

Synthesis and Characterization of pH-Sensitive and Biodegradable Hydrogels Prepared by γ Irradiation Using Microbial Poly(γ -glutamic acid) and Poly(ϵ -lysine)

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SYNOPSIS

Poly(γ -glutamic acid) (PGA) and poly(ϵ -lysine) (PL) solutions were used as components to prepare mixed hydrogels by γ irradiation. A PGA and PL mixed solution was crosslinked to form a hydrogel with specific water content (weight of absorbed water/weight of dry gel) of 10–100 when the 5 wt % solution of mixed polymer was exposed to γ radiation of 87 kGy dosage under N_2 atmosphere. The specific water content increased with increasing PGA content of the PGA/PL mixed gel. The influence of pH and salt concentration on equilibrium swelling was studied. A characteristic pH-sensitive swelling behavior was obtained using compositional changes of PGA and PL in the gel. PGA/PL 50/50 wt % mixed gel swelled in acid (pH < 4.0) and alkaline (pH > 6.0) conditions and was deswelled between pH 4.0 and 6.0 due to the ionic composition changes of the gel network. With an increase in the ratio of PGA to PL, the hydrogels showed increasing sensitivity to salt solutions (NaCl, Na_2SO_4 , and $CaCl_2$). In addition, degradation of PGA/PL gel by protease produced from *Aspergillus oryzae* was investigated at 40°C and pH 7.0. PL gel was degraded completely within 2 days. An increase in the ratio of PGA in the PGA/PL mixed gel led to a decrease in the degree of degradation as expected. Some subtle degradation changes were found in the 50/50 and 80/20 wt % (PGA/PL) gels that were degraded by only 3.5 and 3.8% by protease, respectively. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Recently, hydrogels have received much attention for applications as biomaterials, controlled release devices,^{1,2} and superabsorbent materials.³ Swelling properties of these hydrogels are important factors for each application. Changes in swelling behavior can affect the releasing patterns of bioactive materials in controlled release devices as well as the mechanical properties of the superabsorbent materials. Among many kinds of hydrogels, pH-sensitive polymer gels are potentially useful as biomedical materials for drug delivery. Park and Hoffman⁴ reported the pH- and/or temperature-sensitive hydrogel

based on poly(*N*-isopropylacrylamide), and Batich et al.⁵ studied the pH-sensitive copolymers based on styrene and 4- (or 2-) vinylpyridine. In addition, the preparation conditions, relevant properties, and applications of many pH-sensitive hydrogels have been well reported. Some pH-sensitive hydrogel systems have been formed by incorporating materials into ionizable polymer networks. Although many hydrogels such as pH-sensitive and water-superabsorbent hydrogels have been extensively reviewed with applications, in most cases chemical synthetic hydrogels have received the bulk of the attention due to their efficiency.^{6–8} In recent years, attention has been given to hydrogels prepared from naturally occurring polymers out of respect for environmental preservation. Thus, much work has been carried out on natural materials such as poly(amino acids) and polysaccharides.^{9,10} Moe et

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al.¹¹ studied some swelling properties of covalently crosslinked alginate gels. Choi and Kunioka^{12,13} also reported work on the crosslinking of poly(amino acids) such as microbial poly(γ -glutamic acid) (PGA) and poly(ϵ -lysine) (PL).

Microbial PGA is a water soluble and biodegradable polymer (MW 100,000–1,000,000), which is crosslinked to form a hydrogel by γ irradiation. It has a carboxyl group in its polyanion structure. Relevant studies on the optimization of fermentation, physical and biological properties, and applications of PGA have been well reviewed.^{14,15} Recently, some modifications of this naturally occurring polymer have been attempted for the preparation of hydrogels, and their swelling properties were reported. PGA hydrogels show high water sorption ability (PGA gel can absorb over 1400 times its weight in water) and it is possible to control the water sorption ability by changing the preparation conditions.

