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Synthesis of super-high-molecular-weight poly-γ-glutamic acid by *Bacillus subtilis* subsp. *chungkookjang*

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Abstract

Poly-γ-glutamic acid (PGA) with high molecular weight is a most promising biomaterial in industrial uses; however, it generally diverse in molecular structure and co-produced with polysaccharides and various other biopolymers. In this study, it was ascertained that *Bacillus subtilis* subsp. *chungkookjang* cells are superior to *B. subtilis* (*natto*) cells as the biocatalyst for the synthesis of super-high-molecular-weight PGA (over 2000 k). We effectively purified PGA and fractionated according to its molecular weight by anion-exchange chromatography, and further developed a simple method for determination of the molecular weight of PGA on the basis of numbers of glutamate monomers generated by hydrolysis and a free amino group quantified with 1-fluoro-2,4-dinitrobenzene (FDNB). The molecular weight determination with FDNB was available even for a super-high-molecular-weight PGA, e.g. the 2000-k polymer. Super-high-molecular-weight PGAs (average 2000 k and 7000 k), which were synthesized by the use of *B. subtilis* subsp. *chungkookjang* cells in the presence of a high concentration of ammonium sulfate, were rich in L-glutamate rather than in the D-enantiomer.

Keywords: Poly-γ-glutamic acid; *Bacillus subtilis* subsp. *chungkookjang*; Purification; Molecular weight; Chemical determination; Stereochemistry

1. Introduction

Based on the fact, namely that poly-γ-glutamate (PGA) possesses multi-functionalities [1–3], its application – biodegradable substitutes (as thermoplastics, fibers, films and membranes), hydrogels with very high water-absorption capability, flocculants, heavy metal- and radionuclide-binding agents, cryoprotectants, bitterness-relieving agents, thickeners, animal feed additives, osteoporosis-preventing factors, humectants, drug deliverers, gene vectors, curative biological adhesives, dispersants, and enzyme-immobilizing materials, for example – now attracts particular attention.

PGA is a multi-anionic γ-polypeptide (or isopolypeptide) that usually contains both enantiomers of glutamate at various ratios [4]; for the time being, it is almost impossible to chemically synthesize such the high-molecular-weight polymer. In contrast, the polymer can be synthesized from glutamate monomers by the uses of biocatalysts, such as whole cells of *Bacillus subtilis* (*natto*) [1,5], viable cells of the *Escherichia coli* clone that bears the PGA synthetase complex-encoding *pgsBCA* genes [3,6], and enzyme-associated cell membranes of *B. subtilis* subsp. *chungkookjang* [7]. *B. subtilis* (*natto*) and *B. subtilis* subsp. *chungkookjang* are actually utilized as the starters of Natto and Chungkookjang, traditional

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Japanese and Korean fermented sticky foods made from soybeans, respectively [8–10], and the former typically produces PGA, the predicted molecular weight of which ranges widely from 10 k to 1000 k, with various other biopolymers including polysaccharides. It has been assumed that PGA is changeable in molecular structure [2], depending on the bacterial strains used, broth components, culture conditions and other unknown reasons, and many attempts demonstrated that it is difficult to obtain highly homogenous PGA from the bacterial cultures, owing to such the instability and molecular complexity. Nevertheless, a recent study implied that super-high-molecular-weight PGA (over 2000 k) exhibits unexpectedly attractive functionalities, e.g. an immunological enhancement effect on mammals [11]. Hereafter, it is requested to establish a systematic conversion of commercially inexpensive L-glutamate into super-high-molecular-weight PGA, the value-added new biomaterial.

Here we present the utility of *B. subtilis* subsp. *chungkookjang* cells as the biocatalyst for the synthesis of super-high-molecular-weight PGA, the effective purification of the polymer, and its structural assays using the some new methods proposed recently [7].

2. Experimental

2.1. Materials

1-Fluoro-2,4-dinitrobenzene (FDNB) was purchased from Sigma, St. Louis, MO; Proteinase K from TaKaRa Shuzo, Kyoto, Japan; a Mini-ProteanII Ready Gel J (linear gradient of the gel concentration, 5–15%) and a protein assay kit from Bio-Rad, Richmond, CA; an HMW marker kit containing myosin (200 k), α-2-microglobin (170 k), β-galactosidase (116 k), transferrin (76 k), and glutamate dehydrogenase (53 k) from Amersham Pharmacia Biotech, Buckingham, UK; vials of distilled HCl from PIERCE, Rockford, IL. All other chemicals were of analytical grade.

