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An Unusual Bioconjugate of Glycerol and Poly(γ-Glutamic Acid) Produced by *Bacillus subtilis* C1

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Abstract A bacterium capable of poly(γ-glutamic acid) production was isolated from nonpasteurized soy sauce. It was judged to be a variety of *Bacillus subtilis* and designated as *B. subtilis* C1. *B. subtilis* C1 produced γ-PGA in the absence of exogenous glutamic acid; therefore, it is a *de novo* PGA-producing bacterium. The product produced by *B. subtilis* C1 was characterized by amino acid analysis to be composed of solely glutamic acid. However, the H^1-NMR spectra showed chemical shifts of glycerol protons in addition to those of authentic γ-PGA, indicating that the product is in fact a bioconjugate of γ-PGA. The finding is unique, because the microbial production of γ-PGA bioconjugate has never been reported before. The molecular mass of the product was over 10,000 kDa as determined by GPC, and 97% of the product was D-glutamate, indicating that L-glutamate was converted to its D-form counterpart by *B. subtilis* C1.

Key words: *Bacillus subtilis* C1, poly(γ-glutamic acid), bioconjugate

Poly(γ-glutamic acid), γ-PGA, is an unusual anionic, naturally occurring homopolyamide that is made of D- and L-glutamic acid residues connected by amide linkages between α-amino and γ-carboxylic acid groups. It is water-soluble, biodegradable, edible, and nontoxic toward humans and environment. Therefore, potential applications of γ-PGA and its derivatives have been of interest in the past few years in a broad range of industrial fields such as food, cosmetics, medicine, and water-treatment [19].

γ-PGA was first discovered as a component of capsules of *Bacillus anthracis* [10], and it is also present in the mucilage of "natto" (fermented soybeans, a traditional food in Japan) [6]. Since it was shown that γ-PGA was freely secreted into the growth medium of *B. subtilis* as a product on fermentation, several *Bacillus* species have been shown to produce γ-PGA outside the cells [1, 13]. Lately, several γ-PGA producers have been isolated from highly salty seasoning of fermented soybean foods [2, 9]. However, no γ-PGA derivatives produced directly by microbial fermentation have ever been reported.

γ-PGA derivatives produced by chemical or physical modification of microbial γ-PGA are known, and they offer a wide range of unique applications, including as drug carriers [15, 17], curable biological adhesive [18], biodegradable fibers [4, 11], and highly water absorbable hydrogels [12].

In this paper, we describe the isolation of a *Bacillus subtilis* strain from soy sauce and its production of a biopolymer, which proved to be an unusual glycerol conjugated γ-PGA derivative.

Materials and Methods

Isolation of Bacteria

Soy sauce samples drawn from the fermentation mash without pasteurization were obtained from a local soy sauce manufacturer. The nonpasteurized broth was diluted with sterilized water. After dilution, the aliquots were spread on nutrient agar (NA, Difco Laboratories, Detroit, MI, U.S.A.), containing agar (15 g l^{-1}), beef extract (3 g l^{-1}), and peptone (5 g l^{-1}), and the agar was incubated at 37°C and pH 7.4 for 24 h. The colonies that developed were randomly selected and purified by repetitive dilution and cultivation on NA. The pure colonies were picked up and further cultivated in NA supplemented with 0.4% L-glutamic acid and 4% citric acid. After incubation at 37°C for 24 h, highly mucoid colonies were picked up and inoculated into 100 ml of medium F composed of glutamic acid (65 g l^{-1}), citric acid (22 g l^{-1}), glycerol (170 g l^{-1}),

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NH_4Cl (7.0 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g l^{-1}), $FeCl_3 \cdot 6H_2O$ (0.04 g l^{-1}), K_2HPO_4 (0.5 g l^{-1}), and $CaCl_2 \cdot 7H_2O$ (0.15 g l^{-1}) [20]. The highest γ-PGA producer was chosen, inoculated into 100 ml of 2-XYT medium containing 1.5% tryptone, 1% yeast extract, and 0.5% NaCl, and cultivated at 37°C for 24 h. Cells were collected by centrifugation at 6,000 ×*g* for 15 min and then suspended in 20 ml of 20% glycerol solution. The cell suspension was stored at −20°C until use.

Biopolymer Production, Purification, and Characterization

The stored suspension (1 ml) was first inoculated into 100 ml of 2-XYT medium and incubated at 37°C and 150 rpm for 15 h. The cell suspension (0.4 ml) was transferred to medium F and incubated aerobically at 37°C and 150 rpm for 4 days. The biopolymer was purified by the ethanol precipitation as described previously [20]. The absence of free L-glutamate and polysaccharide was confirmed by the L-glutamate dehydrogenase-coupled assay [2] and by the phenol-sulfuric acid method [5], respectively. The purified material was characterized by amino acid analysis, gel permeation chromatography (GPC), and H^1-NMR spectrometry.

