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Journal of Molecular Catalysis B: Enzymatic 23 (2003) 249–255
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Poly-γ-glutamate depolymerase of *Bacillus subtilis*: production, simple purification and substrate selectivity

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Received 7 February 2003; accepted 18 April 2003

Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

The *Bacillus subtilis pgdS* gene, which is located at the immediate downstream of the *pgs* operon for poly-γ-glutamate (PGA) biosynthesis, encodes a PGA depolymerase. The *pgdS* gene product shows the structural feature of a membrane-associated protein. The mature form of the gene product, identified as a *B. subtilis* extracellular protein, was produced in *Escherichia coli* clone cells. Since the mature PGA depolymerase has been modified with the histidine-tag at its C-terminus, it could be simply purified by metal-chelating affinity chromatography. This purified enzyme digested PGAs from *B. subtilis* (D-glutamate content, 70%) and from *Bacillus megaterium* (30%) in an endopeptidase-like fashion. In contrast, PGA from *Natrialba aegyptiaca*, which consists only of L-glutamate, was resistant to the enzyme, suggesting that, unlike fungal PGA *endo*-depolymerases, the bacterial enzyme recognizes the D-glutamate unit in PGA.

Keywords: Poly-γ-glutamate; Depolymerase; Endopeptidase; Substrate selectivity; *Bacillus subtilis*

1. Introduction

Poly-γ-glutamate (PGA) is an unusual anionic polypeptide in that glutamate is polymerized via γ-amide linkage [1] and synthesized mainly by microorganisms [2–6]. As shown in Fig. 1, stereochemically different three types of PGA have been found: the homopolymer composed of D-glutamate (D-PGA), the homopolymer of L-glutamate (L-PGA), and the copolymer composed of D- and L-glutamate (DL-PGA). The starter bacteria of *natto*, a traditional Japanese fermented food made from soybeans, e.g. *Bacillus subtilis* (*natto*), produce DL-PGA. Although PGA is highly resistant to protease because of its structural features, PGAs with various molecular masses (10–1000 kDa) are found in the culture of *B. subtilis* (*natto*) [1,7,8]. Hereafter, it has been believed that, during PGA production, PGA-degrading enzymes also are secreted [7]. A gene (formerly the *ywtD* gene) is located at the immediate downstream

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doi:10.1016/S1381-1177(03)00087-0

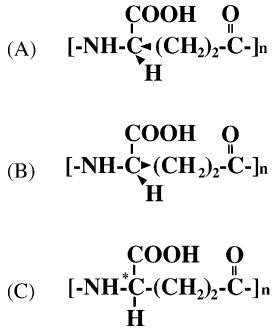

Fig. 1. Chemical structures of PGA. (A) Poly-γ-D-glutamate (D-PGA). (B) Poly-γ-L-glutamate (L-PGA). (C) Poly-γ-DL-glutamate (DL-PGA). The term "*n*" indicates the number of the γ-glutamyl linkages in PGA, and the range of the *n* value is estimated to be 100 to over 10,000.

of the *pgs* operon, composed of *pgsB*, *-C*, and *-A* genes, in the *B. subtilis* chromosome and designated *pgdS* (the DDBJ/EMBL/GenBank accession no. AB085821) [1,9]. The *pgdS* gene product consists of 413 amino acids (molecular mass, 45 kDa) and is similar in primary structure to enzymes belonging to the DL-endopeptidase II family [10], such as LytF (a peptidoglycan hydrolase) [11]. It, however, showed no activity for peptidoglycan degradation. We recently found the PGA depolymerase activity in cell extracts of the *Escherichia coli* clone where the *pgdS* gene was highly expressed [1,9]. In addition to the structural features of the *pgdS* gene product, e.g. the existence of both a hydrophobic cluster (^{10}F-L-L-V-A-V-I-I-C-F-L-V-P-I-M^{24}) and a possible cleavage site by signal peptidases (^{30}A-E-A^{32}) at the N-terminus, a recent study of *B. subtilis* extracellular proteins [12] implies that the *pgdS* gene encodes a membrane-associated form of PGA depolymerase. The membranous PGA depolymerase would be cleaved behind the site ^{30}A-E-A^{32} so that the mature enzyme, composed of 381 amino acids (42 kDa), could be liberated into culture medium from cell membranes in *B. subtilis* cells.

