Characterization of the *Bacillus subtilis* ywtD Gene, Whose Product Is Involved in γ-Polyglutamic Acid Degradation

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The *ywtD* gene, which codes for an enzyme that degrades γ-polyglutamic acid (PGA), was cloned from *Bacillus subtilis* IFO16449. The gene is located immediately downstream of *ywsC* and *ywtABC*, a PGA operon involved in PGA biosynthesis, and it showed partial similarity to genes coding for *dl*-endopeptidase, a peptidoglycan-degrading enzyme. The *ywtD* gene, from which signal sequence is excised, was inserted into pET15b, and the recombinant plasmid was then transformed into *Escherichia coli*. Histidine-tagged YwtD was purified from sonicated cells of the transformant. The purified YwtD degraded PGA to yield two hydrolyzed products, a high-molecular-mass product (490 kDa with nearly 100% γ-glutamic acid) and an 11-kDa product (with γ-glutamic acid and γ-glutamic acid in an 80:20 ratio). This finding and results of enzymatic analysis of the two products with carboxypeptidase G suggest that YwtD is a novel enzyme cleaving the γ-glutamyl bond only between *d*- and *l*-glutamic acids of PGA, and it may be designated γ-*dl*-glutamyl hydrolyase.

γ-Polyglutamic acid (PGA), an amino acid polymer that consists of only *d*-glutamic acid or *d-* and *l*-glutamic acids polymerized through γ-glutamyl bonds, is produced by several strains of *Bacillus* (4, 11, 19, 21). The genes required for PGA biosynthesis have also been cloned as *ywsC* and *ywtABC* (*capBCA* or *pgsBCA*) from *Bacillus anthracis* (14) and *Bacillus subtilis* (2, 26, 27), and *ywsC* was found to code for PGA synthetase (EC 6.3.2), which is a crucial enzyme in PGA production, catalyzing the biosynthesis of PGA from *l*-glutamate in the presence of ATP and Mn2+ ions (26). Sequencing of the complete genome of *B. subtilis* 168 (12) revealed that there is a gene (*ywtD*) located just downstream of *ywsC* and *ywtABC* that encodes a protein of 413 amino acids with a molecular mass of 45 kDa, and YwtD has been identified as an extracellular protein with a signal peptide of 32 amino acids, though its function remains unknown (6). Furthermore, a homology search revealed that a partial amino acid sequence of YwtD consists of only *D*-glutamic acid or *D-* and *L*-glutamic acids and it may be designated γ-*dl*-glutamyl hydrolase.

**Construction of a plasmid containing *ywtD* with a histidine-tag codon.** Plasmid pYWTD for expression of *ywtD* from which a signal sequence (6) was excised was constructed as follows. A sense primer, 5'-CTCAGGATATCATCAGAATTG-3' (*XhoI* site underlined), and an antisense primer, 5'-CTCGAGTTATTGGACCCGATATCCTC-3' (*XhoI* site underlined), were designed on the basis of the sequence of the *ywtD* gene of *B. subtilis* 168 (11). A DNA fragment was amplified by PCR with the two primers and *B. subtilis* IFO16449 (26) chromosomal DNA as a template, and the amplified 1.2-kb fragment was inserted into the *HincII* site of pUC19, generating pUC-ywtD. After the sequence had been checked for amplification errors, it was inserted into the *XhoI* site of pET15b. The resulting plasmid, named pYWTD, was transformed into *Escherichia coli* BL21(DE3) by the method of Inoue et al. (8).

**Production and purification of YwtD in *E. coli*.** *E. coli* BL21(DE3) harboring pYWTD was grown at 37°C with shaking in Luria-Bertani medium containing 50 μg of ampicillin per ml. When cell growth had reached an optical density at 600 nm of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, followed by cultivation for 5 h. Cells were harvested from 1,000 ml of culture by centrifugation, resuspended in 50 ml of 10 mM phosphate buffer (pH 7.0), and then disrupted by sonic oscillation for 20 min at 180 W. The sonicated cell suspension was centrifuged at 28,000 × g for 30 min, and the supernatant was applied to a nickel-nitrilotriacetic acid-agarose column (15 ml; Qiagen) equilibrated with 10 mM imidazole buffer (10 mM imidazole, 20 mM Tris-HCl, 0.3 M NaCl, 20% glycerol [pH 8.0]). The column was washed with 10 mM imidazole buffer, and then histidine-tagged protein was eluted with imidazole buffer (20 mM Tris-HCl, 0.3 M NaCl, 20% glycerol [pH 8.0]) containing a stepwise gradient of imidazole from 100 to 300 mM. The purified YwtD protein exhibited a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2), indicating that the molecular mass of 44 kDa is in agreement with that calculated from the deduced amino acid sequence of the *ywtD* gene from which signal sequence was omitted (6, 12).

