J. Microbiol. Biotechnol. (2001), **11**(2), 342–345

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Enzymatic Degradation of Poly(γ-glutamic acid) Hydrogel Prepared by γ-Ray Irradiation

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Received: October 30, 2000
Accepted: March 20, 2001

Abstract A bacterial strain PH-4, which produces an enzyme catalyzing the degradation of crosslinked poly(γ-glutamic acid) hydrogels, was isolated and identified as a *Flavobacterium* sp. The enzyme was obtained by the sonication of the bacterial cells preincubated in a Bouillon medium with shaking, without adding of poly(γ-glutamic acid) as an inducer. The products of the hydrogel degraded by the crude enzyme agreed closely with the depolymerized materials in SDS-polyacrylamide gel electrophoresis using methylene blue staining, and with a glutamic acid monomer on thin-layer chromatography, thereby suggesting that strain PH-4 produced a kind of exohydrolase.

Key words: Crosslinked poly(γ-glutamic acid), *Flavobacterium* sp., hydrogel, γ-ray irradiation, degradation enzyme, exohydrolase

There has been a growing interest in the biosynthesized polymer poly(γ-glutamic acid) (PGA) [2, 9], which is currently being used in various fields of application [1, 5]. Recently, crosslinked PGAs were produced from PGA molecules by γ-ray irradiation [4, 10] and an electronic beam, along with the use of a crosslinking reagent, such as dihalogenoalkanes [1] or epoxide [6]. The resulting crosslinked PGAs show a high water-sorption capacity with a specific water content of 3,000–5,000 times their dry volume depending on the preparation conditions. Crosslinked PGAs can be used as environmental-friendly materials, for example, waste-water absorbents of livestock and country-side industry, controlled drug delivery systems in bioactive materials such as somatotropin [1], and so on.

The crosslinked PGA, previously prepared by the current authors from PGA using γ-ray irradiation, is now being developed as a new "green plastic" that is degradable in nature, which can be used for making food packaging containers and for "dessert greening" [5]. The work reported

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Table 1. Microbiological characteristics of strain PH-4.

Characteristics	Strain PH-4
Gram staining	Negative
Shape	Rod (0.5±0.1×2.8±0.2 μm)
Motility	-
Poly-β-hydroxybutyrate accumulation	-
Pale yellow colony on NA[a]	+
Visid deposit in NA	+
Spreading growth on CA[b]	-
Growth at 4°C	-
at 42°C	+
at pH 3.6	-
at 4% NaCl	-
on MacConkey agar	+
on Simmons' citrate agar	+
Inorganic salts +glucose medium	-
Amino-acids-containing medium	+
Gelatin hydrolysis	+
Nitrate reduction	+
Catalase	+
Oxidase	+
Starch (agar) hydrolyzed	-
Oxidative in glucose O-F medium	+
Indole production	-
Urease production	-
H_2S production	-
Pigment production on tyrosine agar	-
Tween 80 hydrolysis	+
Fluorescent pigment production on King's medium [8]	-

[a]Nutrient agar. [b]Modified Cytophaga agar [7].

here confirms the enzymatic degradation of crosslinked PGA hydrogels and clarifies the PGA hydrogel-degrading enzyme from strain PH-4, which was isolated from a contaminated PGA hydrogel kept in an open environment.

The morphological and physiological characteristics of strain PH-4 are as follows (Table 1): The cells were Gram negative rods, approximately 0.5±0.1 μm by 2.8±0.2 μm in size, occurring singly and in pairs. No endospores were formed. Intracellular granules of poly-β-hydroxybutyrate were absent. The cells were also nonmotile and glided on a modified Cytophaga agar [7]. The colony was circular, low convex, and shiny with entire edges on the nutrient agar. The color of the colony was a nonfluorescent pale yellow, yet the pigment did not diffuse into the King's B medium [8] and into tyrosine agar. Acid was produced from the glucose in an O-F medium aerobically. D-Glucose, D-mannose, D-fructose, sucrose, maltose, ethanol, and glycerol were utilized as sole carbon sources, however, no growth was observed in acetate or succinate in the synthetic medium when using 0.1% ammonium nitrate. Amino acids and supplementary growth factors were not required for growth.

Electron microscopic observation of strain PH-4 showed flagella-like polar and amphitrichous material (Fig. 1) of about 7.1±0.3 nm in width, which is comparatively thinner than bacterial flagella, such as in Pseudomonad, whose flagella range from 14.5±0.3 to 19.4±0.5 nm in width, which determines their motility [3]. Pseudoflagella have been previously identified in *Flavobacterium* sp., such as *Flavobacterium aquatile* [14] and *F. multivorum* [11]. Populations of *F. aquatile* F36 [15] treated with the impregnation technique also exhibit pseudoflagella that are faintly stained with thin appendages for long filaments. Accordingly, these properties indicate that strain PH-4 belongs to the genus *Flavobacterium*.