Microbial PL can also be crosslinked to form a hydrogel by γ irradiation. PL is a homopolymer of L-lysine consisting of 25–30 residues connected with α -carboxyl and ϵ -amino group linkages. It has a molecular weight of about 4000.¹⁶ PL is a water soluble, hydrodegradable, and biodegradable polymer. It is a cationic polymer with an amino group in its structure. It has antibiotic and antiphage functions and the ability to combine with organic pigments and metal ions such as copper.¹⁷ Formed PL gel has low water sorption ability (150 times) compared with other ionic hydrogels such as PGA and poly(acrylic acid) gel. PL gel has the advantage of little shrinkage when put in a salt solution. PGA and PL hydrogels exhibit different degrees of equilibrium and pH response patterns due to the differences in ionic composition and molecular structure.

In this study, a pH-sensitive, biodegradable hydrogel based on crosslinked PGA and PL natural polymers was developed and its swelling properties studied. These gels were prepared from a mixture of PGA and PL by γ irradiation in order to improve pH sensitivity, specific water content, and equilibrium swelling in a salt surrounding medium. Biodegradability of PGA/PL hydrogels by protease are also discussed.

EXPERIMENTAL

Materials

PGA fermented by *Bacillus subtilis* F-02-1 was obtained from Meiji Seika Kaisha, Ltd. (Japan). This was allowed to react with sodium hydroxide in

deionized water to form water soluble PGA at room temperature. After glass filtration and lyophilization, PGA powder was used to prepare the hydrogel. PL solution (solid content 27.1 wt %), produced by *Streptomyces albulus*, was obtained from Chisso Corporation (Japan). The number-average molecular weight, \bar{M}_n , of PGA and PL were about 5.5×10^5 , 4.0×10^3 , respectively. The average molecular weight was analyzed by gel permeation chromatography [poly(styrene sulfonic acid, sodium salt) standard used]. IR analysis indicated the presence of a carboxyl band at 1408 cm^{-1} in the PGA hydrogel. Methylene bands at 2858 and 2936 cm^{-1} and amide bands at 1550 and 1655 cm^{-1} in the PL gels were also observed. Elemental analysis was used to determine the composition of the prepared PGA/PL mixed hydrogel.

Preparation of PGA Hydrogel

Aqueous solutions of 5 wt % PGA and 5 wt % PL were mixed thoroughly and then poured into a glass bottle at room temperature. Crosslinking of these microbial polymer mixtures (2 mL) by γ irradiation was carried out in capped 10-mL glass bottles (1.5 cm diameter) under N_2 atmosphere. The glass bottles were irradiated with a ^{60}Co γ radiation system at a dose rate of 1.6 kGy/h at room temperature. The gels produced were washed and swollen in deionized water at 4°C for 1 week. Deionized water for gel swelling was changed once a day to remove any uncrosslinked PGA, PL, and residues that remained inside the gels.

Determination of Equilibrium Swelling

To determine specific water content, the swollen gel was transferred into a glass bottle after eliminating gel surface water, and was freeze-dried. The weight of water absorbed by the gel was calculated from the weights of the gel before and after freeze-drying. The specific water content was expressed by the following equation:

$$\text{specific water content} = [(W_0 - W_d)/W_d]$$

where W_0 and W_d were the gel weight before freeze-drying and the weight of dried gel, respectively.

Twenty-five millimoles of citric acid/ Na_2HPO_4 buffer was used for pH 3.0–8.0 and the ionic strength was adjusted with KCl to 25 mM (McIlvaine buffer¹⁸); and 25 mM NaOH/HCl was used for the remaining pH values. A total of 0.5 g of PGA/PL mixed gels was immersed in 50 mL of buffer solu-

tions ranging from pH 2.0 to 12.0 and in 50 mL of NaCl, Na₂SO₄, and CaCl₂ solutions of between 0 and 3 wt % concentrations to observe swelling equilibrium after storage at 4°C for 1 week. Thereafter the degree of swelling of the gel was determined gravimetrically at room temperature after removing gel surface water with filter paper.