2.2. Production and preparation of PGA

B. subtilis (*natto*) IFO 3336 [8] and *B. subtilis* subsp. *chungkookjang* KCTC 0697BP [10] were used as PGA producers in the experiments. The growing cells (wet weight, 1 g) were inoculated into 50 ml of a modified GS medium comprising 2% L-glutamate, 5% sucrose, 0.27% KH_2PO_4, 0.42% Na_2HPO_4, 5% NaCl, 0.5% $MgSO_4{\cdot}7H_2O$, a Murashige-Skoog vitamin solution (PhotoTechnology Laboratories, Shawnee Mission, KS), and 1% ammonium sulfate. Cultivation was carried out at 30 °C for 1 week under shaking. The culture medium was collected, then adjusted to pH 3.0 with 6 M sulfuric acid and incubated at 4 °C for 12 h, and poured into three volumes of ethanol [9]. The precipitate was lyophilized, dissolved in 10 mM Tris–HCl buffer (pH 7.5), and treated with Proteinase K [7,10]. The resulting mixture was then dialyzed against distilled water and used as a PGA sample.

2.3. Purification of PGA

PGA was purified by anion-exchange chromatography as follows. The dialyzed PGA sample was subjected to a Sep-Pak Plus Waters Accell^{TM} Plus QMA cartridge (Millipore, Bedford, MA) equilibrated with distilled water. After washing with 10 ml of distilled water, the cartridge column charged with PGA was stepwise developed with 5 ml of NaCl solutions from 0.1 M to 1.0 M. The fractions bearing PGA were lyophilized. The decontamination of glutamate, polysaccharides, and proteins was verified by the glutamate dehydrogenase-coupling assay [6,8], the phenol–sulfate method [6], and the application of the protein assay kit [7], respectively.

2.4. PGA assay

PGA was first hydrolyzed, and the hydrolysate thus formed was subjected to high-performance liquid chromatography (HPLC) with a chiral carrier, according to the procedures described previously [6,10]. The amount and stereochemistry of PGA were estimated from total content and the DL-ratio of glutamate generated in the hydrolysate, respectively [7].

2.5. Physico-chemical estimation of molecular weight of PGA

The molecular weight of PGA was estimated from the visualized data of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [1,7,8,10,12] with a Digital Science EDAS 120 LE densitometry system (Gibco BRL, Grand Island, NY). It was alternatively determined by gel permeation chromatography (GPC) as follows. PGA (dry weight, 10 mg) was suspended in 1 ml of 0.1 M $NaNO_3$ and dissolved by the addition of 15 μl of 5 M KOH followed by centrifugation at 12,000 × *g* at 25 °C for 10 min. The resulting supernatant (1 ml) was diluted with 9 ml of 0.1 M $NaNO_3$, and subjected to HPLC with a Visco GEL GMPWXL column (7.8 mm × 30 cm; from Viscotek, Houston, TX), which had been equilibrated with 0.1 M $NaNO_3$, at 30 °C. The column was developed with 0.1 M $NaNO_3$ at a flow rate of 0.8 ml min^{-1}. PGA was detected with a Viscotek LR 25 Laser Refractometer, and the average molecular weight was estimated from the retention time of PGA. Polyethylene oxide was used as a tentative standard marker for molecular weight estimation.

2.6. Preparation and determination of dinitrophenyl glutamate (DNP-Glu)

Glutamate was dissolved in 100 μl of 0.1 M borate buffer (pH 8.5). After centrifugation at 12,000 × *g* for 10 min at 25 °C, the supernatant was mixed with 10 μl of 10 mM FDNB

solution (in acetone) [13]. The mixture was incubated at 65 °C for 45 min in the dark. The modification was terminated by incubation in 3.3 M HCl at 105 °C for 12 h. DNP-Glu thus formed was dissolved in 100 μl of distilled water and determined by monitoring an increase in the absorbance at 356 nm against a reagent blank (FDNB-free) and a negative control (glutamate-free) with an Ultrospec 2100 Pro spectrophotometer (Amersham Bioscience, Uppsala, Sweden) [7].

