Efficient Production of γ-Polyglutamic Acid by Bacillus subtilis (natto) in Jar Fermenters

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The large scale fermentation of γ-polyglutamic acid (γ-PGA) by Bacillus subtilis (natto) was done using a 30-liter jar fermenter. A stable cultivation without foaming could be done with addition of 3% NaCl to the medium. The γ-PGA productivity became higher with increasing speed of agitation and amounts of glutamic acid added to the broth. Finally, we were able to obtain about 35 mg/ml of γ-PGA under the optimum conditions. The glutamic acid added to the medium was efficiently converted into γ-PGA in the stationary phase. To discover the role of L-glutamic acid added to the medium for γ-PGA biosynthesis by Bacillus subtilis (natto), the radioactivity incorporated into γ-PGA from 14C-L-glutamic acid was measured. As a result, radioactive γ-PGA was detected in the medium. Then, the glutamic acid in the medium was transported into the cells and actually polymerized as the glutamic acid unit of γ-PGA.

Key words: Bacillus subtilis (natto); γ-polyglutamic acid; salvage pathway

γ-Polyglutamic acid (γ-PGA) is the main constituent of the sticky material of natto, a traditional food in Japan, i.e., cooked soybeans fermented with Bacillus subtilis (natto).γ-PGA is a water-soluble and biodegradable substance which comprises D- and L-glutamic acid polymerized through γ-glutamyl bonds. Its use in the fields of food, cosmetics, or medicine has been proposed, and biodegradable fibers7 and hydrogels8,9 have already been made from γ-PGA.

γ-PGA was discovered as a component of the capsules of Bacillus anthracis10 and Bacillus mesentericus,11) and was isolated from a culture filtrate of Bacillus subtilis.12,13) γ-PGA producing bacteria are divided into two types as to the requirement of glutamic acid for γ-PGA biosynthesis. Glutamic acid-independent production of γ-PGA using B. subtilis SE, B. licheniformis A3515 and B. subtilis TAM-4 have been reported as de novo γ-PGA producing bacteria. On the other hand, other bacteria such as B. subtilis ATCC9945,13) B. anthracis,17) B. subtilis F-2-0118 and B. subtilisIFO333519,20 were stimulated to produce γ-PGA by the addition of L-glutamic acid to the medium. In these bacteria, saliva γ-PGA production was suggested, but there was no evidence that the glutamic acid units in γ-PGA were formed from the glutamic acid in the medium. Kunioka and Goto reported that glutamic acid added to the medium appeared to regulate the de novo γ-PGA production from citrate, and not to be incorporated into γ-PGA.20

To produce γ-PGA on an industrial scale, we searched for suitable conditions for γ-PGA fermentation in a jar fermenter. Moreover, to clarify the role of glutamic acid in the medium, we examined the incorporation of 14C-glutamic acid into γ-PGA.

Materials and Methods

Bacterial strains. Among many γ-PGA producing strains in our stock culture collection, Bacillus subtilis (natto) strain MR-141 was used. This strain was selected as a mutant defective in ammonia production derived from strain MR-1, and produced the most sticky material on natto fermenter when compared with other strains in our stock culture collection.21 The parent strain, MR-1, was isolated from the Miyagino strain (a stock culture of Miyagino Natto Seisakujo) as a commercially useful strain as a natto starter.

Culture conditions. B. subtilis (natto) strain MR-141 was cultured on Nutrient Broth (Difco Laboratories) plates containing 1.5% agar for 7 days at 40°C to induce spore formation. After cultivation, 10 ml of sterilized standard saline (0.9% NaCl) was added to each plate, and the spores were suspended. One-m1 portions (5 × 108 cell/ml) of the spore suspension were divided into several micro tubes and stored in −80°C until use for cultivation.

