

Biochemical and Molecular Characterization of the Polyhydroxybutyrate Depolymerase of *Comamonas acidovorans* YM1609, Isolated from Freshwater

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Comamonas acidovorans YM1609 secreted a polyhydroxybutyrate (PHB) depolymerase into the culture supernatant when it was cultivated on poly(3-hydroxybutyrate) [P(3HB)] or poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] as the sole carbon source. The PHB depolymerase was purified from culture supernatant of *C. acidovorans* by two chromatographic methods, and its molecular mass was determined as 45,000 Da by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme was stable at temperatures below 37°C and at pH values of 6 to 10, and its activity was inhibited by diisopropyl fluorophosphate. The liquid chromatography analysis of water-soluble products revealed that the primary product of enzymatic hydrolysis of P(3HB) was a dimer of 3-hydroxybutyric acid. Kinetics of enzymatic hydrolysis of P(3HB) film were studied. In addition, a gene encoding the PHB depolymerase was cloned from the *C. acidovorans* genomic library. The nucleotide sequence of this gene was found to encode a protein of 494 amino acids (M_r , 51,018 Da). Furthermore, by analysis of the N-terminal amino acid sequence of the purified enzyme, the molecular mass of the mature enzyme was calculated to be 48,628 Da. Analysis of the deduced amino acid sequence suggested a domain structure of the protein containing a catalytic domain, fibronectin type III module as linker, and a putative substrate-binding domain. Electron microscopic visualization of the mixture of P(3HB) single crystals and a fusion protein of putative substrate-binding domain with glutathione *S*-transferase demonstrated that the fusion protein adsorbed strongly and homogeneously to the surfaces of P(3HB) single crystals.

Poly(3-hydroxybutyrate) [P(3HB)] and its copolymers are accumulated as an intracellular storage compound within the cells of a wide variety of bacteria (2, 12, 43). These bacterial polyesters have attracted industrial attention as environmentally degradable thermoplastic used for a wide range of agricultural, marine, and medical applications (18). Thus, P(3HB) and its copolymers are a biodegradable material that serves as an exogenous carbon source for many microorganisms in the environment (24). The microorganisms secrete extracellular polyhydroxybutyrate (PHB) depolymerases to degrade environmental P(3HB) and utilize the decomposed compounds as nutrients (11). A number of P(3HB)-degrading microorganisms have been isolated from various environments such as soil (10, 11, 22, 25, 31, 33, 34), laboratory atmosphere (48), seawater (27, 35), lake water (37), activated sludge (44), and anaerobic sludge (19). Many extracellular PHB depolymerases have been purified from different microorganisms and characterized (9, 10, 22, 25, 27, 31, 35, 37, 38, 44, 46, 48). Analysis of structural genes of several PHB depolymerases has shown that the enzymes are comprised of an N-terminal catalytic domain-containing lipase box as an active site, a C-terminal putative substrate-binding domain (SBD), and a linker domain con-

necting the two domains (8, 20, 21, 23, 24, 28, 29, 40, 42). The presence of both catalytic and SBDs was originally observed in depolymerizing enzymes such as cellulase, xylanase, and chitinase, which hydrolyze water-insoluble polysaccharides (6, 15–17). It is well known that the SBDs in cellulases play a role in the promotion of the adsorption of enzyme onto the surface of insoluble polysaccharide (17). On the other hand, the C-terminal domain of PHB depolymerases from *Alcaligenes faecalis* and *Pseudomonas lemoignei* has been suggested to be capable of binding the enzyme onto the surface of water-insoluble P(3HB) (4, 14). Recently, Jendrossek and coworkers (24, 28) have demonstrated that extracellular PHB depolymerases can be classified into three types, based on the location of the active site (lipase box) in the catalytic domain and on the sequence structure of the linker domain.

In a previous study (37), we isolated 10 different strains of gram-negative bacteria capable of degrading P(3HB) from the freshwater of Lake Biwa. The strain YM1609, identified as *Comamonas acidovorans*, grew well on P(3HB) and secreted an extracellular PHB depolymerase. In this study, we purified the extracellular PHB depolymerase from the culture supernatant of *C. acidovorans* and determined its biochemical and kinetic properties. In addition, the corresponding gene coding for the enzyme was cloned and sequenced. To elucidate the function of the putative SBD of the depolymerase, a fusion protein with glutathione *S*-transferase (GST) was formed in *Escherichia coli* and reacted with P(3HB) single crystals.

