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Preparation and Swelling Characteristics of Hydrogel from Microbial Poly(γ-glutamic acid) by γ-Irradiation

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Abstract: Microbial hydrogel was prepared by γ-irradiation of poly(γ-glutamic acid) (PGA) which was produced from *Bacillus subtilis* BS 62 and it's physico-chemical characteristic was examined. The hydrogel, prepared from 10% PGA with the dose of 48 kGy, was swollen up to 1,370 times of specific water content as dry weight basis. The hydrogels obtained above the dose of 48 kGy appeared to have higher compressive strength but lower specific water content. The period to reach a swelling equilibrium for the hydrogel in deionized water at the temperature range of 4 to 45 °C was about 10 h. The swollen hydrogel was shrunk in ionic solutions with the increase of ionic strength, and the rate of shrinkage was greater in calcium chloride solution than in sodium chloride. Specific water content of the hydrogel was quickly decreased at 80 °C, showing a thermally hydrodegradable property.

Keywords: poly(γ-glutamic acid), PGA, *Bacillus subtilis* BS 62, γ-irradiation, hydrogel, biopolymer.

Introduction

In recent years, considerable attention has been focused on research pertaining to hydrogels that are able to alter their volume and properties in response to environmental stimuli such as solvent, temperature, pH and ionic strength.[1] Hydrogels that can absorb several thousand times of water of their dry weight are useful for controlled release system, functional carrier and prosthetic material.[2,3] Attempts have been made to develop new hydrogels, such as poly(vinyl methyl ether),[4] poly(vinyl alcohol)[5] and poly(*N*-isopropylacrylamide) hydrogels[6] by using the technology of irradiation, repetitive freezing or chemical cross-linking. However, chemical methods generally involve different reactive species, initiators, and catalysts.[7] Hydrogels formed by γ-rays are a result of recombinance among macroradical which is a kind of free radical formed by the irradiation. Some applications have already found in free-radical-induced hydrogel of poly(vinyl alcohol) and poly(acrylic acid) based on γ-ray irradiation.[5]

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Biodegradable hydrogels related to controlled drug delivery have been widely investigated,[8] because degradation of the gel matrix allows a release of entrapped molecules. Biodegradable polymers such as poly(γ-glutamic acid) (PGA) and ε-polylysine (PL) which produced extracellularly by certain microorganisms have been focused in recent years.[9]

PGA is a polymer contained in the traditional Korean food, Chungkookjang,[10] and Japanese food Natto[11] that are made from soy beans fermented by the strains of bacilli. PGA is polymerized from D- and L-glutamic acid, and connected between α-amino and γ-carboxyl groups, with a molecular mass as widely dispersed. Hydrogels originating from microbial PGA can be prepared by γ-irradiation.[1]

PGA hydrogel prepared by Choi and Kunioka[1] showed high sorption ability and varied in its equilibrium swelling characteristics according to the surrounding solution. The equilibrium swelling behavior in various aqueous solutions was investigated as a function of pH and concentration of electrolytes. These PGA hydrogels exhibited reduced swelling when exposed to low pH or electrolyte solutions.

In the previous paper, the PGA which produced by a Chungkookjang strain, *Bacillus subtilis* BS 62 was charac-

terized.[10] In this work, we report on the preparation of hydrogels from the PGA using γ-irradiation and on their swelling characteristics by various parameters such as pHs, electrolytes, and heating in aqueous solution, for the potential application in biomedical fields.