The control of molecular sizes of PGA is important for its application, since those with different molecular sizes are requested for different purposes [1]. It is assumed that PGA depolymerase serves as a potent tool for such purposes. Here we describe the production, simple purification, and substrate selectivity of this useful enzyme.

2. Experimental

2.1. Materials

All restriction enzymes used, isopropyl-β-D-thiogalactopyranoside (IPTG), and SUPREC-02 ultrafiltration system (excluding compounds with the molecular masses of less than 30 kDa) were from Takara Shuzo, Kyoto, Japan. A PRISM kit was from Perkin-Elmer, California, USA. A plasmid pTrc99A (an *E. coli* expression vector having the Trc promoter operating in the presence of IPTG) and a HisTrap column were from Amersham Pharmacia Biotech, Buckingham, UK. Mini-ProteanII Ready Gel J (linear gradient of the gel concentration, 5–15%), an SDS-PAGE HMW marker kit comprising myosin (200 kDa), α-2-microglobin (170 kDa), β-galactosidase (116 kDa), transferrin (76.0 kDa) and glutamate dehydrogenase (53.0 kDa), an SDS-PAGE LMW marker kit containing phosphorylase b (97.0 kDa), bovine serum albumin (BSA) (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa), and a protein assay kit were from Bio-Rad, California, USA. A histidine-tag staining kit and vials of distilled HCl (6N) were from PIERCE, Illinois, USA. PGAs composed of 70% D- and 30% L-glutamate and of 30% D- and 70% L-glutamate were prepared from viscous materials of *B. subtilis* (*chungkookjang*) [13] and of *Bacillus megaterium* WH320 [4], respectively. PGA, which consists only of L-glutamate, was produced by *Natrialba aegyptiaca* under the conditions described previously [6]. All other chemicals were of analytical grade.

2.2. Microorganisms

E. coli JM109 competent cells were from Takara Shuzo. *B. megaterium* WH320 was from MoBiTec Co., Göttingen, Germany. *N. aegyptiaca* was a kind gift from Prof. Dr. A. Steinbüchel of Westfälische Wilhelms-Universität, Germany.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of PGA

PGA was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the

Mini-ProteanII Ready Gel J with the HMW marker kit and visualized by methylene blue staining [13].

2.4. PGA analysis and determination

To examine the stereochemical compositions of PGA and determine the polymer yields, PGA was first hydrolyzed with 6N HCl at 105 °C for 8 h in vacuo using a Hydrolysis Station AHST-1 (Shimadzu, Kyoto, Japan). Hydrolysates were lyophilized, dissolved in 0.2 ml of distilled water, and analyzed by high performance liquid chromatography (HPLC) with a CHIRALPAK MA(+) column (4.6 mm × 50 mm; from DAICEL, Tokyo, Japan) under the conditions described previously [9]. Both DL ratio and yield of PGA can be determined by the use of the standard curves for D- and L-glutamate (showing relationships of the amounts and the apparent peak area on the HPLC profiles): $y_{\text{d-Glu}} = 2.97x$ (fmol) and $y_{\text{l-Glu}} = 2.91x$ (fmol), where x represents each peak area. These curves gave a good linearity in a range of 0.5–100 nmol of glutamate.

2.5. PCR amplification and gene cloning

The internal *pgdS* gene encoding the mature PGA depolymerase was amplified by LA-PCR [14,15] with a sense primer PPGDSi-NF (5′-GCG**CCATGG**-AGGAGGATAGGATCCATGGATACATCATCAGA-ATTGATTGTCAGC-3′), in which an *Nco*I site (bold types), the ribosome-binding site (underlined) and an initial codon (double-underlined) were designed, and an antisense primer PPGDS-CR (5′-GCG**GTC-GAC**TTAGTGGTGGTGGTGGTGGTGTTGCACC-CGTATACTTCCTGCGTA-3′) having a *Sal*I site (bold types) and the histidine-tag sequence (underlined). The amplified fragment (1.2 kb) was digested by both *Nco*I and *Sal*I and ligated into the same site of pTrc99A. The constructed plasmid was designated pTPD42.