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TABLE 1. Bacterial strains and vectors used

Strain, vector, and plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i>		
DH5	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA</i>	Nippongene, Toyama, Japan
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)F'[traD36 proAB⁺ lacI^q lacZ ΔM15</i>	49
DH5α	<i>supE44 ΔlacU169(φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Toyobo
<i>C. acidovorans</i> YM1609	Degradation of extracellular P(3HB)	37
<i>R. eutropha</i> ATCC 17699	Source of P(3HB)	13
Cosmid pWE15	Amp ^r , T3 and T7 promoters	Stratagene
Plasmids		
pUC18	Amp ^r , <i>lacZ</i>	49
pUC118	Amp ^r , <i>lacZ</i>	47
pGEX 4T-1	Amp ^r , <i>tac</i> promoter, GST fusion vector	Pharmacia
pGEX-SBD	The binding domain gene (<i>sbd</i>) in pGEX 4T-1	This study
pCA1	2.0-kbp <i>Bam</i> HI- <i>Sph</i> I fragment in pUC118	This study
pCA2	1.6-kbp <i>Pst</i> I- <i>Sph</i> I fragment in pUC18	This study
pCA3	0.9-kbp <i>Pvu</i> II- <i>Sph</i> I fragment in pUC18	This study

MATERIALS AND METHODS

Chemicals. P(3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [(P(3HB-co-3HV)] were produced by *Ralstonia eutropha* (13). Films of P(3HB) were prepared by solvent-casting techniques from chloroform solution of P(3HB) (1). The X-ray crystallinity of P(3HB) film was 60% ± 5% (mean ± standard deviation). Other chemicals were purchased from Kanto Chemicals (Tokyo, Japan) or Wako Chemicals (Osaka, Japan).

Bacterial strains and vectors. The bacterial strains of *C. acidovorans*, *R. eutropha*, and *E. coli* as well as the cosmid and plasmids used in this study are listed in Table 1.

Media and culture conditions. *C. acidovorans* was grown at 30°C in a mineral salt medium containing 0.46% KH₂PO₄, 1.16% Na₂HPO₄ · 12H₂O, 0.15% P(3HB) granules, 0.05% MgSO₄ · 7H₂O, 0.01% FeCl₃ · 6H₂O, 0.005% CaCl₂ · 2H₂O, and 0.1% NH₄Cl. The mineral agar plates were prepared by the addition of 1.5% agar to the above liquid culture. *E. coli* cells harboring cosmid or plasmids were grown in Luria-Bertani broth or M9 medium containing 0.01% ampicillin at 37°C. Solid media were prepared by the addition of 1.2% agar. Indicator solid media for the identification of *E. coli* clones expressing PHB depolymerase genes were prepared by pouring an overlay of M9 medium containing 0.01% ampicillin and 0.15% P(3HB) granules onto a bottom layer of the same agar without polymer.

Enzyme assay. The activity of PHB depolymerase was determined as follows. The standard assay mixture contained 400 mg of P(3HB) granules per liter and 1 mM CaCl₂ in 50 mM Tris-HCl buffer (pH 7.5). A stable suspension of purified P(3HB) granules was prepared with a sonic oscillator (20 kHz, 250 W) for 10 min. The reaction was started by the addition of enzyme, followed by a decrease in the turbidity of the P(3HB) granules, which was measured at 650 nm and 37°C with 1-cm-diameter light-path cuvettes. One unit of the enzyme was defined as the amount of protein required to decrease the value of *A*₆₅₀ by 1 per min.

Effect of inhibitors. Purified PHB depolymerase (5 μg) was preincubated with five different inhibitors for 10 min at 30°C in a final volume of 100 μl. Inhibitors except for phenylmethylsulfonyl fluoride (PMSF) were dissolved in deionized water, and the solutions were added into enzyme solution to yield a given concentration. PMSF was dissolved in ethanol, and its solution was added to the enzyme solution. The remaining enzyme activity was determined by the standard assay procedure described above.

Purification of enzyme. *C. acidovorans* was cultivated for 48 h at 30°C in 3,000 ml of mineral media containing 0.2% P(3HB) granules under aerobic conditions on a reciprocal shaker (120 strokes/min) in 15 500-ml Sakaguchi flasks. The liquid culture of *C. acidovorans* was centrifuged in a Hitachi R10A rotor at 10,000 × g for 30 min, and the supernatant (3,000 ml) was used for enzyme preparation. All enzyme purification procedures were carried out at 4°C. The supernatant was applied to a Butyl-Toyopearl column (3 by 30 cm; Tosho, Tokyo, Japan) equilibrated with 10 mM phosphate buffer (pH 7.0). The column was washed with 2 bed volumes of the buffer, and the enzyme was eluted with a linear gradient (800 ml) of 0 to 50% ethanol in the same buffer. The fractions with high activity were pooled and dialyzed against 20 mM sodium phosphate buffer (pH

7.0). These fractions were concentrated with Sumikagel (Sumitomo Chemical Industrial, Osaka, Japan) to 1 ml and dialyzed against Tris-HCl buffer (pH 7.5) for 24 h. Concentrated solution was applied to Sephadex G-200 (1 by 60 cm; Pharmacia, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl. Eluted enzyme from the Sephadex G-200 column was dialyzed against 20 mM Tris-HCl buffer (pH 7.5) and stored at 4°C.

