

Note

Characterization of γ -Glutamyl Hydrolase Produced by *Bacillus* sp. Isolated from Thai Thua-nao

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γ -Glutamyl hydrolase with a molecular mass of 28 kDa was purified from the culture broth of *Bacillus* sp. isolated from Thai Thua-nao, a natto-like fermented soybean food. The purified enzyme hydrolyzed chemically synthesized oligo- γ -L-glutamates but not oligo- γ -D-glutamates and degraded γ -polyglutamic acid to a hydrolyzed product of only about 20 kDa (with D- and L-glutamic acid in a ratio of 70:30), suggesting that the enzyme is a γ -glutamyl hydrolase that cleaves the γ -glutamyl linkage between L- and L-glutamic acid of γ -polyglutamic acid.

Key words: γ -polyglutamic acid; oligo- γ -L-glutamates; γ -glutamyl hydrolase; natto; *Bacillus* sp.

γ -Polyglutamic acid (PGA), a polymer that consists of D- and L-glutamic acid polymerized through γ -glutamyl bonds, is produced by certain *Bacillus* strains as a capsular or an extracellular viscous material.^{1–3} PGA is a main constituent of the sticky material in natto, a Japanese fermented food made from soybeans. Natto-like fermented soybean foods have also been manufactured rurally in mountainous areas of South Asia and Southeast Asia.^{4–6} Thua-nao, a natto-like fermented soybean product, is produced in rural areas of northern Thailand, but the viscosity is apparently less than that of natto, probably due to the process of natural fermentation, in which steamed soybeans are wrapped in teak leaves and then fermented by natural organisms at ambient temperature, suggesting that some organisms in Thua-nao are able to produce PGA-degrading enzymes during the process of fermentation. In screening bacteria producing the PGA-degrading enzyme from Thua-nao, we isolated a bacterium, identified as *Bacillus* sp., that produces an extracellular γ -glutamyl hydrolase. In this paper, we describe the purification, properties, and PGA-

cleavage manner of the enzyme.

Bacillus sp. CMU29 was isolated and selected by the following method: Bacteria were randomly isolated from Thua-nao, the PGA content of which is half of that of Japanese commercial natto, and each vegetative bacterium was sprayed on steamed soybeans simultaneously with a natto-producing *B. subtilis* NBRC16449,⁷ in a ratio of 1:1. After incubation at 40 °C for 2 d, 30 strains were selected from fermented products that were visually low in viscosity. The selected strains were then cultured with shaking at 37 °C in LB medium, and then the activity of the PGA-degrading enzyme in the culture broth was assayed by the methods described below. Three strains were found to have the activity; two of the strains produced an exo-type PGA-degrading enzyme and one, designated CMU29, yielded a PGA-degrading enzyme in an endo-type manner. The PGA-degrading activity level in the culture broth of *B. subtilis* NBRC16449 was much lower than that of *Bacillus* sp. CMU29. Strain CMU29 is an aerobic, rod-shaped, endospore-forming, Gram-positive bacterium but does not produce PGA. From these and other characteristics of the strain, the organism was classified as a *Bacillus* species.

PGA-hydrolyzing activity was assayed by measuring free glutamic groups of the products.⁸ The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 50 μ g PGA prepared from *B. subtilis* NBRC16449 as described previously,⁹ and an enzyme source in a total volume of 1.0 ml, and the reaction was carried out at 37 °C. One unit of activity was defined as the amount of enzyme which 1 μ mole of free glutamic groups liberated from PGA per min. The hydrolysis of PGA was also visualized by SDS-PAGE analysis using 10% polyacrylamide gel.¹⁰

The bacterium was grown at 37 °C with shaking at

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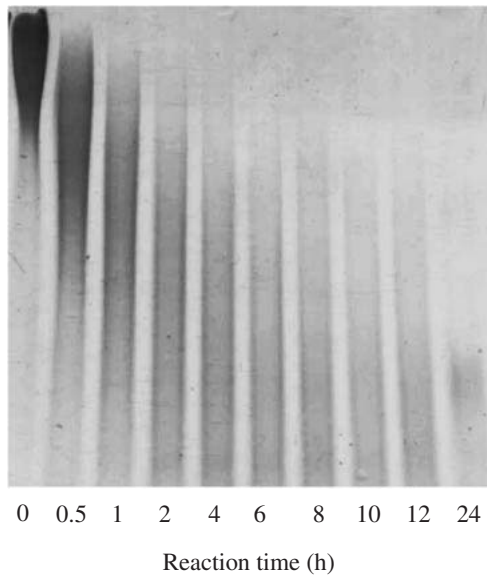


Fig. 1. SDS-PAGE Analysis of the Hydrolyzed Products of PGA by the Enzyme.

