Biodegradation of Chemically Modified Gelatin Films in Lake and River Waters

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ABSTRACT: Gelatin was chemically modified by crosslinking samples with one of a number of bifunctional reagents as was done earlier in a processing technique used to improve mechanical properties through chain orientation. The effects of this crosslinking on the biodegradability of the resulting films were evaluated in the laboratory by exposing them to lake and river waters for 10 days with or without inoculation with periphyton organisms. Biodegradabilities were assessed by weight losses of the films and by measurements of dehydrogenase activity of biomasses taken from their surfaces. The extent of biodegradation depended on the type of crosslinking agent and the presence or absence of the periphyton. The gelatin films crosslinked with formaldehyde, glyoxal, or glutaraldehyde were the slowest to biodegrade; complete degradation required 8–10 days. In contrast, the most biodegradable was the gelatin crosslinked with hexamethylene diisocyanate, which required only 3–4 days. The uncrosslinked gelatin and the gelatin crosslinked with butadiene diepoxide and diepoxyoctane were intermediate, degrading in 5–7 days. The dehydrogenase activity paralleled the weight losses but rapidly decreased when the amount of gelatin remaining was small. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 76: 29–37, 2000

Key words: crosslinked gelatin; biodegradability; dehydrogenase activity; weight loss; periphyton

INTRODUCTION

The biodegradation of synthetic polymers based on petroleum products can be extremely slow. In fact, under natural conditions they can remain as waste products and serious pollutants for years.[1] A possible solution to this problem is replacement of these commonly used plastics with other polymers that can undergo rapid biodegradation. Interest in the production of biodegradable polymers has thus increased as a result of increased demand for environmentally friendly products.[2,3] Gelatin, a biodegradable and environmentally friendly biopolymer, is a high molecular weight protein derived from collagen, which is the protein component of animal connective tissues (tendon), bone, and skin.[4] Collagen occurs in all types of multicellular animals, invertebrates, and vertebrates and forms the major fibrous elements of the extracellular connective tissue. Gelatin is now manufactured from the waste products obtained

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in the processing of animal meats and skins and is mainly used in the food industry.

The main reason for the current interest in gelatin for the production of materials with a wide varieties of applications is its biodegradability under the action of enzymes (proteinases) that are present in microorganisms. Selected strains of such microorganisms, such as *Bacillus subtilis*, produce a very active alkaline proteinase, which can break almost all peptide bonds in a polypeptide chain because of its very low specificity. It has been much studied in a wide variety of applications.[5] However, in order to transform gelatin into a plastic with desirable properties, it is necessary to increase its density of crosslinks (beyond those physical associations of the chains in its usual thermoreversible network). There are, of course, a number of methods for introducing more flexible chemical crosslinks.[6–9] Of particular interest is crosslinking with a bifunctional reagent containing aldehyde, isocyanate, or epoxide groups using existing free ε amino groups from lysine residues. Because the peptide bonds of gelatin would not be involved in such crosslinking, the chains would still be biodegradable from proteolytic enzymes of naturally existing microorganisms widely present in the environment.

Earlier we studied formaldehyde (FA) crosslinked gelatin's biodegradative behavior in the presence of alkaline proteinase[10,11] and gelatin crosslinked with FA, 1,2,3,4-butadiene diepoxide (BDE), or hexamethylene diisocyanate (HMDIC) employing sewage water as the media and periphyton organisms from sewage as the inoculum.[12] Most probably under natural conditions the synthesis of proteinase enzymes was accomplished with the microflora of the periphyton. Thus, its determination was of importance in understanding the biodegradation process. This was accomplished by plating the inoculum from the total periphyton biomass, which completely covered the gelatin films during the biodegradation process. Periphyton was plated on petri dishes with various broth media in order to enhance the growth of the microflora species. The following media were used for growing the molds: the broth medium of Sabouraud containing meat, peptone, and glucose for bacteria; the broth medium with meat extract containing NaCl for yeast; and Gausee's broth medium containing starch and salts for actinomyces.[13,14] The purpose was to determine whether biodegradability was affected by the crosslinking required in a processing technique used to improve the mechanical properties of a polymer through chain orientation.[9] In the present work, we investigate the biodegradability of gelatin that was chemically crosslinked with FA, glyoxal (GO), glutaraldehyde (GA), HMDIC, BDE, or 1,2,7,8-diepoxyoctane (ODE). The conditions used were very similar to the natural environment, specifically, employing water and periphyton samples taken either from a lake in an urban Cincinnati park or from the Ohio River near downtown Cincinnati.

