Preparation and Characterization of Liposomal Microencapsulated Poly- γ -glutamic Acid for Prevention of Ca-Phosphate Precipitation under Intestinal Environment

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Poly- γ -glutamic acid (γ -PGA) from *Bacillus subtilis* (natto) with a high molecular weight (Av. M.W.=350,000) effectively inhibited the formation of insoluble calcium phosphate at Ca/P=0.5 (12.5 mM/25 mM) under the condition of 37°C and pH 7.4. Since decrease in inhibitory activity in γ -PGA was observed after the treatment by simulated gastric fluid (pH 1.2, 0.5% pepsin), γ -PGA was encapsulated by egg lecithin liposome to keep the inhibitory activity under gastric conditions. Encapsulation was done by dehydration-rehydration (DR) and reverse-phase-vesicle (REV) methods. Higher encapsulation efficiency (57.0%) was achieved by DR method. Turbidity measurement at 450 nm indicated the high stability of DR microcapsule, prepared with trehalose, against gastric juice. Encapsulated γ -PGA by DR method showed 93.3% residual inhibitory activity after the gastric juice treatment.

Keywords: liposomes, microencapsulation, poly- γ -glutamic acid, Ca-solubilizing property

Intestinal calcium (Ca) adsorption plays an important role in maintaining mineral homeostasis in human. Precipitation (insolubilization) of calcium phosphate (CaP) decreases Ca absorption in the distal small intestine. Several studies have been made on the inhibition of CaP precipitation. Casein phosphopeptide (CPP) is well known to increase soluble Ca in small intestine. Lee *et al.* (1980) showed that casein phosphopeptides, which are formed during *in vivo* digestion of casein, well prevented calcium ion from CaP precipitation. Naito (1986) reported that CPP increase Ca solubility *in vitro* by preventing the formation of an insoluble complex of Ca with phosphate. Yamamoto *et al.* (1992) reported inhibitory activity of alginates, a dietary fiber, against the formation of CaP. They suggested that the interaction between the uronate residue of the alginate and calcium ion was important in the inhibition of CaP formation.

Poly-glutamic acids are one of the potent inhibitors against CaP precipitation in the distal small intestine. Naito (1986) reported that poly- α -glutamic acid (α -PGA) as well as CPP increased Ca solubility *in vitro* by inhibiting the formation of an insoluble CaP. The inhibitory effect of oligo- and poly-L-glutamic acids against CaP formation was investigated by Yamamoto *et al.* (1994). They showed that the inhibitory activity of oligo- and poly-L-glutamic acids would increase with their molecular weight. Poly- γ -glutamic acid (γ -PGA), natural poly-anion peptides known as the main constituent of the sticky material of *natto* (fermented soybeans), has been expected for inhibitor of the CaP precipitation. Tanimoto *et al.* (2001) reported that calcium solubility *in vitro* and *in vivo* increased by the inhibition of CaP precipitation with *natto* mucilage containing γ -PGA. Tanimoto *et al.* (2003) have investigated the stimulatory effect of Ca supplement containing γ -PGA (Av.

M.W.=26,000) on Ca absorption by measuring urinary Ca excretion. They suggested that the Ca supplement containing γ -PGA increases Ca absorption in humans.

In this study, we investigated the inhibition of CaP formation by γ -PGA from *Bacillus subtilis* (natto) with a high molecular weight (Av. M.W.=350,000). The inhibitory effect of the γ -PGA *in vitro* was compared with those of α -PGAs with various molecular weights. In addition, γ -PGA was encapsulated by microcapsule to reach the small intestine without degradation and/or aggregation by gastric juice. Liposomal microencapsulation is a method that enables intact biological material to reach the small intestine without affecting the quality of foods. We attempted to encapsulate γ -PGA by liposomal microcapsule by reverse-phase evaporation vesicle (REV) and dehydration-rehydration (DR) methods, which possess several advantages for encapsulation of valuable water-soluble materials, and to determine the effect of liposomal microencapsulated γ -PGA on the inhibition of insoluble CaP precipitation.

Materials and Methods

Materials γ -PGA from *Bacillus subtilis* (natto) (Av. M.W.=350,000) was donated by Dr. T. Hara of Kyushu University. γ -PGA, which is composed of D- and L-glutamate in the proportions of 6:4, is water-soluble and biodegradable. α -PGAs (Av. M.W.=7,700, 14,300, 39,900, and 71,700) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Egg lecithin was purchased from Nacalai Tesque Co. (Kyoto, Japan). The bile salts, glycocholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid, taurocholic acid, taurochenodeoxycholic acid, and taurodeoxycholic acid were purchased as sodium salts from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trehalose and all other chemicals were obtained from Nacalai Tesque Co. and were of analytical reagent grade.