Enzymatic Degradation

Protease A produced by *Aspergillus oryzae* was obtained from Amano Pharmacy Ltd. (Japan). The optimum pH of this enzyme is 7.0 and it has 10,000 U/g. Buffers at pH 7.0 were prepared by using NaH₂PO₄ and Na₂HPO₄ (25 mM) in order to investigate enzymatic degradation of the PGA/PL mixed gel.

Enzymatic degradation studies with protease for PGA/PL mixed hydrogels were performed at 40°C. PGA/PL hydrogel samples, 1.5 g, were placed in 50-mL glass bottles; 20 mL buffer solution with enzyme (6 mg) was then added to the samples. The degree of degradation of the hydrogel after enzymatic reaction for 48 h at 40°C was measured using a total organic carbon amounts analyzer (Shimadzu TOC 5000).

RESULTS AND DISCUSSION

Preparation of Hydrogels from PGA/PL Polymer Mixture

The equilibrium swelling of polymer networks is a result of the balance of osmotic forces determined by the affinity to the solvent and the network elasticity. Crosslinked polymers with high equilibrium swelling in water or aqueous solutions can be based on polymers with high hydrophilicity and flexibility. The degree of swelling of hydrogels can be controlled by changing the crosslink density in the network when the gels are prepared. The crosslinking conditions of PGA and PL by γ irradiation and the swelling behavior of their resultant hydrogels were reported previously.^{12,13} When a PGA aqueous solution was irradiated by γ irradiation, PGA was crosslinked to yield high water sorption materials with specific water contents (weight of absorbed water/weight of dry gel) of 100–1400 depending on the preparation conditions, that is, γ -irradiation dose (> 20 kGy) and PGA concentration (2–10 wt %). PL was also crosslinked by γ irradiation under the conditions of 1–7 wt % concentrations and an irradiation dose of over 70 kGy, but their specific

water content was lower (20–160) than that of the PGA hydrogel.

Aqueous PGA/PL polymer mixed solutions of 5 wt % concentration were crosslinked when exposed to γ radiation under N₂ atmosphere. PGA and PL are polyanion and polycation, respectively, but were not precipitated under these preparation conditions. Upon irradiation with 90 kGy, the water-soluble microbial polymer mixture was converted into a series of water-swallowable hydrogels with various specific water contents. The composition ratio of PGA and PL calculated from the ratio of C/N by elemental analysis in the obtained hydrogel was almost coincident to the polymer composition of the irradiated solution as shown in Table I. From this result it can be seen that the composition of hydrogel produced as a result of γ irradiation reflected the composition of the starting materials. Preferential crosslinking of PGA or PL was not observed.

Figure 1 depicts the effect of composition ratio on the specific water content of hydrogels of 5 wt % aqueous PGA and PL mixed solution. As shown in Figure 1, PGA and PL exhibited different specific water contents of 90 and 26, respectively. The specific water content of PGA/PL hydrogel increased upon increasing the ratio of PGA in the PGA/PL polymer mixture. This was caused by the difference of ionic group composition in the gel network, that is, the carboxyl and amino group. Tanaka et al.¹⁹ reported that ionization of the polymer network plays an essential role in swelling behavior. In deionized water (pH 7.0), the PGA molecule has an anionic carboxyl group (—COO⁻) but an almost nonionic amino group (—NH₂) is observed in the PL molecule. The overall equilibrium swelling is thus mainly dependent on the PGA ionic group content in gels because the Donnan effect can be considered as the main driving force for the swelling of these ionizable PGA/PL mixed gels. Upon increasing the PGA content, the concentration of carboxyl ions and thus, the charge density in the PGA/PL hydrogel is increased. This leads to an increase in the extent of swelling due to weaker ionic swelling pressure. The specific water content of the PL gel with no carboxyl group is higher than that of some mixed hydrogels such as 10/90 and 20/80 (PGA/PL) formulation gels. When the PGA content is over 30 wt % in mixture gels, the amino groups are assumed to exhibit a negligible effect on the concentration of the ionizable groups of the resulting gel network in deionized water. It is believed that a fraction of the ionic groups of PL remain in the gel network, and can undergo an ionic interaction with the carboxyl groups of PGA. This ionic interaction