The nucleotide sequence of the cloned gene was verified by the use of the PRISM kit with an Applied Biosystems 373A DNA sequencer.

2.6. Enzyme purification

E. coli JM109 cells were transformed with the plasmid pTPD42 and the clone was grown at 37 °C in 200 ml of Luria-Bertani broth [16] containing ampicillin (50 μg ml^{-1}). When the turbidity of the culture at 600 nm had reached 0.6, 1 mM IPTG was added. The cultivation was continued at 37 °C for another 12 h. The harvested cells (wet weight, 0.6 g) were suspended in 1.2 ml of a TGS buffer (20 mM Tris–HCl, 10% glycerol, and 0.3 M NaCl, pH 8.0), and disrupted by sonication at 4 °C. After centrifugation at 12,000 × *g* for 1 h, the resulting supernatant was applied to the HisTrap column (bed volume, 1 ml) equilibrated with the same buffer. The column was washed with 10 ml of the TGS buffer and then with 30 ml of the TGS buffer with 10 mM imidazole. It was developed with the TGS buffer containing a stepwise gradient of imidazole of 30, 60, and 200 mM (each 5 ml). The histidine-tagged protein (corresponding to the mature PGA depolymerase) was eluted with the TGS buffer comprising 200 mM imidazole.

Protein concentrations in the enzyme fractions were determined by the use of the protein assay kit with BSA as a standard.

2.7. Enzymatic PGA digestion

Enzymatic PGA degradation was performed as follows: The reaction mixture (400 μl) consisted of 40 μmol of Tris–HCl buffer (pH 7.0), 2 μmol of dithiothreitol, 100 μg of PGA, and enzyme and was incubated at 37 °C. After termination of the reaction by heating the reaction mixture at 100 °C for 5 min, the mixture was centrifuged at 8000 × *g* for 10 min. A 2.5 μl of the supernatant was withdrawn and subjected to SDS-PAGE (see above).

3. Results

3.1. Production and simple purification of the mature PGA depolymerase

In order to obtain effectively the mature PGA depolymerase, we first studied the construction of its producer. As shown in Fig. 2A, the mature enzyme with a molecular mass of about 42 kDa was produced in cells of the *E. coli* JM109 clone harboring the plasmid pTPD42.

As the C-terminus of the enzyme was modified with the histidine-tag, the enzyme was rapidly and simply

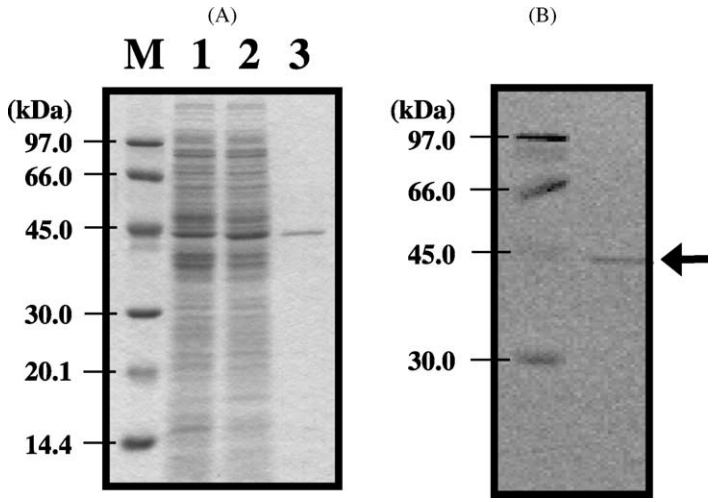

Fig. 2. Production and purification of the mature PGA depolymerase. (A) Protein visualization by the Coomassie-blue staining of the SDS-PAGE gel (12.5% polyacrylamide). Lane M, the LMW marker kit; lane 1, the crude extract of the *E. coli* JM109/pTrc99A clone (10 μg of protein); lane 2, the crude extract of the *E. coli* JM109/pTPD42 clone (10 μg); and lane 3, the purified enzyme (2 μg). The corresponding band to the mature PGA depolymerase produced (42 kDa) in the lane 2 partially lies upon that of a genuine 43 kDa protein of *E. coli* JM109 in the lane 1. (B) Visualization of the purified enzyme by the histidine-tag staining of the SDS-PAGE gel. The corresponding band to the histidine-tagged enzyme is shown by an arrow.

purified by the nickel-chelating affinity chromatography (Fig. 2A, lane 3). By means of histidine-tag staining, the purified enzyme was verified to be a histidine-tagged protein (Fig. 2B).

