Biosynthesis and Hydrolysis of Poly(γ-glutamic acid) from Bacillus subtilis IFO3335

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Poly(γ-glutamic acid) (PGA) production in Bacillus subtilis IFO3335 was studied. When citric acid as a carbon source was added to a glutamic acid medium containing L-glutamic acid and ammonium sulfate, a large amount of pure PGA was produced. On the other hand, when glucose was added to the glutamic acid medium, a by-product was produced, which seemed to be a polysaccharide. Moreover, the mode of hydrolysis was investigated with PGA in aqueous solutions at 80, 100, and 120°C by monitoring the time-dependent changes in the molecular weights. Hydrolytic degradation of PGA was found to proceed through a random chain scission.

It is reported that several bacteria produce poly(γ-glutamic acid) (PGA) (1) outside of the cells.1–4 PGA, which is water-soluble and biodegradable, can be expected to be used for thickeners, humectants, substained release materials, or drug carriers with biodegradability in the fields of food, cosmetics, or medicine.

\[ \text{COOH} \quad \text{O} \\
\text{\(-\text{NH}-\text{CH}-\text{CH}_2-\text{CH}_2-\text{C}-\)}_n \]  

(1)

Ivánovics and co-workers5,6) discovered PGA as a capsule of Bacillus anthracis in 1937. Since Bovarnick7) showed that PGA was accumulated in a culture broth of B. subtilis as a product on fermentation in 1942, much research on PGA has been done. On the other hand, some researchers had been interested in a viscous material called Natto, a traditional food product. In 1905, Sawamura8) isolated a bacterium from Natto and named it B. natto Sawamura. Then Fujii9) showed that Natto contains PGA and a mucin, and that the mucin from Natto consists of a polysaccharide (levan-form fucatan). The PGA-producing bacteria have different nutritional requirements for PGA production. Many of these bacteria require L-glutamic acid for PGA production,10,11) while Murao and co-workers12) isolated B. subtilis var. polysuglumic which does not require L-glutamic acid. It was also shown that some PGA-producing bacteria require biotin, one of the vitamins.13)

In this paper, culture conditions for PGA production without by-products (polysaccharides, etc.) were investigated by using Bacillus subtilis IFO3335 requiring biotin. Addition of citric acid to a glutamic acid medium containing L-glutamic acid and ammonium sulfate resulted in a higher yield of PGA without any by-product. Moreover, the mode of hydrolysis of PGA in aqueous solutions was analyzed by following the time-dependent changes in the molecular weights.

Materials and Methods

Strain and culture condition: Bacillus subtilis IFO3335 (obtained from the Institute for Fermentation, Osaka) was used in this study. The bacterium was first grown aerobically in a medium (100ml) containing 1g of poly glucose, 0.2g of yeast extract, and 0.1g of MgSO_4·7H_2O at 37°C for 20hr. The culture broth was added to the same volume of 20% glycerol aqueous solution. Before being frozen, the bacterial cells were annealed at 60°C for 10 min so that they formed spores to maintain the activity of PGA production.11) They were kept at -20°C. The stored spore suspension was inoculated in 100ml of media,14) adjusted to pH 7.5, in 500-ml Sakaguchi flasks containing 0 to 5g of L-glutamic acid, 0 to 2g of ammonium sulfate, 0 to 5g of a carbon source (glucose, acetic acid, citric acid, l-malic acid, succinic acid, or fumaric acid), 0.1g of K_2HPO_4, 0.1g of Na_2HPO_4·12H_2O, 0.05g of MgSO_4·7H_2O, 0.002g of MnSO_4·nH_2O, 0.005g of FeCl_3·6H_2O, 0.02g of CaCl_2, and 50ug of vitamin H. The media were used after autoclaving at 121°C for 15 min. Cultivation was done aerobically with shaking (120rpm) at 37°C.

Preparation of pure PGA. The methods of isolation and purification of PGA are shown in Fig. 1. The cells were separated from a culture broth by centrifugation. The cells were lyophilized, and the dried cell weights were measured. The supernatant containing PGA was poured into 4 volumes of methanol and left overnight, and the resultant precipitate was collected by centrifugation. The crude product thus obtained was lyophilized, and crude PGA afforded. After the crude PGA was dissolved

<table>
<thead>
<tr>
<th>Culture broth</th>
<th>Centrifuged at 20,000 × g for 15 min.</th>
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<tbody>
<tr>
<td>Culture supernatant</td>
<td>Cell</td>
</tr>
<tr>
<td>Poured into 4 volumes of methanol, and precipitate is centrifuged at 20,000 × g for 15 min. Precipitate is dried in vacuo.</td>
<td></td>
</tr>
<tr>
<td>Crude PGA</td>
<td>Dissolved in 100–200 volumes (w/v) of distilled water, and centrifuged at 25,000 × g for 15 min</td>
</tr>
<tr>
<td>Sup.</td>
<td>Desalted by using IE-Labo for 30min—5hr, and dried in vacuo.</td>
</tr>
<tr>
<td>Pure PGA</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 1. Preparation of Pure PGA.](image-url)
in distilled water (0.5–1 w/v%), any insoluble materials were removed by centrifugation. The aqueous solution of the crude PGA thus obtained was desalted by electrodialysis (IE-Labo, a desalter apparatus from Tosoh Co.) and lyophilized to give pure PGA.

