REVIEW

New Biological Functions and Applications of High-Molecular-Mass Poly-γ-glutamic Acid

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Ultra-high-molecular-weight poly-γ-glutamic acid (γ-PGA) is a most promising biodegradable polymer that is produced by *Bacillus subtilis (chungkookjang)*. Attractive properties of γ-PGA are that it is water soluble, anionic, biodegradable, and edible. Development of γ-PGA has pursued in terms of cosmetics/skin care, bone care, nanoparticle for drug delivery system, hydrogel, and so on. Very recently, our research has shown that γ-PGA can be used as an immune-stimulating agent, especially at high molecular weight. This review presents the synthesis and production of high-molecular-weight γ-PGA and its various applications in industrial fields.

Introduction. – Poly-γ-glutamic acid (γ-PGA) is an unusual anionic polypeptide in which D- and/or L-glutamate is polymerized *via* γ-amide linkages [1] and, therefore, is an optically active polymer with a stereogenic center in every glutamate unit. γ-PGA is a biopolymer for which a large range of applications has been suggested. It can be characterized by its molecular weight and the ratio of D- and L-glutamate monomers. The use of natural compounds and a biocatalyst in an aqueous solvent for synthesis of a polymer is clearly an appropriate approach to reducing the corresponding toxic chemicals used and formed during a production process. Therefore, the research conducted during the past year has explored the exciting potential of the biological synthetic route to produce the water-soluble γ-PGA. γ-PGA can be produced as a product of fermentation by a number of microbial species, most prominently various *Bacilli* [2][3]. So far, three stereochemically different types of γ-PGA have been found in biopolymers [4][5]. A homopolymer composed of D-glutamate (D-PGA), a homopolymer of L-glutamate (L-PGA), and a copolymer in which the D- and L-glutamate units are lined up at random (DL-PGA). Some strains of *Bacillus subtilis*, including the starters of *natto*, a traditional Japanese fermented food made from soybeans, and of *chungkookjang*, a traditional Korean fermented seasoning made from soybeans, produce DL-PGA as a main component of the extracellular mucilage [6][7].

DL-PGA from *B. subtilis (natto)* typically has a variable molecular weight (10–1,000 kDa), whereas high-molecular-weight DL-PGAs (>2,000 kDa) can be obtained from the culture filtrate of *B. subtilis (chungkookjang)* [6]. At present, the most important step is to construct a mass-production system for γ-PGA by applying molecular-biology techniques, and hence an extensive knowledge of both the producers and the synthetases of γ-PGA are indispensable. A unique, membrane bound, three-subunit complex that equally accepts D- and L-glutamate as substrate has been identified as the sole machinery of γ-PGA synthesis in *B. subtilis*. Moreover, resolution of the ternary structures of γ-PGA synthetases and, subsequently, detailed analyses of their catalytic actions at the molecular level will provide major insights into the highly elongated γ-PGA. It is likely that public opinion will also push for the development of such environmentally friendly technology. The potential industrial and scientific benefits that might accrue from extensive investigations of structurally and functionally unique, high-molecular-weight γ-PGA are described. In this review, we give an overview of this highly promising field of research, as there have been many interesting applications.

Microbiological Synthesis of High-Molecular-Mass γ-PGA. – Poly-γ-glutamic acid (γ-PGA) includes repeating units of glutamic acid that are linked between α-amino and γ-carboxylic acid functional groups of the glutamic acid. γ-PGA has the structural formula as shown in *Fig. 1*, where '*n*' generally is in the range of between *ca.* 1,000 and over several 10,000, and a salt of γ-PGA (*Fig. 1,b*) 'M' can be, for example, a suitable metal, such as Na. Naturally produced γ-PGA is degraded over time during isolation and purification, thereby significantly diminishing the molecular weight of naturally-produced γ-PGA which can be recovered. *Bacillus subtilis (chungkookjang)* was isolated from the traditional Korean fermented soybean paste, *chungkookjang*, a highly salty seasoning containing abundant γ-PGA [8]. The apparent viscosity of the culture medium decreased dramatically, as the salt concentration was increased. γ-PGA with relatively high molecular weight (>2,000 kDa) was synthesized at low NaCl concentrations (<0.5%). It is classified as a glutamic acid-dependent producer along with *B. subtilis (natto)* IFO 3335, *B. licheniformis* ATCC 9945A, *B. subtilis* F-2-01 [9–11]. γ-PGA Productivity of *B. subtilis (chungkookjang)* remains unchanged even after long-term storage and repeated subcultivation, which we also reported previously. It is likely that *B. subtilis (chungkookjang)* will serve as a potent tool not only in the development of a mass-producer of γ-PGA, but in the elucidation of the γ-PGA synthetic mechanism in *B. subtilis* in its entirety. Production of high-molecular-weight γ-PGA is one of the unique characteristics of *B. subtilis (chungkookjang)*. A kinetic model for γ-PGA production by *B. subtilis* has been suggested [12]. *B. licheniformis* and *B. subtilis* can easily be cultivated and are generally regarded as safe (GRAS). This has led to much research being conducted on γ-PGA production by these species. *B. megaterium*, *B. halodurans*, *B. amyloliquefaciens*, *Natrialba aegyptiaca*, and *Staphylococuus epidermidis* were also found to produce γ-PGA [13–15]. *Natrialba aegyptiaca,* an extremely halophilic archaeon, produces highly elongated γ-L-PGA (>1,000 kDa) extracellularly. However, *Natrialba aegyptiaca* seems unsuitable for the industrial production of γ-PGA, because it is said to be difficult to cultivate [8]. A main chain of γ-PGA would be formed and highly elongated, when a series of the reaction occurred