results in a reduction of the overall netcharge, that is, a decrease in the ionic repulsive force and an enhancement of elastic forces (flexibility) in the gels. It is apparent that the specific water contents of 10/90 and 20/80 wt % (PGA/PL) formulation gels are decreased more than that of PL as a result of these changes in net charge and elastic forces. It can thus be seen that the crosslinking density and ionic properties of gels are changed by the preparation conditions. Gels showing different specific water content may be prepared.

Formation of hydrogels by means of radiation can be explained as a result of radical interaction. As a consequence of the interaction of γ irradiation with matter, reactive radicals such as hydroxy radicals are formed. These radicals then undergo various mutual reactions with PGA, PL, or the surrounding medium. As a result of these reactions, reactive free radicals at methyne carbons of PGA and PL molecules are generated by the cleavage of the C—H bonds. Subsequently, it seems that intermolecular radical recombination may lead to stable crosslinking formation between polymer chains. When the amount of these new crosslinking bonds is sufficiently great, a gel is formed in the system. The detailed crosslinking mechanism and structure of the PGA/PL hydrogel will be studied in the future.

Swelling Equilibrium in Solutions of Various pHs

In polyelectrolyte hydrogels, the effect of the ionic composition of the surrounding medium is markedly pronounced. Swelling or deswelling in response to changes of ionic strength and pH are typical phenomena of polyelectrolyte gels. The effect of the ratio of PGA/PL on the swelling behavior of PGA/PL

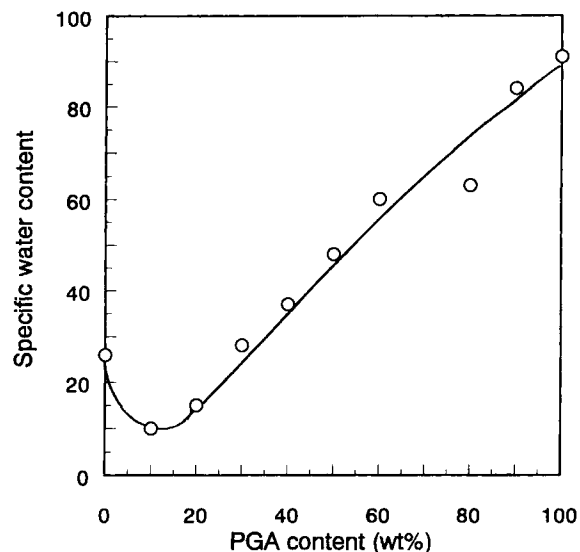


Figure 1 Specific water contents of poly(γ -glutamic acid) (PGA) and poly(ϵ -lysine) (PL) mixed hydrogels as a function of PGA content. These hydrogels of 5 wt % aqueous PGA and PL mixed solutions were irradiated with 90 kGy γ irradiation.

mixed gel prepared with a 90-kGy irradiation dose is shown in Figure 2. The specific water contents for gels with ratios of 100/0, 80/20, 50/50, 20/80, and 0/100 wt % (PGA/PL) were measured as a function of pH. As shown, the specific water content of the swelled PGA gel was about 67–80 over pH 5.0, and under 18 below pH 4.0. At a pH near 5.0, the specific water content abruptly decreased to 18 for PGA gel. This was in agreement with previous reports that hydrogels containing anionic groups exhibit an abrupt phase transition at a particular pH.¹² It was also previously reported that PGA hy-

Table I Production of Poly(γ -glutamic acid) (PGA) and Poly(ϵ -lysine) (PL) Mixed Hydrogels by γ Irradiation

Sample No.	Polymer Composition (wt %) ^a		Elemental Analysis (wt %)			Gel Composition (wt %) ^b	
	PGA	PL	C	H	N	PGA	PL
1	100	0	35.4	4.8	8.7	100	0
2	80	20	39.2	5.5	10.4	79	21
3	50	50	43.9	6.7	13.3	45	55
4	20	80	48.7	8.1	16.8	17	83
5	0	100	52.7	9.3	19.8	0	100

5 wt % PGA and 5 wt % PL aqueous solutions were mixed. Hydrogels were irradiated with 90 kGy γ irradiation under N_2 atmosphere.

