

Biodegradation of Gelatin-g-Poly(ethyl Acrylate) Copolymers

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Synopsis

Gelatin-g-poly(ethyl acrylate) copolymers were prepared in an aqueous medium, using $K_2S_2O_8$ initiator. The composition of the graft copolymers was dependent upon temperature and duration of the reaction. The number of grafting sites was small and molecular weight of the grafted poly(ethyl acrylate) branches was high. Three copolymer samples with grafting efficiencies of 33.3%, 61.0%, and 84.0%, were tested for their microbial susceptibility in a synthetic medium employing a mixed inoculum of *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Serratia marcescens* and the percent weight losses were 12%, 10.1%, and 6.0%, respectively, after 6 weeks of incubation. The extent of degradation seems to decrease with increasing grafting efficiency. There was initial rapid weight loss accompanied by the exponential increase in bacterial population and pH of the culture medium during the first week. The nitrogen analysis also showed the utilization of the polymer. A parallel set of experiments, carried out by employing the samples as the only source of both carbon and nitrogen, showed a marginal but definite increase in the utilization of the polymer.

INTRODUCTION

In recent years there has been an increasing interest in the design of biodegradable polymers for specialized applications such as controlled-release formulations of drugs and insecticide and pesticide carriers, as well as nontoxic surgical implant materials.¹ An interesting application of these plastics is in the field of agriculture, where plastics are being used to an increasing extent as mulches. Degradable plastics may find acceptability in such applications as an economic way of disposing of the used film. Biodegradable containers for transplanting trees and other plants is also of economic interest.²

Chemical modification of natural polymers by grafting serves the twofold purpose of utilizing renewable, naturally derived products, such as polysaccharides and proteins, as replacements for petroleum-based polymers and as biodegradable compositions which can be tailored for the slower or faster rates of degradation.

Gelatin, an animal protein, consists of 19 amino acids joined by peptide linkages and can be hydrolyzed by a variety of the proteolytic enzymes to yield its constituent amino acid or peptide components.³ This nonspecificity is a desirable factor in intentional biodegradation. However, little information is available regarding the modification of gelatin by graft copolymerization.⁴⁻⁸ Moreover, the biodegradative behavior of protein graft copolymers has not yet been reported in the literature. Hence work was taken up to study systematically the modification of gelatin, the physical and mechanical behavior of different copolymers, and their biodegradability by different bacterial and fungal strains.

In an earlier communication preliminary results were reported.⁹ This paper reports the studies on the biodegradation of gelatin-*g*-poly(ethyl acrylate) by a mixed bacterial inoculum.

EXPERIMENTAL

Materials

Gelatin, bacteriological (BDH) ($\bar{M}_n = 90,000$) was used in this investigation. Monomer ethyl acrylate (BDH) was washed with 5% NaOH, and then with water, and finally dried under nitrogen. $K_2S_2O_8$ (E. Merck) was used as the initiator, and other solvents used were of AR grade and were used as such. The bacterial cultures were obtained from the cultural collections of the Microbiology and Cell Biology Laboratory, Indian Institute of Science, Bangalore, India, and routinely maintained on nutrient agar slants.

Preparation of Gelatin-*g*-Poly(ethyl Acrylate) Copolymers

Gelatin (10 g) was added to 200 mL of water and heated to 40°C in a thermostat under nitrogen till it dissolved. Distilled ethyl acrylate (10 g) was added to the aqueous solution of gelatin, and the grafting sites were initiated on the protein backbone by the addition of 0.54 g of $K_2S_2O_8$. All the grafting experiments were carried out with constant stirring at the required temperatures. The product was collected by filtration, washed several times with hot water till the unreacted gelatin was removed, and dried in air. The final water extract was subjected to the Biuret test.¹⁰ The absence of any reddish violet color indicated the absence of free gelatin (protein). The occluded homopolymer was extracted with toluene.