iteratively and successively at an active site of the γ-DL-PGA synthetase complex (PgsBCA) due to the cooperative operation of the PgsBC components as a glutamic acid ligase and the PgsA component as a γ-PGA transporter [16]. γ-PGA has been produced recombinantly in various species. In *E. coli*, γ-PGA was produced in a strain harboring a plasmid containing *pgsB*, *pgsC*, and *pgsA* from *B. subtilis* IFO 3336 [17]. Coryneform bacteria were reported to be suitable hosts for the recombinant production of γ-PGA [16]. There are also *Gram*-positive bacteria like the *Bacillus* strain so the rigid structure of their cell walls seem to be more suitable for the display of pgsBCA in γ-PGA production.

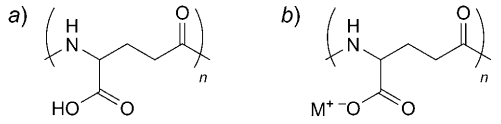

Fig. 1. *Structures of poly-γ-glutamic acid. a*) Acid form and *b*) salt form.

Cultivation for High-Molecular-Mass γ-PGA. – Almost strain of *Bacillus* depends greatly on the composition of the media formulations to produce γ-PGA. *B. subtilis (chungkookjang)* requires glutamic acid addition to the media to stimulate γ-PGA production (*Fig. 2*). The appropriate balance between production media and culture

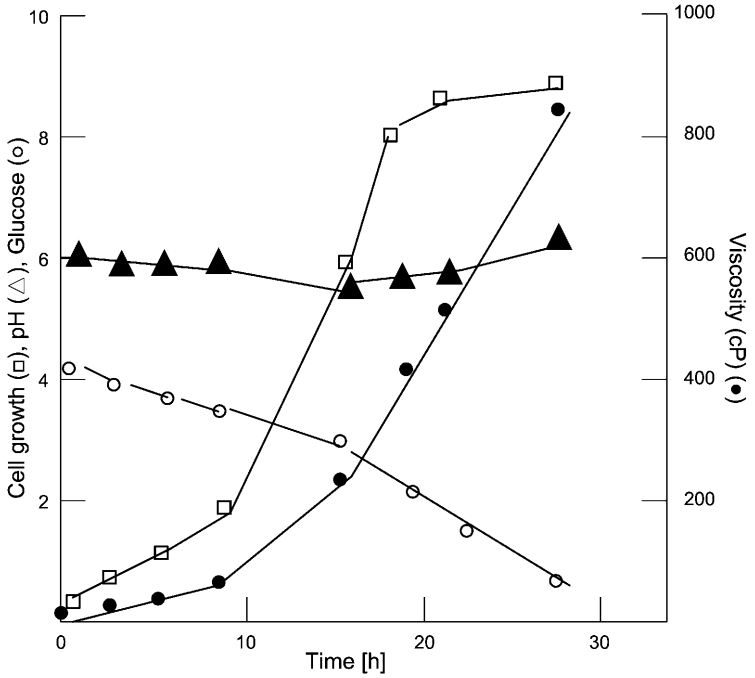

Fig. 2. *Fermentation profile of γ-PGA* (5-ton scale). Viscosity (●), glucose (○), cell growth (□), pH (▲).