^a Polymer composition of irradiated solution.

^b Gel composition of the obtained hydrogel is the composition ratio of PGA and PL calculated from the ratio of C/N by elemental analysis.

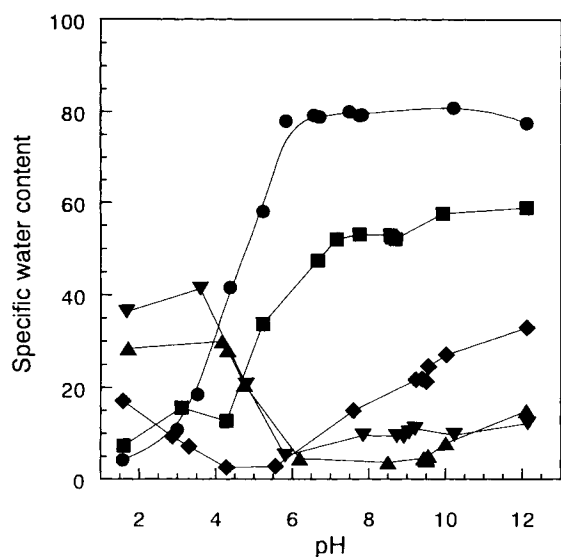


Figure 2 Swellings of PGA/PL mixed hydrogels (90 kGy) in aqueous solutions of various pHs. (●) 100/0; (■) 80/20; (◆) 50/50; (▲) 20/80; (▼) 0/100 wt % (PGA/PL) as shown in Table I.

drogels swelled above pH 4.0, but shrank below pH 4.0 due to the incorporation of negatively charged carboxyl groups into the polymer network. The PL gel however showed the opposite pH response. The specific water content was about 40 below pH 4.0, and 10 above pH 6.0.¹³ With a decrease of PGA in the PGA and PL mixed formulation, a gradual increase of specific water content was observed below pH 5.0. It is clear that this increase is due to the increase of PL content in the mixed gel because the increase of specific water content in low pH (<5.0) was a characteristic property of PL gel. In the case of 50/50 wt % (PGA/PL) formulation gel, the swelling response to pH was characteristic (Fig. 2). Swelling occurred at low pH (<4.0) and also in the pH range 6.0–12.0; however, deswelling occurred in the range of pH 4.0–6.0. From the above observations, we may infer that 50/50 wt % (PGA/PL) gel contains both carboxyl and amino groups, and thus shows characteristic swelling behavior described above. This is to be expected from the variation of ionic composition with pH as shown in Figure 3.

The presence of carboxyl groups in the structure of PGA resulted in swelling in the pH region over 5.0 due to ionic repulsion of unprotonated carboxyl groups (negatively charged, COO^-). Below pH 4.0 deswelling occurred due to protonation of the carboxyl groups (no charge). In the case of PL, the increase of equilibrium swelling with decrease in pH is due mainly to the presence of protonated amino groups (NH_3^+). As pH is lowered at low ionic

strength (25 mM), the NH_2 group is protonated to NH_3^+ . As a result of this change, the internal osmotic pressure and mutual repulsion of charged groups are increased, thus increasing the degree of swelling at low pH (<4.0). Gels with networks containing both carboxyl and amino groups can be prepared by γ irradiation as a result of mixing the two polymers with oppositely charged structures as shown in Figure 3. Because the charge state of the ionic groups varies with pH, the dominant charges in the PGA/PL mixed gel are the protonated amino group [Fig. 3(a)] and the unprotonated carboxyl group [Fig. 3(c)] at pH < 4.0 and pH > 6.0, respectively. In this pH region, the PGA/PL gel is swelled due to the increase in ionic swelling pressure. In contrast, at pH 4.0–6.0, most of the ionic groups are absent due to protonation of the carboxyl group and deprotonation of the amino group as shown in Figure 3(b). The gel is thus deswelled in this region. For