**Analytical procedures.** Analysis of PGA was done with amino acid autoanalyzer (Hitachi 835). Samples were prepared by hydrolysis of the PGA obtained in 6N HCl at 150°C for 3 hr in a glass vial under a nitrogen atmosphere with a Pico-Tag apparatus. It was confirmed that lyophilized samples were pure PGA by detection of only glutamic acid.

The molecular weight of PGA was measured at 25°C by using a Shimadzu 6A GPC (Gel Permeation Chromatography) system and a 6A retractive index detector with Asahipak GS-620H and Tosoh TSKgel GMWP4E columns. Fifty mNaCl aqueous solution-acetonitrile (4:1) was used as eluant at a flow rate of 0.7 ml/min, a sample concentration of 1 mg/ml was used. Two hundred ml of the sample solution was injected. Poly(styrene sulfonic acid, sodium salt) standards were used to make a calibration curve.

The ratio of L-isomer and D-isomer of the PGA monomer unit was measured by liquid chromatography of the hydrolysate of PGA. The sample PGA was hydrolyzed in 6N HCl at 110°C for 24 hr in a glass vial under a nitrogen atmosphere with a Pico-Tag apparatus. A Crownpak CR+ column (Daicel Chemical Industries, Ltd., Japan) was used. Percoll acid solution (pH 2) was used as an eluant at a flow rate of 0.4 ml/min, and a UV detector (200 nm) was used.

**Hydrolysis of PGA in the aqueous solution.** Three ml of a PGA aqueous solution (sample No. 12, 1 mg/ml) was put into a sealed pressure-proof glass tube, and heated in an oil-bath at 80, 100, or 120°C. The time-dependent changes in molecular weight were followed by GPC.

**Results and Discussion**

**Investigations of various carbon sources for PGA production**

*Bacillus subtilis* IFO3335 produced no PGA without L-glutamic acid in the media. The PGA productivity was investigated by using 6 compounds as carbon sources in media (100 ml, pH 7.5) containing 3 g of L-glutamic acid and 0.5 g of ammonium sulfate (glutamic acid medium). The bacterial cells were cultivated aerobically at 37°C for 70 hr. The results are shown in Table I. When 2 g of citric acid (Sample No. 4) was used as a carbon source added to the glutamic acid medium, the highest yield of purified PGA, 0.96 g/100 ml, was produced. It was confirmed that the PGA was pure by amino acid analysis. When 2 g of glucose (Sample No. 2) was used as an additional carbon source, a by-product polymer (it seems to be a polysaccharide) having about 15,000 number-average molecular weight, $M_\text{n}$, was detected by the GPC analysis in the purified polymer. In the case of citric acid, no by-product was detected. When no compound (Sample No. 1), t-malic acid (Sample No. 5), succinic acid (Sample No. 6), or fumaric acid (Sample No. 7) was used as an additional carbon source to the glutamic acid medium, PGA was hardly produced and the by-product was dominant. When acetate acid (Sample No. 3) was used as an additional carbon source to the glutamic acid medium, the cells did not grow. It is thus apparent that citric acid was the best additional carbon source to the glutamic acid medium on PGA production of *B. subtilis* IFO3335.

Figure 2 shows a schematic pathway of PGA synthesis in *B. subtilis* IFO3335. In the case of no additional carbon source in the glutamic acid medium, PGA was hardly produced (Sample No. 1); in the case of the addition of citric acid, a large amount of PGA was produced (Sample No. 4). From the results, it can be presumed that the glutamic acid unit in PGA is mainly produced from citric acid and ammonium sulfate. Therefore, in the case of the addition of citric acid to the medium, glutamic acid is produced from citric acid through isocitric acid and α-ketoglutaric acid in the TCA cycle. Thus, the concentration of glutamic acid in the cells rises. Thereby a large amount of PGA is produced. On the other hand, in the case of the addition of t-malic acid, succinic acid, or fumaric acid (Sample No. 5, 6, or 7) present in the TCA cycle, a polysaccharide is produced from these carbon sources through glyconeogenesis. Thus, the concentration of glutamic acid in the cells does not rise. Furthermore, in the case of the addition of glucose to the medium (Sample No. 2), some of the glucose forms the polysaccharide through the path of polysaccharide production, and the rest enters the TCA cycle via glycolysis. However, as the concentration of glutamic acid is lower than the case of citric acid, a small amount of PGA is produced.

