J. Microbiol. Biotechnol. (2005), **15**(4), 855–858

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effect of High-Molecular-Weight Poly-γ-Glutamic Acid from *Bacillus subtilis* (*chungkookjang*) on Ca Solubility and Intestinal Absorption

PARK, CHUNG[1], YOON-HO CHOI[1], HYUN-JIN SHIN[2], HARYOUNG POO[3], JAE JUN SONG[3], CHUL-JOONG KIM[2], AND MOON-HEE SUNG[1,4]*

[1]*BioLeaders Corporation, Yongsandong, Yuseong-gu, Daejeon, Korea*
[2]*Laboratory of Infectious Diseases, College of Veterinary Medicine, Chungnam National University, Daejeon, Korea*
[3]*Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea*
[4]*Department of Bio & Nanochemistry, Kookmin University, Seoul, Korea*

Received: December 28, 2004
Accepted: January 17, 2005

Abstract The bioavailability of Ca is currently one of the most important topics in nutrition research and is correlated with gastrointestinal solubility. Thus, to increase the solubility of calcium, this study was undertaken to examine the effect of γ-PGA on intestinal Ca solubility. The calcium solubility increased when the amount of γ-PGA was increased, due to the inhibition of the formation of an insoluble Ca complex with phosphate. Therefore, when γ-PGA-500 (avg. MW 5,000 kDa) was added at 0.5 mg/ml, 75% of the total Ca was soluble. The amount of soluble Ca uptake in the small intestine was investigated using Balb/c mice as an animal model system. The soluble Ca uptake in the mice orally administered with γ-PGA-500 (avg. MW 5,000 kDa) was significantly higher than that in the γ-PGA-100 (avg. MW 1,000 kDa)-administered mice ($P<0.05$). Accordingly, these results strongly support the notion that the molecular size of γ-PGA is correlated with Ca solubility. The effects of other factors, such as casein phosphopeptide and vitamin D, on intestinal Ca absorption have also previously been investigated. Therefore, it is hoped that the present observations will help clarify the role of γ-PGA in Ca solubility and its industrial application as an additive.

Key words: *Bacillus subtilis* (*chungkookjang*), poly-γ-glutamic acid (γ-PGA), high molecular weight, calcium solubility, intestinal absorption

The bioavailability of Ca is currently one of the most important topics in nutrition research, and mineral absorption is generally correlated with gastrointestinal solubility, which depends on many factors, such as physiologic status of the body and food composition. The intestinal absorption of Ca is relatively low and mainly influenced by Ca intake, vitamin D, casein phosphopeptide, dietary fiber, phytate, oxalate, fat, and lactose [7, 9, 12, 16, 24]. Poly-γ-glutamic acid (γ-PGA), which is water-soluble and biodegradable, has shown potential to be used for thickeners, humectants, sustained-release materials, or drug carriers with biodegradability in the fields of food, cosmetics, and medicine [3, 6, 23]. Furthermore, several applications of γ-PGA have already been established in the food industry [19], where it is used to relieve bitter tastes (amino acids, peptides, quinine, caffeine, minerals) and improve the quality of foods and drinks; for example, as an ice-cream stabilizer, thickener, and functional supplement in animal feeds [4, 10, 18]. We recently developed a manufacturing system for very highly elongated γ-PGA (avg. MW 2,000 kDa–5,000 kDa), based on culturing *B. subtilis* chungkookjang in a fermentor to produce γ-PGA on a commercial scale [20].

Tanimoto *et al.* [21] reported recently that γ-PGA increases Ca solubility *in vitro* and *in vivo*, as well as intestinal Ca absorption, and that γ-PGA has the effect of natto mucilage in relation to increase the solubility and intestinal absorption of Ca. We also investigated the effect of molecular weight of γ-PGA on Ca solubility, and the results strongly supported the notion that the average molecular weight of γ-PGA has an effect on Ca solubility [15]. In addition, Ikeda and Doi [8] identified from the culture broth of *B. subtilis* vitamin K-binding factors responsible for the formation of water-soluble vitamin K, and proved that these factors consisted mainly of glutamic acids. Therefore, these findings would seem to imply that natural γ-PGA and functional foods supplemented with adequate quantities of γ-PGA may be useful as therapeutic agents to prevent osteoporosis [1]. However, despite several studies on the effect of γ-PGA on Ca absorption, there has been no previous investigation of a correlation between the molecular weight

*Corresponding author
Phone: 82-42-934-7671; Fax: 82-42-934-7670;
E-mail: smoonhee@kookmin.ac.kr, smoonhee@bioleaders.co.kr

of γ-PGA, Ca solubility, and small-intestine absorption. Therefore, this study was undertaken to examine the effect of high-molecular-weight γ-PGA from *Bacillus subtilis* (*chungkookjang*) on Ca solubility and small-intestine absorption.