PGA was hydrolyzed under the conditions described in the text. SDS-PAGE was performed using a 10% polyacrylamide gel by the method of Yamaguchi *et al.*¹⁰⁾

150 rpm in 150 ml of LB medium dispensed into a 500-ml Sakaguchi flask. Cells were removed at the early stationary phase, and the enzyme was purified from the supernatant by ammonium sulfate precipitation, consecutive column chromatographies on DEAE-Sephacryl CL-6B and Hydroxylapatite, and Sephacryl S-200 gel filtration. The purified enzyme exhibited a single band on SDS-PAGE, and the molecular mass of the enzyme was estimated to be 28 kDa by comparison with marker proteins. The N-terminal amino acid sequence of the enzyme was found to be QTGGS. The specific activity of the purified enzyme was 3.5 units per mg protein, and 15 μ g of the purified enzyme was obtained from 150 ml of the culture broth. Its optimum pH and temperature were 8.0 and 37 °C. The enzyme was stable up to 40 °C for 1 h in 50 mM Tris-HCl buffer (pH 8.0). The addition of a divalent cation such as Ca^{2+} , Co^{2+} , Mg^{2+} , Mn^{2+} , or Ni^{2+} and EDTA at 5 mM had little effect on the enzymatic activity. Neither phenylmethylsulfonyl fluoride nor iodoacetic acid at 5 mM affected the activity, but 5 mM 4-(hydroxymercurio)benzoate, a sulfhydryl inhibitor, inhibited 40% of the activity.

Figure 1 shows the results of SDS-PAGE analysis of the hydrolyzed products from PGA by the enzyme. The products were degraded to low-molecular ones concomitantly with incubation time, indicating that the enzyme hydrolyzes PGA in an endo-type manner. Next, we examined for the cleavage site of the enzyme using chemically synthesized oligo PGAs, di- γ -L-Glu (L2), tri- γ -L-Glu (L3), tetra- γ -L-Glu (L4), di- γ -D-Glu (D2), tri- γ -D-Glu (D3), and tetra- γ -D-Glu (D4), which are products of Multiple Peptide Systems (San Diego, CA). The PGA-hydrolyzing enzyme was active on L2, L3, and L4,

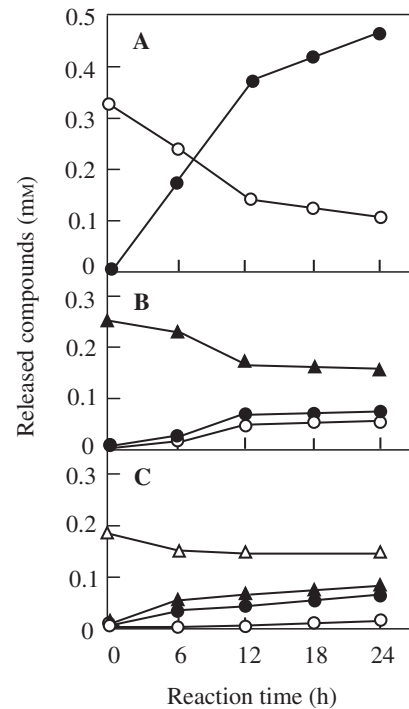


Fig. 2. Hydrolysis of Oligo- γ -L-glutamates by the Enzyme.

Fifty μ g of L2 (A), L3 (B), or L4 (C) was incubated with the purified enzyme (100 μ g protein) at 37 °C in 0.5 ml of 50 mM Tris-HCl buffer (pH 8.0) solution. The reaction was stopped by boiling, and aliquots (each 40 μ l) were analyzed by HPLC using a 320HQ column (250 \times 4 mm, Shodex, Tokyo, Japan). Samples were eluted with a linear gradient of 0.05–0.3 M NaCl in 0.1 M Tris-HCl buffer (pH 8.4) at a flow rate of 0.5 ml/min, and monitored by absorbance at 220 nm. Peaks were assigned by coelution with authentic L-glutamate, L2, L3, and L4, and the amounts were estimated by computerized integration of the peak areas. Symbols: Δ , L4; \blacktriangle , L3; \circ , L2; \bullet , L-glutamate.

but not on the D-isomer of oligo PGAs. The hydrolyzed products of L2, L3 and L4 were identified by reverse HPLC analysis (Fig. 2). The enzyme cleaved L3 into a dimer and a monomer, and cleaved L4 into a trimer and a monomer and a small amount of a dimer.