Analysis of the Graft Copolymers

Grafted side chains were removed from the protein backbone by the procedure given by Rao et al.¹¹ Acid hydrolysis was carried out by heating the graft copolymers with 6N HCl for 24 h. The insoluble residue was filtered off, washed, and dried. The intrinsic viscosities of poly(ethyl acrylate) (PEA) branches were determined in benzene at 30°C. The \bar{M}_n was calculated from the following equation:

$$[\eta] = 27.7 \times 10^{-5} \bar{M}_n^{0.67} \text{ dL/g}$$

The grafted samples were analyzed for total nitrogen. The gelatin content of the graft copolymers was also calculated from total nitrogen content of gelatin.

The infrared spectrum of polymers were recorded with a Carl-Zeiss Model Sp 700 Infrared Spectrophotometer using KBr pellets. The percent grafting, the efficiency of grafting, total conversion, and the number of grafting sites were calculated from the following relations^{11,12}:

$$\% \text{ grafting} = \frac{(\text{total wt of grafted copolymers}) - (\text{wt of gelatin})}{\text{wt of gelatin}} \times 100$$

$$\text{Efficiency of grafting (\%)} = \frac{\text{wt of PEA grafted}}{(\text{wt of PEA grafted}) + (\text{PEA homopolymer})} \times 100$$

$$\text{No. of grafting sites} = \frac{\text{wt of PEA grafted}}{\text{mol. wt of PEA}} \bigg/ \frac{\text{wt of used gelatin}}{\text{mol. wt of gelatin}}$$

$$\text{Total conversion (\%)} = \frac{\text{wt of polymer grafted and homopolymer}}{\text{wt of monomer charged}} \times 100$$

Film Preparation

The dry polymer was hot pressed into films of uniform thickness (2 mm) at a pressure of 100 kg/cm². The die was maintained at a temperature of 145–150°C. However, the melt flow of the samples containing more than 50% by weight of the protein was low.

Microbiological Testing

For testing biodegradability, mineral salts medium with the polymer in question as the sole source of carbon was used.¹³ The composition of the medium was: KH₂PO₄, 0.7 g; K₂HPO₄, 0.7 g; MgSO₄, 7H₂O, 0.7 g; NH₄NO₃, 1.0 g; NaCl, 0.005 g; FeSO₄, 0.002 g; ZnSO₄, 0.002 g; and MnSO₄, 0.001 g, in 1 L of distilled water.¹⁴ A parallel set of experiments were carried out by using the polymer as the only source of both carbon and nitrogen by employing the above medium with the deletion of NH₄NO₃. The above medium was taken in 10 mL amounts in 150 × 18 mm test tubes, and 0.3 g of the sample was added in each tube. The tubes were autoclaved at 120°C for 15 min. The weight loss was negligible in the controls after autoclaving. In the present investigation a mixed bacterial inoculum of *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Serratia marcescens* was used. All the tubes were inoculated with 0.1 mL of the mixed bacterial suspension under sterile conditions along with the required controls and incubated at 30°C for 6 weeks on a rotary shaker.

Three tubes were removed every week from each set, and the samples were submerged in 0.1% solution of HgCl₂ for 10 min to halt further action, thoroughly rinsed with water, and placed in a dessicator for 36 h for drying. The samples were then weighed, and the weight loss was recorded.

The growth of the organisms was followed by recording absorbance of the culture every 24 h in a Bausch and Lomb Spectronic 20 Calorimeter at 600 nm. pH measurements of the culture medium were also carried out periodically.

RESULTS AND DISCUSSION

The grafting experiments were conducted at 60°C and 70°C for 30 min, 60 min, and 120 min, in each case. These experimental conditions were specifically chosen to study the changes in graft copolymer compositions at lower and higher temperatures as well as at shorter and prolonged polymerization times. Proof of grafting was obtained by IR analysis. Pure gelatin has amide absorption centered around 1660 cm⁻¹. Pure poly(ethyl acrylate) has strong absorption due to the ester carbonyl group centered around 1725 cm⁻¹. The IR spectra of graft copolymers showed characteristic absorption bands due to amide groups of gelatin (1660 cm⁻¹) and ester carbonyl groups (1725 cm⁻¹) of poly(ethyl acrylate).