MATERIALS AND METHODS

Preparation of High-Molecular-Weight Poly-γ-Glutamic Acid

Bacillus subtilis (*chungkookjang*) [2, 11, 14] was inoculated into 30 l of a sterile LB medium composed of 1% bacto-tryptone, 0.5% bacto-yeast extract, and 1% NaCl, and they were incubated at 37°C for 8 h. The resulting seed culture was transferred to a 500-l jar fermentor (Korea Fermentor Co., Korea) containing 300 l of a production medium composed of 4% Na-glutamate, 4% glucose, 2% citrate, 1% $(NH_4)_2SO_4$, 0.27% KH_2PO_4, 0.42% $Na_2HPO_4 \cdot 12H_2O$, 0.05% NaCl, 0.5% $MgSO_4 \cdot 7H_2O$, and 0.05% vitamin solution. The cells were separated from the culture broth by centrifugation. The supernatant containing γ-PGA was then poured into 3 volumes of ethanol and left overnight at 4°C. Thereafter, the resultant precipitate of γ-PGA was collected by centrifugation, dissolved in water, and the pH adjusted to 2.0 with H_2SO_4. After washing 3 times with water, the γ-PGA was lyophilized to give pure γ-PGA. The number and weight-average molecular weights (M_n and M_w, respectively) along with the polydispersity (M_w/M_n) of the γ-PGA were measured by gel permeation chromatography (GPC) using a GMPWXL column (Viscotek) and LR125 Laser Refractometer (Viscotek, Texas, U.S.A.). Polyethylene oxide (PEO) standards with a narrow polydispersity were used to construct a calibration curve from which the molecular weights of the γ-PGA were calculated.

Assay for Ca Solubility *In Vitro*

The effect on Ca solubility *in vitro* was evaluated as the inhibitory effect on the formation of an insoluble complex of Ca with phosphate by the method similar to that of Naito [13]. The sample solution (0.5 ml) and 10 mM calcium chloride solution (0.5 ml) were mixed, 1.0 ml of 20 mM phosphate buffer (pH 8) was added to the solution, and the mixture was incubated at 37°C for 2 h. The solution was then centrifuged at 2,000 ×*g* and 25°C for 30 min. Ca concentration in the supernatant was analyzed as described below, and Ca solubility (%) was calculated using the following formula: (Ca concentration in the supernatant/ total Ca concentration in the solution)×100.

Animals and Feeding

The present study used 30 four-week-old Balb/c male mice that were kept in cages under temperature control and a 12-h light-dark cycle. All the mice were fed on a basal diet and allowed to drink distilled water.

Experimental Design

The mice were divided into three groups of ten: γ-PGA-100 group (avg. MW 1,000 kDa), γ-PGA-500 group (avg. MW 5,000 kDa), and control group. For the experimental groups, the γ-PGA was administered orally, while PBS was administered to the control group.

Sampling and Sample Preparation

Two hours after the γ-PGA administration, all the mice were anesthetized by inhalation of ether. Subsequently, the entire small intestine was removed from the abdomen and divided into two parts: the upper and lower halves.

The intestinal contents from each half of the small intestine were then completely flushed out with an ice-cold saline solution. After flushing, the intestinal tissues were homogenized in an adequate volume of ice-cold saline, and the homogenates were centrifuged at 8,000 ×*g* for 20 min at 4°C. Both the supernatants (soluble fraction) and precipitates (insoluble fraction) were collected and stored at −20°C until analyzed.

Analyses and Statistics

Ca concentrations were measured using a colorimetric method based on a commercial kit (Calcium, Sigma, U.S.A.), and differences in different times and molecular weights of the γ-PGA were compared between the samples from the experimental and control groups. A statistical analysis was performed, and the differences between the means were evaluated by the least significant difference test, where $P<0.05$ was considered as significant.

