

Chelation of Calcium Ions by Poly(γ -Glutamic Acid) from *Bacillus subtilis* (*Chungkookjang*)

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Many studies have clarified that poly(γ -glutamic acid) (PGA) increases the solubility of Ca^{2+} , suggesting that PGA enhances calcium absorption in the small intestine. However, there has been no report on the specific interaction between PGA and Ca^{2+} in water. We studied the aqueous solution properties of PGA calcium salt (PGA–Ca complex). The chelating ability and binding strength of PGA for Ca^{2+} were evaluated. The PGA–Ca complex was soluble in water, in contrast to the insolubility of poly(acrylic acid) (PAA) calcium salt, and the chelating ability of PGA for Ca^{2+} was almost the same as that of PAA. The globular conformation of the PGA–Ca complex in water was estimated by SEC and viscosity measurements. The chelation ability of PGA for Ca^{2+} was examined by ¹H NMR. The present study showing the characteristics of the PGA–Ca complex will provide useful information about the calcium absorption by PGA *in vivo*.

Keywords: Poly(γ -glutamic acid), *Bacillus subtilis* (*chungkookjang*), chelation, calcium ion

Poly(γ -glutamic acid) (PGA) is an anionic, water-soluble, safe, and edible biomaterial that is naturally synthesized by *Bacillus subtilis*, in which the α -amino and γ -carboxy groups of glutamic acid are polymerized by γ -amide linkage. The multifunctionalities of PGA, such as its biodegradability, nontoxicity, compatibility, and edibility, have made it a promising biopolymer for use as a health food, a thickener,

an osteoporosis-preventing factor, a stabilizer in food industry, a moisturizer in cosmetics, and in various biomedical product applications [1, 3, 11, 12].

Adequate calcium intake is recommended for the development of high peak bone-mass and for the prevention of osteoporosis, and consideration should be given not only to the intake of an adequate amount of calcium, but also to the absorptive efficiency of the ingested calcium, because intestinal calcium absorption is influenced by a lot of factors [5, 6, 9, 13, 14]. There were many reports to improve the Ca^{2+} solubility by PGA in aqueous solutions [4, 8, 10], suggesting that PGA enhances calcium absorption in the small intestine. We previously reported that PGA increases the solubility of dietary calcium *in vitro* in a molecular mass-dependent manner [7]. However, no report on the specific interaction between PGA and Ca^{2+} in aqueous solutions has appeared. In this study, we have fundamentally investigated the aqueous solution properties of PGA calcium salt (PGA–Ca complex). The results will be beneficial for understanding calcium absorption through PGA.

MATERIALS AND METHODS

Materials

PGA (50 kDa, 500 kDa, and 5,000 kDa) derived from *Bacillus subtilis* (*chungkookjang*) was prepared by following the previously reported method [2], and poly(acrylic acid) (PAA, 25 kDa) was purchased from Wako Pure Chemicals. All other chemicals were of analytical grade.

Measurements

Dynamic light scattering (DLS) measurement was carried out using an Otsuka Electronics DLS-6006US He-Ne laser (632.8 nm) system at 90° angle. Ca^{2+} and proton concentrations were measured by a Horiba D-52 pH meter equipped with a Horiba 6583-10C calcium

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ion electrode and a Horiba 9611-10D pH electrode, respectively. Size-exclusion chromatographic (SEC) analysis was performed using a Tosoh HLC-8020 apparatus using a Tosoh TSK-gel (α -M) column with 1.0 ml/min flow rate at 40°C. Viscosity was measured using a Toki Sangyo TV 20 viscometer at 25°C. ^1H NMR spectra were obtained by a Bruker DPX 400 spectrometer.

RESULTS AND DISCUSSION

Aqueous Solution of PGA Calcium Salt

The appearance change by the addition of CaCl_2 to the solution of PGA sodium salt (PGA–Na complex) was examined. PAA, one of the most typical carboxylate polymers, was used for comparison. The aqueous solution of the calcium salt of the carboxylate polymers was prepared by the addition of a given amount of 0.1 M CaCl_2 solution to 10 mM (unit of the carboxylate polymer) of PGA–Na or PAA sodium salt (PAA–Na complex) solution. The molar ratio of Ca^{2+} and the carboxylate group of the polymer varied from 0.1 to 0.5. For PGA, the apparently homogeneous solution was formed in all the cases examined. Furthermore, no nano-size coagulant was detected by the DLS measurement (Table 1). These data suggest that the PGA–Ca complex was homogeneously dissolved in water and no aggregates were formed. On the other hand, PAA calcium salt (PAA–Ca complex) showed a different behavior. When the molar ratio of Ca^{2+} and the carboxylate group of PAA was 0.1, a homogeneous solution was obtained. In the case of the ratio of 0.2, the nanoparticles were formed even in the apparently homogeneous solution. The turbid dispersion solution was obtained in the ratio of 0.3 and the precipitates of the PAA–Ca complex were formed in the ratio higher than 0.4. These data indicate the formation of the PAA–Ca complex aggregates insoluble in water.