Figure 2 shows the time course of the hydrogel-degrading enzyme production from strain PH-4 cultured in

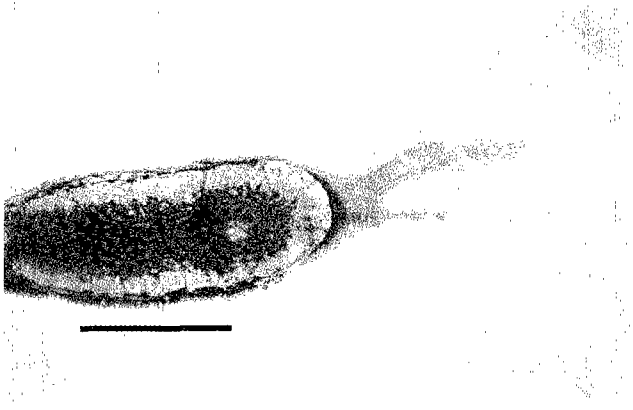

Fig. 1. Scanning electron micrograph of *Flavobacterium* sp. PH-4. Bar represents 0.4 mm.

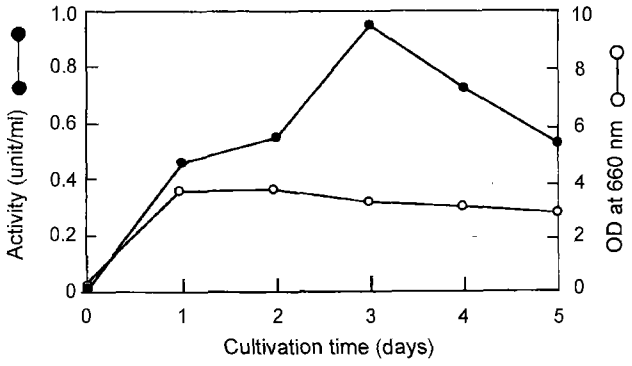

Fig. 2. Time course of hydrogel-degrading enzyme production by *Flavobacterium* sp. PH-4.

a Bouillon medium (pH 7.0). The cultivation was carried out in a 500-ml Erlenmyer flask with shaking at 32°C. For the enzyme assay, 0.1 g of crosslinked PGA powder prepared by γ-ray irradiation was added to 300 ml of distilled water, kept overnight in a cold room to form a hydrogel, and then filtered through an 80-mesh sieve (polyethylene; Tanaka Sanjiro Co. Ltd, Japan) so as to drain remaining water. Two grams of hydrogel were put into a bottle (Wheaton vial, 6 cm×ϕ2.5 cm) and 0.1 ml of crude enzyme solution was added. The crude enzyme solution was prepared by lysozyme treatment and sonication of the bacterial cells; wet cells were treated with lysozyme (Sigma Co. U.S.A.) dissolved in 20 ml of 25 mM sodium phosphate buffer (pH 7.0) at 30°C for 60 min, sonicated with an ultrasonic disruptor (UD-201, Tomy Co. Japan) at 0–5°C, and then centrifuged at 10,000 ×g for 90 min to remove any cell debris. The resulting clear, slightly viscous supernatant constituted the crude enzyme preparation. The hydrogel and enzyme mixture were incubated at 45°C for 18 h, and filtered through an 80-mesh sieve to measure the filtrate quantity released from the hydrogel, which was kept at room temperature for 30 min. The heat-denatured enzyme (by boiling for 5 min) was used as a control. One unit of the hydrogel-degrading enzyme was defined as the amount of enzyme releasing 1 ml of filtrate passed through an 80-mesh sieve under the above conditions. As shown in Fig. 2, the maximum enzyme activity in the culture broth was reached after 72 h; thereafter, the activity declined. Whereas no enzyme activity was detected in the culture filtrate of strain PH-4, it was in the sonicated bacterial cells, thereby suggesting that strain PH-4 produced the enzyme intracellularly. The hydrogel prepared according to the above description was not degraded by commercial enzymes such as papain, trypsin 250, pancreatin, pepsin, Zymolyase 20T, cellulase, α-amylase, and pectinase (data not shown).