Enzymatic hydrolysis of P(3HB) film. Enzymatic hydrolysis of P(3HB) films by the purified PHB depolymerase from *C. acidovorans* was carried out at 37°C in 0.1 M potassium phosphate buffer (pH 7.4). The enzyme solution (10 μl) of different concentrations was added to the reaction cuvette containing 1 ml of 0.1 M potassium phosphate buffer. The cuvette was maintained at 37°C. The reaction was started by the addition of a P(3HB) film (film dimensions, 10 by 10 by 0.1 mm) into 1 ml of enzyme solution. The number of 3HB units liberated from P(3HB) film during the enzymatic hydrolysis was determined by the UV method as described in previous studies (26, 36). The water-soluble products after enzymatic degradation were analyzed with a Shimadzu LC-9A high-pressure liquid chromatography (HPLC) system with a gradient controller and an SPD-10A UV spectrophotometric detector (1). The stainless steel column (250 by 4 mm) containing LiChrospher RP-8 (5 mm) was used at 40°C. Sample solutions after the enzymatic degradation were acidified to pH 2.5 with HCl solution, and 50-μl samples were injected into the column. The gradient of distilled water (pH 2.5, adjusted by the addition of HCl solution) to acetonitrile for 40 min was carried out with a pump speed of 1.0 ml/min. In this method, the monomer, dimer, trimer, and tetramer of 3-hydroxybutyric acid were detected at 210 min and eluted at 6.1, 13.2, 17.2, and 19.5 min, respectively (1). In the reaction solution, both the monomer and dimer were detected as the water-soluble products, while trimer and tetramer were not detected.

Analytical procedures of enzyme. Polyacrylamide gel electrophoresis of enzyme in the presence of sodium dodecyl sulfate (SDS) was carried out by the method of Laemmli (30) with a molecular weight calibration kit (Pharmacia). After electrophoresis, proteins were silver stained. Protein concentrations were determined by the method of Bradford (7) with the protein assay kit II (Japan Bio-Rad Laboratories, Tokyo, Japan) with bovine serum albumin as the standard. For N-terminal sequencing, the purified enzyme was blotted onto Prospin (polyvinylidene difluoride membrane module; Perkin-Elmer Corp., Norwalk, Conn.) by centrifugation (10,000 × g for 10 min). A membrane strip was cut off and applied for sequencing. ABI 473 A protein sequencer (Perkin-Elmer) was used.

DNA preparation and manipulation. *E. coli* was grown aerobically in a Luria-Bertani medium, and cells were transformed by the calcium chloride procedure (3). Recombinant plasmid DNA was isolated by the method of Birnboim and Doly (5) or with a Flexi-prep kit (Pharmacia). For sequencing, this DNA was further purified by polyethylene glycol precipitation (3). Restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan) or Toyobo (Osaka, Japan), and calf intestinal alkaline phosphatase was purchased from Boehringer GmbH (Mannheim, Germany). The enzymes were used according to the manufacturers' instructions.

Construction of *C. acidovorans* genomic DNA library. Genomic DNA of *C. acidovorans* was isolated as described previously (3) and partially digested with restriction endonuclease *Sau3A*I. The partially digested DNA fragments were ligated into *Bam*HI-digested pWE15-vector, and the recombinant molecules were packaged *in vitro* with a DNA packaging kit (Stratagene, La Jolla, Calif.).

DNA sequencing and sequence analysis. Nucleotide sequence was determined by the dideoxy-chain termination method (41) with the Takara Taq cycle sequencing kit version 2 for automated sequencing with a model DSQ 1000 sequencer (Shimadzu, Kyoto, Japan). A series of deletion fragments were generated and subcloned into pUC118 or pUC18. The DNA and deduced amino acid sequences were analyzed with the sequence analysis program GENETYX (Software Development Co., Tokyo, Japan). Database searches were performed with the BLAST program via GenomeNet World Wide Web server.

Construction of a GST-SBD fusion protein plasmid. A DNA fragment encoding the 66-amino-acid C-terminal region with a putative SBD of the *C. acidovorans* PHB depolymerase was obtained by PCR amplification of *C. acidovorans* genomic DNA. For amplification of the sequence encoding SBD, the 5' primers with a *Bam*HI site (5'-ATGTTGGATCCCTGGCCGCATCGGGCGGTGGC-3') and the 3' primers with a *Sal*I site (5'-ATCCAGTCGACCCCGCACTTTAAGGACAAGTGCC-3') were used (Fig. 5). PCR was carried out with the ExTaq kit (Takara Shuzo) under the conditions recommended by the manufacturer. The reaction mixture contained 100 ng of template DNA and 40 pmol of each oligonucleotide primer, and 25 reaction cycles (1 min at 95°C for denaturation and 5 min at 68°C for annealing and extension) were used. The resulting product was digested with *Bam*HI and *Sal*I and then ligated into *Bam*HI-*Sal*I-digested pGEX 4T-1 vector (Pharmacia) to generate a translational fusion between *gst*, the gene for GST, and *sbd*, the gene for SBD. The correct insertion of the *sbd* gene was verified by DNA sequencing for both strands. The resulting plasmid, pGEX-SBD, expressed a GST-SBD fusion protein that includes the putative SBD of *C. acidovorans* PHB depolymerase in *E. coli* DH5 α .