EXPERIMENTAL

Materials

The gelatin employed in this study had a number-average molecular weight (M_n) of 90,000. The crosslinking agents were FA, GO, GA, HMDIC, BDE, and ODE. These agents were specifically chosen to study the effects of their chain length and chemical nature on the biodegradation process. Thus, the numbers of —CH_2— groups in the crosslinking bridges would be 1 (FA), 2 (GO), 3 (GA), 4 (BDE), 6 (HMDIC), or 8 (ODE). In addition, these bifunctional compounds were of three rather different types: dialdehydes, diepoxides, and diisocyanates. All of the crosslinking agents and solvents employed were AR grade and were used as obtained. The correspondingly crosslinked gelatin samples were designated by the crosslinking agent designation followed by G for gelatin (e.g., FAG, GOG, etc.) with NG specifying no crosslinking agent at all (the uncrosslinked gelatin).

Lake water was taken from an artificial urban lake in Burnet Woods in Cincinnati (Hamilton County, OH). River water was taken from the Ohio River near downtown Cincinnati. Periphyton organisms were scraped from stones in the lake or river from an area of approximately 2 dm^2 and were then brought up to 500 mL using the same water. They were then guided through a metal sieve (2-mm openings) using a knife. The suspensions were permitted to settle for 30 min, and the resulting sediment was used as the inoculum.

Crosslinking of Gelatin

Two grams of gelatin was soaked overnight in distilled water at 5°C and then dispersed at 45°C in 20 mL of 0.02*M* phosphate buffer (pH 5.8). Solutions of FA, GO, or GA in the same buffer

were added with thorough mixing to the gelatin solution in an appropriate quantity to obtain a $0.012M$ concentration of crosslinking agent. Crosslinking was performed at the same temperature (45°C) for 120 min.

Crosslinking with BDE and ODE was performed in the same way as the aldehydes, specifically under identical conditions (concentrations of crosslinking agent and gelatin, temperature, solvent, and reaction time).

The crosslinking with HMDIC was performed by adding appropriate amounts of HMDIC and triethylamine (1/3 part of the HMDIC quantity) to a 5.5 wt % solution of gelatin in 2,2,2-trifluoroethanol (TFE) at room temperature (25°C). In all cases the resulting solutions were poured into 10-cm diameter aluminum dishes and were then dried at room temperature for 24 h to obtain isotropic films with a thickness of approximately 0.25 mm.

Evaluation of Crosslink Density

The isotropic dry gelatin films (10 × 10 × 0.25 mm squares, five pieces for each crosslinking agent) were swollen at 20°C for 48 h in distilled water in order to achieve equilibrium swelling. The gelatin volume fraction (ν_G) was determined assuming the additivity of specific volumes of gelatin and water:

$$\nu_G = W_o\rho_w/[W\rho - W_o(\rho - \rho_w)] \quad (1)$$

where ρ_w is the density of the water at 20°C and ρ is the density of the dry, chemically uncrosslinked gelatin. In the first approximation this latter value was also used for the crosslinked gelatin samples. The swollen sample was weighed after removing the excess liquid on the surface of the sample with a piece of filter paper.

The ν_G thus obtained was used to estimate the network crosslink density as given inversely by the average molecular weight between crosslinks M_c. It was calculated from the Flory–Rehner equation:[15]

$$M_c = -\rho V_1 \nu_G^{1/3}/[\chi\nu_G^2/2 + \ln(1 - \nu_G) + \nu_G] \quad (2)$$

where V_1 is the molar volume of the solvent and $\chi = 0.49$ is the polymer–solvent interaction parameter.[15]

Biodegradation Experiments

A shallow rectangular plastic tray (30 × 40 × 15 cm) provided with an electromagnetic stirrer and an aeration device was filled with either lake or river water. Each gelatin sample (circular disks with approximate 8-cm diameters) was cut into a four approximately equal segments. These pieces were then individually weighed, placed into petri dishes (4-cm diameters), and submerged in the tray. In some experiments a periphyton suspension was added to the tray after the samples were submerged. On alternate days one-half of the water was replaced with fresh portions from the same batch. Photographs of the samples were taken periodically.