For cultivation in a 30-liter jar fermenter, 20 liters of MSM medium comprising 6% maltose, 7% soy sauce, 3% sodium L-glutamate, 0.25% K2HPO4, 0.05% MgSO4, 7H2O, and 3% NaCl or GSG medium containing 6% glucose instead of maltose was used. As an antifoaming agent, 0.1% silicone oil (SH200-100s; Toray Dow Corning Silicone Co.) was added. After sterilization, an appropriate volume of 20% NaOH sterilized by autoclaving was added to the medium to adjust the pH to 8. The spore solution (1 ml) was diluted with 10 ml of standard saline, and then added to the medium to start the cultivation. The temperature and agitation were maintained at 40°C and 400 rpm, respectively, for every cultivation time. The aeration was maintained at 0.1vvm until 18 h and then at 1 vvm after 18 h to prevent loss of the medium as foam. Fifty ml of the culture broth was removed from the jar fermenter at appropriate times and stored at −20°C until analysis.

γ-PGA analysis. The amount of glutamic acid in the broth was measured with an amino acid analyzer (Model L-8500; Hitachi Co.). Cells were removed from the culture broth by centrifugation, and each supernatant was filtered through a membrane filter (0.45 μm) to obtain a culture filtrate. γ-PGA in the culture filtrate was hydrolyzed with 6 N HCl at 100°C for 4 hours, and the amount of glutamic acid after hydrolysis was regarded as the total glutamic acid. The amount of free glutamic acid derived from sodium L-glutamate in the culture filtrate was measured without hydrolysis. The amount of γ-PGA was calculated as glutamic acid equivalent to the difference between the total and free glutamic acid.

The measurement of glucose and maltose in the medium. The glucose remaining in the culture filtrate was measured using a Glucose Test Wako (Wako Pure Chemical Industries, Ltd.) and calculated the maltose concentration, a 10 μl sample was incubated with 10 μl (1.4 U) of α-glucosidase (AGH-211; Toyobo Co., Ltd.) at 37°C for 30 min. After
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14C-Glucose and 14C-l-glutamic acid incorporation into γ-PGA. For a small-scale culture in a 50-ml flask, 10 ml of GYG medium comprising 3% glucose, 2.5% yeast extract, 3% sodium l-glutamate, 0.25% K3HPO4, 0.05% MgSO4·7H2O, and 3% NaCl was used. The spore solution (10 μl) was inoculated into the GYG medium containing 185 kBq of [1-14C] D-glucose (New England Nuclear, U.S.A., 37-185 MBq/μmol) or [1-14C] L-glutamic acid (New England Nuclear, U.S.A., 10.95 MBq/μmol). The cells were grown at 37 °C at 120 rpm on a rotary shaker for 72 h. Fifty μl of the broth was removed from the flask at appropriate times and mixed with 50 μl of the SDS-sample buffer (2% SDS, 30% glycerol, and 0.25 M Tris hydroxymethylammonium, pH 6.8), and then boiled for 2 min. Each sample solution (20 μl) was put on a 4-15% gradient acrylamide slab gel (Daichi Pure Chemicals, Ltd.), and then electrophoresis was performed to separate the labeled γ-PGA from free 14C-glucose or 14C-glutamic acid. The γ-PGA was detected by staining with alcan blue as described by Yamaguchi et al., and the radioactivity of γ-PGA was detected by autoradiography. The spots corresponding to γ-PGA were cut from the gel and the radioactivity was counted in a toluene scintillation solution.

Results
Effects of the NaCl concentration on γ-PGA fermentation
At first, γ-PGA production in a jar fermenter could not be done, because the viscous broth foamed out of the jar fermenter. To prevent foaming, several anti-foaming agents were examined, but the foaming of the viscous medium was not suppressed by any anti-foaming agent, such as oil or silicone. The most effective treatment for preventing foaming was the addition of NaCl to the medium.