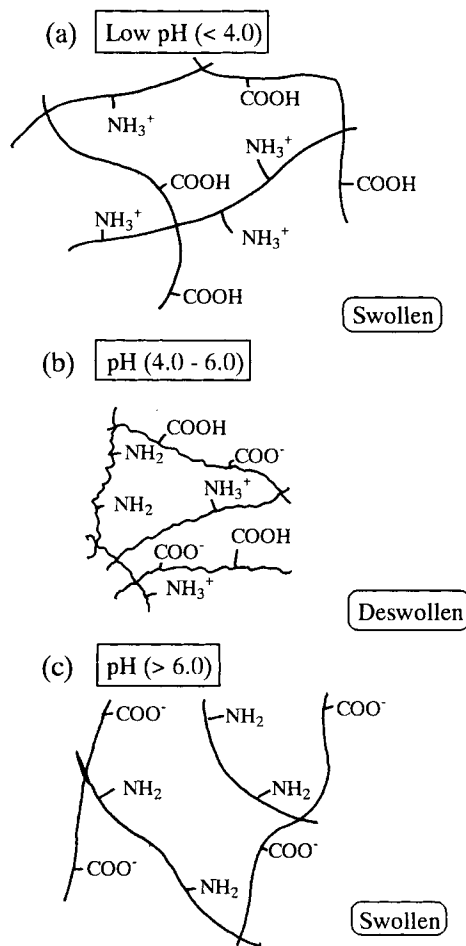


Figure 3 Hypothetical sketch of swelling-deswelling molecular structure of 50/50 wt % (PGA/PL, 90 kGy) hydrogel in aqueous solutions at < 4.0, 4.0–6.0, and > 6.0 pH region.

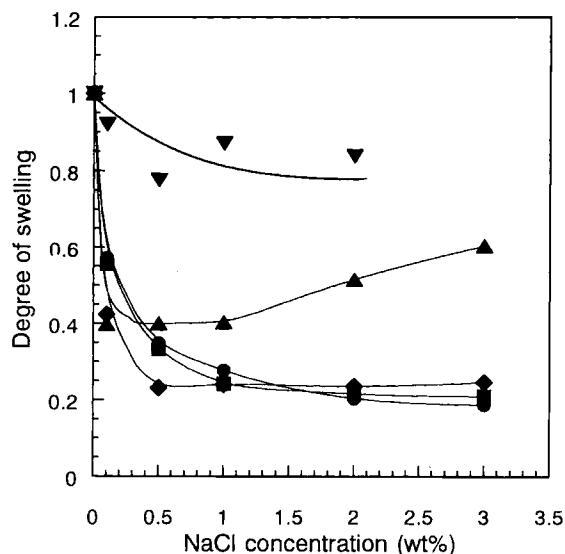


Figure 4 Swelling of PGA/PL mixed hydrogels (90 kGy) in aqueous solutions of various NaCl concentrations. (●) 100/0; (■) 80/20; (◆) 50/50; (▲) 20/80; (▼) 0/100 wt % (PGA/PL) as shown in Table I.

this reason, it is believed that the 50/50 wt % (PGA/PL) gel shows characteristic equilibrium swelling dependent on pH and that the swelling transition is induced by the ionization of the mixed gels. As the ionic groups of PGA in mixed gel were decreased, a broad transition for curve of equilibrium swelling as a function of pH was observed. Although PGA/PL mixed gels show characteristic pH sensitivity, it is necessary to improve on the broad transition for future applications.