Ca^{2+} Chelating Ability of PGA

The Ca^{2+} chelating ability of PGA was evaluated to measure free Ca^{2+} in its aqueous solution. When the molar ratio of Ca^{2+} and PGA was 0.5, the chelation ratio was 87%. Under the same condition, the chelation ratio of PAA for Ca^{2+} was 86%. These data indicate that both carboxylate polymers possess a high chelating ability for Ca^{2+} .

Table 1. Particle size of PGA and PAA in water by the addition of calcium salt to the carboxylate polymer sodium salt^a.

PGA					
$[\text{Ca}^{2+}]/[\text{COO}^-]$	0.1	0.2	0.3	0.4	0.5
Particle size (nm)	–	–	–	–	–
PAA					
$[\text{Ca}^{2+}]/[\text{COO}^-]$	0.1	0.2	0.3	0.4	0.5
Particle size (nm)	–	80±30	110±30	>1000	>1000

^aParticle size was determined by DLS.

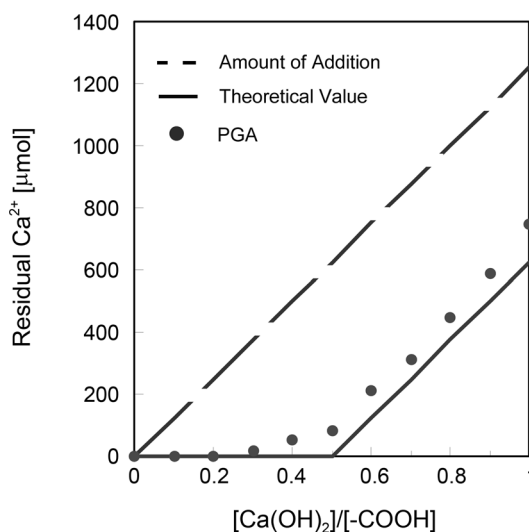


Fig. 1. Residual Ca^{2+} amount in the solution by the addition of Ca^{2+} to the PGA–Na complex.

The PGA–Na complex or PAA–Na complex (12.5 mmol of the monomer unit) was dissolved in 50 ml of ultrapure water, and a given amount of 1 M CaCl_2 solution was added to the solution. The solution was kept under gentle stirring at 25°C for 24 h, and the concentration of free Ca^{2+} was measured by using a calcium ion electrode.

Fig. 1 shows the residual Ca^{2+} amount by the addition of Ca^{2+} to PGA Na. In the low region of the unit ratio of Ca^{2+} and glutamate of PGA, free Ca^{2+} was not detected and the experimental data were well fitted to the theoretical value on the assumption of the quantitative chelation of PGA for Ca^{2+} . When the ratio was in the range from 0.3 and 0.4, a small amount of free Ca^{2+} was found. In the region of the molar ratio higher than 0.5, the concentration of free Ca^{2+} was a little larger than the theoretical one. In all the cases measured, the chelation ratio was higher than 80%. These data support the high chelating ability of PGA for Ca^{2+} .

Fig. 2 shows SEC traces of PGA or PAA in the absence and presence of a small amount of Ca^{2+} (the unit ratio of Ca^{2+} and carboxylate of PGA or PAA = 10%). In the case of PGA, the presence of Ca^{2+} hardly affected the spectral pattern, meaning that the strong inter- and/or intramolecular interaction of PGA and Ca^{2+} was not detected by the SEC analysis. On the other hand, the trace was slightly shifted to the shorter elution time for PAA in the presence of Ca^{2+} , showing the formation of the aggregates *via* the intermolecular interaction of PAA and Ca^{2+} . These data strongly suggest that the conformation of the PGA molecule in the Ca^{2+} -containing aqueous solution is different from that of PAA, despite the similar chelating abilities of PGA and PAA.