Expression and purification of GST-SBD fusion protein. pGEX-SBD expressing a GST-SBD fusion protein was introduced into *E. coli* DH5 α by transformation. Overnight culture of *E. coli* DH5 α , harboring pGEX-SBD, was diluted 1:10 in 2 \times yeast extract-tryptone medium (3). After 3 h of cultivation at 25°C, the cultures were induced with 0.05 mM isopropyl-1-thio- β -D-galactopyranoside. After 4 h of further cultivation at 25°C, the cells were spun down and resuspended in phosphate-buffered saline. The cells were lysed by sonication, and Triton X-100 was added to a final concentration of 1%. Whole cells and cell debris were removed by centrifugation for 30 min in a Hitachi RPR 20-2 rotor at 12,000 \times g at 4°C. Purifications of the GST-SBD fusion protein from the supernatant were performed with Bulk GST purification module (Pharmacia) according to the manufacturer's instructions.

Preparation of P(3HB) single crystals. The single crystals of P(3HB) were grown from dilute solution according to a method derived from that of Marchessault et al. (32). One milligram of P(3HB) was dissolved into 0.5 ml of chloroform at 30°C, and then 3.5 ml of ethanol at 60°C was added. The mixture was then brought quickly to 73°C and maintained there for 10 min, after which slow cooling was applied until 60°C and maintained there for 6 h. A slow cooling was applied by cutting the heating element of a silicone oil bath. The crystals were collected by centrifugation and washed three times with ethanol at room temperature.

Immunogold labeling. Antiserum (anti-GST) and anti-goat immunoglobulin G (whole molecule) gold conjugate (10-nm nominal particle) were purchased from Pharmacia LKB and Sigma Chemical Company (St. Louis, Mo.), respectively. A 4- μ l aliquot of a solution of GST-SBD fusion protein in 20 mM Tris-HCl buffer (pH 7.5) was mixed with 1 ml of P(3HB) single crystal suspension (0.4 mg/ml) in Tris-HCl buffer. The mixture was incubated at room temperature for 15 min to allow the adsorption of GST-SBD into the P(3HB) single crystals and was then washed three times with buffer 1 (0.1 M maleic acid and 0.15 M NaCl, pH 7.5) supplemented with 1% skim milk and 0.5 M NaCl by centrifugation and resuspension. The washed P(3HB) single crystals were resuspended in 1.0 ml of buffer 1 supplemented with 1% skim milk and 0.5 M NaCl, mixed with 10 μ l of goat antiserum (anti-GST), and incubated at room temperature for 3 h. The P(3HB) single crystals were washed once with buffer 1 supplemented with 1% skim milk and 0.5 M NaCl, twice with buffer 1 supplemented with only 0.5 M NaCl, and once with buffer 1 alone to remove unbound antiserum. The crystals were then resuspended in 200 μ l of buffer 1 supplemented with 0.1% polyethylene glycol 20000 (PEG 20000). Ten microliters of gold conjugate was added and incubated at room temperature for 30 min. The gold-labeled preparation was washed once with buffer 1 supplemented with 0.05% PEG and once with buffer 1 supplemented with 0.02% PEG and finally was resuspended in 200 μ l of buffer 1 supplemented with 0.02% PEG, for deposition on carbon-coated electron microscope grids.

Electron microscopy. Drops of P(3HB) single crystal suspension, with and without gold labeling, were deposited on carbon-coated grids and then allowed to dry. These grids were observed with a JEM-2000FX II electron microscope operated at an acceleration voltage of 200 kV. Images were recorded on Kodak SO-163 films developed with Kodak D-19 developer.

Nucleotide sequence accession number. The DDBJ accession no. for the *C. acidovorans* PHB depolymerase gene is AB003186.

TABLE 2. PHB depolymerase activities in the culture supernatants of *C. acidovorans* grown on various carbon sources

Carbon source ^a	Growth ^d	Activity ^e (U/ml)
P(3HB) ^b	++	1.7
P(3HB-co-7mol%3HV) ^b	++	1.2
P(3HB-co-9mol%3HV) ^b	++	0.9
P(3HB-co-22mol%3HV) ^b	++	1.0
P(3HB-co-39mol%3HV) ^b	++	0.4
P(3HB-co-65mol%3HV) ^b	++	0.1
(R)-3-hydroxybutyrate ^c	+	0
(S)-3-hydroxybutyrate ^c	+	0
Malate ^c	+	0
Gluconate ^c	+	0
D-(-)-Fructose	+	0
Acetate ^c	+	0
4-Hydroxybutyrate ^c	-	0
Citrate ^c	-	0
Valerate ^c	-	0
Octanoate ^c	-	0
Propionate ^c	-	0
Nutrient broth	++	0
Nutrient broth containing 0.2% P(3HB)	++	0

^a *C. acidovorans* cells were grown in mineral medium containing 0.2% (wt/vol) carbon source at 30°C for 48 h.

^b P(3HB) and its copolyesters were added into the mineral medium as films.

^c This carbon source was added into the mineral medium as sodium salt.

^d ++, optical density at 650 nm (OD₆₅₀) of more than 1.0; +, OD₆₅₀ between 0.1 and 1.0; -, OD₆₅₀ of less than 0.1.

^e PHB depolymerase activities in the culture supernatant of cells were measured by the turbidimetric method with P(3HB) granules.