3.2. Substrate selectivity of the PGA depolymerase

In the characterization of PGA depolymerase, it is essential to examine whether the enzyme exhibits a certain selectivity for PGA that is stereochemically various, as such information possibly leads to the processes of the structurally controlled PGAs. In this work, the enzymatic degradation of three types of PGAs was conducted. As shown in Fig. 3, *B. subtilis* PGA containing the D-glutamate units in considerable amounts was the best substrate, followed by *B. megaterium* PGA comprising highly the L-glutamate units. However, *N. aegyptiaca* PGA composed only of L-glutamate was apparently inert as a substrate (Fig. 3C). The results indicated that the PGA depolymerase recognizes the D-glutamate unit in PGA. Moreover, by the use of the DL-glutamate assay system with both glutamate racemase and L-glutamate dehydrogenase [17], we obtained evidence that no glutamate monomer was included in either the digested preparation from *B. subtilis* PGA or from *B. megaterium* PGA. This suggests that the enzyme digests PGA in an endopeptidase-like fashion.

3.3. Probable recognition site of the PGA depolymerase

Both PGAs from *B. subtilis* and *B. megaterium* are copolymers composed of D- and L-glutamate but not of a mixture of the D- and L-homopolymers [4,18]. Therefore, it remained to be investigated whether the PGA depolymerase cleaves the γ-amide linkages between the D- and L-glutamate units or those between the D- and D-units (Fig. 4). If the enzyme recognizes only the γ-amide linkages between the D- and L-units of PGA, it could not digest either region composed mainly (or only) of D-glutamate or of L-glutamate in PGA. A mixture of the fragments composed mainly of D-glutamate and those of the L-isomer would remain in the fraction of high-molecular-mass products (Fig. 4A and B). Eventually, there may be little difference in the stereochemical compositions of the substrate PGA and the high-molecular-mass products. Its low-molecular-mass products (corresponding to the recognition sites) are theoretically composed of 50%

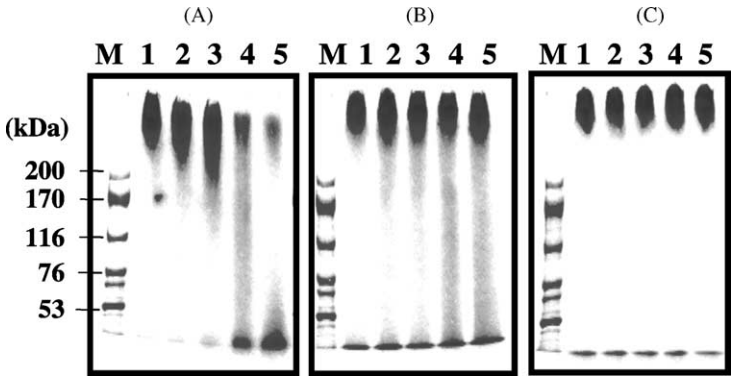

Fig. 3. Substrate selectivity of the mature PGA depolymerase. As described in Section 2, the purified enzyme (1 μg) was incubated with either PGA (100 μg) of *B. subtilis* (A), *B. megaterium* (B) or *N. aegyptiaca* (C) at the indicated times (lane 1, 0 h; lane 2, 0.5 h; lane 3, 1 h; lane 4, 6 h; and lane 5, 24 h), and these reaction mixtures were subjected to SDS-PAGE with the HMW marker kit (lane M) to observe changes in the molecular size of each PGA during the enzyme reaction.