Experimental

Production and Purification of PGA. The bacterium (*Bacillus subtilis* BS 62, KACC91064) was grown on agar plates from which the most mucoid colony was selected to inoculate in the fermentation medium and grown for 6 d at 37 °C.[10] After fermentation, the culture broth was centrifuged at 6,000 × *g* for 15 min to remove the bacterial cells. The PGA from the culture broth was purified by precipitation in ethanol and subsequently lyophilized, and the weight average and number average molecular weight of the purified PGA were 6.24×10^5 and 2.14×10^5 Da, respectively, when they were measured by gel permeation chromatography using poly(ethylene oxide) standards with narrow polydispersity.[10]

Synthesis of the PGA Hydrogel. High purity PGA hydrogel having diverse structures was synthesized by the method of Choi and Kunioka.[1] Gelation of PGA was performed by ^{60}Co irradiation in aqueous solution. PGA solution was irradiated by the γ-rays to construct crosslink structure among the molecules. Aqueous solutions from 2 to 10 w/v% were poured into glass vials (H 5.5 cm × ϕ2.0 cm), in which N_2 gas were filled. The bottles were irradiated with ^{60}Co γ-ray at the dose rate of 1.6 kGy h^{-1} for 5 d at room temperature. And then the hydrogels obtained were swollen in deionized water by exchanging water every day for one week at 4 °C to remove any unreacted molecules of PGA. Networked and swelled PGA hydrogels were filtered through a sieve of 80 meshes,[12] and lyophilized.

Specific Water Content. Specific water content was determined by weighing the swollen gel content at room temperature after sieving the surface water of the hydrogel through a sieve of 80 meshes and calculated using following equation:

$$\text{Specific water content} = (W - W_d)/W_d \quad (1)$$

Here, W_d and W are the weight of dried hydrogel and swollen hydrogel in deionized water at 4 °C, respectively.

Swelling and Shrinking of Hydrogel. PGA hydrogels were placed in various buffer solutions ranging from pH 2.5 to 11.0 at 4 °C, and in NaCl, Na_2SO_4, $CaCl_2$ or glucose solutions between 0 and 3.0 wt% concentrations to observe the swelling equilibrium. Each of 25 mM McIlvaine (citrate/Na_2HPO_4) buffer was prepared for the pH range of 2.5 to 8.0, and 25 mM sodium borate-NaOH buffer was used for the remaining pH values. The gel weight in the equilibrium state was determined by weighing the hydrogel after removing surface water through a sieve of 80 meshes. Degree of swelling was calculated from the ratio of the remaining gel weight to initial amount of PGA hydrogel.

Hydrolytic Degradation of Hydrogel. Heat stability of PGA hydrogel was estimated at 40 and 80 °C. The hydrogel was placed in a glass bottle with deionized water, and heated. Then the wet hydrogel samples were taken from the bottle by appropriate interval and filtered though a sieve of 80 meshes. By weighing the remained gel on the sieve, the degree of swelling was calculated for estimating the thermal hydrolytic degradability.

Hardness Measurement. The hardness of the PGA hydrogel was measured by the texture analyzer, TA-XT2 (England) with Texture Export Software (Stable Micro System Ltd., Version 1.22). The compression plunger descended at a rate of 1 mm/s to compress the sample.

Results and Discussion

Hydrogel Preparation by γ-Irradiation. PGA obtained from *B. subtilis* BS 62 was used to prepare the hydrogel. When the aqueous solution of PGA was irradiated by ^{60}Co γ-ray with the dose rate of 1.6 kGy h^{-1} for 5 d, various types of hydrogels having weak or elastic feature were formed (data not shown). As shown in Figure 1, the hydrogels were produced from 6 and 10 w/v% solution of PGA in deionized water. For gelation of PGA under above conditions, the aqueous solution of 10% was more effective than that of 6.0%. The specific water content of the hydrogel prepared from 10% PGA solution was maximal, 1,370 times as dry