Assay for the inhibitory activity of PGA on CaP precipita-

tion in vitro Inhibitory effects of PGAs (α - and γ -PGA) on the precipitation of insoluble CaP were assayed as follows. Each of PGA, CaCl_2 , and Na_2HPO_4 was dissolved in 50 mM Tris-HCl buffer (pH 7.4). At the beginning of the reaction, 0.5 ml of PGA solution (final conc. 0.5–2 mg/ml) and 0.5 ml of 50 mM calcium chloride solution was mixed by vortexing. Then, 1.0 ml of 50 mM Na_2HPO_4 solution was added (Ca/P mol ratio=1/2), and the mixture was incubated at 37°C. After incubation, precipitated CaP was removed by centrifugation at 3,000 $\times g$ for 5 min. The concentration of calcium in the supernatant was determined by an *o*-cresolphthalein complexon method (Gitelman, 1967), of the Calcium C-test kit (Wako Pure Chemicals Co., Osaka, Japan).

Inhibition (%) of PGA was calculated by the following formula:

$$\text{Inhibition (\%)} = \frac{C_B - C_C}{C_A - C_C} \times 100$$

where C_A is initial Ca concentration, C_B is Ca concentration after the incubation for 30 min with PGA, and C_C is Ca concentration after the incubation for 30 min without PGA.

Assay for the inhibitory activity of free and encapsulated PGA treated with simulated gastric fluid. Free and encapsulated PGA was suspended in 1 ml of a 0.3% solution (w/v) of pepsin, dissolved in HCl solution containing 0.2% NaCl (pH 1.2). The suspension was incubated at 37°C for 4 h, then was neutralized with NaOH to pH 7.4. Inhibitory effects of free α - and γ -PGA on CaP precipitation were assayed as described above. Inhibitory effect of encapsulated γ -PGA was similarly assayed after degradation of microcapsule by Hoffman bile acid solution (Hofmann, 1963). This solution, which simulated human intestinal content, was prepared by mixing of the six bile salts at the following ratio (by volume): sodium glycocholate, 30%; sodium glycochenodeoxycholate, 30%; sodium glycodeoxycholate, 15%; sodium taurocholate, 10%; sodium taurochenodeoxycholate, 10%; sodium taurodeoxycholate, 5%.

Residual inhibition (%) of PGA was calculated by the following formula:

$$\text{Residual inhibition (\%)} = \frac{B}{A} \times 100$$

where A is the inhibition (%) of intact PGA and B is the inhibition (%) of PGA treated with simulated gastric fluid.

Liposomal microencapsulation of PGA Dehydration-rehydration (DR) microcapsules were prepared with trehalose according to the method described by Kim *et al.* (1999) with minor modifications. Egg lecithin in 150 ml of ethanol was dried under reduced pressure at 37°C in a rotary evaporator to leave a thin film on the wall of a 500 ml volume round-bottomed flask. The dried lipid film was suspended in 20 ml of deionized water, and then sonicated by a bath-type sonicator for 10 min to produce small unilamellar vesicles (SUV). PGA was dissolved in 10 mM sodium citrate solution and 10 ml of the PGA solution was mixed with the SUV suspension. The mixture was then lyophilized. For rehydration, 1.5 ml of 1% trehalose solution was added to the freeze-dried preparation and vortexed for 10 min (in encapsulation without trehalose, 1.5 ml of deionized water was added for the rehydration.) The suspension was lyophilized again. The freeze-dried preparation was rehydrated by the addition of deionized water (30 ml). DR microcapsules were separated from the suspension by centrifuga-

tion at 10,000 $\times g$ for 10 min. Unencapsulated PGA was hydrolyzed with 6 M HCl at 110°C for 20 h, and then determined by high-performance liquid chromatography with 6-*N*-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as described by Reverter *et al.* (1997). Encapsulation efficiency was calculated by the following formula:

Encapsulation efficiency (%) =

$$\frac{\{(\text{weight of added PGA} - \text{weight of unencapsulated PGA}) / \text{weight of added PGA}\} \times 100}{\text{weight of added PGA}} \times 100$$