Analytical Methods

The number-average molecular weight (M_n) of the γ-PGA was measured by gel permeation chromatography (GPC) using a Hitachi L6200 system controller equipped with Shodex KB800 series columns (two KB80M, one KB802.5) and a refractive index (RI) detector (Bischoff, Model 8110). Dextrans standards (Phenomenex, Torrance, CA, U.S.A.; Mw. 7,200, 16,230, 35,600, 74,300, 170,000, 535,000, 1,580,000, 2,754,000) were used to construct a calibration curve. The eluant H_2O was set at a flow rate of 1.0 ml/min, and the column oven was at 50°C. The total carbohydrate contents of the products were determined by the phenol-sulfuric acid method. H^1-NMR spectroscopy was performed with a Varian Unity Inova 600 spectrometer. Samples for NMR were dissolved in D_2O solution. The D-/L-glutamate composition of γ-PGA was determined by liquid chromatography with a Crownpak CR(+) column (Daicel Chemical Industries Ltd.) as described previously [7].

Results and Discussion

Identification of the Isolated Bacterium

A bacterium, strain C1, which produced γ-PGA derivatives, was isolated from nonpasteurized soy sauce as described in Materials and Methods. It was aerobic, Gram-positive, spore-forming rods, and used citrate. The morphological observation and physiological characteristics (Table 1) indicated that strain C1 was a strain of *Bacillus subtilis* according to the description in *Bergy's Manual of Systematic Bacteriology*. Furthermore, the 16S ribosomal DNA sequence of the isolated strain showed 97% similarity to that of *Bacillus subtilis*. The *B. subtilis* C1 did not require biotin for its growth, in contrast to many γ-PGA-producing strains classified as *B. subtilis* (natto). In addition, the strain could grow in the presence of 12% NaCl, whereas *B. subtilis* from commercially available Natto foods could not grow and produce γ-PGA in the presence of 5% NaCl.

Table 1. Taxonomic characteristics of C1 isolated from nonpasteurized soy sauce.

Gram strain	+[a]
Shape	Rod, 0.7–0.8×2–3.0 mm
Sporulation	+
Endospore	Cylindrical, central
Mobility	+
Anerobic growth	−[b]
Citrate utilization	+
Catalase test	+
Oxidase test	+
Voges-Proskauer test	+
Nitrate reduction	+
Biotin required	−
Hydrolysis of casein	+
starch	+
Gelatin liquefaction	+
Growth on pH 6.8	+
Growth in 12% HNaCl	+

[a]Positive.
[b]Negative.

Effect of Glutamate on Cell Growth and Biopolymer Production

γ-PGA-producing bacteria are divided into two groups; glutamate-dependent bacteria requires the addition of L-glutamic acid to the medium to stimulate γ-PGA production and cell growth, and glutamate-independent bacteria does not require L-glutamic acid for γ-PGA production because of the operation of the *de novo* pathway of L-glutamate synthesis [19]. We examined the effect of glutamate on PGA production by *B. subtilis* C1 by cultivating the bacteria in medium F, a modified medium E, which is a medium of

Table 2. Effect of L-glutamate concentration on biopolymer production.

Concentration of L-glutamate (g l^{-1})	Yield of biopolymer (g l^{-1})
0	8.2
1	1.3
10	3.0
20	3.6
50	3.8
65	4.1

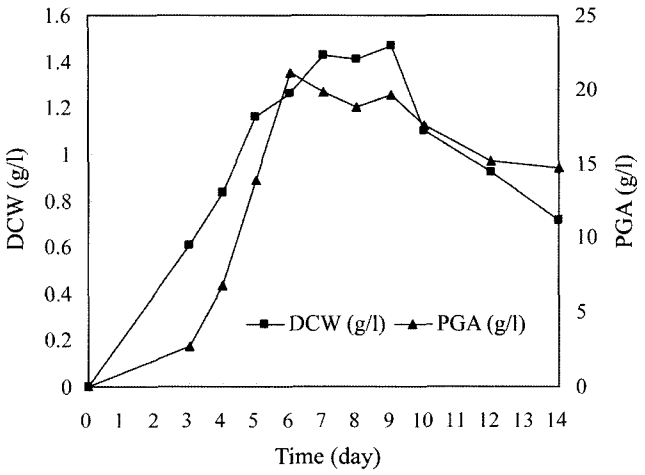

Fig. 1. The profile of cell growth and γ-PGA production by *B. subtilis* C1 in T1 medium.

choice for many researchers to investigate production of γ-PGA by *Bacillus* species [14], with various concentrations of L-glutamate. As shown in Table 2, *B. subtilis* C1 produced γ-PGA in the absence of exogenous glutamic acid; therefore, it is a *de novo* PGA-producing bacterium. The addition of L-glutamic acid in the medium did not have any effect on γ-PGA productivity. Accordingly, medium T, a medium F deprived of glutamic acid, was chosen for the following study.