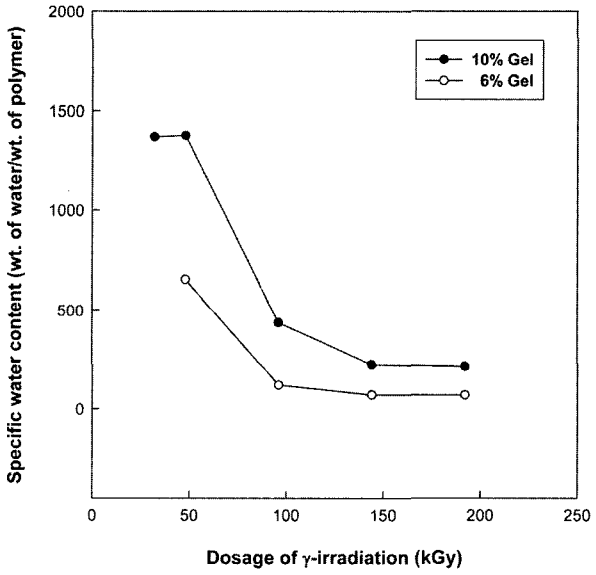

Figure 1. Changes in specific water contents during the formation of PGA hydrogels by irradiation. PGA aqueous solutions, at 6 and 10 w/v%, were irradiated by ^{60}Co γ-ray for 120 h at the dose rate of 1.6 kGy h^{-1}.

basis, at a dose of 48 kGy. And with irradiation at a dose of 48 to 100 kGy, it decreased in steep gradient, and above 100 to 192 kGy in gentle gradient, showing 440 and 220 times as dry basis at a dose of 96 and 144 kGy, respectively.

Generally, γ-ray-induced cleavage of the C-H bonds may generate free radicals at the methyne carbons of PGA, and the subsequent intermolecular radical combination may lead to crosslinking.[1] Water absorption ability may be due to the increment of crosslink density inside the PGA chains.

The hardness of hydrogels formed by γ-ray irradiation is shown in Figure 2. The hardness of both hydrogels from 6 and 10 wt% solutions was low at the dose of 48 kGy and moreover gelation was not occurred below 30 kGy. Gel strength increased in proportion to the dosage of γ-rays upto 144 kGy, wheresas the specific water content decreased. The textural units of hardness reached to 90 at the dosage of 144 kGy in 10% PGA solution and 67 in 6% PGA solution.

The specific water content of 10% hydrogel was higher than that of 6% hydrogel, suggesting that the numbers of PGA molecules in 6% solution are not sufficient to construct crosslinking structure, and the density of crosslink inter PGA molecules is at low level. Hydrogel was not formed below 30 kGy dosage, which implies that the minimum dosage value to construct hydrogel is above 30 kGy. Conclusively, to obtain high specific water content of the hydrogel formed by γ-irradiation using the PGA from *Bacillus subtilis* BS 62, the dosage of 48 kGy in the 10% solution is recommended.

The Swelling Changes at Various Temperatures. To compare the changes in swelling according to the temperature, the hydrogel from 10% PGA solution was swelled in deionized water (pH 7.0) and subsequently adjusted to various temperatures such as 4, 25, 35 and 45 °C (Figure 3). As shown in Figure 3, swelling of the hydrogel reached to equilibrium within 10 h at all kinds of temperature, and after that only negligible change was observed. Moreover the time taken to reach the swelling equilibrium in deionized water at 25 °C was the shortest than those of others.

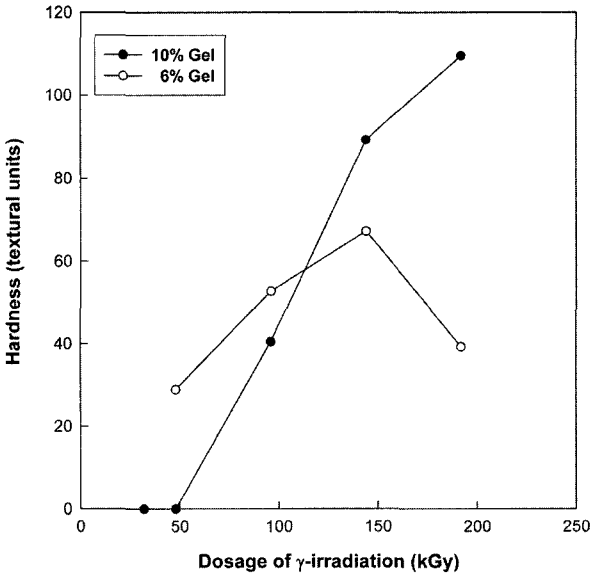

Figure 2. Change in hardness during the formation of PGA hydrogels by irradiation. Notes refer to Figure 1.