Reverse-phase evaporation vesicle (REV) method was done as described by Yoshimaru *et al.* (1999) with minor modification. The dried egg lecithin film was prepared by a method similar to that of DR method. After the dried film was dissolved in 50 ml of ethanol, 10 ml of the γ -PGA solution was added to the suspension. The resulting two-phase system was sonicated briefly (1 min $\times$ 5) in a bath-type sonicator until the mixture became a clear one-phase dispersion. Ethanol in the mixture was removed under reduced pressure at 37°C in a rotary evaporator. The resultant REV microcapsules were separated from the suspension by centrifugation at 10,000 $\times g$ for 10 min. Similar to the above DR method, unencapsulated γ -PGA was determined and encapsulation efficiency was calculated.

Turbidity measurement of the microcapsule suspension

Stability of the microcapsule against simulated gastric fluid (pH 1.2) was investigated by turbidity measurement. The microcapsule was suspended in simulated gastric fluid at the initial turbidity of approximately 1.0, and then incubated at 37°C for 240 min. The turbidity was measured spectrophotometrically at 450 nm. The percentage of degradation in the microcapsule was calculated from the formula:

$$\text{Degradation (\%)} = \frac{A - B}{A - C} \times 100$$

where A is the turbidity at 0 min, B is the turbidity during the treatment, and C is the turbidity after complete degradation by treating with 25 mM Hofmann bile acid solution at 37°C for 60 min.

Degradation of DR microcapsule by 10 mM Hofmann bile acid solution was monitored by the turbidity measurement in a similar manner. The percentage of degradation was calculated from the above formula.

Statistical analysis Results are presented as means $\pm$ SD and analyzed using one-way analysis of variance followed by Tukey-Kramer test. All statistical analyses were performed using Stat View software version 5.0J (SAS institute Inc. USA).

Results and Discussion

Effect of PGA on CaP precipitation in vitro Inhibition of CaP precipitation at pH 7.4 and 37°C with various amounts of γ -PGA is shown in Fig. 1. When γ -PGA was not added, CaP precipitation was observed just after starting the incubation. Soluble Ca concentration decreased sharply to less than 1 mM within 5 min. The CaP precipitation was inhibited by the addition of γ -PGA. Inhibitory effect of γ -PGA on CaP precipitation was shown at the concentration of above 0.5 mg/ml and soluble Ca concentration increased with increase in the concentration of γ -PGA. Little decrease in soluble Ca concentration was observed for 30 min at the γ -PGA concentration of 2.0 mg/ml. In addition, the Ca concentration with 2.0 mg/ml of γ -PGA was kept at above 11 mM for at least 2 h (data not shown).

Fig. 1. Inhibition of calcium phosphate precipitation by addition of poly- γ -glutamic acid (γ -PGA) at pH 7.4 and 37°C. γ -PGA was incubated with 12.5 mM CaCl_2 and 25 mM Na_2HPO_4 ($\text{Ca/P}=0.5$). Solid circles, 0 mg/ml; open circles, 0.5 mg/ml; solid triangles, 1.0 mg/ml; open triangles, 1.5 mg/ml; solid squares, 2.0 mg/ml.

Table 1. Inhibition of calcium phosphate precipitation by α - and γ -PGA.

Compounds	(Av. M.W.)	Inhibition (%) [*]
α -PGA	(7,700)	21.2 ± 3.32^a
α -PGA	(14,300)	29.4 ± 5.18^a
α -PGA	(39,900)	55.4 ± 8.83^b
α -PGA	(71,700)	75.7 ± 5.75^c
γ -PGA	(350,000)	93.0 ± 2.10^d

^{*}Each assay was done by addition of PGA (2 mg/ml) at pH 7.4 and 37°C for 30 min ($\text{Ca/P}=0.5$) and values (mean $\pm$ SD of 5 experiments) in a column sharing a common letter are not significantly different ($p > 0.05$).

These results suggest that γ -PGA would be able to effectively inhibit the Ca precipitation even at low Ca/P.

