring was continued overnight. The slurry-like mixture was filtered by suction and washed with *n*-hexane. The white solid was washed in a 50/50 (v/v) solution of methanol–water. The solid was again filtered by suction and washed repeatedly with portions of anhydrous methanol. Soaking and stirring in methanol (anhydrous) at 50°C was continued overnight. After filtering and washing with fresh hot methanol and drying in vacuo, the yield of polymer was 3.68 g (68.0%), mp 191–199°C.

ANAL. Calcd for $C_{28}H_{36}N_4O_6$ (repeating unit): C, 64.10; H, 6.92; N, 10.68. Found: C, 63.88; H, 6.98; N, 10.66.

Degradation Testing Procedure

While our degradation testing procedure[12] has included culturing methods with microorganisms, we here use only the direct exposing of model compounds and polymers to enzyme solutions. This procedure accelerates the degradation and simplifies the chemistry of the degradation reaction by eliminating complicating side reactions originating in the microorganism.

Powdered samples (100–200 mg) were placed into 24-ml borosilicate corrosion-resistant glass vials. Duplicates or triplicates of samples to be degraded and of the two controls were prepared. Concentrated enzyme solutions (1 mg/ml) were made as needed and kept under appropriate storage conditions of pH and temperature. Enzyme solutions were prepared fresh every second or third day. In order to begin a degradation sequence, 10 ml of a 0.2*M* buffer of pH at which the enzyme exerts maximum proteolytic activity was pipetted into each vial containing samples. The buffer was supplemented with a low concentration of a bactericidal agent, usually sodium azide or toluene. In addition, an equal amount of buffer was added to vials which contained no samples but were to receive a daily 1-ml dosage of the concentrated enzyme solution. These vials served as controls necessary in the ninhydrin analytic assay for amino end groups. Vials containing sample without enzyme served as a second set of controls for determining the amount of acid- or base-catalyzed hydrolysis of samples due to the buffer alone. All vials were placed on a variable-speed shaker table (30 rpm) at ambient temperature for six to ten days. During this period,

1 ml concentrated enzyme solution was added to the sample vials and buffer–enzyme control vials every 24 hr. Control vials containing buffer but no enzyme received daily 1 ml of the buffer in which the enzyme was stored. (The two buffers need not necessarily be the same.) At the end of the last day of exposure to enzymes, all vials containing samples were filtered in predried, tared sintered glass funnels or cellulose filter paper. Filtrates were saved for ninhydrin or spectroscopic analysis, and the remaining solids were washed with equal amounts (50 ml total) of distilled water. The solids were dried in vacuo for 48 hr at 60°C. The distilled water washed were also saved for analysis.

It is noteworthy to point out that at the start of a degradation study all samples appeared "unwettable" by the buffers and consequently floated on top of the liquid. During the course of the degradation, samples receiving enzyme were observed to sink to the bottom of the vials or become suspended in the buffered media, whereas control samples typically retained most of this nonwettability characteristic.

Analytic Techniques to Determine Extent of Degradation

Weight Loss. After carefully filtering solid residues in sintered glass funnels or cellulose analytical filters, the solids were washed with 50 ml distilled water and dried in vacuo at 60°C for 48 hr. The filters plus solids were weighed and the weight loss for each sample was determined. The overall weight loss due to enzyme degradation was corrected for weight loss in enzyme-free controls due to buffer hydrolysis.

Ninhydrin Analysis. This spectrophotometric assay can detect primary amine end groups in the micromolar range. The technique is not restricted to amino acids but is applicable to primary amines in general.[15] Control measurements on the buffer/enzyme and buffer/substrate solutions were necessary to correct for their contribution to degraded sample supernatants. This correction permitted the overall increase or decrease (due to any enzyme adsorption onto solid samples) in amine groups to be determined with the aid of a standard plot obtained from solutions of known amine concentration. Phenylalanine was convenient to use for this purpose.

Ultraviolet Analysis. Chymotrypsin degradation of the low molecular weight poly(ester urea) (**2**) was expected to yield soluble products containing a benzyl aromatic group which absorbs strongly in the UV region. By first determining the molar extinction coefficient for a known concentration of L-phenylalanine in the buffer used, it was possible to determine the total amount of L-phenylalanine present in the buffer-soluble degradation fragments. Two wavelengths (290 and 258 nm) were chosen as arbitrary reference points for calculations on spectra. At 290 nm, the enzyme showed an absorption, whereas L-phenylalanine did not. Both showed an absorption at 258 nm, which also happens to be the wavelength of maximum absorption for the aromatic group of L-phenylalanine. All transmittances at these two wavelengths were corrected for a slight absorbance in the buffer due to the presence of an added bactericidal agent, toluene.