Properties of PGA–Ca Complex Aqueous Solution

Fig. 3 shows the viscosity of the aqueous solution of PGA–Na complex and PGA–Ca complex with different molecular weights. The viscosity increased as a function of

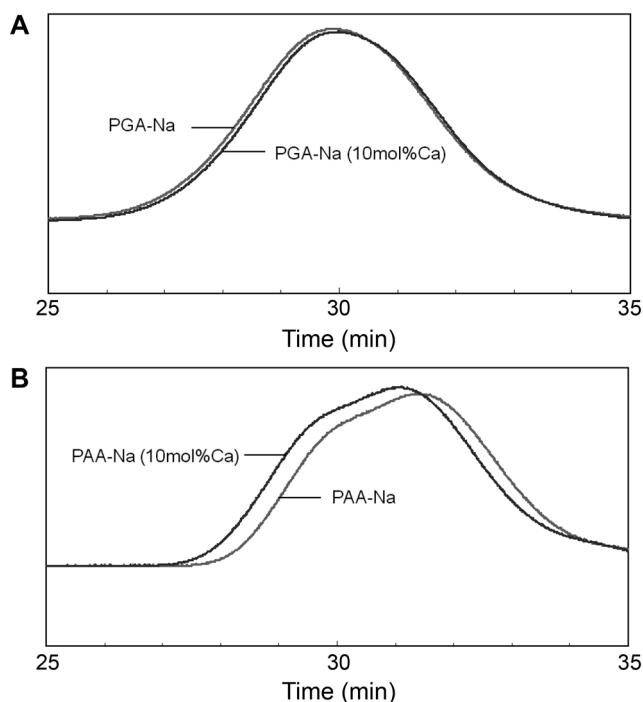


Fig. 2. SEC traces of the (A) PGA–Na complex with and without Ca^{2+} (10 mol% for Na) and (B) PAA–Na complex with and without Ca^{2+} .

The solution for the SEC analysis was prepared as follows. The PGA–Na complex or PAA–Na complex (10 μmol of monomer units) was dissolved in 10 ml of 0.02 M NaCl aqueous solution, and a given amount of 1 M CaCl_2 solution was added to the solution.

the molecular weight. Interestingly, the viscosity of the PGA–Na complex was much higher than that of the

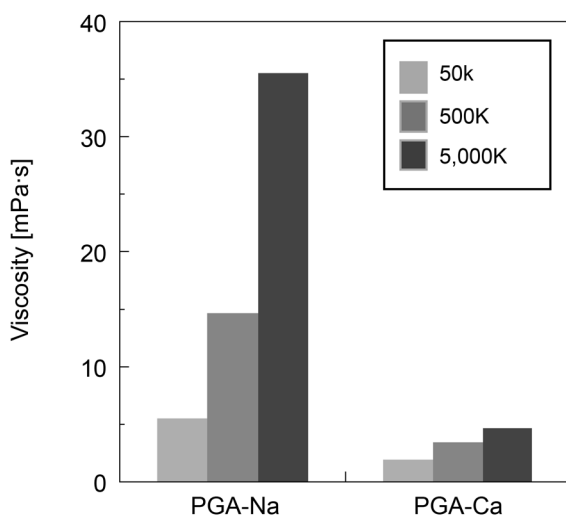


Fig. 3. Viscosity of the PGA–Na complex and PGA–Ca complex with different molecular weights.

The sample solution was prepared by mixing PGA (80 mM of glutamate unit) with NaHCO_3 or $\text{Ca}(\text{OH})_2$ in deionized water. The solution was gently stirred for 24 h and the viscosity was measured at 25°C.

Table 2. ^1H NMR chemical shift of sodium and calcium salts of PGA.

Sample	Chemical shift (ppm)				
	1	2	3	4	
PGA Na	7.95	4.02	1.90	2.25	
PGA Ca	7.99	4.04	1.91	2.26	

PGA–Ca complex, suggesting the globular conformation of the PGA–Ca complex in water, probably due to the intramolecular interaction of the PGA–Ca complex.

To investigate the chelating behavior of PGA, ^1H NMR measurement of the PGA–Na complex and PGA–Ca complex in D_2O was carried out (Table 2). The amide proton of the PGA–Ca complex in the main chain was observed in a slightly lower magnetic field than that of the PGA–Na complex, meaning there was electron donation of the amide group of PGA to Ca^{2+} . These data suggest that the amide group participates in the chelating ability of PGA for Ca^{2+} .