D- and 50% L-glutamate. On the other hand, when the enzyme cleaves the bonds between the D- and D-units, high-molecular-mass and low-molecular-mass products probably consists mainly of L-glutamate and mainly of the D-enantiomer, respectively (Fig. 4C). The digested fragments from *B. subtilis* PGA or from *B. megaterium* PGA were each subjected to the SUPREC-02 system and the high-molecular-mass products (more than 200 kDa; see Fig. 3, lane 5) were collected (by removing low-molecular-mass products). As shown in Fig. 5, the L-glutamate ratios of the high-molecular-mass products gradually increased during the enzymatic digestion (also resulting in a gradual decrease in the amounts of high molecular mass PGAs). We further found that the low-molecular-mass products generated by the digestion of *B. subtilis* PGA (average 15 kDa; see Fig. 3, lane 5) consisted mainly of D-glutamate (Fig. 6). Moreover, although the theoretical linkage numbers between the D- and L-units of *B. subtilis* PGA (the D/L

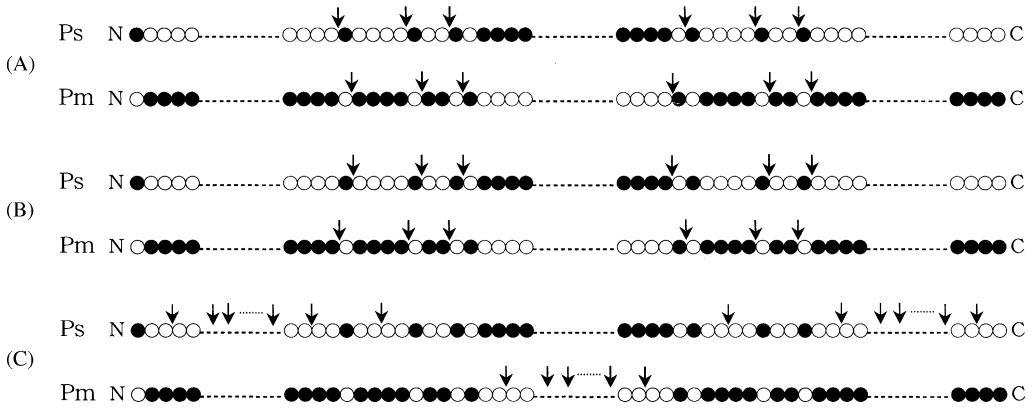

Fig. 4. Proposed mechanisms of the enzymatic PGA digestion. The D- and L-glutamate units in PGA are shown by the open and closed circles, respectively. *B. subtilis* and *B. megaterium* PGAs (4, 18) were abbreviated as Ps and Pm, respectively. N and C show the amino and carboxyl termini of PGA, respectively. Dots indicate the internal regions being lined with the identical units. (A) The cleavage between the D- and L-units in PGA. (B) The cleavage between the L- and D-units in PGA. (C) The cleavage between the D- and D-units in PGA. Probable recognition sites are indicated by arrows. Because the PGA depolymerase is an endopeptidase, the reaction products should be γ-glutamyl peptides with certain molecular masses but not glutamate monomers and dipeptides.