RESULTS

Secretion of PHB depolymerase from *C. acidovorans*. The effect of various carbon sources on the growth of *C. acidovorans* was investigated by measuring the turbidity at 650 nm of the culture media. As shown in Table 2, the growth level of the bacterium depended on which kind of carbon substrate was added into the culture media. *C. acidovorans* secreted PHB depolymerase upon incubation with carbon sources such as P(3HB) and P(3HB-co-3HV), with 3HV compositions varying from 7 to 65 mol%. When malate, gluconate, fructose, acetate, and 3-hydroxybutyrate were used as the sole carbon source, *C. acidovorans* grew well, but PHB depolymerase activity was not detected in the culture supernatants. Though *C. acidovorans* also allowed good growth in both the nutrient broth and nutrient broth containing 0.2% P(3HB), PHB depolymerase activity was not detected in the culture supernatants. In the copresence of P(3HB) and other carbon sources which the bacterium utilized with greater facility than P(3HB), no synthesis of PHB depolymerase was detected, suggesting a catabolite repression of the PHB depolymerase gene.

Purification and properties of PHB depolymerase. A PHB depolymerase was purified to electrophoretic homogeneity from the culture supernatant of *C. acidovorans* YM1609 by hydrophobic interaction chromatography (Butyl-Toyopearl), followed by gel filtration (Sephadex G-200). Details of the PHB depolymerase purification are given in Table 3.

The purified enzyme appeared on SDS-polyacrylamide gel electrophoresis as a single band with a relative molecular mass of 45 kDa. The optimum pH of this enzyme was 9.0. After incubation at 37°C for 5 h at different pH values, the enzymatic activity was stable for pH values from 6.0 to 10.0. The enzyme was stable for at least 30 min at temperatures below 37°C, while the stability of the enzyme decreased sharply at temperatures over 40°C. No influence on the activity of PHB depoly-

TABLE 3. Purification of a PHB depolymerase from *C. acidovorans*^a

Step	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Recovery (%)	Purification (times)
1. Supernatant	3,000	65.3	46	100	1.0
2. Butyl-Toyopearl	518	4.5	115	17	2.5
3. Sephadex G-200	252	0.6	420	8	9.1

^a Purification was carried out with 3.0 liters of culture medium.

merase in the presence of 10 mM EDTA was observed after 30 min of incubation at 37°C. On the other hand, the addition of 10 mM diisopropyl fluorophosphate, 10 mM PMSF, 10 mM dithiothreitol, and 0.1% Tween 20 reduced the activity of PHB depolymerase at levels of 10, 51, 17, and 1%, respectively.

The N-terminal amino acid sequence of purified enzyme was determined as Ala-Val-Asn-Leu-Pro-Ala-Leu-Lys-Ile-Asp-Lys-Thr-Gln-Thr-Thr-Val-Ser-Gly-Leu-Ser by Edman degradation.

The composition of water-soluble PHA-degradation product was measured by HPLC analysis during the enzymatic hydrolysis of P(3HB) film at 37°C in 0.1 M potassium phosphate buffer (pH 7.4) with enzyme concentrations of 0.2, 2.0, and 20 µg/ml. HPLC analysis showed that both the monomer and dimer of 3-hydroxybutyric acid were produced during the enzymatic hydrolysis of P(3HB) film, whereas the 3HB trimer and tetramer were not formed by the enzymatic hydrolysis. Figure 1 shows the time-dependent changes in relative amounts (HPLC peak areas) of 3HB generated as the monomer and dimer of 3-hydroxybutyric acid. The dimer was the major product at low enzyme concentrations of 0.2 and 2.0 µg/ml, while a high enzyme concentration of 20 µg/ml gave rise to the monomer as the major product. The total amount of monomer and dimer increased with reaction time. The rate of monomer and dimer formation was maximal at an enzyme concentration of 2.0 µg/ml.

Kinetic analysis of enzymatic hydrolysis. As reported previously (26, 36), the water-soluble products can be quantitatively detected by monitoring the absorption at 210 nm due to the carbonyl groups of 3HB monomer and dimer. Fig. 2A shows the time-dependent changes in the number of 3HB units liberated as water-soluble product during the course of enzymatic hydrolysis of P(3HB) film at 37°C in 0.1 M phosphate buffers (pH 7.4) containing different amounts of PHB depolymerase from *C. acidovorans*. An induction period of about 30 min was observed at the initial stage of enzymatic hydrolysis, and then the amount of water-soluble products increased proportionally with time. The rate of enzymatic hydrolysis was strongly dependent on the concentration of enzyme.

Figure 2B shows the effect of PHB depolymerase concentration on the liberation rate of 3HB units from P(3HB) film. A maximum rate of enzymatic hydrolysis was observed at an enzyme concentration of 2.0 µg/ml.