Batch culture was carried out to examine the profile of cell growth and γ-PGA production in medium T. As shown in Fig. 1, γ-PGA concentration rapidly increased during active cell growth and started to reach a maximum as the cells entered the stationary phase. After 6-days incubation in the absence of L-glutamate, the product reached a maximum of 22 g/l.

Characterization of Product

The number-average molecular weight (M_n) of the product was determined by gel permeation chromatography and found to be over 10,000 kDa. The 6 N HCl hydrolysate of the purified viscous material was found to be solely composed of glutamic acid. Thin-layer chromatography of the hydrolysate performed on a cellulose thin-layer plate and visualized with 0.2% ninhydrin indicated a single spot with a R_f value identical to that of authentic glutamic acid. Furthermore, the ninhydrin and biuret reactions for the

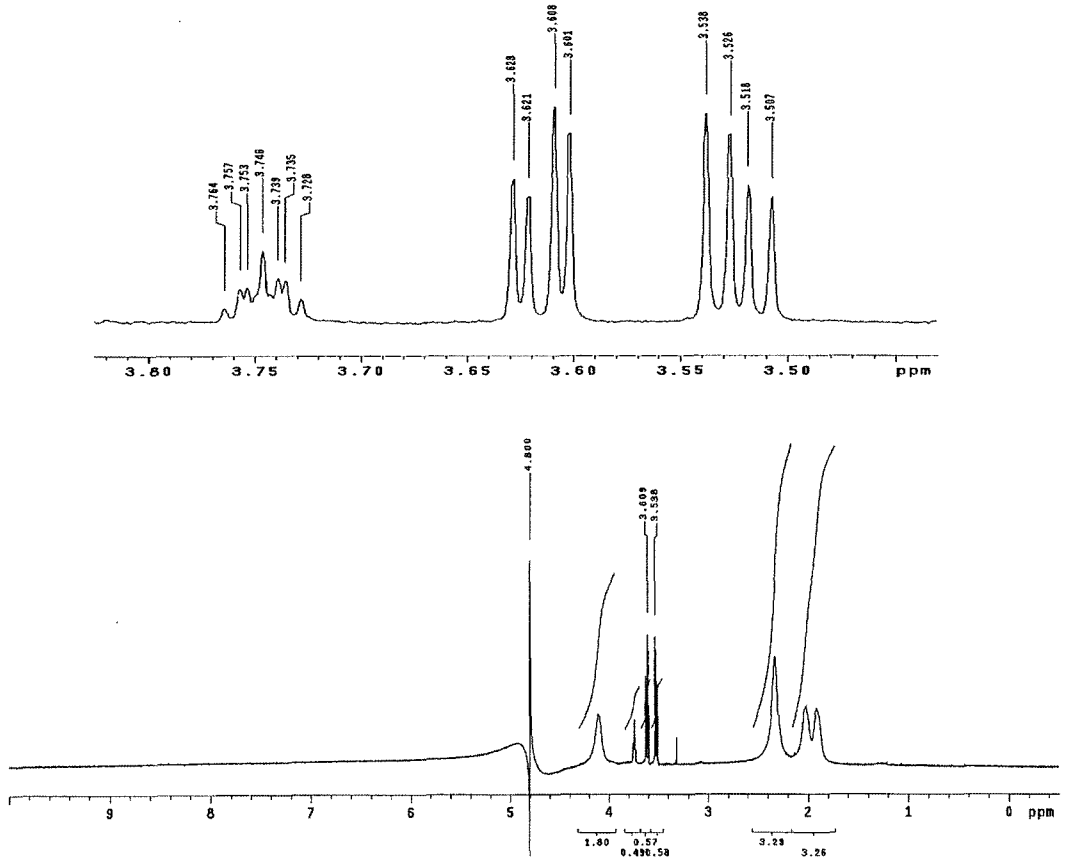

Fig. 2. The H^1-NMR spectra of the product produced by *B. subtilis* C1 (Bottom panel: the whole spectrum; top panel: the amplification of glycerol peaks).

