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Effective synthesis of bio-nylon materials using *Bacillus megaterium*

Makoto ASHIUCHI

Department of Agriculture, Kochi University

Research aims

Poly-γ-glutamate (PGA) is a biopolymer with a nylon-like backbone, and some experiments regarding the esterification of its α-carboxyl side chains indicate that PGA substantially exhibits nylon-like properties.[1)] Unlike chemical nylons, PGA with multiple chirotopic carbons possesses fair biodegradability and other useful functionalities.[2)] PGA is thus considered as a bionylon. Edible PGA is obtained from the mucilage of Japanese *natto* food.[3)] Unfortunately, it is not quality-controlled in D- and L-glutamate sequences (D ratios, 60%±15%; L ratios, 40%±15%) and molecular size distribution (Mw, 10,000 to 1,000,000):[2)] the demerits of *natto* PGA from the industrial point of view. Stereo-regular PGA (L-rich form) was recently isolated from archaeal exopolymers,[4)] but the low productivity of archaeal PGA may be fatal in ensuring its feasibility. Therefore, further research for useful PGA producers has been requested.

A most important determinant for PGA stereochemistry is glutamate racemase, which virtually serves for the formation of intercellular D-glutamate pools.[2)] The *natto* starters of *Bacillus subtilis* indeed produced the enzyme in abundance;[2)] however, *Bacillus megaterium* showed only a little D-glutamate-supplying activity,[5)] *viz*., a point of resemblance to L-rich PGA-producing archaea. This study was aimed at the effective synthesis of stereo-regular PGA as an applicable bionylon material, and here the PGA productivity of *B. megaterium* was investigated.

Methods

B. megaterium WH320 (MoBiTec Co., Göttingen) was used in the experiments, and the synthesis of PGA was carried out as follows. First, growing cells were inoculated at an appropriate concentration ($8\,g\,L^{-1}$) in a standard medium (pH 7.0) containing 5% sucrose (as a sugar), 2% L-glutamate (as a substrate), 5% NaCl (as a salt), 1% $(NH_4)_2SO_4$, 0.27% KH_2PO_4, 0.42% Na_2HPO_4, 0.5% $MgSO_4 \cdot 7H_2O$, and MS vitamin solution (PhotoTechnology Laboratories, KS) and incubated at 30°C for 5 days.

PGA was isolated by the methods of Park *et al*,[6)] and a fraction of the isolated PGA was hydrolyzed with distilled HCl (6 M) at 105°C for 8 h *in vacuo*.[2)] The total amount of glutamate monomers thus generated, which is applicable to the calculation of the polymer yield, was determined by means of high-performance liquid chromatography with a chiral separation column;[2)] typically, D-glutamate is more rapidly eluted than L-glutamate. The standard curve for D- and L-glutamate (showing the relationships of their amounts and the peak area on the high-performance liquid chromatography profiles): $y_{D\text{-}Glu}=2.97x$ (fmol) and $y_{L\text{-}Glu}=2.91x$ (fmol), where x represents each peak area.[5)] On the other hand, a fraction of the isolated PGA was subjected to SDS-PAGE and specifically visualized by the gel staining with methylene blue (a basic dye).[3)]

Results

Optimization of substrate concentration. An essential substrate for the synthesis of *B. megaterium* PGA was an amino acid, glutamate. Based on the tests under various concentrations (0% to 10%), its optimum concentration was defined as 2%. In the standard medium containing 2% L-glutamate, the maximum volumetric yield of *B. megaterium* PGA was $8.6\,g\,L^{-1}$; this value is almost as high as that of *natto* PGA.[2)] On the other hand, *B. megaterium* could not utilize D-glutamate effectively in contrast to some *B. subtilis* strains.[7,8)]

Furthermore, the stereochemistry of *B. megaterium* PGA was analyzed. If L-glutamate was applied as a substrate, stereo-regular PGA, in which the L ratio reached a maximum of 95%, can be synthesized consistently.[5)] In contrast, when D-glutamate (*e.g*., 2%) was used, a random copolymer of PGA with a DL ratio of 45 : 55 was generated.

Effects of salt and osmotic stress. As shown in Fig. 1, *B. megaterium* dramatically accumulated PGA in salt-rich liquid media (>2% NaCl). It is also noteworthy that *B. megaterium* PGA with a greater molecular size (Mw, >2,000,000) was obtained from a medium containing a higher salt concentration (Fig. 1 upper, an SDS-PAGE gel).

In general, salt stress causes osmotic (due to high solute concentrations) as well as ionic stress (due to over-accumulation of salt in cells). The effect of osmotic stress on *B. megaterium* PGA production was examined by the use of sorbitol (0.1 M to 1.0 M)[7)] instead of NaCl. As a result, PGA was not accumulated in media under any sorbitol concentrations tested, suggesting that the PGA productivity of *B. megaterium* is affected by ionic-than-osmotic stress.