Effect of Salt Concentration on Swelling

In most cases, the swelling of polyelectrolyte hydrogels depends only on ionic strength of the solution and not on the size and nature of the ions. PGA gels showed high water sorption ability, and deswelled markedly when put in ionic solution as reported previously. The degree of deswelling of PL hydrogels, however, was less than that of normal polyelectrolyte hydrogels in ionic solution. The main purpose of preparing hydrogels from mixtures of PGA and PL is to improve the swelling behavior in salt solution for applications such as superabsorbent materials and controlled release devices.

Figure 4 shows the swelling equilibrium of PGA/PL hydrogel as a function of NaCl concentration. A marked volume decrease was observed in PGA gel with increasing salt concentration in the surrounding solution as reported previously. Apart from the 20/80 wt % (PGA/PL) hydrogel, the degree of

swelling of other PGA/PL mixed gels was decreased abruptly from 1.0 in deionized water to 0.22 in NaCl solutions. It is well known that the Donnan effect is considered as the main driving force for the swelling of polyelectrolyte gels. Therefore, with an increase in salt concentration outside the gel, ionic swelling pressure is decreased, resulting in a decrease of gel volume. The swelling data of the 80/20 and 50/50 wt % (PGA/PL) mixed gels show similar salt concentration dependence to that for the PGA gel. It appears that the overall equilibrium swelling behavior of these gels is dominated by the PGA molecules in the gel, the change on PGA being greater than that on PL. However, the 20/80 wt % (PGA/PL) mixed gel exhibits an unusual swelling pattern in salt solutions. Upon increasing the salt concentration over 0.5 wt %, the degree of swelling was increased.

As shown in Figure 5, when PGA/PL gels were put in Na_2SO_4 solution, the gels deswelled in the same manner as in the NaCl solution. However, when the gels were put in 0.1 wt % CaCl_2 solution (Fig. 6), the degree of swelling was decreased more to about 0.1 compared with that of 0.4–0.6 in 0.1 wt % monovalent salt ion solutions. Ions present in the solution strongly suppress swelling, and thus the higher the ion charge, the lower the concentration necessary to ensure the same degree of deswelling. Exceptions include H^+ and other ions giving stable associates with the ionized network-fixed (usually carboxylate) groups. It can be seen from above that

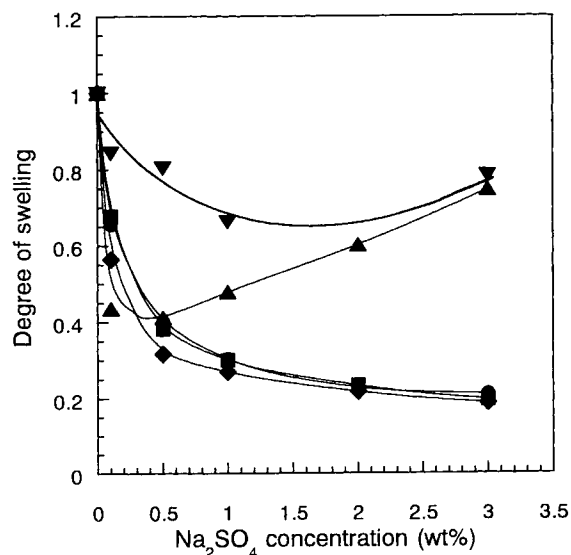


Figure 5 Swelling of PGA/PL mixed hydrogels (90 kGy) in aqueous solutions of various Na_2SO_4 concentrations. (●) 100/0; (■) 80/20; (◆) 50/50; (▲) 20/80; (▼) 0/100 wt % (PGA/PL) as shown in Table I.

the effect of salt ions on the swelling behavior of 50/50, 80/20, and 100/0 wt % (PGA/PL) gels is profound, but that there is little effect on the swelling of 20/80 and 0/100 wt % (PGA/PL) gels.