3. Results and discussion

3.1. Chemical determination of the molecular weight of PGA with FDNB

To establish the chemical determination of the molecular weight of PGA, we focused on the fact that every molecule of PGA has one terminal free amino group irrespective of the number of γ-glutamyl residues. Thus, the ratio of numbers between the amino group and γ-glutamyl residues of PGA is parallel to its molecular weight, as reported for determination of lisinopril molecular weight [13]. As the number of amino group can be estimated by measuring the amount of DNP-Glu from the FDNB-modified PGA [7], the average molecular weight of PGA was calculated by the following Eq. (1), in which the value, 129 corresponds to the molecular weight of one γ-glutamyl residue.

$$\text{Average molecular weight} = \frac{129 \times \text{number of } \gamma\text{-glutamyl residue}}{\text{number of amino group}} \quad (1)$$

Fig. 1 shows the standard curve of DNP-Glu: $y = 0.009x$, where x and y represent the concentration and absorbance at 356 nm, respectively; it gave a good linearity in a range of 1.5–200 μM DNP-Glu. The present method shows a high sensitivity; for example, even when PGA with a molecular weight of 1000 k is analyzed, only a small amount of the polymer sample, such as 0.15 mg, is required for its molecular weight determination.

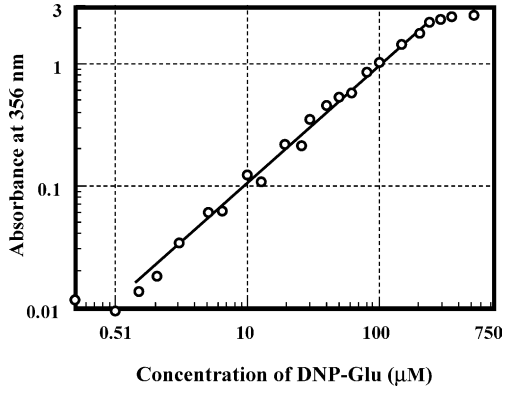

Fig. 1. Standard curve of dinitrophenyl glutamate (DNP-Glu).

3.2. Further utility of the FDNB method in PGA study

In some literatures [14–17], total precipitates formed usually when a high concentration of ethanol was added to the culture of *B. subtilis* (*natto*) had been assuming to be pure PGA with neither additional treatment [5,9,10] nor purity assessment [7,8,13], and directly subjected to its molecular weight estimation using GPC and the analysis of the polymer stereochemistry using HPLC with a chiral carrier. Then, we attempted the one-step preparation of PGA from *B. subtilis* culture according to the method of Nagai et al. [15]. The obtained PGA was fractionated in dependence on the apparent molecular weight by means of GPC as described in Section 2. Every fraction was subjected to the SDS-PAGE analysis; as PGA sample that we can prepare is, in fact, a mixture of different lengths of γ-glutamyl polypeptides, its corresponding bands are not sharp and generally represents as apparently smear staining area on an SDS-PAGE gel [8,10,14]. The result showed that the 50-k, 250-k, 2000-k, 3800-k, and 5000-k PGAs (obtained by the GPC-fractionation) were distributed over the molecular weight ranges of less than 10 k to 170 k, 10 k to 500 k, 100 k to 1000 k, 100 k to 1000 k, and 170 k to over 1000 k, respectively (Fig. 2). It seems likely that the SDS-PAGE analysis is not applicable for the precise assay of high-molecular-weight PGA with over 2000 k. We further examined the average molecular weight of PGA contained in every fraction by the FDNB method and obtained an unexpected result, namely that all the molecular weights were calculated to be less than 1000 (1 k). The one-step preparation of PGA [14–17] may give rise to

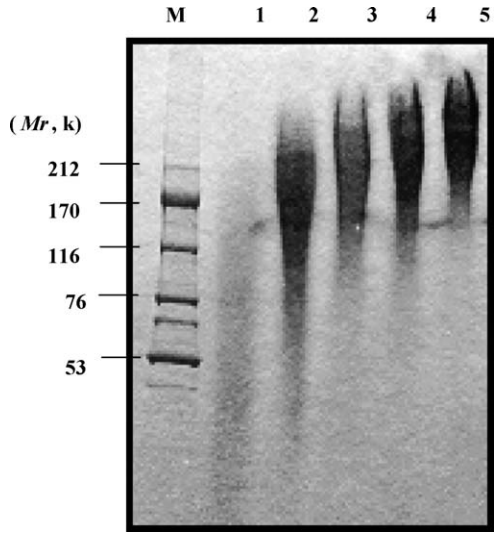

Fig. 2. Molecular weight-dependent fractionation of *B. subtilis* PGA by GPC. According to the procedures described in Section 2, PGA was subjected to SDS-PAGE using a Mini-ProteanII Ready Gel J with the HMW marker kit (lane M) and visualized as smear area by methylene-blue staining [8]. The data represent gel permeation chromatography (GPC)-fractionated PGAs (each 50 μg) with an apparent molecular weight of 50 k (lane 1), 250 k (lane 2), 2000 k (lane 3), 3800 k (lane 4), and 5000 k (lane 5).