The same dependencies of enzyme concentration on the rate of enzymatic hydrolysis of P(3HB) film have been reported with other PHB depolymerases from *Comamonas testosteroni*, *Ralstonia pickettii*, *A. faecalis*, *P. lemoignei*, and *Pseudomonas stutzeri* (26, 36, 45, 46). The rate (R) of enzymatic hydrolysis of P(3HB) film could be expressed as follows:

$$R = ksK[E]/(1 + K[E])^2 \quad (1)$$

where ks is a rate constant for the hydrolysis of P(3HB) chain by an enzyme, K is an adsorption equilibrium constant for the

enzyme, and $[E]$ is the concentration of enzyme. Equation 1 converted to the linear form is as follows:

$$([E]/R)^{1/2} = (K/ks)^{1/2}[E] + 1/(ksK)^{1/2} \quad (2)$$

The values of rate constant ks and adsorption constant K at 37°C were determined to be $7.2 \pm 1 \mu\text{mol/h/cm}^2$ and $0.45 \pm 0.1 \text{ ml}/\mu\text{g}$, respectively, from the slope and intercept of the linear plot on the basis of the data in Fig. 2B. In a previous study (36), the values of ks and K at 37°C with an *A. faecalis* PHB depolymerase were determined to be $4.0 \pm 0.1 \mu\text{mol/h/cm}^2$ and $0.56 \pm 0.2 \text{ ml}/\mu\text{g}$, respectively. The values of adsorption equilibrium constant K for two PHB depolymerases were similar. By contrast, the value of hydrolysis rate constant ks for *C. ac-*

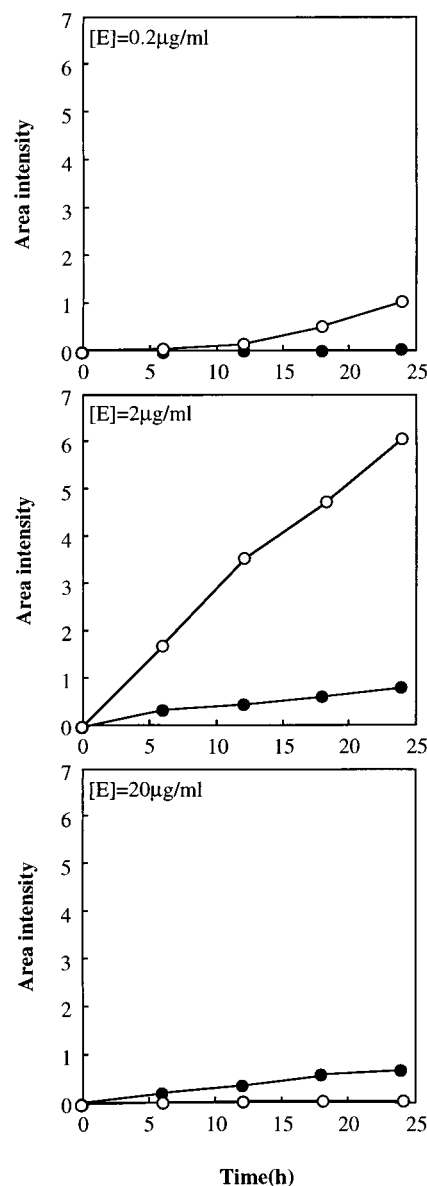


FIG. 1. HPLC analysis of water-soluble products. The relative HPLC peak areas of 3HB monomer and dimer were generated as water-soluble products during the enzymatic hydrolysis of P(3HB) film at 37°C (pH 7.4) with different concentrations of *C. acidovorans* PHB depolymerase. ●, 3HB monomer; ○, 3HB dimer.

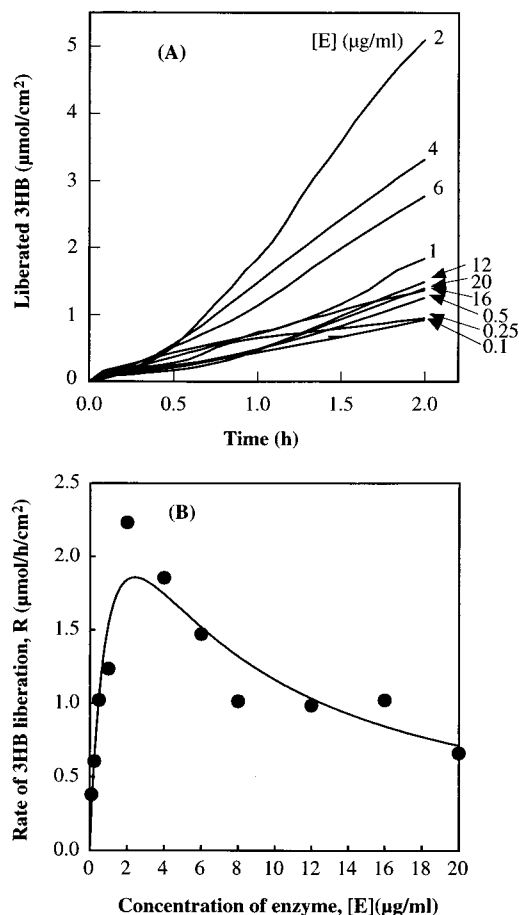


FIG. 2. Effect of PHB depolymerase concentration on the rate of 3HB unit liberation from P(3HB) film. (A) The amounts of 3HB units liberated as monomer and dimer during the enzymatic hydrolysis of P(3HB) film in aqueous solutions containing different amounts of *C. acidovorans* PHB depolymerase at 37°C (pH 7.4) are shown. (B) The rate of 3HB liberation according to the concentration of PHB depolymerase is shown.

idovorans PHB depolymerase was twice as large as the value for *A. faecalis* PHB depolymerase.

