Glutamic Acid Independent Production of Poly(γ-glutamic acid) by Bacillus subtilis TAM-4

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A bacterium that produced a large amount of poly(γ-glutamic acid) (PGA) when it was grown aerobically in a culture medium containing ammonium salt and sugar as sources of nitrogen and carbon, respectively, was isolated from soil. The bacterium, strain TAM-4, was classified as Bacillus subtilis. The maximum PGA production (22.1 mg/ml) was obtained when it was grown in a medium containing 1.8% ammonium chloride and 7.5% fructose at 30°C for 96 h with shaking. Some properties of the PGA obtained at different times of cultivation were investigated by gel permeation chromatography, SDS-PAGE, and measurement of viscosity, and calculation of the d/l ratio of glutamic acid constituting PGA. The results suggested that PGA was elongated with no changes in the diastereoisomer ratio in the molecule.

Key words: Bacillus subtilis; poly(γ-glutamic acid)

It was reported that several strains of Bacillus sp. produce poly(γ-glutamic acid) (PGA) as an extracellular viscous material.1-9 PGA is a water-soluble macromolecular-peptide that consists of D- and L-glutamic acids and is polymerized by γ-glutamyl bonds. The polymer has been expected to be a new industrial biodegradable resource. For example, Yahata et al. reported that PGA benzyl ester is applicable to fiber with a high quality of dynamics,10 and Kunioka reported hydrogels prepared by γ-irradiation in PGA aqueous solutions.11 However, although some tentative pathways of PGA biosynthesis have been proposed,12-15 they are not fully understood.

The PGA-producing bacteria are divided into two types; one produces PGA depending on the existence of L-glutamic acid in the medium, the other does not. The former includes many of these bacteria such as B. subtilis,11 B. subtilis ATCC9945,12 B. subtilis IFO333513 and B. subtilis F-2-01.9 The later includes B. subtilis SE9 and B. licheniformis A35.14 It seems better to choose the latter (de novo PGA-producing bacteria) for the studies on the mechanism of PGA formation and their industrial use. However, relatively little is known about these bacteria.

In this study, we screened for and isolated a bacterium from soil, strain TAM-4, which produced a large amount of PGA when it was grown aerobically in a culture medium containing ammonium salt and sugar as sources of nitrogen and carbon, respectively. The effects of various nitrogen and carbon sources on PGA productivity of the strain TAM-4 and some properties of the PGA obtained at different times of cultivation were investigated.

Materials and Methods

Screening. Soil samples collected from many places were suspended in sterile distilled water and boiled for 5 min. The diluted suspensions were spread onto TSBr agar plates composed of 3.0% Trypticase Soy Broth (BBL), 0.5% potassium nitrate, 1.0% glucose, and 1.2% agar, pH 7.4. After incubation at 30°C for 2 days, highly mucoid colonies that appeared on the plates were picked up, inoculated into 5 ml of GB medium composed of 1.0% peptone (Kyokuto), 1.0% bouillon (Kyokuto), 0.5% NaCl, and 1.0% glucose, pH 7.0 in a 30-ml test tube, and then incubated at 30°C for 24 h with shaking at 300 rpm. One ml of the culture was inoculated into 100 ml of medium composed of 1.8% ammonium chloride, 7.5% glucose, 0.15% K2HPO4, 0.035% MgSO4·7H2O, 0.005% MnSO4·SH2O, and 3.0% CaCO3 (pH 7.2) in a 500-ml flask and inoculated at 30°C for 96 h with shaking at 150 rpm. A bacterium, strain TAM-4, which produced a large amount of viscous material was obtained. The viscous material was purified by the procedures described below and analyzed by the method described by Cheng et al.16 The 6N HCl hydrolyzate of the purified viscous material was composed solely of glutamic acid. Furthermore, the ninhydrin and biuret reactions for the viscous material were negative. From these results, it was concluded to be poly(γ-glutamic acid).

Preparation of pure PGA. Culture broth was centrifuged at 20,000 x g for 30 min. The supernatant was poured into 3 volumes of ethanol, then the resulting precipitate was collected by centrifugation and washed with ethanol. After drying in vacuo, the precipitate was dissolved and dialyzed against deionized water. The dialyzed solution was centrifuged and the supernatant was lyophilized to give pure PGA.