Samples of each film were taken from the petri dishes and weighed for measurements of weight loss (WL) and dehydrogenase activity (DA).[16–18] The periphyton was carefully separated with a plastic knife from the surfaces of the films and then used for the DA determination. The residues of the films were dried at room temperature (22–24°C) for 24 h and then at 60°C for 12 h and reweighed.

The DA determinations were performed as described by Boyanovski[19] with some modifications. Briefly, 2,3,5-triphenyl tetrazolium chloride (TTC) was used at an incubation temperature of 28°C. The reduction of TTC, which is catalyzed by the periphyton dehydrogenases, leads to the formation of triphenyl formazan (TF), which absorbs at 470 nm. To avoid interference from photosynthetic pigments, the measurements were carried out at 520 nm (where their absorbance is negligible but TF still has considerable absorbance). The TF formed was extracted with absolute alcohol for 10 min. The results were expressed as the amount of TF formed in 1 h per unit volume using a standard curve plotted for pure TF.

RESULTS

Sample designations, specifications of crosslinking agents, and the conditions for crosslinking are presented in Table I. The appearances of the gelatin films after various days of biodegradation in lake and river water are shown in Figures 1 and 2, respectively. Portion A of each figure shows the results obtained using periphyton, but each photograph was taken after the periphyton was removed from the sample. Portion B shows the corresponding results obtained in the absence of periphyton. Even with just the naked eye one can see the low biodegradability of gelatins crosslinked with aldehydes, the moderate biodegradability of gelatins crosslinked with diepoxides

Table I Sample Designations, Crosslinking Agent (CA), and Preparative Conditions

Gelatin Sample	CA	CA Concn (M)	Solvent	Temp. (°C)	M_c
FAG	Formaldehyde	0.003307	Phosphate buffer, pH 5.8	45	13000 ± 1140
GOG	Glyoxal	0.003202	Phosphate buffer, pH 5.8	45	850 ± 64
GAG	Glutaraldehyde	0.003202	Phosphate buffer, pH 5.8	45	1250 ± 63
HMDICG	Hexamethylene diisocyanate	0.01282	Trifluoroethanol	45	1500 ± 69
BDEG	1,3-Butadiene diepoxide	0.01210	2M KSCN	25	3200 ± 255
ODEG	1,2,7,8-Diepoxyoctane	0.01210	2M KSCN	45	3474 ± 225
NG	None	—	Distilled water	45	6890 ± 323

All crosslinked samples were obtained from a 5.5 wt % solution of gelatin at 25°C with a crosslinking reaction time of 120 min.

(which coincide with the biodegradability of uncrosslinked gelatin), and the very high biodegradability of those crosslinked with diisocyanate. The increased biodegradability in the last case suggests that the diisocyanate either degraded some of the chains during the crosslinking or made them more vulnerable during the biodegradation process. In the course of the biodegradation the films became thinner and lost their regular shapes. At the end it was very difficult to separate the biomasses from the remaining parts of the films.

The weight losses for the films in lake water biodegradation are shown in Figure 3. Even on the first day the differences in WL for all seven samples were substantial. This was true for both the presence and absence of the periphyton. It is notable that the WL did not depend significantly on the presence of periphyton. In its absence a layer of it was formed in the water environment to an extent comparable with those in the inoculated samples. Apparently there were enough microflora present in the lake water to form periphyton on the first day of degradation, which is necessary for the biodegradation.

The WL in the HMDICG is 2 times that in NG, ODEG, and BDEG and more than 3 times that in FAG, GAG, and GOG. Complete degradation in the HMDICG occurred on the third day, both with or without periphyton, at which point the WL of NG, ODEG, and BDEG was between 70 and 85% and for FAG, GOG, and GAG it was between 40 and 60%. In the NG, BDEG, and ODEG complete degradation occurred between days 5 and 6 at which point the WL for the aldehyde crosslinked gelatin was 70–90%. With the exception of GAG (complete degradation without periphyton on day 6), all the samples of dialdehyde crosslinked gelatins completely degraded between days 8 and 10.

The decreasing order of biodegradability in lake water (with or without periphyton) was HMDICG > NG, ODEG, BDEG > FAG, GOG, GAG. Within the aldehydes the order was FAG > GOG > GAG, and within the diepoxides it was ODEG > BDEG. In the latter case the longer the —CH_2— crosslinking chains, the greater the biodegradability of the film.