condition is very important for mass production of γ-PGA. Much research has been conducted on identifying the optimal culture conditions for the production of γ-PGA [1][18][19]. The culture conditions used can have a great impact on the production yield and average molecular weight of γ-PGA produced. *B. subtilis (chungkookjang)* was shown to have an absolute requirement of Mn^{2+} for sporulation, but produced abundant γ-PGA without Mn^{2+}. The γ-PGA productivity of *B. subtilis (chungkookjang)* remains unchanged even after long-term storage and repeated subcultivation [10]. It is likely that *B. subtilis (chungkookjang)* will serve as a potent tool not only in the development of a mass-producer of γ-PGA but in the elucidation of the γ-PGA synthetic mechanism in *B. subtilis* in its entirety. Production of high-molecular-weight γ-PGA is one of the unique characteristics of *B. subtilis (chungkookjang)*, and molecular weight of γ-PGA can be controlled by varying the NaCl concentration in the medium [16]. For high-molecular-weight γ-PGA produced, a method was designed for the free amino group quantification and measurement of glutamic acid concentration after HCl hydrolysis [20]. Generally, γ-PGA is an exocellular capsular polymer produced by several members of the genus *Bacillus* with molecular weights ranging from 100 to over 1,000 kDa. In the case of *B. subtilis (chungkookjang)*, the molecular weight of γ-PGA in culture broth reaches over 10,000 kDa, which is the highest molecular weight reported so far.

Biological Safety. – γ-PGA is a safe and edible biomaterial that is naturally synthesized by *Bacillus subtilis*. However, for the use of γ-PGA in food supplements, cosmetics, and pharmaceuticals, and for medicinal purposes, its potential toxicity needs to be examined. The toxicity of γ-PGA on the human B-cell line EHRB and on mice has been evaluated. No toxic effect on the EHRB cells was reported at 20 mg/l, and there was only a weak effect at 100 mg/l. Furthermore, no toxic effect was observed in mice following the injection of 1 mg of γ-PGA [21]. The acute toxicity of γ-PGA developed by *BioLeaders Corp*. (Korea) for cosmetic uses and *B. subtilis (chungkookjang)* were investigated. The molecular weight of γ-PGA in this study was 2,000 kDa. γ-PGA (2.5 g/kg) and 0.1 ml of 10^{10} cfu/ml of *B. subtilis (chungkookjang)* were administered to the Balb/c mice orally. The mice were monitored for clinical signs or death for 2 weeks. In both cases, LD_{50} value was not accessible as there were no deaths. Between the treated and the control groups, there were no statistically significant differences in body-weight changes, clinical signs, or pathological changes during the 14-d observation period. Accordingly, the γ-PGA and *B. subtilis (chungkookjang)* are considered not to have acute toxicity in mice [16]. Repeated oral administration of γ-PGA to rats for 13 weeks exhibited diarrhea and soft stool in males and females treated with 2,000 mg/kg, and increased blood-chemistry value in BUN in males treated with 2,000 mg/kg. These differences were considered to be related to treatment with test substance, but to be of no toxicological importance. Therefore, no observed adverse effect level (NOAEL) was considered to be 2,000 mg/kg both in males and females SD rats. Also, significantly increased values in BUN in males treated with 2,000 mg/kg of test substance revealed no distinct differences when compared to controls after recovery period, and, therefore, it was considered to be a reversible effect. Other tests including bacterial reverse-mutation test, *in vivo* micronucleus test, *in vitro* chromosome aberration test, cumulative skin-irritation test, photosensitization test, and eye-

irritation test were performed in accordance with standard operating procedures of *Biotoxtech Co., Ltd*. (Korea), and following good laboratory practice and test guideline. According to the results, it was concluded that high molecular weight of γ-PGA ($>2{,}000$ kDa) is a very safe biomaterial.