Inhibitory activity of *natto* mucilage containing γ -PGA on CaP precipitation was compared *in vitro* with that of α -PGA (degree of polymerization=535) by Tanimoto *et al.* (2001). They showed that *natto* mucilage had lower activity than the α -PGA. In this study, the inhibitory effect of γ -PGA *in vitro* was compared with that of α -PGA with various molecular weights (Table 1). Average M.W. of α -PGAs were 7,700, 14,300, 39,900, and 71,700. All PGAs used in this study showed an inhibitory effect against CaP precipitation, and their inhibitory activity increased with their molecular weight. γ -PGA showed the highest inhibitory activity against the CaP precipitation (93% inhibition) with activity over 15 percent higher than that of α -PGA (Av. M.W.=71,700, degree of polymerization=475). This result suggests that γ -PGA with high molecular mass in *natto* mucilage would have powerful inhibition against CaP precipitation. Yamamoto *et al.* (1994) investigated the effect of molecular weight on the calcium binding properties of oligo-L-glutamic acids (<20 mer: M.W. 2598) using a calcium-ion-selective electrode. They showed that the apparent amount of Ca bound to oligo-L-glutamic acids tended to increase with their molecular weight. In the present study, strong binding property of γ -PGA with high molecular mass to calcium-ion resulted in powerful inhibition against CaP precipitation.

Stability of PGA against simulated gastric juice To investigate the stability of α - and γ -PGA in the gastric environ-

Fig. 2. Inhibition of calcium phosphate precipitation with α - and γ -PGA treated by simulated gastric solution at 37°C. Each assay was done by addition of PGA (2 mg/ml). Each point represents the mean $\pm$ SD ($n=3$). solid circles, γ -PGA; open circles, α -PGA (Av. M.W.=71,700).

Fig. 3. Encapsulation of γ -PGA by dehydration-rehydration (DR) and reverse phase evaporation vesicle (REV) methods. Both encapsulations were done by using 25 mg of γ -PGA. DR capsules were prepared with trehalose. Each point represents the mean $\pm$ SD ($n=3$).

ment, each PGA was treated with a simulated gastric juice for 4 h. Residual inhibitory activity of α - and γ -PGA on CaP precipitation was determined after this treatment (Fig. 2). The inhibitory activity of α -PGA decreased to less than 5% within 15 min; in contrast, slower decrease in the activity of γ -PGA was observed. Residual activity of γ -PGA was reduced to 60.1% after the gastric treatment for 4 h. The difference in their tendencies would result from their molecular mass and/or the conformation in the gastric juice. In addition, only the gastric solution containing γ -PGA became turbid after 1 h-incubation, and the turbidity increased with the passage of treatment time. In the absence of pepsin, similar increase in the turbidity was observed and the inhibitory activity of γ -PGA decreased to 83.1% after 4 h (data not shown). These results suggest that peptic degradation and/or aggregation of γ -PGA in the gastric environment would cause a great loss in the inhibitory activity of γ -PGA.

Table 2. Encapsulation of Glu, Glu-Glu, α -PGA, and γ -PGA by DR method.

Compounds	(Av. M.W.)	Encapsulation efficiency (%) ^a
Glu		14.4 $\pm$ 5.26 ^a
Glu-Glu		30.9 $\pm$ 7.17 ^b
α -PGA	(7,700)	58.8 $\pm$ 4.61 ^{cd}
α -PGA	(39,900)	54.6 $\pm$ 8.83 ^c
α -PGA	(71,700)	68.0 $\pm$ 4.00 ^d
γ -PGA	(350,000)	56.9 $\pm$ 5.32 ^{cd}

^aEach capsule was prepared by using 25 mg of glutamyl compound. Encapsulations were done by addition of trehalose. Values (mean $\pm$ SD of 5 experiments) in a column sharing a common letter are not significantly different ($p>0.05$).

Liposomal encapsulation of PGA To prevent loss in inhibitory activity of γ -PGA against CaP precipitation through a gastric environment, γ -PGA was encapsulated by egg lecithin liposomes. Encapsulation was done by DR and REV methods. In both methods, ethanol was used to prepare the liposome microcapsules. Other organic solvents, such as chloroform, diethyl ether, or methanol, carry a possible risk to human health and are unsuitable for industrial food use. Although the organic solvents are usually removed by evaporation, traces may be present in liposome formulation to cause a toxic problem. Figure 3 shows the encapsulation efficiency of γ -PGA by DR and REV methods with various amounts of egg lecithin. The encapsulation efficiency by DR method was higher than that by REV method. Highest encapsulation efficiency of γ -PGA (25 mg) by the DR method was 57.0% using 250 mg of egg lecithin.