The hydrolyzates of the crosslinked PGA hydrogel degraded by the crude enzyme prepared from *Flavobacterium* sp. PH-4 were examined by SDS-polyacrylamide gel

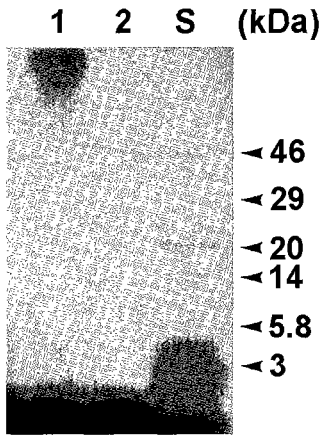

Fig. 3. SDS-PAGE patterns of hydrogel hydrolyzates using crude enzyme from *Flavobacterium* sp. PH-4. A reaction mixture containing 2 g of the hydrogel and 1.0 unit of the crude enzyme was incubated at 45°C for 2 d.
Lanes 1 and 2 show the reaction products with the native and heat-denatured enzyme, respectively; S shows the prestained SDS-PAGE standards from a GibcoBRL.

electrophoresis (SDS-PAGE) and thin-layer chromatography (TLC). The crude enzyme and hydrogel were mixed in a bottle and incubated at 45°C for 2 days. The hydrolyzates were then subjected to SDS-PAGE, which was performed with a 10% acrylamide gel and stained with methylene blue. The methylene blue staining was performed using the protocol described by Yamaguchi *et al.* [16]. Due to its highly anionic properties, PGA can not be stained with Coomassie brilliant blue, the most popular dye for protein staining, but it can be stained with methylene blue. As shown in Fig. 3, the SDS-PAGE pattern of the hydrolyzates showed a smeared image (lane 1). However in the reaction mixture with the heat-denatured enzyme, no depolymerized materials were found (lane 2). These results indicate that strain PH-4 may produce a hydrogel-degrading enzyme.

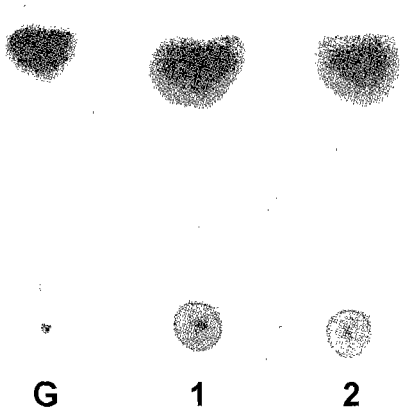

Fig. 4. Thin-layer chromatograms of the hydrogel hydrolyzates using crude enzyme from *Flavobacterium* sp. PH-4.
For reaction conditions, refer to Fig. 3. G, L-glutamic acid; lane 1, the reaction product+L-glutamic acid (1:1); lane 2, the reaction product.

Figure 4 shows the TLC of the reaction products derived from the hydrogel. The same hydrolyzed mixtures that were used for the SDS-PAGE analysis were also spotted on a Cellulose Thin-layer plate (Funacoshi Co, Japan), and developed in a solvent (upper layer of following mixture, n-butanol:CH_3COOH:H_2O=4:1:5). The TLC plate was sprayed with a ninhydrin solution (Wako Co., Japan) and dried at 105°C. The enzyme reaction mixture showed a single spot (lane 2), and the reaction mixture+L-glutamic acid (1:1) also showed a single spot (lane 1), thereby suggesting that the reaction product coincided with the glutamic acid used as a standard in the TLC. The reaction product when using the hydrogel-degrading enzyme from *Flavobacterium* sp. PH-4 was a glutamic acid monomer, exhibiting exohydrolase action. This finding is the first report on a PGA hydrogel-degrading enzyme. In the case of PGA hydrolysis, a PGA hydrolase has already been recognized in the strain of *Flavobacterium polyglutamicum* [13], and its mode of action on PGA has been characterized as an exohydrolase that releases L-glutamic acid at the carboxyl end of the peptide. *Aspergillus oryzae* N-2 is also reported as a strain producing a PGA exohydrolase, because only glutamic acid was recognized as the hydrolysate of L-PGA when using paper chromatography [12].

The PGA hydrogel-degrading enzyme from the strain *Flavobacterium* sp. PH-4 may be a kind of constitutive enzyme, because it could be produced in the medium without addition of PGA as an inducer. With this crude enzyme, no proteins with an α-linkage peptide bond, such as albumin from bovine, casein from milk, and gelatin, were hydrolyzed. However, when used as a substrate, a PGA with a γ-linkage peptide bond was degraded by the crude enzyme. This enzyme could be used as sources for analyzing the structure of water-absorbable biopolymer crosslinked PGAs.

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