The values for the WL for river water biodegradation (not shown) were very close to those for the lake water. However, the differences in the rate of biodegradation for gelatin crosslinked with aldehydes, diepoxides, and HMDIC were more pronounced. The order of the biodegradability was also similar with regard to the crosslinking agents having different functional groups and to crosslinking agents with identical functional groups but with different numbers of methylene groups. Again, no significant differences were observed in the biodegradation with or without periphyton. In river water without added periphyton a layer of it was formed on the first day of biodegradation, which indicates an adequate amount of microflora was also present in the river water.

The DAs of the periphyton for lake water biodegradation are shown in Figure 4. The curves of DA coincided with the WL ones, but only over a certain range of time. The values of the DA gen-

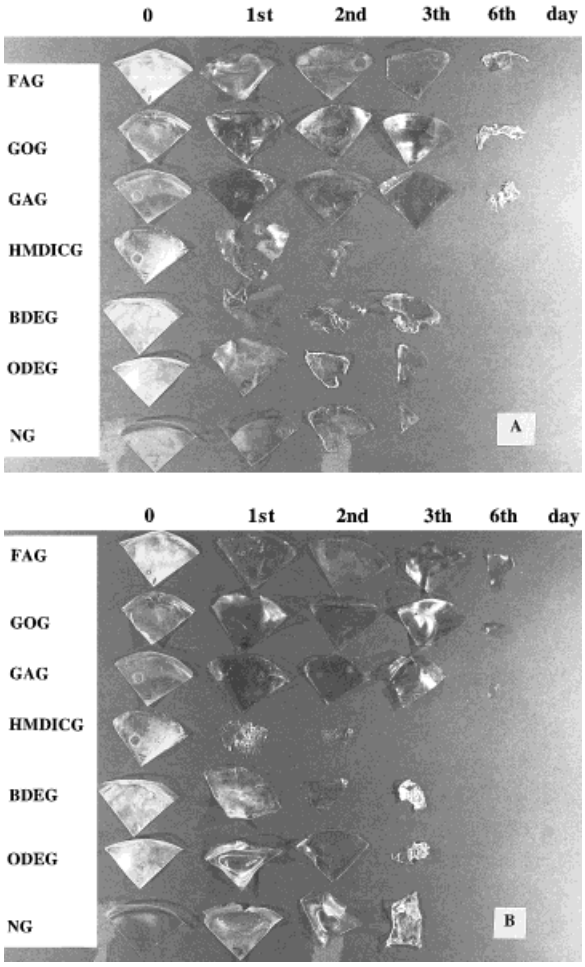

Figure 1 The appearances of the gelatin films after the specified days of biodegradation in lake water either (A) with inoculation of periphyton or (B) without inoculation.

erally increased until nearly complete biodegradation of the films and then gradually dropped to a low value. The exceptions were NG and aldehyde crosslinked gelatin in which it started to decrease well before complete biodegradation. The values of DA in water inoculated with periphyton were considerably higher than those in water without inoculation.

The values of the DA of the periphyton for river water biodegradation were very similar. In the samples without periphyton all curves showed maximal activity on day 2 of biodegradation and then gradually dropped until day 10. In the samples with periphyton the HMDICG, ODEG, and NG showed maximal activity of DA on day 2, whereas for all other samples it was on day 3.

DISCUSSION

The WLs were assumed to be due to the utilization of the gelatin by the periphyton microflora. The rate of biodegradation was high for all the samples (complete biodegradation in 3–10 days) compared to starch-based extruded plastic films,[20] which biodegraded only after 6 or more weeks. Hence, the present samples could be classified as rapidly biodegradable materials. Thus, the biodegradability was not seriously diminished by the crosslinking employed earlier in a processing technique used to improve the mechanical properties of gelatin through chain orientation.[8,9]

Some authors explained the low biodegradability of crosslinked polymers in terms of the enzyme-inhibitory effect of cyano groups.[19,21] Because HMDICG biodegraded at the highest rate