Table 1
Molecular weight determination of PGAs from *B. subtilis* (*natto*) IFO 3336

Samples	Molecular weights	
	Apparent[a]	Found[b]
PGA sample (1)[c]	10 k to 1000 k	39 ± 13 k
0.1-M NaCl fraction (2)	N.D.[d]	146 (L-glutamic acid)[e]
0.2-M NaCl fraction (3)	N.D.	N.D.
0.3-M NaCl fraction (4)	10 k to 20 k	13 ± 4 k
0.4-M NaCl fraction (5)	20 k to 150 k	52 ± 13 k
0.5-M NaCl fraction (6)	50 k to 300 k	130 ± 40 k
0.6-M NaCl fraction (7)	100 k to 1000 k	390 ± 40 k
0.7-M NaCl fraction (8)	200 k to >1000 k	1300 ± 130 k
0.8-M NaCl fraction (9)	N.D.	N.D.
1.0-M NaCl fraction (10)	N.D.	N.D.

[a] The data of the molecular weight distribution of PGA were densitometrically estimated from the data of the SDS-PAGE method.
[b] The data of the average molecular weight of PGA were determined by the FDNB method.
[c] The numeral put in parentheses corresponds to the lane numbered in the SDS-PAGE method (see Fig. 3).
[d] N.D., not detectable.
[e] Free L-glutamate shared 0.14% of total glutamate content in the PGA sample.

some issues, e.g. contamination of free amino acids or other compounds from the used media into the samples.

It is noteworthy that free L-glutamate present in PGA samples, which should give rise to some profound issues not only in the molecular weight determination of PGA by the FDNB method but also in the analysis of the polymer stereochemistry (i.e., determination of the DL-ratio of glutamate generated in the polymer hydrolysate by HPLC with a chiral carrier), was essentially fully eluted with 0.1 M NaCl by the use of anion-exchange chromatography (Tables 1 and 2). In contrast, it was estimated that free L-glutamate shared over several % of total glutamate content in every PGA sample obtained by only the one-step preparation [14–17] and by the direct GPC-fractionation (Fig. 2). The results implicate that the separation of PGA and free glutamate is not achieved by only the GPC method. However, this is not surprising, since PGA, especially a high concentration of the high-molecular-weight polymer, substantially adsorbs various bio-products including bioactive minerals such as Ca^{2+}, heavy metals, small bio-molecules such as amino acids and peptides, and further macromolecules, and is practically applied as the biodegradable flocculant [1]. The use of anion-exchange chromatography is indispensable to obtain the high-purity PGA sample applicable for the precise structural analysis of the useful polymer and its application as a fine chemical, and the FDNB method is useful for assessment of the purity of PGA samples besides the molecular weight determination. Their strategies would give a clue to resolve the conflict of the amount of PGA produced with the enantiomer composition of PGA.

Hereafter, in order to avoid and to resolve the conflict between the data by GPC and the FDNB method, we adopted the multi-step preparation of PGA (see Section 2) for the following experiments.

Table 2
Molecular weights of PGAs from *B. subtilis* subsp. *chungkookjang*

Samples	Molecular weights	
	Apparent[a]	Found[b]
PGA sample (1)[c]	100 k to >1000 k	65 ± 20 k
0-M NaCl fraction (2)	N.D.[d]	N.D.
0.03-M NaCl fraction (3)	N.D.	N.D.
0.1-M NaCl fraction (4)	N.D.	146 (L-glutamic acid)[e]
0.2-M NaCl fraction (5)	N.D.	N.D.
0.3-M NaCl fraction (6)	N.D.	N.D.
0.4-M NaCl fraction (7)	N.D.	N.D.
0.5-M NaCl fraction (8)	N.D.	N.D.
0.6-M NaCl fraction (9)	100 k to 1000 k	910 ± 70 k
0.7-M NaCl fraction (10)	200 k to >1000 k	1800 ± 130 k
0.8-M NaCl fraction (11)	200 k to >1000 k	N.D.