**Investigation of L-glutamic acid, citric acid, or ammonium sulfate concentrations in media for PGA production**

The PGA productivity was investigated with various

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**Table I. Production of PGA by Various Carbon Sources**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Carbon source</th>
<th>Cell dry weight (g/100 ml)</th>
<th>Purified polymer (g/100 ml)</th>
<th>By-product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>0.14</td>
<td>0.03</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Glucose</td>
<td>0.85</td>
<td>0.11</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Acetic acid</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Citric acid</td>
<td>0.57</td>
<td>0.96</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>t-Malic acid</td>
<td>0.57</td>
<td>0.05</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Succinic acid</td>
<td>0.95</td>
<td>0.06</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Fumaric acid</td>
<td>0.98</td>
<td>0.03</td>
<td>+</td>
</tr>
</tbody>
</table>

* a Cells were cultivated for 70 hr at pH 7.5 and 37°C.

* b Carbon source (2 g) in the media (100 ml) with 3 g of L-glutamic acid and 0.5 g of ammonium sulfate.

* c Measured by GPC. +, a by-product was detected; -, not detected.

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Fig. 2. Pathway of PGA Synthesis in *B. subtilis* IFO3335.
concentrations of L-glutamic acid in the media (100 ml, pH 7.5) containing 2 g of citric acid and 0.5 g of ammonium sulfate. The results are shown in Table II. The cell dry weight, in the range of 0.42—0.66 g/100 ml, was independent of L-glutamic acid concentration. When 3 g of L-glutamic acid (Sample No. 12) was added to the medium, the highest yield of purified PGA, 0.89 g/100 ml, was produced. When 1 g of L-glutamic acid was not added to the medium (Sample No. 8), PGA was hardly produced. The amount of PGA produced increased with the increase in L-glutamic acid concentration to 3 g/100 ml. Thus, it was found that L-glutamic acid was an essential component of the medium for PGA production and the most suitable amount was about 2—3 g.

The PGA productivity was also investigated with various concentrations of citric acid in the media containing 3 g of L-glutamic acid and 0.5 g of ammonium sulfate. The results are shown in Table III. The cell dry weight increased with the increase in citric acid concentration. When 2 g of citric acid (Sample No. 12) was added to the medium, the highest yield of purified PGA, 0.89 g/100 ml, was produced. When citric acid was not added to the medium (Sample No. 14), PGA was hardly produced, similar to the case of L-glutamic acid. The amount of PGA produced increased with the increase in citric acid concentration up to 2 g/100 ml. When 3 g or more citric acid was added, the amount of PGA decreased. From these results, it is clear that citric acid was essential for PGA production and that the most suitable amount of citric acid in the medium was approximately 2 g.

In addition, the PGA productivity was investigated with various concentrations of ammonium sulfate in the media containing 3 g of L-glutamic acid and 2 g of citric acid. The results are shown in Table IV. The cell dry weight decreased with the increase in ammonium sulfate concentration. When 1 g of ammonium sulfate (Sample No. 22) was added to the medium, the highest yield of purified PGA, 1.02 g/100 ml, was produced. In contrast to the experiments in which the concentrations of L-glutamic acid (Sample No. 8) and citric acid (Sample No. 14) were zero, when ammonium sulfate was not added to the medium (Sample No. 19), PGA was produced, although only in a small amount. However, a by-product having about 15,000 of $M_n$ was detected by the GPC analysis. When 0.25 g of ammonium sulfate (Sample No. 20) was added, the yield of PGA increased greatly. In this case, the by-product was also detected. On the contrary, when 0.5 g or more ammonium sulfate was added, no by-product was detected. In this way, it was found that the addition of ammonium sulfate had an influence on PGA production. From the results, it was found that the most suitable amount of ammonium sulfate in the medium was about 0.5—1 g.