Analytical procedures. The bacterial growth was monitored by viable-cell counts or measurements of optical density at 660 nm after solubilizing calcium carbonate with an equivalent volume of 1 N HCl and a 20-fold dilution with distilled water. The G+C content of the bacterial DNA was measured by a DNA-GC-kit (Yamasa Syoyu Co., Ltd.). Glucose in the medium was assayed using a F-kit-glucose (Boehringer Mannheim). Ammonia in the medium was assayed by indophenol-blue method.17 Relative viscosity was measured by an Ostwald viscometer at 20°C using distilled water as a standard. The supernatant of the culture medium or PGA solution (5 mg/ml in water, pH 7.0) were used for the measurement. The ratio of D- and L-glutamic acid constituting PGA was measured by HPLC with a Crownpak CR+ column (Daicel Chemical Industries, Ltd.) as described by Goto and Kunioka.18 The concentration of polysaccharides contaminating in purified PGA was estimated by the phenol-sulfuric acid method19 from a calibration curve with glucose as a standard.

Molecular weight measurement. The weight-average, number-average molecular weights and the dispersity (Mw/Mn) of PGA were measured by gel permeation chromatography using a Shimadzu 10A GPC system with two Asahipak GSS-700H columns. The mobile phase contained 50 mm phosphate buffer, pH 6.86, and the flow rate was 1.0 ml/min. Pullulan standards (5.3, 23.7, 48, 100, 186, 380, and 853 kDa) obtained from Shimadzu Co. were used to make a calibration curve.

Electrophoresis. SDS-PAGE was done by the method of Laemmli.20

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Abbreviation: PGA, poly(γ-glutamic acid).
with a 5-15% polyacrylamide gradient gel. The gel was stained by the method of Yamaguchi et al. as described below. First, the gel was stained for protein with Coomassie Brilliant Blue (R-250) and destained in 7% acetic acid 10% methanol. Next, it was stained for PGA with 0.5% methylene blue in 3% acetic acid and destained in water. A molecular weight marker Daiichi (Daiichi Pure Chemicals Co.) was used as a molecular weight standard.

Results and Discussion
Identification of the isolated bacterium
A bacterium, strain TAM-4, which produced a large amount of PGA in the medium containing ammonium chloride and glucose as sources of nitrogen and carbon, respectively, was isolated from soil as described in Materials and Methods.

Since the bacterium produces PGA in the medium without glutamic acid, it is a de novo PGA-producing bacterium. Taxonomic characteristics of the strain TAM-4 are summarized in Table I. The bacterium was Gram-positive, spore-forming rods, and used citrate. The bacterium was grown in nutrient broth containing 10% NaCl. The G+C content (mol%) of the DNA was 45.7%. According to Bergey’s Manual of Systematic Bacteriology, Vol. 2, the bacterium was classified as Bacillus subtilis. The B. subtilis TAM-4 does not require biotin for its growth in contrast with many of PGA-producing strains classified as B. subtilis (natto). Some strains have problems associated with strain degeneration, but the strain TAM-4 has kept the ability of PGA production by maintenance of a semiannual subculture on TS agar slants at room temperature.

Effects of nitrogen and carbon sources on PGA production
The PGA productivity of B. subtilis TAM-4 was investigated using various nitrogen sources. Ammonium chloride in the M medium was replaced by other nitrogen sources at the final nitrogen concentration of 0.47%. The cultivation were done at 30 C for 96 h with shaking at 130 rpm. As shown in Table II, the B. subtilis TAM-4 used several kinds of ammonium salts, nitrates, and organic sources for its growth. However, ammonium carbonate, diammmonium hydrogen phosphate, and urea were not used under these conditions (data not shown). The B. subtilis 5E was able to use urea and ammonium carbonate, but unable to use other ammonium salts. In B. subtilis TAM-4, ammonium chloride was the most useful as a nitrogen source for PGA production, and 13.4 mg/ml accumulation of PGA was observed. On the other hand, when it was grown with the organic nitrogen sources, 3.4–8.0 mg/ml of PGA was produced. The result shows that the nitrogen of glutamic acid was used for formation of PGA as well as components of the cell. It is interesting that some of the inorganic nitrogen sources were more favorable for production of PGA than organic ones, including glutamic acid.