[a] The data of the molecular weight distribution of PGA were densitometrically estimated from the data of the SDS-PAGE method.
[b] The data of the average molecular weight of PGA were determined by the FDNB method.
[c] The numeral put in the parentheses corresponds to the lane numbered in the SDS-PAGE method (see Fig. 4).
[d] N.D., not detectable.
[e] Free L-glutamate shared 0.19% of total glutamate content in the PGA sample.

3.3. Purification and fractionation of PGA by anion-exchange chromatography

As shown in lane 1 of Fig. 3, the PGA sample from *B. subtilis* (*natto*) IFO 3336 [8] contains polymers with a various molecular weight (Mr., less than 10 k to 1000 k). The FDNB method indicated that the average molecular weight of PGA from *B. subtilis* (*natto*) IFO 3336 was 39 ± 13 k (Table 1). Based on the grounds that the acidity of PGA increases with an increase in molecular weight corresponding to the number of γ-glutamyl residues, we attempted the purification of *B. subtilis* (*natto*) PGA by the use of anion-exchange

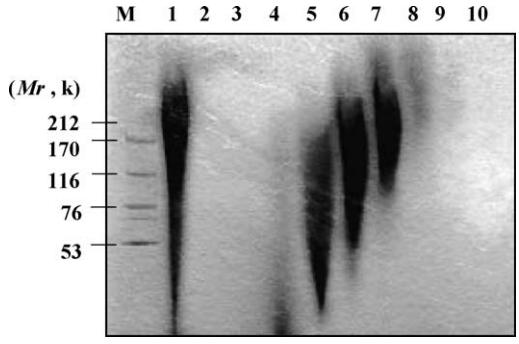

Fig. 3. Molecular weight-dependent fractionation of PGAs from *B. subtilis* (*natto*) IFO 3336 by anion-exchange chromatography. PGA was subjected to SDS-PAGE and visualized by the same way described in Fig. 2.The data represent the HMW marker kit (lane M), the PGA sample (50 μg; lane 1), and the fractions (each 20 μl) eluted with 0.1 (lane 2), 0.2 (lane 3), 0.3 (lane 4), 0.4 (lane 5), 0.5 (lane 6), 0.6 (lane 7), 0.7 (lane 8), 0.8 (lane 9), and 1.0 M (lane 10) NaCl solutions.

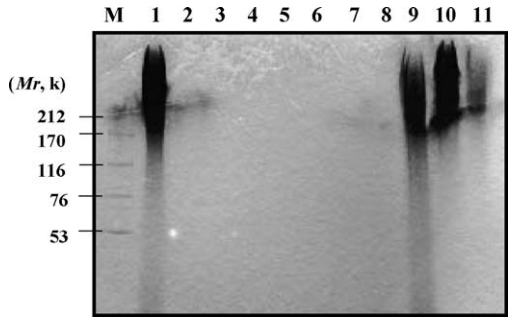

Fig. 4. Molecular weight-dependent fractionation of PGAs *B. subtilis* subsp. *chungkookjang* by anion-exchange chromatography. PGA was subjected to SDS-PAGE and visualized by the same way described in Fig. 2. The data represent the HMW marker kit (lane M), the PGA sample (50 μg; lane 1) and the fractions (each 20 μl) eluted with 0 (lane 2), 0.03 (lane 3) 0.1 (lane 4), 0.2 (lane 5), 0.3 (lane 6), 0.4 (B, lane 7), 0.5 (lane 8), 0.6 (lane 9), 0.7 (lane 10), and 0.8 M (lane 11) NaCl solutions.

chromatography. As a result, PGA was fractionated in dependence on the molecular weight (Fig. 3, lanes 2–10). The data of the molecular weight of PGA contained in every fraction that was determined chemically (Table 1) are in agreement with those estimated by the SDS-PAGE method (Fig. 3, lanes 2–10). Thus, anion-exchange chromatography is relevant and practical to purify and fractionate PGA besides GPC based on the molecular filtration.

By the SDS-PAGE method, we ensured that the apparent molecular weight of the PGA sample from *B. subtilis* subsp. *chungkookjang* (200 k to over 1000 k; Fig. 4, lane 1) is much higher than that from *B. subtilis* (*natto*) (Fig. 3, lane 1). The average molecular weight of PGA from *B. subtilis* subsp. *chungkookjang*, however, was determined to be 65 ± 20 k by the FDNB method (Table 2); this value is obviously small compared with those estimated from the visualized data. The PGA sample from *B. subtilis* subsp. *chungkookjang* was subjected to anion-exchange chromatography as well. The result shows that *B. subtilis* subsp. *chungkookjang* PGA substantially includes little amount of low-molecular-weight polymers, unlike *B. subtilis* (*natto*) PGA (Fig. 4). Tables 1 and 2 revealed that multiple trials to determine the molecular weight of PGAs purified gave reproducible values.