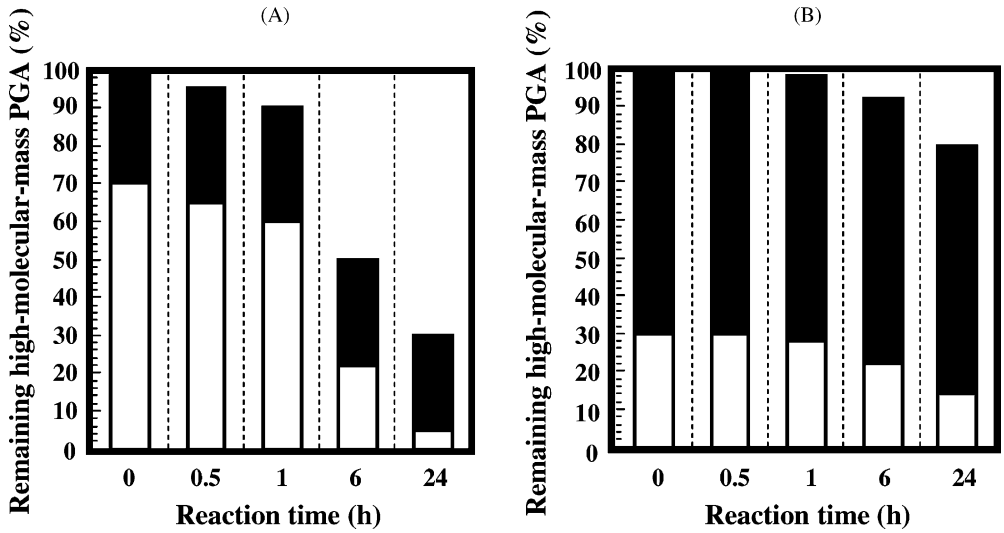

Fig. 5. The quantitative and stereochemical analysis of high-molecular-mass products obtained after the enzymatic PGA degradation. The reaction conditions were the same as those described in Fig. 3. The D- and L-glutamate contents of the reaction products are shown by white and black columns in bars, respectively. (A) Degradation of *B. subtilis* PGA by the purified depolymerase. (B) Degradation of *B. megaterium* PGA by the purified depolymerase.

ratio, 70/30) and of *B. megaterium* PGA (D/L, 30/70) should be similar to each other (Fig. 4A and B), *B. subtilis* PGA was obviously preferable than *B. megaterium* PGA as the substrate of the depolymerase, as shown in Figs. 3 and 5. Accordingly, our observations support the latter mechanism that the enzyme cleaves between the D- and D-glutamate units in PGA (Fig. 4C).

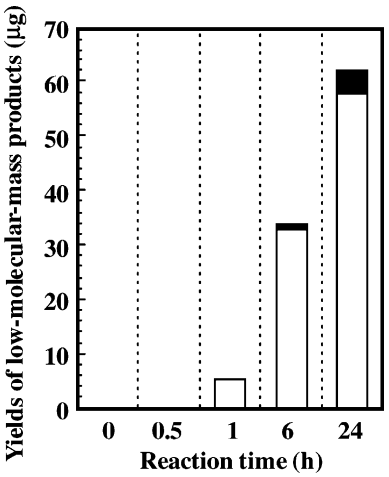

Fig. 6. The quantitative and stereochemical analysis of low-molecular-mass products obtained after the enzymatic degradation of *B. subtilis* PGA. The reaction conditions were the same as those described in Fig. 3. The D- and L-glutamate contents of the reaction products are shown by white and black columns in bars, respectively.

4. Discussion

Until now, both *exo*- and *endo*-types of PGA depolymerases have been identified and clarified to be extracellular enzymes in every case [1]. Abe et al. [8] showed that extracellular γ-glutamyl transpeptidases of *B. subtilis* correspond to the PGA *exo*-depolymerases. The *B. subtilis ggt* gene encodes the enzyme [19]. Similarly to the organization of the gene cluster involved in PGA metabolism of *B. subtilis*, the PGA depolymerase-encoding gene of *Bacillus anthracis*, *dep*, lies immediately downstream of the encapsulation genes at the same direction [20]. The *dep* gene, however, encodes a γ-glutamyl transpeptidase-like enzyme, which degrades PGA in an exopeptidase manner [20]. Tanaka et al. [18] purified the PGA *endo*-depolymerase cleaving between the L- and L-units of PGA from a filamentous fungus, *Myrothecium* sp. TM-4222, and it was then designated as PGA *endo*-L-depolymerase. King et al. [21]

characterized an extracellular enzyme of *B. licheniformis* as the PGA *endo*-depolymerase cleaving between the D- and D-units, and it was then termed as PGA *endo*-D-depolymerase [1]. Our study is the first example showing that the *B. subtilis pgdS* gene encodes PGA *endo*-D-depolymerase. These PGA depolymerases would serve for the detailed structural analyses of the polymer.