Figure 1 shows the effects of the NaCl concentration on γ-PGA fermentation. With 1 or 2% NaCl, γ-PGA production became maximum at 24 and 50 h, respectively, although free glutamic acid remained in the medium. With NaCl at these concentrations, the medium changed into a mousse after 24 and 50 h, respectively, and then foamed out. In the case of 3 or 5% NaCl, foaming of the medium was prevented and the γ-PGA fermentation could be done stably until cultivation was stopped. But the γ-PGA production with 5% NaCl was repressed after 48 h, and the amount of γ-PGA remained at 12 mg/ml. An effective γ-PGA fermentation was possible with 3% NaCl. With this concentrations, glutamic acid added to the medium seemed to be efficiently converted into γ-PGA, because the final amount of γ-PGA was equal with the total glutamic acid level, and free glutamic acid was not detected in the medium.

Efficient conversion was also observed even with increasing amounts of sodium L-glutamate in the medium (data not shown). With every concentration of sodium L-glutamate, the γ-PGA production continued until the free glutamic acid had been consumed. Since the amount of total glutamic acid was maintained at a constant level during these cultivations, it was deduced that strain MR-141 could not use glutamic acid as a carbon source.

Effects of agitation on γ-PGA fermentation
To obtain efficient production of γ-PGA, the effects of agitation on γ-PGA fermentation were examined using MSG medium containing 6% sodium L-glutamate (Fig. 2). Under agitation at 300 or 350 rpm, the rates of γ-PGA production were low, and above 15 mg/ml of free glutamic acid remained in the broth. In the case of 400 or 450 rpm agitation, the rates of γ-PGA production were prominently higher than with 300 or 350 rpm. But, the agitation effect seemed to be maximum at 400 rpm, since 450 rpm gave similar γ-PGA production.

γ-PGA fermentation in a glucose or maltose medium
Figure 3 shows the course of γ-PGA production in medium containing either glucose or maltose as a carbon source. In the GSG medium containing glucose as a carbon source, the cell numbers quickly increased up to 18 h and was maintained at a constant level after 24 h. The glucose concentration in the medium quickly decreased in the logarithmic phase, and was not detected after 30 h. The pH of the broth also decreased while glucose remained. However, when glucose had been consumed, the pH quickly increased for several hours and then was maintained at from 7 to 8. The initial γ-PGA production increased with cell growth in the logarithmic phase. Moreover, after 48 h, when glucose had been consumed, the amount of γ-PGA also gradually increased without cell growth. However, the efficient conversion of free glutamic acid into γ-PGA did not occur within 90 h, because the rate of γ-PGA production was slightly decreased in the stationary phase. Thus, the yield of γ-PGA in the GSG medium remained at only 25 mg/ml.

On the other hand, in the case of cultivation in the MSG medium containing maltose as a carbon source, the cell growth pattern resembled that in the GSG medium, but the speed of assimilation of maltose was very low and maltose remained until 90 h. Then, the pH was maintained at a low
value in the stationary phase, and the total glutamic acid level did not change during the cultivation. The initial γ-PGA production also increased with cell growth in the MSG medium. Moreover, the rate of γ-PGA production in the stationary phase was almost equal to the initial rate. Finally, 35 mg/ml of γ-PGA, the highest yield in our experiment, was obtained.

\(^{14}\text{C}-\text{Glucose and }^{14}\text{C-L-glutamic acid incorporation into }\gamma\text{-PGA}\)

From the effects of glutamic acid addition on γ-PGA production in a jar fermenter described above, the incorporation of the glutamic acid into γ-PGA was apparent. To clarify the role of L-glutamic acid and the pathway of γ-PGA biosynthesis, the incorporation of \(^{14}\text{C-glucose or }^{14}\text{C-L-glutamic acid into }\gamma\text{-PGA was examined. Radioactivity was detected in the }\gamma\text{-PGA produced in the medium containing }^{14}\text{C-glucose or }^{14}\text{C-L-glutamic acid (Fig. 4). The radioactivity of }^{14}\text{C-L-glutamic acid was actually incorporated into }\gamma\text{-PGA and increased concomitantly with }\gamma\text{-PGA production. But, under culture conditions like these in a 50-ml flask, the yield of }\gamma\text{-PGA was maintained at only 7 or 8 mg/ml after 48 h. Then, only about 35% of the total radioactivity of }^{14}\text{C-L-glutamic acid was detected in }\gamma\text{-PGA. On the other hand, the incorporation of radioactivity of }^{14}\text{-glucose into }\gamma\text{-PGA was only about 6% and was maintained at this low level for 72 h. On autoradiography, intermediate compounds of low molecular weight were not detected even in the early period of cultivation (Fig. 5). Moreover, other protein bands labeled with }^{14}\text{-glucose or }^{14}\text{-L-glutamic acid were scarcely detected.}
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Cultivation Time (h)
0 5 8 11 14 17 20 24 28 32 36 40 44 48 54 60 66 72