Specific Applications of High-Molecular-Mass γ-PGA. – *In vivo* tumor regression activity of high-molecular mass γ-PGA from *B. subtilis (chungkookjang)* was analyzed [22]. C57BL/6 Mice were orally administered 10, 100, or 2,000 kDa γ-PGA or β-glucan (positive control), and antitumor immunity [23][24] was examined. The results revealed that higher levels of NK cell-mediated cytotoxicity and IFN-γ secretion in mice treated with 2,000 kDa γ-PGA was observed than those treated with lower-molecular-mass γ-PGA (10 or 100 kDa) or β-glucan. These novel findings indicate that high-molecular-mass γ-PGA mediates antitumor immunity *via* the activation of NK cells, suggesting that γ-PGA may be a good candidate for cancer immunotherapy. Furthermore, γ-PGA induced secretion of tumor necrosis factor (TNF)-α from the bone-marrow-derived macrophages of wild type (C57BL/6 and C3 H/HeN) and *Toll*-like receptor 2 knock-out mice, but not that of myeloid differentiation factor 88 knockout and TLR4-defective mice. In summary, the results demonstrated that treatment with high-molecular-weight γ-PGA (2,000 kDa) initiated innate immune responses, such as iDC maturation, *via* TLR4 signaling in mice. These findings strongly suggest that γ-PGA may be a new immunomodulator targeting TLR4 (*Fig. 3*), and may warrant consideration as a therapeutic agent for cancer and other diseases [25]. Nanoparticles formed from γ-PGA and chitosan have been designed for the oral delivery of hydrophobic drugs and proteins. Gelation was performed simply by an ionic interaction under mild conditions, and zeta-potential and particle size can be controlled by changing the composition and concentration of the reaction mixture [26][27]. A different type of nanoparticle was designed to target refolding of inclusion body protein or using as drug-delivery vehicle. This particle contains a γ-PGA as a hydrophilic part and cholesterol as a hydrophobic part to form amphiphilic nanoparticle as shown in *Fig. 4*. However, detailed information on these promising results has yet to be published. γ-PGA-Based nanofiber sheets were prepared by electrospinning technique to evaluate the ability of the prevention of postoperative tissue adhesion [28]. Electrospinning is a unique process to produce submicron polymeric fibers in the average diameter range of 100 nm–5 μm [29][30]. Currently, as shown in *Fig. 5*, different compositions were studied for the control of dissolving speed of nanofiber for cosmetics use.

Conclusions. – Since γ-PGA is substantially biodegradable in the environment, nontoxic to humans, and even edible, its potential applications have been studied from an industrial standpoint in the past years. From the discovery of *B. subtilis (chungkookjang)* that produces high-molecular-weight of γ-PGA, specific applications have continued to be developed during the last ten years. Potential industrial and scientific benefits might accrue form extensive investigations of structurally and functionally unique γ-PGA. The high-molecular-weight γ-PGA may be a potential antitumor agent which can modulate the immune system against tumors. The coming decade will undoubtedly be devoted to further developments in functional research but