Structurally controlled PGAs are required for its further application [1]. PGAs with variable molecular sizes are indispensable for establishment of a drug-delivery system operating in tissues [1]. Tanimoto et al. [22] reported that PGA from *natto* mucilage, the molecular sizes of which would be various due to digestion by the depolymerases, increased Ca^{2+} solubility in vitro and in vivo and intestinal Ca^{2+} absorption. Such digested PGA may be useful as a therapeutic tool for osteoporosis. The chemical, physical, biological, and biochemical digestions of PGA [1,2,18,21,23] and the molecular size-controlled production of the polymer [13] have been attempted. It seems likely that the utilization of PGA *endo*-depolymerases is the most promising. Our results showed that both high-molecular-mass PGA composed mainly of L-glutamate (over 200 kDa) and low-molecular-mass PGA of the D-isomer (average 15 kDa) were produced by the enzymatic digestion. The PGA *endo*-D-depolymerase identified in this study, the PgdS enzyme, thus, contributes to processing of the useful biopolymer, PGA.

References

[1] M. Ashiuchi, H. Misono, in: S.R. Fahnestock, A. Steinbüchel (Eds.), Biopolymers, vol. 7, Wiley-VCH, Weinheim, 2002, Chapter 6, p. 123.
[2] G. Pérez-Camero, F. Congregado, J.J. Bou, S. Muñoz-Guerra, Biotechnol. Bioeng. 63 (1999) 110.
[3] S. Makino, I. Uchida, N. Terakado, C. Sasakawa, M. Yoshikawa, J. Bacteriol. 171 (1989) 722.
[4] M. Torii, J. Biochem. 46 (1959) 189.
[5] R. Aono, Biochem. J. 245 (1987) 467.
[6] F.F. Hezayen, B.H.A. Rehm, B.J. Tindall, A. Steinbüchel, Int. J. Syst. Evol. Microbiol. 51 (2001) 1133.
[7] C.B. Thorne, C.G. Gómez, H.E. Noyes, R.D. Housewright, J. Bacteriol. 68 (1954) 307.
[8] K. Abe, Y. Ito, T. Ohmachi, Y. Asada, Biosci. Biotechnol. Biochem. 61 (1997) 1621.
[9] M. Ashiuchi, H. Misono, Appl. Microbial. Biotechnol. 59 (2002) 9.
[10] T.J. Smith, S.A. Blackman, S.J. Foster, Microbiology 146 (2000) 249.
[11] R. Onishi, S. Ishikawa, J. Sekiguchi, J. Bacteriol. 181 (1999) 3178.
[12] I. Hirose, K. Sano, I. Shinoda, M. Kumano, K. Nakamura, K. Yamane, Microbiology 146 (2000) 65.
[13] M. Ashiuchi, T. Kamei, D.H. Baek, S.Y. Shin, M.H. Sung, K. Soda, T. Yagi, H. Misono, Appl. Microbiol. Biotechnol. 57 (2001) 764.
[14] M. Ashiuchi, K. Soda, H. Misono, Biochem. Biophys. Res. Commum. 263 (1999) 6.
[15] T. Miyaji, M. Ashiuchi, K. Packdibamrung, S. Nagata, H. Misono, J. Mol. Catal. B: Enzym. 12 (2001) 77.
[16] S. Sakamoto, M. Seki, S. Nagata, H. Misono, J. Mol. Catal. B: Enzym. 12 (2001) 85.
[17] M. Ashiuchi, K. Tani, K. Soda, H. Misono, J. Biochem. 123 (1998) 1156.
[18] T. Tanaka, K. Fujita, S. Takenishi, M. Taniguchi, J. Ferment. Bioeng. 84 (1997) 361.
[19] K. Xu, M.A. Strauch, J. Bacteriol. 178 (1996) 4319.
[20] I. Uchida, S. Makino, C. Sawamura, M. Yoshikawa, C. Sugimato, M. Terakado, Mol. Microbiol. 9 (1993) 487.
[21] E.C. King, A.J. Blacker, T.D.M. Bugg, Biomacromolecules 1 (2000) 75.
[22] H. Tanimoto, M. Mori, M. Motoki, K. Torii, M. Kadowaki, T. Noguchi, Biosci. Biotechnol. Biochem. 65 (2001) 516.
[23] H. Kubota, Y. Nanbu, T. Endo, J. Polym. Sci. Chem. 34 (1996) 1347.