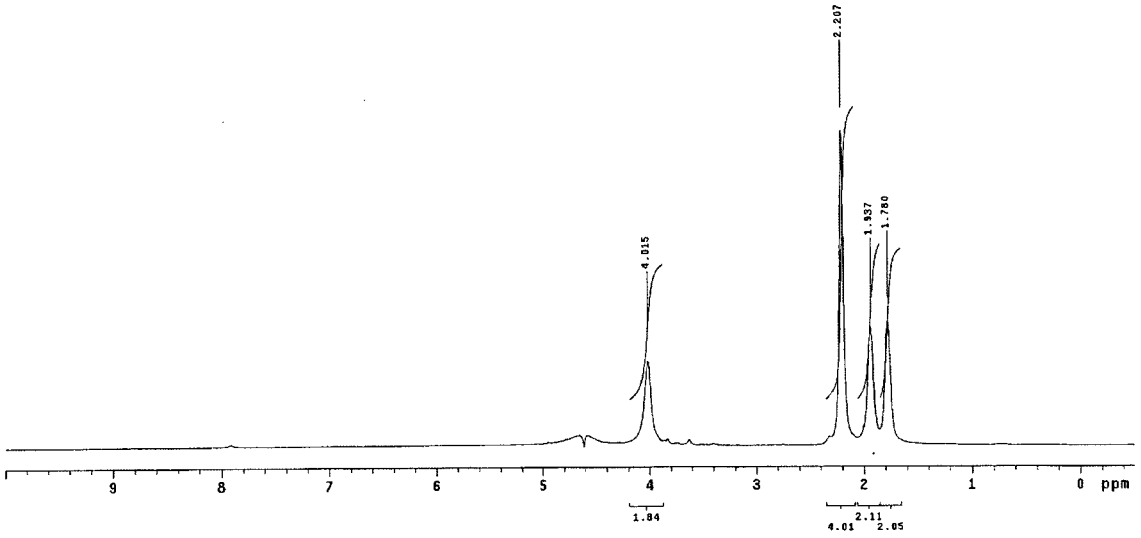

Fig. 3. The H[1]-NMR spectra of the product after mild hydrolysis.

viscous material were negative. Analysis by the phenol-sulfuric acid method showed that the polysaccharide contained less than 1% (w/w) of total sugar. From these results, the biopolymer was first thought to be poly (glutamic acid). However, the H[1]-NMR spectra shown in Fig. 2 indicated otherwise. The chemical shifts in ppm, 1.8–2.1 (m, β, 2H), 2.3–2.4 (b, γ, 2H), 4.1–4.2 (b, γ, 1H) and 7.8 (N-H) shown in Fig. 2 (bottom panel), corresponded to the peak position of authentic γ-PGA previously synthesized [3, 20]. In addition, the chemical shifts in ppm, 3.50–3.54 (dd, 2H), 3.60–3.63 (dd, 2H), 3.72–3.77 (m, 1H) shown in Fig. 2 (bottom and top panels), corresponded to the peak positions of glycerol. The glycerol peaks persisted even after the product was extensively dialyzed against distilled water with a 10-kDa cutoff membrane for 3 days, suggesting a possibility that the glycerol was covalently bonded to γ-PGA. To examine the possibility, the product was mildly hydrolyzed in 0.1% HCl solution (150°C, 30 min), followed by dialysis and freeze-drying. The H[1]-NMR spectra of the hydrolyzed product is shown in Fig. 3, in which only the chemical shifts of γ-PGA appeared without any trace of glycerol. The glycerol-conjugated γ-PGA derivative is unusual in that no such material has ever been reported, although it was synthesized by microbial fermentation. The γ-PGA to glycerol ratio in the conjugate was about 10:1, judged by the proton integration, shown in Fig. 2. The mechanism of how the γ-PGA was derivatized from glycerol is yet to be determined.

It is known that the molecular weight of γ-PGA varies, ranging from 10 kDa to 2,000 kDa, depending upon the species and the cultivation conditions used for its production [13]. Under the conditions used in this study, the molecular size of the γ-PGA derivative produced by *B. subtilis* C1 was over 10,000 kDa, determined by GPC. To the best of our knowledge, γ-PGA with a molecular weight over ten million daltons has rarely been produced. The unusual high molecular size of the product in this study is probably due to intramolecular cross-linking of γ-PGA by glycerol. This was confirmed by the fact that the molecular size of the product was significantly decreased to 6×10^6 daltons after it was mildly hydrolyzed by 0.1% HCl.

Most of the γ-PGA producers have been shown to produce γ-PGA of various stereochemical compositions. The capsule of *B. anthracis* contains PGA composed of only D-glutamate [8], and *B. licheniformis* produces various PGAs (D-glutamate content: 10–100%) in the presence of different metal ions, especially Mn^{+2} [16]. In this study, the D-/L-glutamate composition determined by HPLC with a Crownpak CR(+) column showed that the D-glutamate content was 97% in every product produced under the conditions used, suggesting that the bacterium possesses a metabolic pathway to produce D-glutamate from L-glutamate. Two different pathways have been indicated for the conversion of L-glutamate to its D-form counterpart; one involves D-amino acid amino-transferase and the other involves glutamate racemase [2]. The pathway responsible for the conversion has yet to be determined.

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