It can be seen from Table I that the yield of the graft copolymer reached a

TABLE I
Preparation of Gelatin-*g*-Poly(ethyl Acrylate) Copolymers

Sample ^a	Graft copolymer (%)	Unreacted gelatin (%)	Homopolymer of (%) poly(ethyl acrylate)
At 60°C			
30 min	22.56	45.20	26.57
60 min	65.25	26.56	8.32
120 min	69.65	26.00	5.80
At 70°C			
30 min	40.05	50.35	6.05
60 min	56.00	27.68	11.55
120 min	58.25	26.00	12.00

^a In each case 0.54 g of K₂S₂O₈ initiator, 10 g of gelatin and 10 mL of ethyl acrylate were dispersed in 200 mL of water.

maximum after 1 h at both the temperatures. Table II shows that, at 60°C, the efficiency of grafting and percent grafting increased significantly during the course of polymerization. In other words, the homopolymer formation was greater in the initial stages whereas there was much less homopolymer after 1 h. However, the increase in the efficiency of grafting was only marginal after 1 h. At 60°C, the number of grafting sites per molecule also increased with time, though the molecular weights did not exhibit comparable difference. At a shorter polymerization time (30 min), at 70°C, the grafting efficiency and the number of grafting sites increased with a significant decrease in the molecular weight of the PEA branches. In all cases, after 1 h, the insoluble graft copolymer got completely precipitated out and the reaction reached a stage where it could be considered as complete.

The number of grafted PEA branches reached a high value at a lower temperature for prolonged polymerization time. A relatively short polymerization time at a low temperature was preferable to obtain long grafted branches. It should be noted that the number of PEA branches per gelatin molecule was small, and their molecular weight was high in the present graft copolymerization in conformity with an earlier report on similar work.⁴ When a chain of PEA grows on gelatin backbone, the latter becomes insoluble though it remains swelled. The free radicals generated in aqueous phase, from water-soluble K₂S₂O₈, mutually react with one another, but do not terminate the growing chain, resulting in high molecular weight of the side branches. The small number of grafted branches may be attributed to the structure of gelatin, where the number of -CH(OH)- groups responsible for the generation of the backbone radicals is small in number.³

Three samples of the graft copolymers with grafting efficiencies of 33.3% (8.5% N), 60.9% (7.1% N), and 84.1% (5.6% N), designated as GEA, GEB, and GEC, respectively, were selected for testing bacterial susceptibility. These samples were chosen to study the effect of grafting efficiency and the number of grafting sites on the extent of biodegradation. A single parameter which was found to correlate most closely with all the other indices of polymer degradation by microorganisms is weight loss.¹⁵ The loss in weight of the samples was followed, and the percent weight loss recorded. In the controls, the reduction in weight was negligible, and data have been corrected for the same. The percent weight loss vs. time plots are shown in Figure 1. To ensure that estimates of behavior

TABLE II
Composition of Gelatin-Poly(ethyl Acrylate) Copolymers

Sample ^a	Nitrogen (%)	Gelatin (%)	Poly(ethyl acrylate)(%)	Grafting (%)	Efficiency of grafting (%)	Conversion (%)	\bar{M}_n of PEA $\times 10^{-5}$	No. of grafting sites (mol/mol)
At 60°C								
30 min	8.5	53.2	46.5	88.39	33.29	53.99	9.0	0.0895
60 min	5.6	33.3	65.8	211.5	84.14	73.66	8.0	0.2379
120 min	5.5	33.3	66.0	210.0	84.16	73.60	8.0	0.2379
At 70°C								
30 min	4.5	27.2	72.5	114.2	47.23	36.95	3.5	0.6857
60 min	7.1	44.5	55.0	124.7	60.98	54.73	7.0	0.1603
120 min	7.15	45.2	54.6	123.5	61.05	55.02	7.0	0.1603

^a In each case, 0.54 g of $K_2S_2O_8$ initiator, 10 g of gelatin and 10 mL of ethyl acrylate were dispersed in 200 mL of water.