For analysis of the PGA-hydrolyzed product, PGA (2.5 mg) was completely hydrolyzed by the purified enzyme (100 μ g) at 37 °C for 24 h in 2.5 ml of 50 mM Tris-HCl buffer (pH 8.0) solution. The reaction was stopped by boiling, and an aliquot of the mixture was subjected to gel filtration HPLC. Only the hydrolyzed product was detected, and the molecular mass was estimated to be about 20 kDa in comparison with accurate standards of partially hydrolyzed PGAs (Fig. 3). The hydrolyzed product was separated by Sephacryl S-300HR gel filtration and subjected to acid hydrolysis to measure the glutamic acid optical isomer with an L-glutamic acid assay kit (Roche Molecular Biochemicals, Basel, Switzerland) as described previously.¹¹⁾ The product was composed of D-glutamic acid and L-glutamic acid in a ratio of 70:30, coincident with that of PGA, which was used as substrate.¹²⁾ Bovine γ -glutamyltransferase (Sigma Chemical Co., St. Louis,

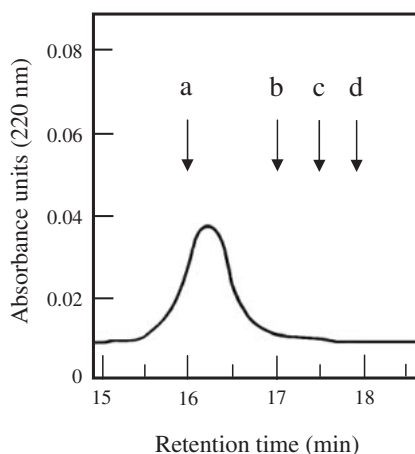


Fig. 3. HPLC Analysis of the Hydrolyzed Product.

The molecular mass of the hydrolyzed product was estimated by gel filtration HPLC using a SB804HQ column (250 × 4 mm, Shodex) with absorbance at 220 nm. Samples were eluted with 0.1 M Tris-HCl buffer (pH 8.4) at a flow rate of 0.5 ml/min at 40 °C. The void volume was 5.5 ml, corresponding to 11 min of retention time with pullulan (1,600 kDa). Peaks were assigned by coelution with low-molecular-mass PGAs, the molecular masses of which were determined to be 26.0 (a), 8.0 (b), 4.8 (c), and 3.6 (d).

MO)^{13,14} hydrolyzed the product, and carboxypeptidase G (γ -L-glutamyl hydrolase, Sigma)¹⁵ also degraded it, indicating that it contains L-glutamic acid in both the N- and C-termini. Furthermore, the 20-kDa product was hydrolyzed by YwtD,¹³ a γ -glutamyl hydrolase that cleaves the γ -glutamyl linkage between D- and D-glutamic acid recognizing adjacent L-glutamic acid toward the N-terminal region of PGA, into two products of about 20 kDa and about 2 kDa in molecular mass. This suggests that the 20-kDa PGA produced by γ -glutamyl hydrolase of *Bacillus* sp. CMU29 is not homogeneous. Considering our previous observation that *Bacillus* PGA consists of an L-glutamate-rich region and a D-glutamate-rich region,^{12,13} the 2-kDa fragment might be derived from the D-isomer-rich region. From these results and the fact that the enzyme hydrolyzes chemically synthesized oligo γ -L-PGAs, as mentioned above, it is conceivable that the enzyme is a unique one that cleaves γ -glutamyl bond between the L- and L-glutamic acids of PGA.

So far, γ -L-glutamyl hydrolase that hydrolyzes the γ -glutamyl linkage between L- and L-glutamic acids has been purified and characterized from a filamentous fungus, *Myrothecium* sp.,^{16,17} humans,¹⁸ rats,¹⁹ and *Arabidopsis thaliana*.²⁰ The latter three were purified from *Escherichia coli* as recombinant enzymes to hydrolyze the γ -glutamyl linkage of folyl-poly- γ -L-glutamates. The rat enzyme hydrolyzed the glutamate polymer between L- and L-glutamic acids, but not between D- and D-glutamic acid of the *Bacillus* PGA as in the case of γ -glutamyl hydrolase of *Bacillus* sp. CMU29.¹³ On the other hand, the *Myrothecium* enzyme purified from the culture broth has been proposed as a γ -

L-glutamyl hydrolase because this enzyme degraded the *Bacillus* PGA into two hydrolyzed products, a high-molecular-mass product (20–40 kDa with D- and L-glutamic acids in ratio of 76:24) and a 0.5-kDa product with only L-glutamic acid. γ -Glutamyl hydrolase, described here, hydrolyzed chemically synthesized oligo- γ -L-PGAs but did not completely cleave the γ -glutamyl linkage between the L- and L-glutamic acids of the *Bacillus* PGA, which is similar to that of the *Myrothecium* enzyme, suggesting that the enzyme acts differently on chemically synthesized oligo PGAs and *Bacillus* PGA.

Acknowledgments

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