Low encapsulation efficiency (>15%) by the REV method would be due to the ethanol used to prepare the liposomes. Cortesi *et al.* (1999) prepared liposomes by REV method using ethanol and determined the encapsulation efficiencies of hydrophilic and hydrophobic model drugs. They suggested that encapsulation of hydrophilic compounds with ethanol would be difficult because hydrophilic compounds would not interact with the phospholipid bilayers formed in a water-ethanol system. In our encapsulation by the REV method, γ -PGA with hydrophilicity interacted little with egg lecithin bilayers in water-ethanol system.

Encapsulation of Glu, Glu-Glu, α -PGAs, and γ -PGA were attempted by DR method (Table 2). Each DR microcapsule was prepared with 250 mg of egg lecithin. High encapsulation efficiencies (>50%) were achieved in all PGAs, and little difference was observed between α -PGAs and γ -PGA. In contrast, encapsulation efficiencies of Glu and Glu-Glu were significantly low. It is likely that strong interaction between a number of carboxyl residues of PGA and hydrophilic groups of lecithin bilayers would result in high encapsulation efficiency in the DR method.

Resistance of DR microcapsules against gastric environment

To investigate the resistance of DR liposomes encapsulating γ -PGA against the gastric environment, turbidity measurement was done as described by Kim *et al.* (1999). Figure 4 shows the degradation ratios of DR liposomes calculated from the turbidity of liposome suspensions. To determine the effect of trehalose on the liposome stability in gastric solution, DR liposomes were prepared with and without trehalose (15 mg/250 mg–lecithin). These liposomes showed high stability against gastric solution. Stability of DR liposomes with trehalose was higher

Fig. 4. Stability of DR microcapsules against simulated gastric juice at 37°C. Stability of capsules was measured by turbidity monitoring at 450 nm. DR microcapsules were prepared with (solid circles) and without (open circles) trehalose. Both encapsulations were done by using 25 mg of γ -PGA. Each point represents the mean $\pm$ SD ($n=3$).

Fig. 5. Degradation of DR microcapsules with Hofmann bile acids (pH 6.4) at 37°C. Degradation (%) was measured by turbidity monitoring at 450 nm. DR microcapsules were prepared with trehalose. Both encapsulations were done by using 25 mg of γ -PGA. Each point represents the mean $\pm$ SD ($n=3$).

than that of the liposomes without trehalose. Degradation ratio of DR liposomes with trehalose was 14.2% even after gastric treatment for 4 h. Similar results were obtained in the encapsulation of α -PGA (data not shown).

Freeze-dried liposomes were stabilized by trehalose (Crowe *et al.*, 1986). In the present encapsulation, therefore, trehalose would stabilize liposomes in a freeze-dried process in the DR method and result in high resistance of DR liposomes against the gastric environment.

Release of γ -PGA from DR liposomes by bile acids Release of γ -PGA from DR liposomes after passing through the stomach is required to inhibit CaP precipitation in the intestinal tract. Degradation of DR liposomes by bile acids, which is responsible for liposome digestion (Rowland & Woodley, 1980; Nagata *et al.*, 1990), was investigated by turbidity. Figure 5 shows the percent of degradation in DR liposomes prepared with trehalose. Rapid degradation was observed just after starting the incubation. Degradation ratio of DR liposomes reached 95% within 15 min. This result suggests that DR liposomes

Fig. 6. Inhibitory effect on calcium phosphate precipitation with free and encapsulated γ -PGA treated by simulated gastric solution at pH 1.2 and 37°C for 4 h. Microcapsules were prepared by DR method with (A) and without (B) trehalose. Both encapsulations were done by using 25 mg of γ -PGA. The results are expressed as means $\pm$ SD of 6 experiments (* p < 0.001).

have an ability to release γ -PGA within their residence time in the bile salts-rich intestinal tract. In addition, DR liposomes encapsulating α -PGA were easily degraded in a similar manner (data not shown).

Inhibition of CaP precipitation with encapsulated γ -PGA Inhibitory effect of free and encapsulated γ -PGA was determined after the treatment by gastric solution for 4 h. Encapsulation was done by DR method with and without trehalose (15 mg/250 mg–lecithin). After the gastric treatment, γ -PGA was released from DR capsules by the Hofmann bile acid solution, and then its inhibitory activity was assayed. Figure 6 shows residual inhibitory activities of free and encapsulated γ -PGAs on CaP precipitation after the treatment with gastric solution. DR liposomes were prepared with and without trehalose. As a result, protective effect of DR liposome against gastric treatment was observed. After the gastric treatment for 4 h, 93.3 and 79.1% of residual inhibitory activities were obtained by encapsulated γ -PGA with and without trehalose, respectively. This suggests that γ -PGA encapsulated by DR method would pass through the stomach while retaining its inhibitory activity, and then, after being released from liposomes by the bile acids, inhibit the CaP precipitation in the intestinal tract.