The effects of carbon sources on PGA production were also investigated. The results are shown in Table III. Galactose, lactose, and glycerol were not favorable for growth. In contrast, glucose, fructose, saccharose, and maltose were favorable for growth and PGA production. Particularly, when B. subtilis TAM-4 was grown in the medium containing fructose as a carbon source, 22.1 mg/ml of PGA was produced in the medium. Such a high productivity of PGA in the medium with no addition of

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Growth (A490)</th>
<th>PGA (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>22.1</td>
<td>13.4</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>18.2</td>
<td>10.9</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>10.3</td>
<td>12.6</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>16.5</td>
<td>2.5</td>
</tr>
<tr>
<td>KNO₃</td>
<td>16.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Peptone</td>
<td>6.8</td>
<td>5.4</td>
</tr>
<tr>
<td>Casamino acid</td>
<td>22.4</td>
<td>3.4</td>
</tr>
<tr>
<td>l-Glutamate*</td>
<td>20.8</td>
<td>8.0</td>
</tr>
</tbody>
</table>

The media contain each nitrogen source corresponding 0.47% nitrogen and 7.5% glucose as a carbon source. The cultivation was done at 30 C for 96 h with shaking at 150 rpm.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Growth (A490)</th>
<th>PGA (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>22.1</td>
<td>13.4</td>
</tr>
<tr>
<td>Fructose</td>
<td>11.6</td>
<td>22.1</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Saccharose</td>
<td>12.0</td>
<td>16.9</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Maltose</td>
<td>16.8</td>
<td>16.1</td>
</tr>
<tr>
<td>Xylose</td>
<td>10.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The media contain 7.5% each carbon source and 1.8% ammonium chloride as a nitrogen source. The cultivation was done at 30 C for 96 h with shaking at 150 rpm.

Table I. Taxonomic Characteristics of TAM-4

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>+</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod, 0.7–0.8 x 2–3 μm</td>
</tr>
<tr>
<td>Endospores</td>
<td>Cylindrical, central, 0.7–0.8 x 1.2–1.5 μm</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>-</td>
</tr>
<tr>
<td>Acid from D-glucose</td>
<td>+</td>
</tr>
<tr>
<td>Gas from D-glucose</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
</tr>
<tr>
<td>Hydrolysis of casein</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of citrate</td>
<td>-</td>
</tr>
<tr>
<td>Propionate</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate reduced to nitrate</td>
<td>+</td>
</tr>
<tr>
<td>Formation of indole</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer test</td>
<td>+</td>
</tr>
<tr>
<td>pH 6.8, nutrient broth</td>
<td>+</td>
</tr>
<tr>
<td>pH 5.7</td>
<td>+</td>
</tr>
<tr>
<td>Growth in NaCl 5%</td>
<td>+</td>
</tr>
<tr>
<td>Growth in NaCl 7%</td>
<td>+</td>
</tr>
<tr>
<td>Growth in NaCl 10%</td>
<td>+</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>45.7%</td>
</tr>
<tr>
<td>Biotin required</td>
<td>-</td>
</tr>
</tbody>
</table>

Table II. Effects of Various Nitrogen Sources on Growth and PGA Production of B. subtilis TAM-4

Table III. Effects of Various Carbon Sources on Growth and PGA Production of B. subtilis TAM-4
glutamate has not been reported before. *B. subtilis* ISO3335 produced PGA together with a by-product, which seemed to be a polysaccharide, when it was grown in the medium containing glucose. Each purified PGA produced by *B. subtilis* TAM-4 from various media was tested by the phenol-sulfuric acid method. Reduced to glucose, less than 1% (w/w) of the compounds were detected in all samples (data not shown). The result suggest that *B. subtilis* TAM-4 scarcely produces polysaccharides.

**Course of PGA production**

When *B. subtilis* TAM-4 was grown at 30°C for a few days on M medium, it was observed that the medium became highly viscous due to the production of PGA along with the bacterial growth. Therefore, we observed the course of PGA production as well as changes of viscosity, pH of the medium, and consumption of ammonia and glucose. As shown in Fig. 1, the growth, the production of PGA, and the relative viscosity of the medium reached a maximum after incubation for 84 h. The production of PGA increased linearly, but the relative viscosity of the medium increased little for the first 54 h, and then increased rapidly. The change of pH was slight because of the addition of CaCO₃. The consumption of glucose corresponded to bacterial growth, and finally it was all consumed. The ammonia was consumed to about half of the amount added. When *B. subtilis* TAM-4 was grown in the M medium limiting ammonium chloride (0.9%), the PGA productivity was lower than that of the control (10.3 mg/ml). Therefore, it is considered that the ammonia which was not consumed affected PGA production.