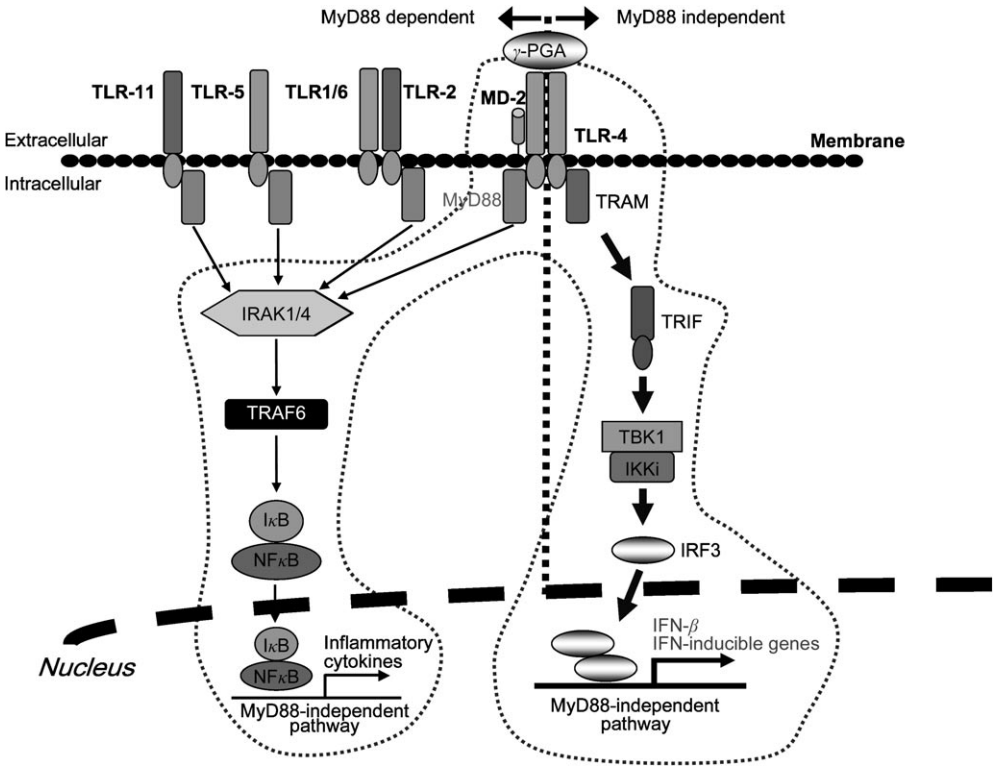

Fig. 3. *γ-PGA initiates innate immune responses* via *TLR4 signaling*

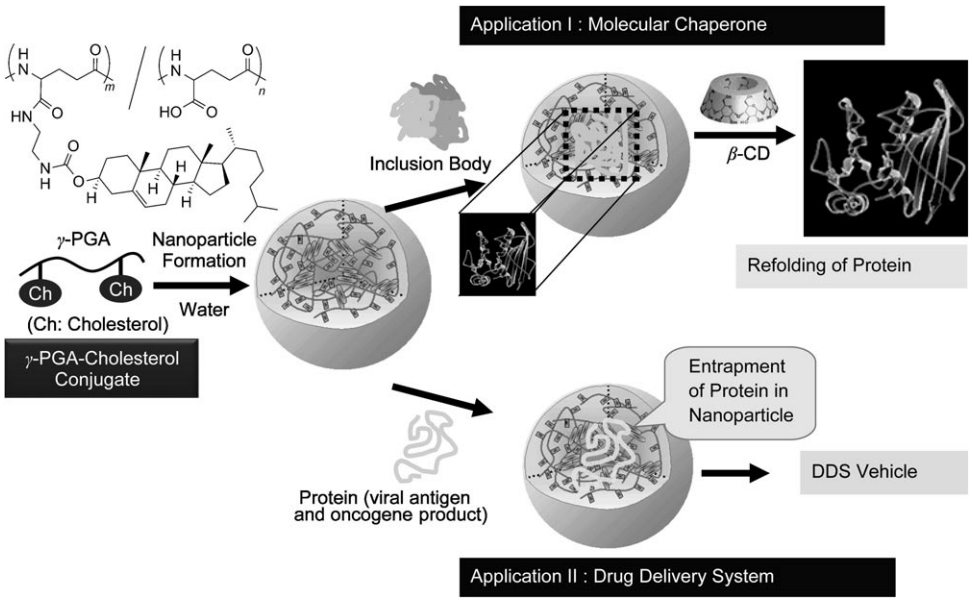

Fig. 4. *γ-PGA–Cholesterol nanoparticle and applications*

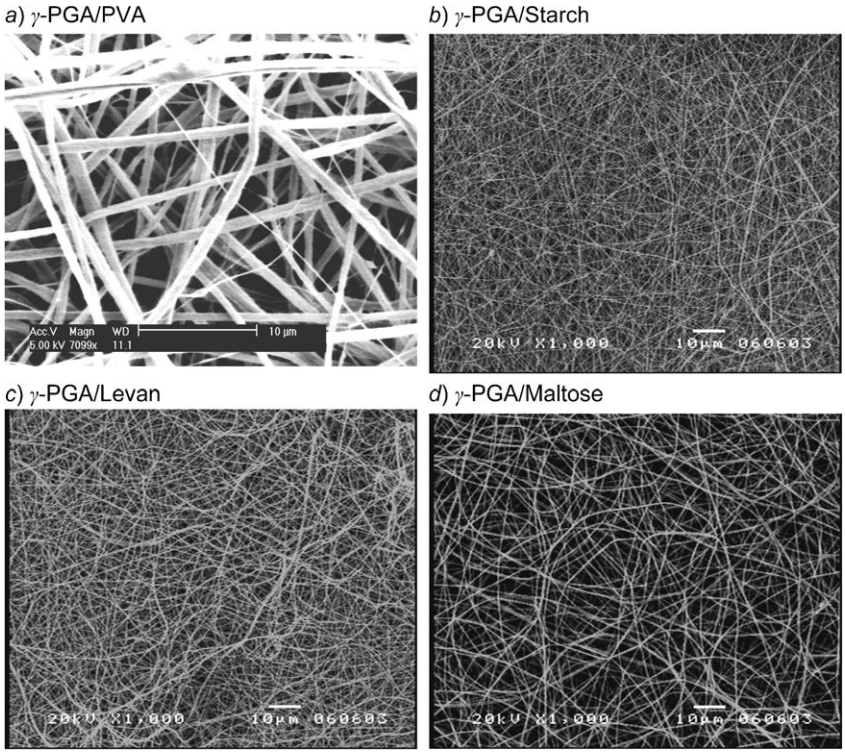

Fig. 5. *SEM Images of various biodegradable nanofiber sheets*

also in numerous areas including fundamental research. Still, much remains to be discovered of this highly promising polymer.

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