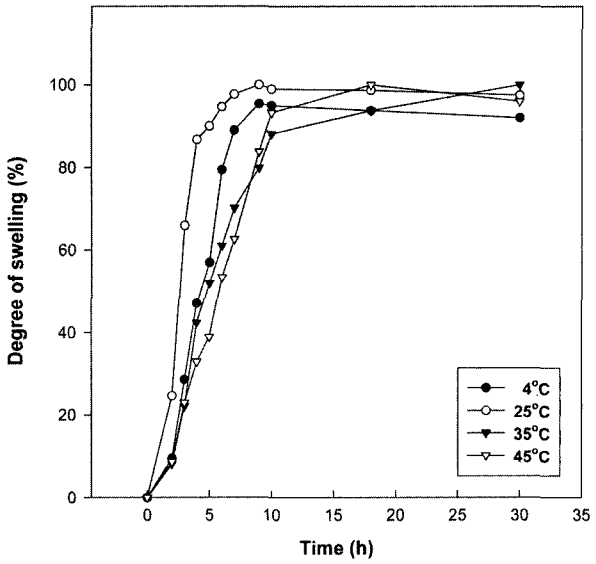

Figure 3. Change in degree of swelling of the PGA hydrogels in deionized water as a function of time. The hydrogels, prepared from 10 w/v% PGA solution by γ-irradiation at the dose of 48 kGy, were examined.

Effects of Salts. Generally, polyelectrolyte hydrogels like PGA undergo continuous or discontinuous capacity change with surrounding environmental parameters such as, ions, temperature, and solvent etc. Because swelling of hydrogel is dependent on solution's ion strength, and the main factor for swelling is the concentration and atomic valence of ions in the solution. In Figure 4, swelling and shrinking patterns of 10% PGA hydrogel made by γ-irradiation are investigated as affected by the presence of salts, NaCl, Na_2SO_4, and $CaCl_2$, or glucose as a non-electrolyte. PGA hydrogel shrunk even in all electrolyte solutions of 0.01% concentration within 30 min, and shrunk below 10% of the fully swollen hydrogel in deionized water at 0.5% concentration. The swelling rates decreased, and maintained a parallel level despite of increasing salt concentration upto 3.0%. Because the Donnan effect[13] is considered as the main driving force for the swelling of PGA hydrogel, it was found that with an increase of the salt concentration outside the gel, ionic swelling pressure decreased, and that lead to a decrease in gel volume.

When the shrunken PGA hydrogel was transferred into deionized water, inner ions of the hydrogel were partially released into deionized water. At the same time, the deionized water outside of hydrogel was absorbed into hydrogel, and therefore the swelling reached to a 90% level of initial

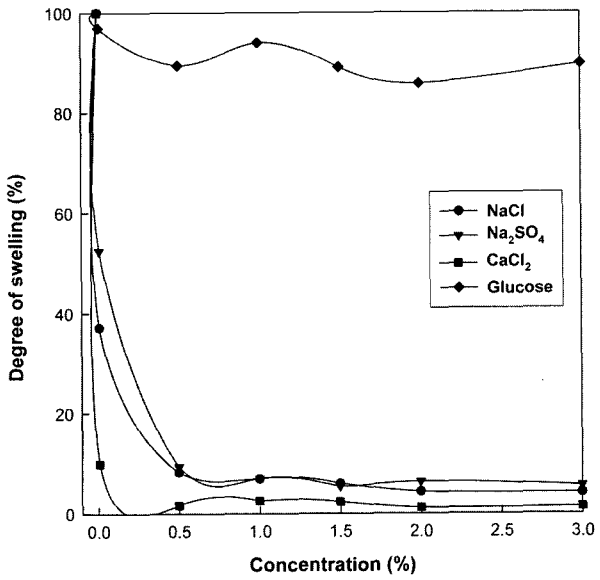

Figure 4. Change in degree of swelling of the PGA hydrogels in various solution as a function of ionic strength. Notes refer to Figure 3. ●, NaCl (1:1 electrolyte); ▼, Na_2SO_4 (2:1 electrolyte); ■, $CaCl_2$ (1:2 electrolyte); ◆, glucose (non-electrolyte).