RESULTS AND DISCUSSION

Preparation of High-Molecular-Weight Poly-γ-Glutamic Acid

A large amount of γ-PGA (35 g/l) without any byproducts were produced using *Bacillus subtilis* (*chungkookjang*) [Fig. 1(A)] and exhibited a weight-average molar mass of 5.34×10^6 g mol^{-1} [Fig. 1(B)]. The ratio of L-isomer to D-isomer of the γ-PGA monomer unit in the γ-PGA hydrolysate was measured by HPLC, where the sample PGA was hydrolyzed in 6 N HCl at 105°C for 10 h in a glass vial under a nitrogen atmosphere using a Pico-Tag apparatus. In addition, a Rexchrome S5-100-ODS column (Regis Chem., 4.6 mm×25 cm×5 m, U.S.A.) along with a UV detector (200 nm) was used, and the column was eluted with a perchloric acid solution (pH 2) at a flow rate of 0.4 ml/min. The polymer was found to contain 60% L-glutamic acid and 40% D-glutamic acid (data not shown).

Assay for Ca Solubility *In Vitro*

In this study, the γ-PGA was found to increase the Ca solubility *in vitro*, strongly suggesting that the molecular

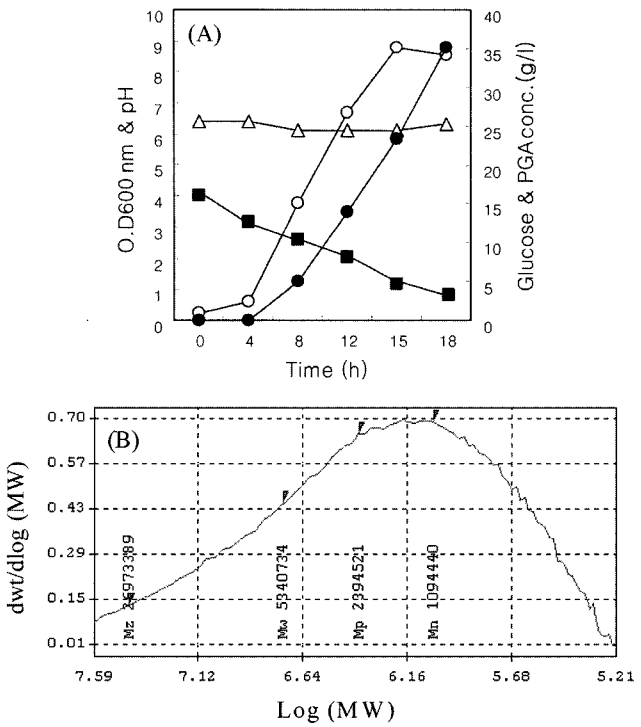

Fig. 1. γ-PGA production (A) and molecular weight distribution of γ-PGA using gel permeation chromatography (B).
γ-PGA (●), cell growth (○), glucose (■), pH (△).

weight of γ-PGA affects the Ca absorption in the small intestine. The effect of the γ-PGA on Ca solubility *in vitro* is shown in Fig. 2: the solubility increased in a dose- and molecular-weight-dependent manner. When the γ-PGA-500 (avg. MW 5,000 kDa) was added at 0.5 mg/ml, 75%

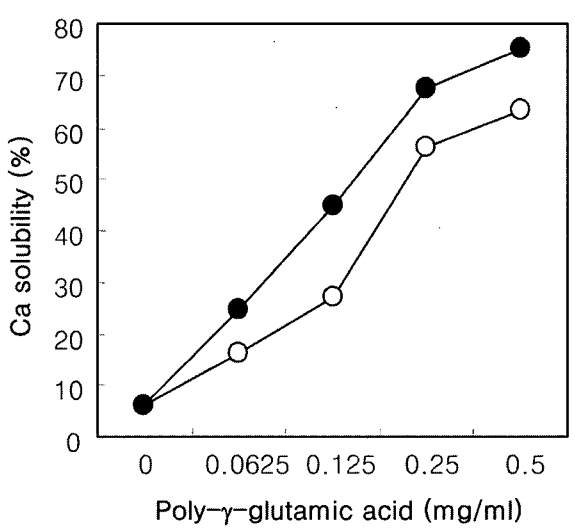

Fig. 2. Effect of poly-γ-glutamic acid on Ca solubility *in vitro*.
Ca solubility *in vitro* was measured, as described in Materials and Methods. γ-PGA-500 (Mw. 5,000 kDa) (●), γ-PGA-100 (Mw 1,000 kDa) (○).