When 0 or 0.25 g of ammonium sulfate (Sample No. 19, 20) was added, a small amount of x-ketoglutaric acid forms glutamic acid because of the low concentration of ammonia in the cells (Fig. 2). Thus, the concentration of glutamic acid is low in the cells, and a small amount of PGA is produced. Furthermore, it can be presumed that a polysaccharide is formed from excess citric acid through the TCA cycle and glyconeogenesis (Fig. 2).
Course of PGA production
Changes in cell dry weight and the yield of PGA were investigated during cultivation. The results are shown in Table V and Fig. 3. The media (100 ml) containing 3 g of L-glutamic acid, 2 g of cistic acid, and 0.5 g of ammonium sulfate were used at initial pH 7.5 and 37°C. At 24 hr of cultivation, the cells had grown, but PGA was not produced. At 40 hr, the highest yield of PGA, 1.04 g/100 ml, was produced. After that time, the yield of PGA decreased with the elapse of cultivation time. On the other hand, the cell dry weight increased up to 47 hr, and became constant (about 0.40 g/100 ml) after that time.

In addition, the number-average molecular weights ($M_n$) and the polydispersities ($M_w/M_n$) of PGA given at each culture time were measured by GPC (see Table V). The $M_n$ values were over 2,000,000 up to 70 hr, but decreased extensively to about 266,000 at 94 hr. B. subtilis IF03335 (wet, about 1 g) was inoculated in a medium containing only PGA (0.1 w/v%) as a carbon and nitrogen source, and was cultivated aerobically with shaking (120 rpm) at 37°C. After 24 hr, PGA was almost all degraded. Furthermore, the culture supernatant (sterilized by filtration) after 70 hr of cultivation was incubated at 37°C. The degradation of PGA in the culture supernatant was faster than its hydrolysis. These results suggest that B. subtilis IF03335 excreted a PGA degradation enzyme with the progress of cultivation and PGA was degraded by this enzyme.

The monomer units of PGA produced at 40 hr and 70 hr of cultivation had about a 2/8 L-isomer/D-isomer ratio. Thorne and co-workers reported that $D$-glutamic acid was formed from $L$-glutamic acid via $a$-ketoglutaric acid by $L$-transaminase, alanine racemase, and $D$-transaminase in B. subtilis ATCC9945. In the case of B. subtilis IF03335 used in this study, it is presumed that $D$-glutamic acid is mainly formed from cistic acid through the same pathway.

Hydrolysis of PGA in aqueous solution
Studies on the hydrolysis of PGA in aqueous solution were investigated at 80°C, 100°C, or 120°C. The results are shown in Table VI and Fig. 4. PGA from the culture condition of Sample No. 12 was used with a number-average molecular weight ($M_n$) of approximately 226,000. The experiments of hydrolysis were done by heating the aqueous solution of PGA (1 mg/ml) and following the time-dependent changes in the $M_n$ values by GPC. At 180 min of heating at 80°C, the $M_n$ value became about a half of the initial $M_n$ value due to hydrolysis. In this case, the rate of hydrolytic degradation at 80°C was much slower than that at 100°C or 120°C. In this way, it was found that PGA in the aqueous solution was hardly hydrolyzed within 60 min of heating at 80°C. On the contrary, PGA was rapidly hydrolyzed by heating at 120°C.

As shown in Fig. 4, the relationship between the reciprocal of the number-average molecular weight at time $t$, $1/M_n(t)$, and time $t$ was linear at each temperature. This result indicated that the hydrolysis of PGA in the aqueous

![Fig. 3. Course of PGA Production by B. subtilis IF03335 in Medium (100 ml) Containing 3 g of L-Glutamic Acid, 2 g of Citric Acid, and 0.5 g of Ammonium Sulfate at 37°C.](image)

![Fig. 4. Relationship between $1/M_n$ and Time $t$ of PGA in the Aqueous Solution at Different Temperatures.](image)

<table>
<thead>
<tr>
<th>Table VI.</th>
<th>Changes in Molecular Weight of PGA* in the Aqueous Solution during Hydrolytic Degradation at Different Temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp. °C</td>
<td>Molecular weight $\times 10^{-3} M_n$</td>
</tr>
<tr>
<td>80</td>
<td>0 min</td>
</tr>
<tr>
<td>100</td>
<td>226</td>
</tr>
<tr>
<td>120</td>
<td>226</td>
</tr>
</tbody>
</table>

* PGA Sample No. 12 was used.

* Measured by GPC.
solution by heating was due to a random chain scission.

Provided that the polymer chain scission is completely random due to the hydrolysis of PGA in the aqueous solution by heating, the number-average degree of polymerization at time $t$, $\bar{P}_{n,t}$, is given by

$$1/\bar{P}_{n,t} - 1/\bar{P}_{n,0} = k_d t$$

where $\bar{P}_{n,0}$ is the initial number-average degree of polymerization and $k_d$ is the rate constant of hydrolysis. The $k_d$ values at each temperature were calculated from the slopes of the plots in Fig. 4. The activation energy of the polymer chain scission due to the hydrolysis of PGA in the aqueous solution by heating was approximately 120 kJ·mol$^{-1}$.

**References**