The conformation change of the PGA–Na complex and PGA–Ca complex was examined by SEC (Fig. 4). The SEC trace of PGA in 0.1 M NaCl aqueous solution was shifted to a longer elution time by changing 0.1 M CaCl_2 aqueous solution as the eluent. The trace pattern of PGA in 0.1 M NaCl aqueous solution was similar to that in 0.3 M NaCl aqueous solution. On the other hand, similar traces were detected for poly(ethylene glycol), a typical nonionic water-soluble polymer (data not shown). These data strongly suggest that the specific behavior of PGA in the 0.1 M CaCl_2 eluent is due to the difference of the molecular conformation between the PGA–Na complex and PGA–Ca complex; the conformation of the PGA–Ca complex in water is that of a globular shape, resulting in the longer elution time of the SEC trace. This is closely related to the viscosity behavior (Fig. 3).

The chelating stability of PGA for Na^+ and Ca^{2+} in the acidic condition was evaluated. The proton concentration

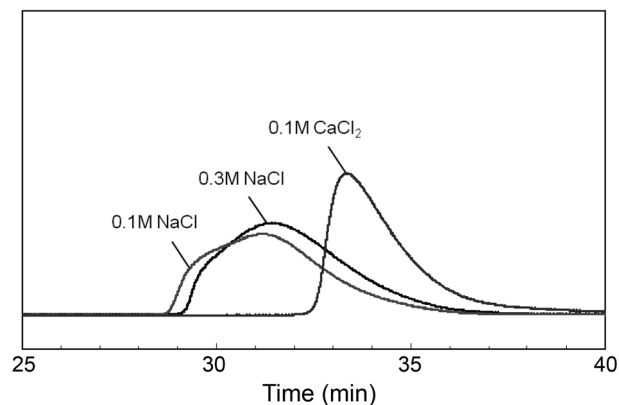


Fig. 4. SEC traces of PGA in aqueous solutions of NaCl or CaCl_2 .

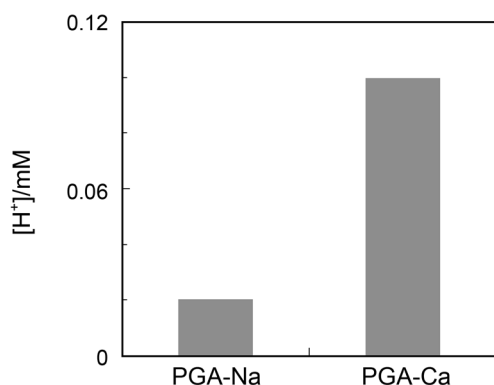


Fig. 5. Residual proton concentration after the addition of half molar of hydrochloric acid to PGA Na or Ca.

To a mixture of PGA (0.50 mmol of glutamate unit) and NaHCO₃ (0.50 mmol) or Ca(OH)₂ (0.25 mmol) in 50 ml of deionized water, 0.25 ml of 0.1 M hydrochloric acid solution was added and the mixture was kept under gentle stirring for 24 h. The pH of the solution was measured and the proton concentration in the solution was calculated from the pH value.

was measured by the addition of half molar of hydrochloric acid to an aqueous solution of PGA–Na complex or PGA–Ca complex, followed by stirring for 24 h. If a carboxylate polymer strongly interacts with cation species, the ion exchange of the cation to proton prevents even by the addition of acid to increase the proton concentration. As shown in Fig. 5, the proton concentration for the PGA–Ca complex was larger than that of PGA–Na complex. These data imply the stronger chelation of PGA for Ca²⁺ owing to the specific interaction between PGA and Ca²⁺.

In this study, the properties of the aqueous solution of PGA–Ca complex were systematically investigated. The summary of the characteristics of the PGA–Ca complex solution is as follows: (i) a high water solubility, in contrast to the insolubilization of the PAA–Ca complex toward water; (ii) the shrinkage of the polymer chain due to the globular conformation by the intramolecular interaction, in contrast to the intermolecular one of PAA–Ca; (iii) the involvement of the amide group for the chelation. Our first findings of the specific properties of the PGA–Ca complex are highly beneficial for understanding the calcium absorption of PGA. Additionally, the present fundamental study on the conformation of the carboxylate polymer calcium salts in water will contribute to applications of these polymers in various industrial fields. Further studies on the specific interaction of PGA with various minerals are under way in our laboratories.

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