Enzymatic Degradation

Most crosslinked polymers with high equilibrium swelling in water are based on synthetic polymers with high hydrophilicity and flexibility, often in combination with the polyelectrolyte properties. Natural polymers like cellulose, starch, lignin, dextran, etc. do not form high water sorption hydrogels, so it is necessary to modify these natural polymers with hydrophilic synthetic polymers. Almost all hydrogels with high water absorbent capacity prepared with synthetic polymer are difficult to degrade naturally, which is an environmental problem all over the world. *Protease A* produced by *A. oryzae* is specific toward the amide bonds of polypeptides and poly(amino acid) such as PL. Because hydrogels prepared from PL have the same degradable bond as PL, the gels can be degraded by *Protease A*. The degree of degradation of PGA/PL mixed gels by protease are listed in Table II. Pure PL gel was degraded completely within 2 days. PGA gel was not degraded because it had a different molecular structure that cannot be degraded by this type of protease. The degree of degradation of 20/80 wt % (PGA/PL) gel was 63.6% in protease solution. PGA/PL gels, 50/50 and 80/20 wt %, were degraded by only 3.8

Table II Enzymatic Degradation of PGA/PL Mixed Gels by *Protease A* at 40°C and pH 7.0 for 48 h

Gel Composition (wt %)	Degree of Degradation (%)
(PGA/PL)	
100/0	0
80/20	3.5
50/50	3.8
20/80	63.3
0/100	100.0

Protease A (6 mg) in a buffer (20 mL). The mixed gels were prepared with 90 kGy γ -irradiation dose. Degree of degradation was calculated from the total organic carbon amounts of filtrated solutions.

and 3.5%, respectively. When the PGA content is over 50 wt % in the PGA/PL mixed gel, the degree of degradation is decreased abruptly. The differences between the PL amount of mixed formulation and the enzymatic degradation amounts of gels were considered as corresponding to the modification of substrate with PGA and other by-products of γ irradiation. We also observed that PGA gel was responsible for biodegradation in soil, and thus we are trying to isolate the microorganism that excreted the PGA degradation enzyme. These results and the degradation mechanism of PGA/PL hydrogel using *Protease A* and the PGA degradation enzyme will be presented in a forthcoming article.

In summary, new pH-sensitive hydrogels that can be degraded by protease and some microorganisms were prepared in aqueous solution of microbial PGA and PL by γ irradiation. Depending on the pH of the surrounding medium and mixed formulation of polymer, the hydrogels showed characteristic equilibrium swelling. In addition, this series of pH-sensitive gels is degraded by enzymes and the rate of degradation can be controlled by changing the hydrogel composition. These pH-sensitive and biodegradable hydrogels will be useful as controlled release devices and matrices for enzyme immobilization. Optimization of the formulation, degradation *in vivo*, strength, and enzyme or drug loading of the gel will be a subject of further studies.

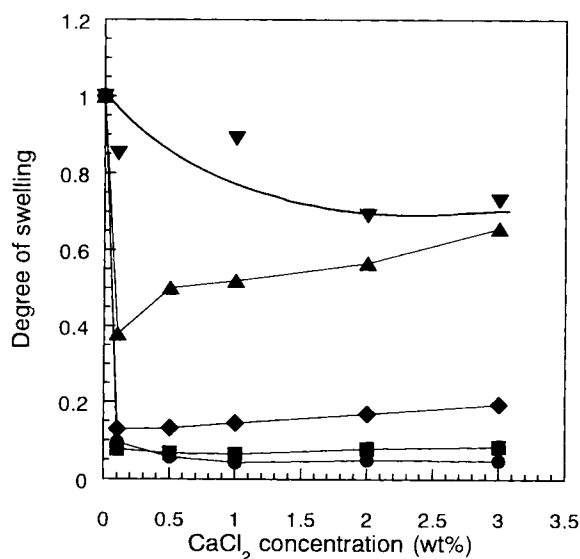


Figure 6 Swelling of PGA/PL mixed hydrogels (90 kGy) in aqueous solutions of various CaCl_2 concentrations. (●) 100/0; (■) 80/20; (◆) 50/50; (▲) 20/80; (▼) 0/100 wt % (PGA/PL) as shown in Table I.

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