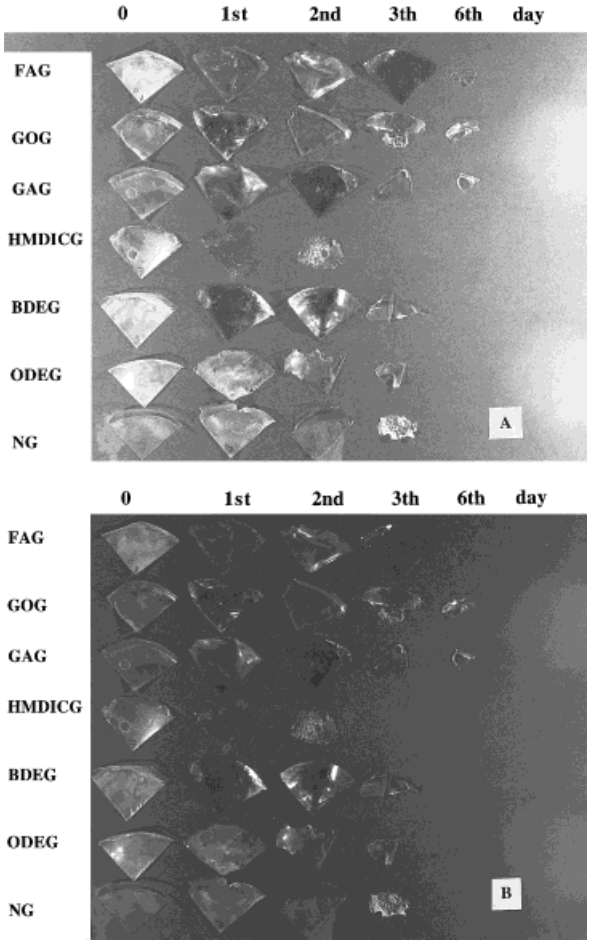

Figure 2 The appearances of the gelatin films after the specified days of biodegradation in river water either (A) with inoculation of periphyton or (B) without inoculation.