Furthermore, physical properties of the PGA produced by *B. subtilis* TAM-4 were investigated. Purified PGA obtained at various cultivation times were analyzed to measure the molecular weight by gel permeation chromatography and SDS-PAGE. As shown in Table IV, the weight-average molecular weight of PGA in early phase was about $6 \times 10^3$ and in later phase was about $1.6 \times 10^6$. In addition, it was observed that the electrophoretic mobility of PGA became low along with the cultivation time in SDS-PAGE (Fig. 2). On the ground of the mobilities of PGA in the later phase, which was lower than that of myosin (200 kDa) and the smear bands of PGA in the early phase, the molecular weight of each PGA was not measured exactly. However, it was confirmed that the PGA was elongated along with cultivation time. Moreover, the relative viscosity of each purified PGA (5 mg/ml in water) was measured (Table IV). The increasing of the value along with cultivation time was supported by the elongation of PGA. In the early phase, the viscosity of the medium increased little, though production of PGA increased linearly (Fig. 1). It was possible that the low viscosity of PGA in the early phase cause the disagreement. It is reported that the number-average molecular weight of PGA from *B. subtilis* ISO3335 and the weight-average molecular weight of PGA from *B. subtilis* TAM-4 is not determined.

**Fig. 2. SDS-PAGE Profiles of PGA Obtained at Different Times of Cultivation.**

The purified PGA (10 μg) was electrophorered as described in Materials and Methods. Lanes M, 36, 42, 48, 54, 60, 66, 72, 78, 84, and 96 indicate the cultivation time. Lane M contains standard proteins: myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa), and myoglobin (17 kDa).

**Table IV.** *Mw*, Viscosity, and Polymer Composition of PGA Obtained at Different Times of Cultivation

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Molecular weight* ($M_w$) ($\times 10^3$)</th>
<th>Relative viscosity*</th>
<th>Polymer composition†</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>560</td>
<td>2.7</td>
<td>78.1</td>
</tr>
<tr>
<td>42</td>
<td>667</td>
<td>3.8</td>
<td>79.1</td>
</tr>
<tr>
<td>48</td>
<td>667</td>
<td>3.8</td>
<td>79.0</td>
</tr>
<tr>
<td>54</td>
<td>84</td>
<td>5.7</td>
<td>78.3</td>
</tr>
<tr>
<td>60</td>
<td>84</td>
<td>5.7</td>
<td>78.3</td>
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<tr>
<td>66</td>
<td>1355</td>
<td>8.1</td>
<td>79.0</td>
</tr>
<tr>
<td>72</td>
<td>1355</td>
<td>8.1</td>
<td>79.0</td>
</tr>
<tr>
<td>78</td>
<td>1556</td>
<td>13.2</td>
<td>77.7</td>
</tr>
<tr>
<td>84</td>
<td>1556</td>
<td>13.2</td>
<td>77.7</td>
</tr>
<tr>
<td>96</td>
<td>1683</td>
<td>13.9</td>
<td>77.9</td>
</tr>
</tbody>
</table>

* Measured by gel permeation chromatography.
† Measured by Ostwald viscometer. Five mg/ml of purified PGA was measured.
‡ Not determined.
molecular weight of PGA from *B. licheniformis* ATCC-9945a,16 were over 10⁶ in early cultivation phase and the value remained almost constant during cultivation. Therefore, the change in molecular weight of PGA suggested by the results of gel permeation chromatography, SDS–PAGE and the viscosity, may be a distinguishing feature of *B. subtilis* TAM-4.

In addition, the D/L ratio of glutamic acid constituting PGA obtained at different times of cultivation was calculated (Table IV). All the monomer unit of PGA had about a constant 78:22 (D-isomer:L-isomer) ratio. This result suggests that PGA produced by *B. subtilis* TAM-4 was elongated with no change in the diastereoisomer ratio of the glutamic acid constituting PGA during cultivation. Kubota et al.9 reported that *B. subtilis* F-2-01 produces PGA mainly consisting of L-isomer in the early cultivation phase, and the ratio of D-isomer increased in the later cultivation phase. The structure of PGA produced by *B. subtilis* TAM-4 may be different from that of *B. subtilis* F-2-01.

Onodera et al.23 already reported that *B. subtilis* TAM-4 had no plasmid and presumed that the gene coding for formation of PGA lies on the genomic DNA. However, further details are still unknown. In addition, to make clear the mechanism of PGA formation, the lower molecular precursor and the enzyme(s) involved in the elongation of PGA remain to be identified. Sawa et al.24 presumed that the precursor of the γ-linked polymer was not glutamic acid but some other compounds having relation with N-acetyl glutamic acid, glutamyl-γ-semialdehyde, or glutamyl phosphate. Ogawa et al.25 presumed that glutamine was polymerized by γ-glutamyl transpeptidase. Recently, effective formation of oligo-γ-glutamic acid by γ-glutamyl transpeptidase from bovine kidney using glutamic acid esters as substrates has been reported.25 Studies including the purification of the enzyme are now in progress.

References

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