Cloning of PHB depolymerase gene of *C. acidovorans*. About 5,000 recombinant strains of *E. coli* DH5 harboring partially

Sau3AI-digested genomic DNA of *C. acidovorans* in cosmid pWE15 were screened for expression of PHB depolymerase activity. One clone produced a large translucent halo on the M9 medium containing P(3HB) granules, indicative of the functional expression of PHB depolymerase gene. Restriction analysis of the recombinant cosmid demonstrated that the subcloned 4.1-kbp *SphI*-*SphI* fragment in pUC118 had a halo-forming ability on the M9 medium containing P(3HB) granules. To determine the minimal size of the region encoding the PHB depolymerase structural gene, three deletion plasmids (pCA1, pCA2, and pCA3) were constructed and their halo-forming abilities were examined in *E. coli* JM109 (Fig. 3). As a result, a 2.0-kbp *Bam*HI-*SphI* region was found to confer halo-forming ability.

Analysis of the PHB depolymerase gene and the deduced protein. Three plasmids (pCA1, pCA2, and pCA3) and their derivatives produced by nested deletions of the three plasmids were sequenced by the strategy illustrated in Fig. 3. The nucleotide sequences obtained were aligned by their overlaps to form a single contig (Fig. 4). As a result, one open reading frame of 1,485 bp was assigned to the PHB depolymerase gene. The assumed coding sequence started with the initiation codon ATG at position 1, which was preceded by a potential ribosomal binding site at position -14 to 10, and extended to stop codon TAA at position 1483. The encoded polypeptide is a preprotein of 494 amino acids and has a deduced molecular mass of 51,018 Da. Amino acid residues 26 to 45 of the deduced gene product were identical to the N-terminal amino acid sequence determined for the mature PHB depolymerase secreted by *C. acidovorans*, suggesting that the 25-amino-acid polypeptide was a signal peptide. The molecular mass deduced for the mature PHB depolymerase of *C. acidovorans* was 48,628 Da, which was in agreement with that (45 kDa) determined by SDS-polyacrylamide gel electrophoresis for the purified PHB depolymerase from *C. acidovorans*.

The predicted amino acid sequence of PHB depolymerase from *C. acidovorans* was compared with those of other PHB depolymerases. In consequence, the primary structure of PHB depolymerase from *C. acidovorans* revealed the presence of three domains (catalytic, binding, and linker domains, as shown in Fig. 4) which were observed in other PHB depolymerases (24). The sequence with the highest level of homology to *C. acidovorans* PHB depolymerase was that of PHB depolymerase from *Comamonas* sp. (20) (level of identity, 56%),

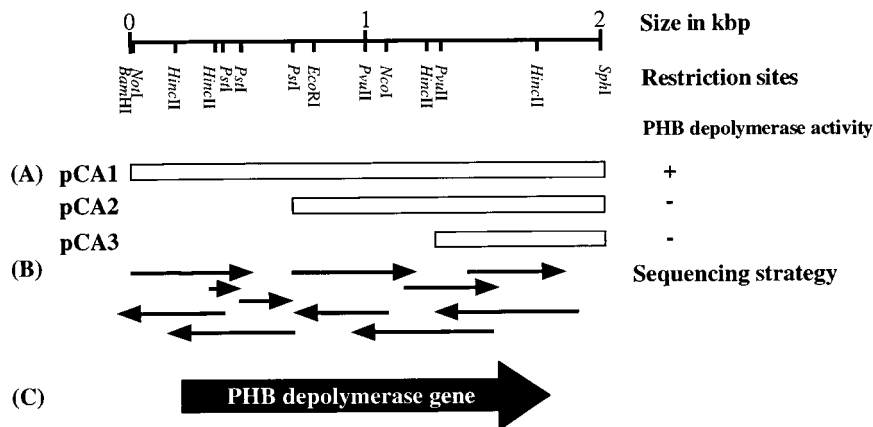


FIG. 3. Genetic structure of 2.0-kbp *Bam*HI-*SphI* fragment that shows PHB depolymerase activity in *E. coli* JM109. (A) Three subclones were prepared and tested for their ability to form clearing zone on indicator medium. (B) Sequencing strategy for determination of the nucleotide sequence. (C) Wide arrow shows the open reading frame of the PHB depolymerase gene.