RESULTS AND DISCUSSION

The L,L- and D,L-isomers of a phenylalanine-based diester diurea model compound 1 were prepared according to reactions (1) (Ph = C_6H_5):

$$\begin{array}{l} H_2NCHCOOH \\ \quad CH_2PH \end{array} \xrightarrow{SOCl_2 \cdot CH_3OH} \begin{array}{l} HCl \cdot H_2NCHCOOCH_3 \\ \qquad\quad CH_2Ph \end{array}$$

$$\xrightarrow[\text{2. } OCN(CH_2)_6NCO]{\text{1. aq. } NaHCO_3} \underset{\text{toluene}}{CH_3OOCCHNHCONH(CH_2)_6NHCONHCHCOOCH_3} \quad (1)$$
$$\qquad\qquad\qquad\qquad\quad CH_2Ph \qquad\qquad\qquad\qquad CH_2Ph$$

diester diurea 1; L,L-isomer = 92%, D,L-isomer = 95%

Approximately 150 mg of the water-insoluble diester diurea model compound was exposed to 10 ml buffered enzyme solutions (0.1 mg/ml) for ten-day periods at room temperature. Extents of enzymatic hydrolysis were determined in two ways. First, the compound's weight loss was compared to controls in which samples were exposed to buffer solutions free of enzymes. Secondly, the supernatant solutions filtered from the remaining solids were analyzed for primary amino end-group content by the ninhydrin method. A net increase would result if cleavage occurred at the urea linkages, while no amine increase would result from only ester cleavage. The degradation results for the model compounds are listed in Table I.

Of the six enzymes used, chymotrypsin was found to be the most effective enzyme in cleaving model compound 1. The L,L-isomer of 1 was three times more degraded than the D,L-isomer. Undoubtedly, differences in the stereochemistry of the L,L-isomer and D,L-isomer mixture was the controlling factor here. Methanol was detected in the supernatant mixture by vapor-phase chromatography. No ester-containing compound was detected in the supernatant, and because there was little or no amino end-group increase, it is assumed that hydrolysis of the diester to the soluble diacid was responsible for the weight loss. This is in agreement with the known substrate specificity of chymotrypsin in preferentially cleaving the acyl site of aromatic side chains in polypeptides.[16] Papain, a plant protease with a chymotrypsin-like specificity, was equally as effective as chymotrypsin in cleaving the D,L-isomer. A very small amount of

TABLE I
Enzyme Degradation of Diester Diurea Model Compounds 1[a]

		L,L-Isomer		D,L-Isomer	
Enzyme	Buffer, pH	% Weight loss[b]	% —NH_2 increase[c]	% Weight loss[b]	% —NH_2 increase[c]
Chymotrypsin	imidazole, 7.8	79.9	[d]	27.0	[d]
Papain	phosphate, 6.5	1.2	4.5	24.7	2.7
Elastase	Tris, 8.8	12.1	3.7	18.3	1.0
Acid protease	glycine HCl, 3.1	—	—	0.5	—
	phthalate HCl, 3.1	1.5	[e]	—	—
Pepsin	HCl, 2.2	[d]	—	—	—
Urease	phosphate, 7.0	[f]	1.8	—	—

[a] Data are averages for triplicates. Tris = tris(hydroxymethyl)aminomethane.

[b] Weight loss (±0.3%) after exposure to buffered enzyme solution in excess of that from exposure to buffer only.

[c] Expressed as percentage of total possible primary amine which could have appeared if all urea linkages were completely hydrolyzed. Analyzed by ninhydrin assay, ±0.1%.

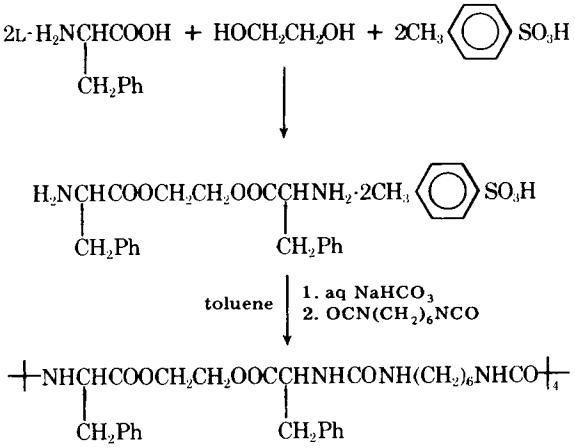
[d] Negligible.