Fig. 5. Autoradiogram of γ-PGA Labeled with 14C-L-Glutamic Acid. Culture broth (10 μl) of B. subtilis (natto) strain MR-141 was analyzed by SDS-PAGE. The radioactivity incorporated into γ-PGA from 14C-L-glutamic acid was detected by autoradiography.

Discussion
To produce γ-PGA on an industrial scale, we searched for suitable conditions for γ-PGA fermentation in a jar fermenter. We succeeded in obtaining very efficient production of γ-PGA (about 35 mg/ml). However, even under our suitable conditions, supplemented air was not efficiently dispersed in the broth due to its high viscosity, such as above 1500 mPas. Thus, more effective γ-PGA production may be possible with extra oxygen.

From the results of 14C-glucose and 14C-glutamic acid incorporation, it was deduced that γ-PGA may be synthesized via both de novo and salvage pathways in the logarithmic stage with cell growth, but in the stationary stage when glucose has been consumed, γ-PGA may be produced independently of cell growth and exclusively synthesized from free glutamic acid in the medium via a salvage pathway. We obtained the same results in the case of strain NR-1 isolated from the Naruse strain, which is a strain commercially used as a natto starter (data not shown). But, on a small scale cultivation such as in a 50-ml flask, efficient γ-PGA production like that in a jar fermenter was not possible, because the dissolved oxygen concentration may be limited.

Kunioka and Goto reported that glutamic acid added to the medium was not assimilated by B. subtilisIFO3335, and may be a regulator of the enzyme involved in γ-PGA biosynthesis.20 Then, the glutamic acid unit in γ-PGA was assumed to be synthesized via a de novo pathway from citric acid and ammonium sulfate. But, our cultivation results suggested that effective conversion of glutamic acid added to the medium into γ-PGA was very important for producing a large amount of γ-PGA (Fig. 3). Moreover, the radioactivity of glutamic acid was actually incorporated into γ-PGA (Fig. 4). Therefore, in the case of B. subtilis (natto), L-glutamic acid in the medium is likely to serve as a material for γ-PGA biosynthesis, rather than as a regulator. The difference between B. subtilis (natto) and B. subtilisIFO3335 suggests that the mechanism of γ-PGA biosynthesis may be different in these strains or under each set of culture conditions.

Ito et al. suggested an intermediate of γ-PGA existed in the medium of B. subtilis TAM-4, which synthesized γ-PGA via a de novo pathway.16 But, in our experiments, an intermediate was not detected in the autoradiogram (Fig. 5). Therefore, in the case of B. subtilis (natto), γ-PGA seems to be secreted into the medium at a high molecular mass. Moreover, other proteins bands labeled with 14C-glutamic acid were scarcely detected under the γ-PGA bands. Thus, glutamic acid was efficiently incorporated into γ-PGA rather than protein.

Tory suggested that polyglutamyl synthetase activity detected in membrane particles required ATP for γ-PGA biosynthesis.23 In our case, γ-PGA biosynthesis appeared to require energy for the polymerization reaction, because high productivity was obtained by using maltose, which was gradually used as an energy source during cultivation (Fig. 3), and by increasing the agitation speed to increase aerobic metabolism (Fig. 2). Thus, we consider that the γ-PGA productivity in the stationary phase will be further increased by the addition of suitable amounts of sugar and glutamic acid as energy and material sources, respectively.

References