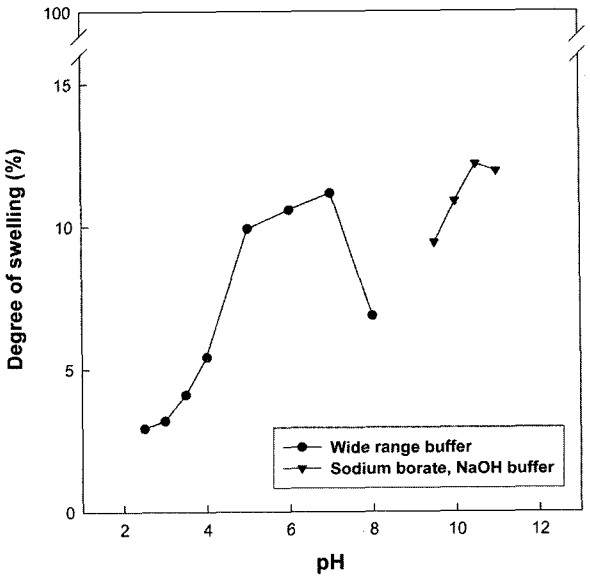

Figure 5. Change in degree of swelling of the PGA hydrogels as a function of pH at constant ionic strength (25 mM). Notes refer to Figure 3. ●, Wide range buffer (pH 2.5-8.0); ▼, Sodium borate, NaOH buffer (pH 9.5-11.0).

equilibrium status in 2 h, and was completed in 4 h (data not shown). Generally, it is known that the shrinking speed was faster than swelling speed.[7]

Shrinking of PGA hydrogel by Ca^{2+} ions was faster than that of Na^+ ion. The swelling rate decreased to 53, 38, and 10% of the swollen gel in 0.01% of Na_2SO_4, NaCl, and $CaCl_2$, respectively. Ions such as H^+, Ca^{2+}, and Na^+ could shrink hydrogel by connecting each electric charge. The larger the ion's atomic valency, the lower is the ion's concentration required to shrink the hydrogel. In other word, divalent cation is more effective to neutralize hydrogel electrically than monovalent cations, therefore, Ca^{2+} ion can shrink the hydrogel at half the concentration to that of Na^+ ion. The shrinking of PGA hydrogel fainted in non-electrolyte glucose solution, and thus transition phenomenon did not happen in the network. Likewise, PGA hydrogel could also characterize swelling and shrinking, if there were changes in the surrounding environmental condition, because it could differ in the properties according to type of ions and their concentrations in the solution.

To optimize the strength of hydrogel prepared from biopolymer, modified polypeptide that contain γ-benzyl glu-tamic acid as the common structural backbone was reported.[14] Modification of structural attributes by random copolymerization of the D- and L-isomers of γ-benzyl glutamic acid produced poly(γ-benzyl- D,L-glutamic acid).

Effect of pH. The swelling-equilibrium of polymer such as found in PGA hydrogel was affected by the property of network caused by elasticity or osmotic pressure. It was reported that PGA hydrogel having fixed electrical charges in the structure was influenced by pH of the surrounding solution due to the systematic property.[1] It is shown in Figure 5 that the properties of swelling and shrinking of the hydrogel could be estimated by changing the pH of hydrogels from 2.5 to 11.0. The hydrogel was shrunken in acidic range markedly below pH 5.0. In the pH range 5.0 to 7.0 it maintained a constant volume, and the highest swelling rate was found at pH 10.5. Based on the result of Donnan equilibrium,[13] PGA hydrogel in acidic pH range has a low swelling status due to decrease in the density and osmotic pressure that fixes the electrical charges in the hydrogel continuously. Choi[15] reported that the properties of the hydrogel mixed with PGA and PL could be shown in response of hydrogels according to the pH changes.