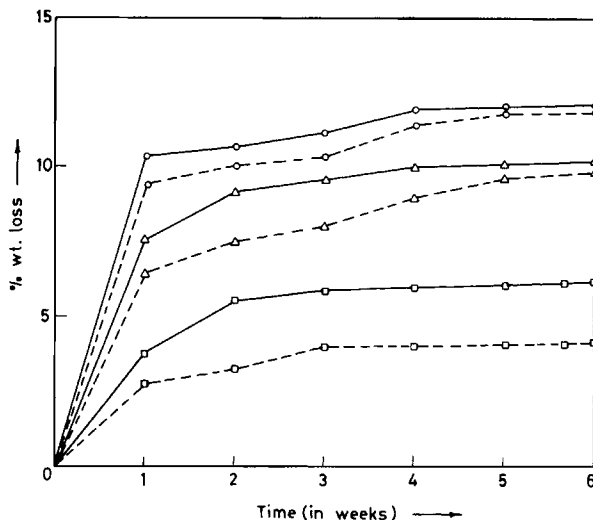


Fig. 1. Percent weight loss vs. time curves of gelatin-*g*-poly(ethyl acrylate) samples incubated with *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Serratia marcescens* in nitrogen-free (—) and nitrogen-rich (---) media. (O) GEA, (Δ) GEB, (\square) GEC.

are not too optimistic, the greatest degree of deterioration is reported, which has been the convention.¹³ Figure 1 shows that the extent of degradation is in the following order:

$$\text{GEA} > \text{GEB} > \text{GEC}$$

From Table II, it is clear that the efficiency of grafting and the number of grafting sites per molecule are in the following order:

$$\text{GEC} > \text{GEB} > \text{GEA}$$

Weight loss was assumed to be due to the utilization of the gelatin portion of the copolymer because of the relatively short incubation period used in this study and the well-known ability of gelatin to biodegrade. The results indicate that the more the number of grafting sites on gelatin backbone, the slower will be the rate of biodegradation. All the plots (Fig. 1) clearly indicate rapid initial loss of the material in the first week of the incubation period. It is also obvious that when the samples were used as the only sources of both carbon and nitrogen, there was a marginal but definite increase in the utilization of the polymer, the difference being quite significant in the first half of the test period. Also, the difference is more prominent in the case of GEC, which contains the least amount of nitrogen.

TABLE III

Nitrogen Content of the Samples Inoculated with *Pseudomonas Aeruginosa*, *Bacillus Subtilis*, and *Serratia Marcescens* before and after Testing

Sample	Initial nitrogen %	After 15 days nitrogen (%)		After 45 days nitrogen (%)	
		N(+) medium	N(-) medium	N(+) medium	N(-) medium
GEA	8.53	5.8	5.5	4.5	4.0
GEB	7.15	4.5	4.8	3.6	3.9
GEC	5.6	4.0	3.9	3.0	3.6

Further evidence for the degradation of the polymer was obtained from the estimation of nitrogen content of the samples before and after testing. Though the results indicated (Table III) positively the utilization of the gelatin portion, the trend observed in weight-loss time curves could not be found in this case.

The growth of bacteria, monitored by reading the absorbance at 600 nm, showed a sharp increase during the first week (Fig. 2). The lag period, being less in nitrogen-free medium compared to nitrogen-rich medium, indicates a better attack on the polymer in the absence of added nitrogen source.

Protein hydrolysis results in the release of amino acids. The amino acids, ultimate products of proteolysis, are subject to a variety of pathways for microbial decomposition. Many microorganisms can deaminate amino acids and produce ammonia. The production of ammonia in this way is referred to as ammonification.¹⁶ The results of pH measurements indicated a significant increase in the pH of the medium over a period of 1 week in all the cases, and it remained constant afterwards (Fig. 3). It is quite logical to get pH changes towards the alkaline side because of ammonification. There was no notable difference in this regard between the nitrogen-free and nitrogen-rich media.