Table 1. Amount of soluble Ca in the upper and lower halves of the small intestine.

Group	Small intestine	Ca amount (mg)
Control	Upper half	0.18±0.04
	Lower half	0.15±0.06
γ-PGA-500	Upper half	0.35±0.08
	Lower half	0.28±0.04
γ-PGA-100	Upper half	0.23±0.05
	Lower half	0.19±0.04

of the total Ca was soluble, which was significantly higher than that in the other groups. As a consequence, these results strongly support the notion that the average molecular weight of γ-PGA has an effect on Ca solubility. However, this is the first time that the molecular weight of γ-PGA has been shown to affect Ca solubility and intestinal absorption; therefore, the results need to be validated under other conditions, since many other factors, such as the dietary compounds and experimental procedures, could also be involved.

Soluble Ca in the Small-Intestine Contents

The amounts of soluble Ca in the upper and lower halves of the small intestine are shown in Table 1. The soluble Ca uptake was higher in the γ-PGA-500 (avg. MW 5,000 kDa) group than in the γ-PGA-100 (avg. MW 1,000 kDa) group ($P<0.05$), demonstrating that the difference in the Ca absorption was due to the difference in the molecular weight of the γ-PGA.

Casein phosphopeptide (CPP) and vitamin D3 have already been known to increase Ca solubility and absorption in the small intestine. CPP is a natural component of milk and released by proteolytic digestion of casein during food processing or gastrointestinal digestion. A previous *in vitro* rat study showed that the amount of soluble Ca in the intestine increased with addition of CPP, indicating that CPP may increase Ca absorption [17]. However, other animal studies have produced inconsistent results on the effect of CPP on Ca absorption [5, 22, 25]. We also investigated the effect of γ-PGA on the sustained release of Ca in the small intestine (data not shown), and the results suggested that the Ca absorption in the small intestine increased dramatically with time. Consequently, this observation may suggest new methods of Ca supply for the food and drink industry. To have a better insight on this matter, further studies are needed to examine the effect of γ-PGA with other dietary compounds.

Acknowledgment

This work was supported by the 2003 research fund of Kookmin University in Korea.