Conclusions

The inhibitory effect of γ -PGA with high molecular weight on CaP precipitation was determined *in vitro*. Strong inhibitory effect of γ -PGA was shown at Ca/P=0.5, and a decrease in the

inhibitory activity was observed after incubation in gastric solution with pepsin. To avoid the loss in the activity, γ -PGA was effectively encapsulated in liposomes by DR method. The liposome-microcapsules showed high resistance against gastric solution. Liposome-encapsulated γ -PGA showed 93.3% of intact inhibitory activity after the gastric juice treatment for 4 h.

References

- Cortesi, R., Esposito, E., Gambarin, S., Telloli, P., Menegatti, E. and Nas-truzzi, C. (1999). Preparation of liposomes by reverse-phase evapora-tion using alternative organic solvents. *J. Microencapsul.*, **16**, 251–256.
- Crowe, L.M., Womersley, C., Crowe, J.H., Reid, D., Appel, L. and Rudolph, A. (1986). Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. *Biochim. Biophys. Acta*, **861**, 131–140.
- Gitelman, H.J. (1967). An improved automated procedure for the deter-mination of calcium in biological specimens. *Anal. Biochem.*, **18**, 521–531.
- Hofmann, A.F. (1963). The function of bile salts in fat absorption. *J. Bio-chem.*, **89**, 57–68.
- Kim, C.K., Chung, H.S., Lee, M.K., Choi, L.N. and Kim, M.H. (1999). Development of dried liposomes containing β -galactosidase for the digestion of lactose in milk. *Int. J. Pharm.*, **183**, 185–193.
- Lee, Y.S., Noguchi, T. and Naito, H. (1980). Phosphopeptides and soluble calcium in the small intestine of rats given a casein diet. *Br. J. Nutr.*, **43**, 457–467.
- Nagata, M., Yotsuyanagi, T. and Ikeda, K. (1990). Bile salt-induced dis-integration of egg phosphatidylcholine liposomes: A kinetic study based on turbidity changes. *Chem. Pharm. Bull.*, **38**, 1341–1344.
- Naito, H. (1986). The mechanism of enhancement in intestinal calcium absorption with phosphate derived during casein digestion (in Japa-nese). *J. Jpn. Soc. Nutr. Food Sci.*, **39**, 433–439.
- Reverter, M., Lundh, T. and Lindberg, J.E. (1997). Determination of free amino acids in pig plasma by precolumn derivatization with 6-*N*-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and high-performance liquid chromatography. *J. Chromatogr. B.*, **696**, 1–8.
- Rowland, R.N. and Woodley, J.F. (1980). The stability of liposomes *in vitro* to pH, bile salts and pancreatic lipase. *Biochim. Biophys. Acta*, **620**, 400–409.
- Tanimoto, H., Nozawa, H., Okada, K. and Miyano, R. (2003). A calcium supplement containing poly- γ -glutamic acid increases human calcium absorption (in Japanese). *Nippon Nougai Kagaku Kaishi*, **77**, 504–507.
- Tanimoto, H., Mori, M., Motoki, M., Torii, K., Kadowaki, M. and Nogu-chi, T. (2001). Natto mucilage containing poly- γ -glutamic acid increases soluble calcium in the rat small intestine. *Biosci. Biotechnol. Biochem.*, **65**, 516–521.
- Yamamoto, K., Kumagai, H., Sakiyama, T., Song, C.M. and Yano, T. (1992). Inhibitory activity of alginates against the formation of calcium phosphate. *Biosci. Biotechnol. Biochem.*, **56**, 90–93.
- Yamamoto, K., Kumagai, H., Suzuki, A. and Arai, S. (1994). Inhibitory activity of oligo- and poly-L-glutamic acids against calcium phosphate insolubilization and calcium binding with special relevance to their molecular weight dependence. *Biosci. Biotechnol. Biochem.*, **58**, 1662–1665.
- Yoshimaru, T., Nakayama, M., Ohmachi, K. and Matsumoto, K. (1999). Preparation of liposomes for disintegration in the intestinal environ-ment. *J. Fac. Agric., Kyushu Univ.*, **43**, 441–449.