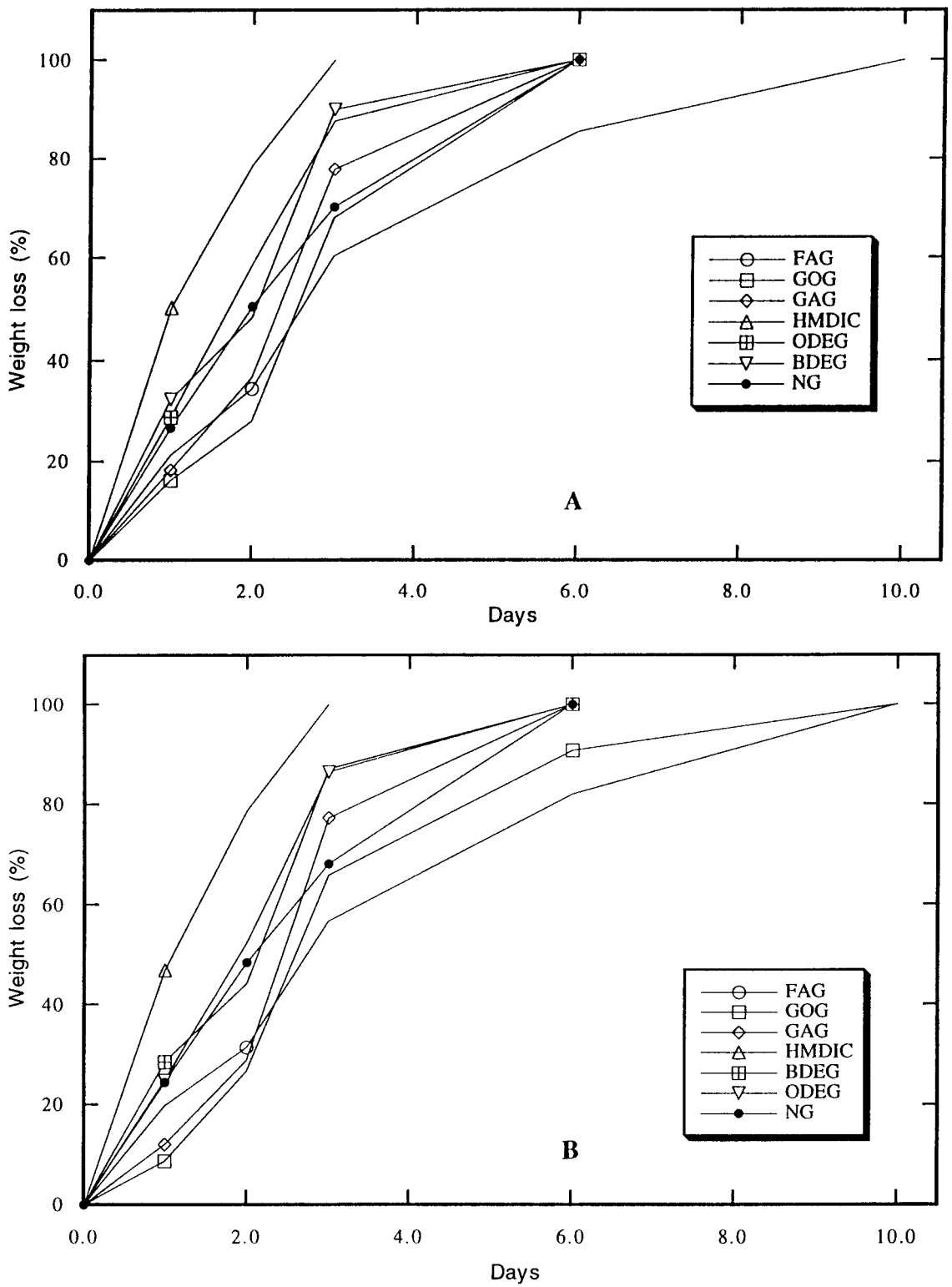

Figure 3 The weight losses of the films in lake water (A) with inoculation of periphyton and (B) without inoculation. Not all data points are shown.