Thermal Hydrolytic Degradability. The temperature has been known as the important factor in swelling of the hydrogel, and Kunioka and Choi[4] studied the thermal hydrolytic degradability of hydrogel prepared from microbial PGA and poly(ε-lysine) by γ-irradiation. Hara *et al.*[12] reported the enzymatic degradation of crosslinked PGA hydrogel by the enzyme from strain pH-4, which was isolated from a contaminated PGA hydrogel kept in an open environment.

As shown in Figure 6, changes in swelling of PGA hydrogels were related to the temperature both at 40 and 80 °C. The hydrogel was unstable at 80 °C, and the swelling rate increased to two folds for 30 min and then quickly decreased to below 10% of the swollen hydrogel, suggesting that the hydrogel could have a thermally hydrodegradable property.

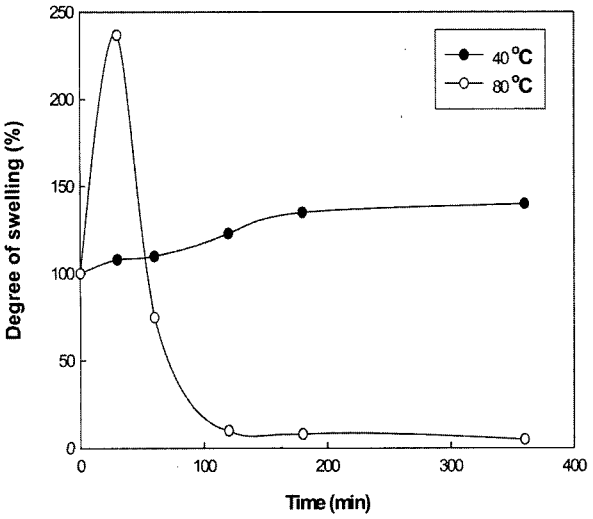

Figure 6. Change in degree of swelling of the PGA hydrogels in deionized water as a function of temperature. Notes refer to Figure 3.

Conclusions

Microbial PGA hydrogel having hydrodegradable characteristics was prepared using 10 w/v% PGA aqueous solution by means of γ-irradiation with dose rate of 1.6 kGy h^{-1} for 30 h, and its specific water content was maximal, 1370 times as dry weight basis. The hydrogel swelled in deionized water (pH 7.0) and reached to equilibrium within 10 h at various temperatures such as 4, 25, 35, and 45 °C. When the swollen hydrogel was put into 0.01% of Na_2SO_4, NaCl, and $CaCl_2$ solutions, the specific water content decreased to 53, 38, and 10% of the swollen hydrogel, respectively, suggesting that the hydrogel have a very sensitive response for the electrolytes. Choi *et al.*[1] reported that the specific water content of PGA hydrogel by their own preparation from *Bacillus subtilis* F-02-1 decreased within 10 min from 100 to 65, 58, and 10 for 0.1 w/v% of Na_2SO_4, NaCl, and $CaCl_2$, respectively. The hydrogel shrunk in acidic range markedly below pH 5.0. In the pH range 5.0 to 7.0, it maintained a similar volume, and the highest swelling rate was found at pH 10.5.

The specific water content of hydrogel was quickly decreased at 80 °C showing a thermally hydrodegradable property. The hydrogel was stable at 40 °C for 6 h but slowly degraded by lasting the reaction time above 15 h same as other PGA.[9] The slow hydrolysis of the gels at neutral pH is very important for application in slow-release systems. For the purpose of designing a slow delivery system, control of the hydrogel degradation through both cross-linker type and degree is desirable because the gels also have different swelling degrees.[16] These properties may be used as a new material adapting for slow release system of drug.

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