3.4. Isolation of super-high-molecular-weight PGA

Our observations suggest that it is essentially difficult to isolate super-high-molecular-weight PGA (over 2000 k) from the PGA samples of *B. subtilis* (*natto*). Then, high-molecular-weight PGAs from *B. subtilis* subsp. *chungkookjang* (Fig. 4, lanes 10 and 11) were re-subjected to the GPC method and fractionated to PGA samples I (apparent molecular weight, 500 k), II (1000 k), III (2000 k), and IV (7000 k) (Table 3). Average molecular weights of these PGA samples were verified by the FDNB method; the result was in good agreement with those by GPC over a range of 500–2000 k, whereas it was impossible to determine accurately PGA with an average molecular weight of 7000 k.

Table 3
Determination of super-high-molecular-weight PGA

PGAs	Average molecular weights	
	Found A[a] (k)	Found B[b] (k)
Sample I	500	390 ± 40
Sample II	1000	780 ± 70
Sample III	2000	1600 ± 130
Sample IV	7000	N.D.[c]

[a] The data were estimated by analysis using GPC.
[b] The data were determined by the FDNB method; 0.8 mg of each polymer was used for the molecular weight determination.
[c] N.D., not detectable.

The result, conversely, implicates that the molecular weight of the sample IV is at least higher than the value of 2000 k.

3.5. Stereochemistry of super-high-molecular-weight PGA

We additionally analyzed the stereochemistry of the 2000-k and 7000-k PGAs, which were produced by *B. subtilis* subsp. *chungkookjang* in the ammonium sulfate-supplemented GS medium, and found that both polymers consisted of 42.5 ± 7.5% D- and 57.5 ± 7.5% L-glutamate (the six independent experiments were carried out in duplicate). As we previously found that the bacterium synthesizes D-glutamate-rich PGA (D-isomer content, 72.5 ± 7.5%) in the original GS medium [7,10], the finding that it could synthesize even such L-glutamate-rich DL-PGA is unexpected. Low-molecular-weight PGAs from *B. subtilis* subsp. *chungkookjang* in the other fractions were rich in L-glutamate as well (data not shown). This is thus the first example showing that ammonium sulfate may also be a determinant for the stereochemistry of PGA besides manganese ions [15,18,19].

Structurally designed PGAs, e.g. high-molecular-weight PGA that consists only of one enantiomer of glutamate, contribute to the development of new green-technology [1]. In general, the thermoplastic functionality of biomaterials (polymers) increases with an increase in the enantiomeric homogeneity and degree of polymerization [20]. Low-molecular-weight PGA composed of only D-glutamate will serve as food additives with various functions [1,3], since D-glutamate is almost tasteless [21] and D-glutamyl oligomers also have little taste and odor. *B. subtilis* subsp. *chungkookjang* cells (as biocatalyst) are thus useful for the further study of PGA synthesis, which helps developing the structure designing of PGA-based biomaterials. On the other hand, the biochemical techniques presented in this issue may also provide insights into the structural assays of other attractive biomaterials derived from polyamino acids, e.g. ε-poly-L-lysine from *Streptomyces albulus* [22].

4. Conclusion

Until now, many literatures have dealt in characteristics of the biocatalysts for PGA synthesis

[1,5–9,10,14–16,18,23–25]. However, attempts have been made to develop the effective production of super-high-molecular-weight PGA (over 2000 k) with no success, due to the disadvantageous nature seen in usual PGA producers including *B. subitlis* (*natto*), namely that PGA degradation initiates with the synthesis (or elongation) of the polymer [16,17,25], the low PGA productivity of the *E. coli* clone cells harboring the membranous PGA synthetase complex from *B. subtilis* [6], the extreme instability of the PGA synthetase itself [7], and other factors. In this study, on the basis of the important finding that PGA degradation is not observed during the polymer synthesis in *B. subtilis* subsp. *chungkookjang* [10], we for the first time demonstrated that cells of the strain actually served as the good biocatalyst in the synthesis of useful super-high-molecular-weight PGA [11].

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