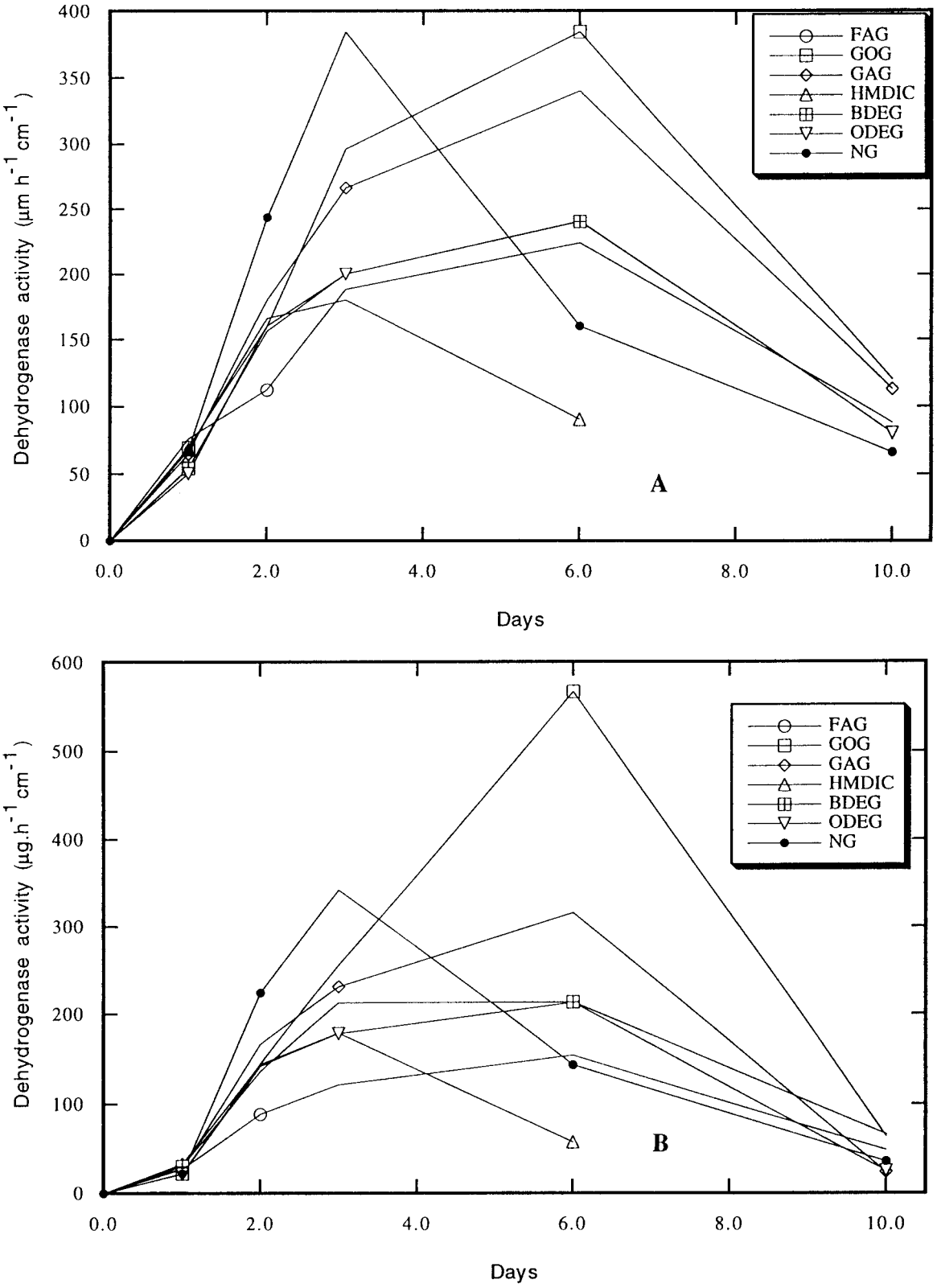

Figure 4 The dehydrogenase activities of the biomasses taken from the surfaces of films biodegraded in lake water (A) with inoculation of periphyton and (B) without inoculation. Not all data points are shown.

observed, this assumption cannot be supported for these materials. Furthermore, the active functional groups of the crosslinking agents would be used up in the crosslinking process. The access to the proteolytic and oxidative enzymes of their substrate groups depends on the crosslink density of the network, because high crosslink density impedes swelling and ingress of any small molecules. Another factor that could influence the biodegradation rate is the type of network present. Gelatin can contain both chemical networks (consisting of covalent bonds and bonds obtained by using a chemical agent) and physical networks (consisting of collagen triple helices formed by hydrogen and noncovalent bonds). It is well known that rigid structures (such as the collagen triple helix) are more resistant than disordered ones, because enzymes need proper spatial interaction with the substrate to function.[22] After a pepsin attack on collagen, the helical parts were found to be resistant while the disordered chains were degraded.[23] More recently a similar behavior was observed using trypsin.[24] These observations could explain the highest rate of biodegradation found in the case of HMDICG.

The decreasing order of biodegradability in lake water (HMDICG > NG, ODEG, BDEG > FAG, GOG, GAG) is in accordance with the crosslink density results. As mentioned, the absence of physical junctions (collagen triple helices) explains the highest biodegradability of HMDICG. The lowest values of M_c (i.e., the densest networks) were obtained for aldehyde crosslinked gelatins, which were slowest to biodegrade. The observed biodegradability of aldehyde crosslinked gelatin samples could be explained by the peculiarities of the crosslinking reactions[18] performed using the three aldehydes. Tabor et al.[18] showed that the order of increasing reaction rate for these three aldehydes is glutaric aldehyde > GO > FA. However, there is another factor affecting the network density, namely, the length of the crosslinking agent chain. Glutaric aldehyde has the longest and the FA has the shortest chains. For equal crosslinking agent concentrations and an identical mechanism of the crosslinking reaction (two aldehyde molecules involved in one crosslink formation[18]) the differences between the M_c values seems to be due to the differences in crosslinking chain length. The increasing order of biodegradability (GAG > GOG > FAG) is in accordance with this suggestion.

The addition of periphyton in the biodegradability experiments did not have a large effect on the process of biodegradation. Particularly in polluted environments, such as the lake and river waters in question, periphyton was formed after the first day and preferentially on the surfaces of the gelatin films.

The high values observed for the DA represent evidence for the facile assimilation of gelatin by periphyton microflora. The rapid increase in DA on the first day in all samples was due to the large amount of nutritive material. After complete exhaustion of the gelatin the enzyme activity decreased to its lowest level. The maximal values were reached in NG, ODEG, and BDG (i.e., in samples with a comparatively moderate biodegradability rate). In these cases the biomasses developed sufficiently rapidly and the enzymatic activity was pronounced in the presence of sufficient gelatin. The low activity for HMDICG could be due to rapid exhaustion of the nutritive material before complete development of the oxidative enzyme systems. The moderate enzymatic activity for FAG, GOG, and GAG could be due to the gradual assimilation of the gelatin. The development of DA was due to the growth of periphyton on the surfaces of the gelatin films, which allows microflora to recognize the modified gelatin as a nutritive protein source. It can be concluded that DA depends on the biodegradability and it could be used as a measure for biodegradation for modified gelatin and probably for other modified biopolymers as well.

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