REFERENCES

1. Ashiuchi, M. and H. Misono. 2002. *In* A. Steinbüchel (ed.), Biopolymers, vol. 7. Wiley-VCH, Weinheim, Chapter 6.
2. Ashiuchi, M., T. Kamei, D.-H. Baek, S.-Y. Shin, M.-H. Sung, K. Soda, T. Yagi, and H. Misono. 2001. Isolation of *Bacillus subtilis (chungkookjang)*, a poly-γ-glutamate producer with high genetic competence. *Appl. Microbiol. Biotechnol.* **57:** 764–769.
3. Cheng, C. Y., Y. Asada, and T. Aida. 1989. Production of γ-polyglutamic acid by *Bacillus subtilis* A35 under denitrifying conditions. *Agric. Biol. Chem.* **53:** 2369–2375.
4. Daninippon Pharmaceutical Co, Ltd., 1972. Ice cream stabilizer, JP Patent 19735/72.
5. Erba, D., S. Ciappellano, and G. Testolin. 2002. Effect of the ratio of casein phosphopeptides to calcium (w/w) on passive calcium transport in the distal small intestine of rats. *Nutrition* **18:** 743–746.
6. Goto, A. and M. Kunioka. 1992. Biosynthesis and hydrolysis of poly(γ-glutamic acid) from *Bacillus subtilis* IFO 3335. *Biosci. Biotech. Biochem.* **56:** 1031–1035.
7. Heaney, R. P., C. M. Weaver, and M. L. Fitzsimmons. 1991. Soybean phytate content: Effect of calcium absorption. *Am. J. Clin. Nutr.* **53:** 745–747.
8. Ikeda, H. and Y. Doi. 1990. A vitamin-K_2-binding factor secreted from *Bacillus subtilis*. *Eur. J. Biochem.* **192:** 219–224.
9. Kim, Y. J., K. W. Lee, and H. J. Lee. 2003. Increase of conjugated linoleic acid level in milk fat by bovine feeding regimen and urea fractionation. *J. Microbiol. Biotechnol.* **13:** 22–28.
10. Kunno, A., T. Taguchi, and T. Yamaguchi. 1988. New use of polyglutamic acid for foods. EP 0284386 B1.
11. Lee, M. H., J. J Song, Y. H. Choi, S. P. Hong, E. Rha, H. K. Kim, S. G. Lee, H. Poo, S. C. Lee, Y. B. Seu, and M-H. Sung. 2003. High-level expression and secretion of *Bacillus pumilus* lipase B26 in *Bacillus subtilis* Chungkookjang. *J. Microbiol. Biotechnol.* **13:** 892–896.
12. Loennerdal, B., A. S. Sandberg, B. Sandstroem, and C. Kunz. 1989. Inhibitory effects of phytic acid and other inositol phosphate on zinc and calcium absorption in suckling rats. *J. Nutr.* **119:** 211–214.
13. Naito, H. 1986. The mechanism of enhancement in intestinal calcium absorption with phosphate derived during casein digestion. *J. Jpn. Soc. Nutr. Food Sci.* **39:** 433–439.
14. Paik, H. D., S. K. Lee, S. Heo, S. Y. Kim, H. H. Lee, and T. J. Kwon. 2004. Purification and characterization of the fibrinolytic enzyme produced by *Bacillus subtilis* KCK-7 from Chungkookjang. *J. Microbiol. Biotechnol.* **14:** 829–835.
15. Park, C., K.-S. Kim, C.-M. Jung, H.-J. Shin, C.-J. Kim, M. Ashiuchi, K. Soda, and M.-H. Sung. 2003. Effect of poly-γ-glutamic acid on calcium solubility *in vitro* and *in vivo*. The society of deep-sea water and health science. *Kyoto Japan* **3:** 71–75.
16. Pingle, U. and B. V. Ramasastri. 1978. Absorption of calcium from a leafy vegetable rich in oxalates. *Br. J. Nutr.* **39:** 119–125.
17. Saito, Y., Y.-S. Lee, and S. Kimura. 1998. Minimum effective dose of casein phosphopeptide (CPP) for enhancement of calcium absorption in growing rats. *Int. J. Vit. Nutr. Res.* **68:** 335–340.
18. Sakai, K., C. Sonoda, and K. Murase. 2000. Bitterness relieving agent. JP Patent WO0021390.
19. Shih, I. L. and Y. T. Van. 2001. The production of poly(γ-glutamic acid) from microorganisms and its various applications. *Bioresour. Technol.* **79:** 207–225.
20. Song, J. J., J.-J. Kim, S.-P. Hong, Y.-H. Choi, C. Park, and M.-H. Sung. 2001. Production of a high molecular weight of poly-γ-glutamic acid. Theories and applications of chemical engineering. Proceedings of KIChE meetings. **7:** 3903–3906.
21. Tanimoto, H., M. Mori, M. Motoki, K. Torii, M. Kadowaki, and T. Noguchi. 2001. Natto mucilage containing poly-γ-glutamic acid increases soluble calcium in the rat small intestine. *Biosci. Biotech. Biochem.* **65:** 516–521.
22. Tsuchita, H., T. Susuki, and T. Kuwata. 2001. The effect of casein phosphopeptides on calcium absorption from calcium-fortified milk in growing rats. *Br. J. Nutr.* **85:** 5–10.
23. Yahata, K., J. Sadanobu, and T. Endo. 1992. Preparation of poly-α-benzyl-γ-polyglutamate fiber. *Polym. Prepr. Jpn.* **41:** 1077.
24. Yamanaka, S. 1991. New gamma-polyglutamic acid, production therefore and drinking agent containing the same. JP Patent 3047087.
25. Yang, J. K., S. H. Lee, D. H. Hahm, I.-L. Kim, and S.-Y. Choi. 2004. Enhancement of calcium-binding quality of proglycinin peptides by chemical phosphorylation. *J. Microbiol. Biotechnol.* **14:** 607–611.