[e] Enzyme was later shown to bind to cellulose filter paper, causing incorrect control values.

[f] Solids removed by centrifugation, not weighed.

peptide hydrolysis was detected by the ninhydrin assay. Hydrolysis of the L,L-isomer, however, was surprisingly low. Apparently, proper positioning of one L end within the enzyme's long catalytic site[17] is prevented because the adjacent urea linkage and methylene groups make is impossible for the second L segment to form needed secondary attachments to the enzyme. Elastase enzyme was somewhat effective in ester cleavage of both D,L- and L,L-isomers. This result may follow from the fact that the enzyme's natural substrate, elastin, is high in nonisomeric glycine. Three other enzymes, a bacterial acid protease (*R. chinensis*), pepsin, and urease, were ineffective in degrading compound **1** at the concentrations used.

With the promising result from the model compound degradation, we proceeded to prepare the related poly(ester urea), poly(L-phenylalanine/ethylene glycol/1,6-diisocyanatohexane), **2**, according to scheme (2):



(2)

poly(L-phenylalanine/ethylene glycol/1,6 diisocyanatohexane) **2**

Since chymotrypsin was the most effective enzyme in cleaving model compound **1**, powdered samples of the low molecular weight polymer **2** ($\bar{M}_n$ 1930) were subjected to chymotrypsin digestion at pH 7.8 (Table II). After a ten-day exposure, a 19.3% weight loss of the solid polymer was observed. In confirmation of this value, ultraviolet spectroscopy revealed that 22.9% of the maximum possible phenylalanine originally present in the polymer backbone was now present in the water-soluble portion of the degradation mixture. Only a 0.6% increase in amino end groups was detected in the soluble portion by the ninhydrin assay. These results indicate that significant degradation of the phenylala-

TABLE II
Chymotrypsin Degradation of Poly(ester Urea) **2** and **3**, Ten-Day Exposure

Polymer	Buffer, pH	% Weight loss[a]	% —NH_2 increase[b]	% benzyl in solution[c]
2	Tris, 7.8	19.3	0.6	22.9
3	imidazole, 7.6	0.6	1.1	—

[a] See Table I.
[b] See Table I.
[c] By UV spectroscopy, ±1%.

nine-based polymer occurred, with the enzyme-catalyzed hydrolysis occurring mainly at the acyl sites of the phenylalanine residues. The water-soluble degradation fragments are presumably a phenylalanine-capped urea and ethylene glycol. In agreement with this result is the finding that a nonaromatic poly(ester urea)[18] prepared in similar fashion from glycine (**3**, $\overline{M}_n$ 1600) was found to resist chymotrypsin degradation under similar conditions of pH, temperature, and exposure time (Table II).

$$\text{-HNCH}_2\text{COOCH}_2\text{CH}_2\text{OOCCH}_2\text{NHCONH(CH}_2)_6\text{NHCO-}_5$$

poly(glycine/ethylene glycol/1,6-diisocyanatohexane) **3**

It is noteworthy that the extent of degradation of the L,L-diesterdiurea model **1** (containing *two* ester linkages in each molecule) was four times that of the L-poly(ester urea) oligomer containing an average of *eight* ester linkages in each molecule. It is tempting to conclude that the susceptibility to attack of ester linkages both in model compound **1** and in the less crystalline polymer **2** are similar. However, further research on the effects not only of molecular structure but also polymer morphology, crystallinity, and wettability have to be carried out before definite guidelines for degradability can be made. Several points can nevertheless be made from the data. Chymotrypsin exhibited stereospecificity on isomers of the model compound, and it exhibited chemical side-group specificity on the related low molecular weight polymers. The unsubstituted poly(ester urea) **3** resisted chymotrypsin hydrolysis presumably due to lack of the hydrophobic interaction which can exist between an aromatic group and the enzyme active site.[19] We have found that other substituted polymers (methylated nylon 2,10 and benzylated nylon 6,3, for example) exhibit higher biodegradability than the corresponding unsubstituted polymers,[8,9] and studies are continuing to determine other factors controlling biodegradation.

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