In all the cases, there was an initial rapid weight loss during the first week, followed by slow but steady disappearance of the substrate. This was accompanied by the exponential growth in bacterial population and pH. All these observations "superimpose" well and indicate an early and rapid exhaustion of more accessible material. Though the weight loss-time curves seem to indicate a levelling off of the degradation of the polymer, it cannot be concluded that degradation has stopped. If the degradation had stopped after 10 days or so,

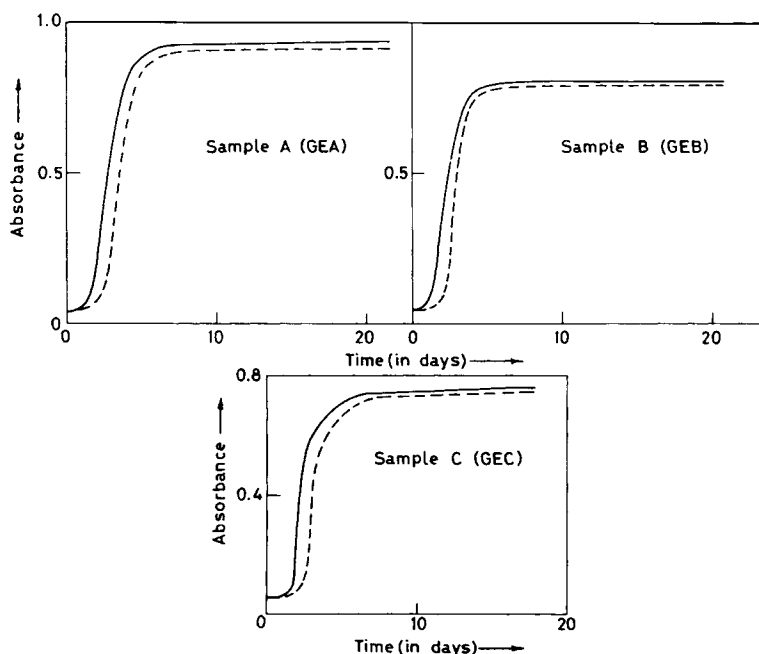


Fig. 2. Absorbance of the culture medium at 600 nm vs. time plots of gelatin-*g*-poly(ethyl acrylate) samples incubated with *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Serratia marcescens* in nitrogen-rich (---) and nitrogen-free media (—).

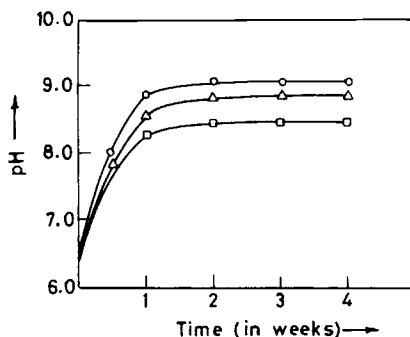


Fig. 3. pH of the culture medium vs. time plots. (O) GEA, (Δ) GEB, (\square) GEC.

then absorbance of the culture medium should have decreased. But the fact that the absorbance remains stationary shows the sustaining growth of bacteria, which can only be due to the continuing utilization of the polymer

The observed phenomena might be due to rapid hydrolysis of more susceptible peptide bonds in the early stages and then the slow hydrolysis of others. The increase in pH due to initial rapid hydrolysis might have inhibited further proteolysis. It is also known that limitation of ammonia in the medium depresses proteolytic enzyme action.¹⁷ Another reason for the inhibited rate would be possibly due to the presence of some degradation products which might have inhibited further proteolysis.

CONCLUSIONS

The characteristic feature of grafting of ethyl acrylate onto gelatin was the small number of PEA branches per gelatin molecule and their high molecular weight. The extent of degradation decreased with the efficiency of grafting. There was an initial rapid utilization of the polymer by the bacteria followed by slow but steady degradation. When the polymer samples were used as the only sources of both carbon and nitrogen, there was marginal but definite increase in the utilization of the polymer.

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