**Characterization of YwtD.** Purified YwtD (30 μg) and PGA (600 μg), prepared from *B. subtilis* IFO16449 as described previously (9), were mixed in 300 μl of 50 mM citrate buffer (pH 5.0) and then incubated at 37°C. Aliquots (30 μl each) were removed at various intervals for monitoring of the hydrolysis products by high-pressure liquid chromatography (HPLC) using a gel filtration column with authentic *α*-PGA (Sigma...
Chemical Co.) as standards for molecular mass. The enzyme activity was also assayed by measuring free amino acid groups of the products (16). As shown in Fig. 3, PGA was degraded by YwtD to generate two separately hydrolyzed products: a high-molecular-mass product of about 490 kDa and a low-molecular-mass product of about 11 kDa. The latter product was gradually depolymerized to a lower-molecular-mass one by the enzyme reaction. Neither free glutamic acid nor γ-glutamyl dipeptide was detected through the reaction, indicating that YwtD cleaves the γ-glutamyl bond of PGA in an endo-type manner. The PGA hydrolase acts neither on α-L- and α-D-PGAs (Sigma) nor on cell wall peptidoglycan prepared from B. subtilis 168 by a previously described method (5).

The optimum pH for enzymatic activity was 5.0 in 50 mM citrate buffer, and the optimum temperature for the activity was 45°C. The enzyme was stable between pH 4 and 11 at 4°C for 16 h and retained 50% of its activity at 45°C for 1 h in 50 mM citrate buffer (pH 5.0). The addition of a divalent cation such as Ba²⁺, Ca²⁺, Mg²⁺, Mn²⁺, or Ni²⁺ at 5 mM had little effect on the enzymatic activity. Neither 5 mM EDTA nor 1 mM phenylmethylsulfonyl fluoride affected the activity, but pretreatment with 1 mM 4-(hydroxymercuri)benzoate, a sulfhydryl inhibitor, remarkably inhibited the enzymatic activity, indicating that YwtD may be a cysteine enzyme similar to DL-endopeptidase II (15, 22).

Analysis of hydrolyzed products. PGA (20 mg) was completely hydrolyzed by purified YwtD (2.5 mg protein) at 37°C for 48 h in 5 ml of 50 mM citrate buffer (pH 5.0) solution. The reaction was stopped by boiling, and the mixture was subjected to ultrafiltration (Ultracent-30; Tosoh, Tokyo, Japan) for separation of a high-molecular-mass product (F-1) and a low-molecular-mass product (F-2). Each product was further purified by Sephacryl S-300HR gel filtration, and separation of both products was checked by HPLC analysis as described above. PGA, F-1, and F-2 were subjected to acid hydrolysis for measuring total glutamic acid content with an amino acid analyzer and glutamic acid optical isomer with an L-glutamic acid assay kit (Roche) as described previously (3). As shown in Table 1, PGA used as a substrate consisted of D- and L-glutamic acids in a ratio of 70:30, the molecular mass of which was estimated to be about 1,500 kDa. F-1, whose molecular mass was about 490 kDa, contained nearly 100% L-glutamic acid, whereas F-2, whose molecular mass was about 11 kDa, was composed of both D-glutamic acid and L-glutamic acid in a ratio of 80:20. The fact that F-1 consists almost only of L-glutamic acid and the fact that F-2 is rich in the D-isomer of glutamic acids suggest that the PGA hydrolase cuts neither...
between L- and D-glutamic acids nor between D- and L-glutamic acids of the γ-glutamyl bond of PGA. In order to elucidate which glutamic acid isomer exists at the carboxyl termini of the polymers, PGA, F-1, and F-2 (0.3 mg of each) were incubated with 0.3 U of carboxypeptidase G (Sigma) at 30°C for 12 h in 0.3 ml of 10 mM phosphate buffer (pH 7.2) solution. Free L-glutamic acid was released from PGA and F-1 but not from F-2, indicating that the carboxyl terminus of F-2 consists of D-glutamic acid (Table 1). It is conceivable that L-glutamic acid of F-2 may exist as a cluster at the amino terminus on account of the action of the enzyme. These results strongly suggest that the PGA hydrolyase exclusively cleaves the γ-glutamyl bond between D- and L-glutamic acids, and the enzyme can be designated γ-DL-glutamyl hydrolase (EC class 3.4.19) according to the recommended nomenclature (17).

Tanaka et al. purified an endo-type PGA-degrading enzyme that cleaves only the γ-glutamyl bond between L- and D-glutamic acids from a filamentous fungus (24), and they demonstrated by analysis of the enzymatic products that B. subtilis PGA is composed of an optically heterogeneous peptide in which each cluster of D- and L-glutamic acids is copolymerized into a single chain (23). Their finding is consistent with our results showing that the major product, F-2, is made up of both D- and L-glutamic acids in a ratio of 80:20, along with the occurrence of F-1, a high-molecular-mass product that consists almost entirely of L-glutamic acid (Table 1).

Other PGA-degrading enzymes have been isolated from bacteria (1, 10, 25, 28) and phage-infected cells of bacilli (7, 29), including an endo-type enzyme purified from phage-infected cells and B. licheniformis (10), but the enzyme actions on PGA were not analyzed in detail. γ-Glutamyltranspeptidase (GGT) from B. subtilis was reported to possess an exo-type PGA-hydrolyzing activity (1, 18). Dep of lytF encodes a γ-D-glutamyltranspeptidase gene, LytF, which is essential for encapsulation in Bacillus subtilis: gene cloning and biochemical analysis of γ-D-glutamyltranspeptidase gene, LytF, which is essential for encapsulation in Bacillus subtilis. Nature 263:147–142.

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