Effect of sugar-solute concentration. To further examine the effect of sugar-solute, the structural features of *B. megaterium* PGA produced at various concentrations of sucrose (0% to 10%) were examined. There was no difference in the molecular size distribution of PGA, whereas the effect on the polymer stereochemistry was not negligible. The

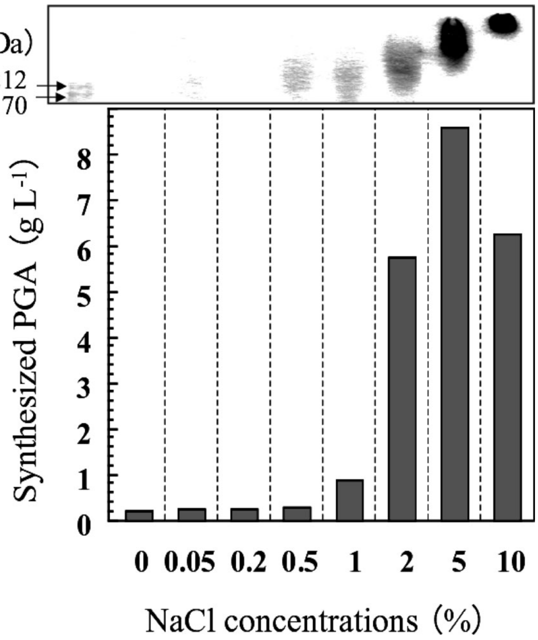

Fig. 1. Salt-responsive PGA production by *B. megaterium*.[5] Upper, SDS-PAGE and visualization of PGA. Lower, PGA accumulation in the standard medium containing the indicated concentration of NaCl (0% to 10%).

addition of a sugar-solute (preferably >5% or at least >2%) to media allowed for the production of PGA with a high content of L-glutamate (*viz*., >90%). In fact, if a sugar-less liquid medium was used, the L ratio of *B. megaterium* PGA was decreased to 75%. Indeed, the DL ratio of *Bacillus* PGA often was shown to be affected by coexisting metal ions;[2] however, this is the first example showing that the apparently similar phenomena are induced by a sugar-solute as well.

Ability for sugar utilization. Probably, sugar plays an important role in *B. megaterium* PGA production. Further research for such sugar effects thus helps to develop an effective synthesis system for stereo-regular PGA. In this work, *B. megaterium* was found to utilize glucose, fructose, xylose, arabinose, galactose, sucrose, maltose, cellobiose, lactose, and starch (5% each). Cellobiose is a main digestive product of cellulose, whereas xylose and arabinose are generated by hemicellulose decomposition.

Identification of preferable sugars. Besides sucrose, the effects of other sugars on *B. megaterium* PGA production were examined. In this work, the PGA productivity in the presence of sucrose was defined as 100% (Fig. 2, bar ②), and the L ratio of the polymer was about 92%. As shown in an SDS-PAGE gel of Fig. 2 (upper), there were no differences in the molecular size distribution among all PGAs synthesized here. When a sugar-less liquid medium was used (Fig. 2, bar ①), the PGA yield reduced by half. Among sugars tested, arabinose (bar ⑥) and fructose (bar ⑦) significantly increased the PGA yield; the productivity was about 1.8-fold higher than that under the standard conditions (bar ②). Considering the effectiveness of the stereo-regular PGA synthesis, it seems likely that the use of arabinose is superior to those of other sugars; the L ratios (%) of the polymers synthesized are indicated in the parentheses of Fig. 2.

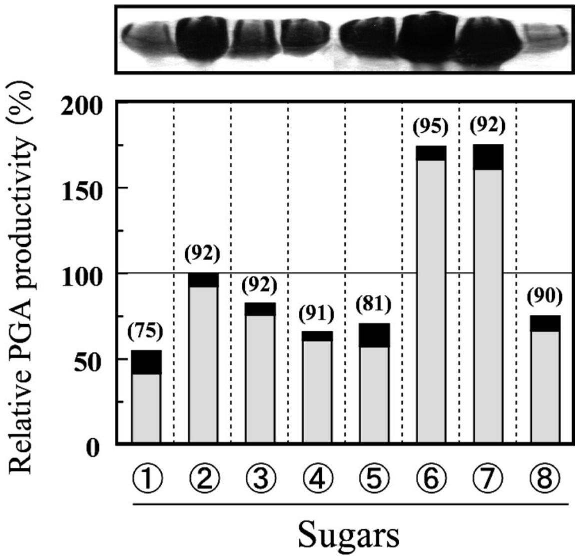

Fig. 2. Sugar effects on *B. megaterium* PGA production. *Upper*, SDS-PAGE and visualization of PGA. *Lower*, Relative PGA productivity against the use of various sugars: ①, sugar-less; ②, sucrose; ③, glucose; ④, xylose; ⑤, galactose; ⑥, arabinose; ⑦, fructose; and ⑧, lactose. The values in the parentheses represent the L ratios (%) of the synthesized PGA.

Conclusion

The study showed that *B. megaterium* PGA production is induced by ionic-than-osmotic stress and verified the feasibility of the addition of a high concentration of salt to the culture media. In addition, the optimization of the substrate and sugar concentrations in media and the identification of the preferable sugars allowed for the effective synthesis of stereo-regular PGA, a promising bionylon material. As the preferable sugars identified are essentially the same as small products derived from woody biomass and garbage, *B. megaterium* will serve as one of the most useful microorganisms to realize a strategy for biomass polymer refinery. The development of a genetically engineered *B. megaterium* that exhibits a high biomass-degradation activity is now in progress.

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