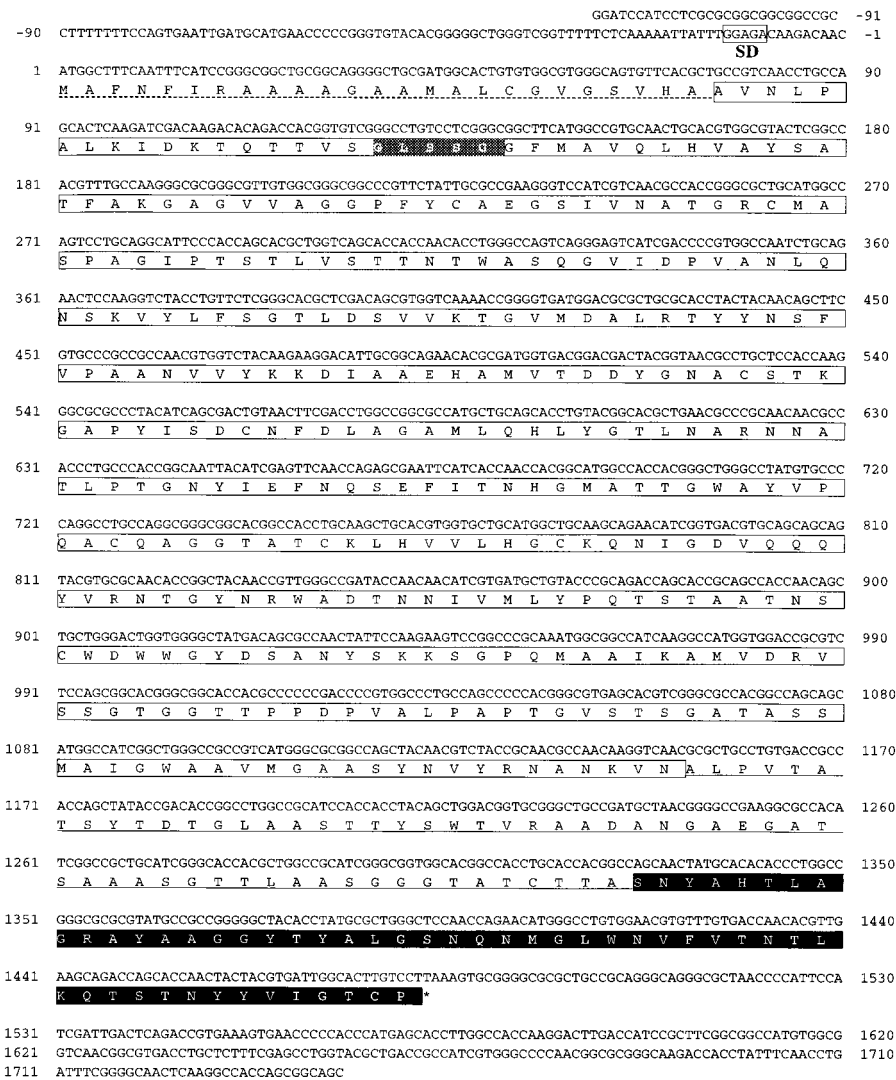


FIG. 4. Nucleotide sequence of a 2.0-kbp *Bam*HI-*Sph*I fragment from *C. acidovorans* containing the open reading frame coding for PHB depolymerase. The putative ribosome binding site is boxed. The deduced amino acid sequence of PHB depolymerase shows the signal peptide (broken line), a catalytic domain (boxed) containing the lipase consensus sequence (shaded), a linker domain (underlined), and a putative SBD (white letters on black background).

followed by the PHB depolymerases from *C. testosteronei* (42) (level of identity, 55%), and *Streptomyces exfoliatus* (28) (level of identity, 48%). The 359-amino-acid N-terminal catalytic domain contained a catalytic triad (Ser45, Asp132, and His166) and oxyanion hole (His258) similar to the sequential order of four active amino acid residues in PHB depolymerases from *Comamonas* sp., *C. testosteronei*, and *S. exfoliatus*, indicating that the PHB depolymerase from *C. acidovorans* is type II (24, 28). The C-terminal putative SBD is 52 amino acids long, and the amino acid sequence exhibits a high level of homology against those of other PHB depolymerases (level of identity, 60 to 73%) except for two PHB depolymerases from *P. lemoignei* (PhaZ1 and PhaZ4). Furthermore, there is a fibronectin type III like sequence as a linker domain between the catalytic and SBDs.

Functional analysis of putative SBD. In order to investigate the function of the putative SBD, we prepared a fusion protein of SBD with the GST and performed the adsorption experiment on P(3HB) single crystals. To clone the gene encoding the putative SBD, we designed two primers that flank the domain as shown in Fig. 5. Following the amplification and cloning into GST

fusion vector pGEX 4T-1, the resulting plasmid, pGEX-SBD, was transformed into *E. coli* DH5 α . The fusion protein, GST-SBD, was purified to electrophoretic homogeneity from the recombinant strain, as described in Materials and Methods. The purified GST-SBD fusion protein and the GST protein were reacted with P(3HB) single crystals and visualized by gold-conjugated antibody and transmission electron microscopy (TEM). Figure 6 shows the images of the adsorption of GST-SBD and GST proteins to a P(3HB) single crystal, respectively. The fusion protein GST-SBD adsorbed onto the surfaces of P(3HB) single crystals uniformly. By contrast, GST alone did not adsorb there. These results indicate that the putative SBD has the ability to adsorb onto P(3HB) single crystals.

DISCUSSION

In this study we report the synthesis, purification, biochemical and kinetic properties, primary structure, and function of a PHB depolymerase from *C. acidovorans* YM1609.

The results of a homology search of the deduced amino acid

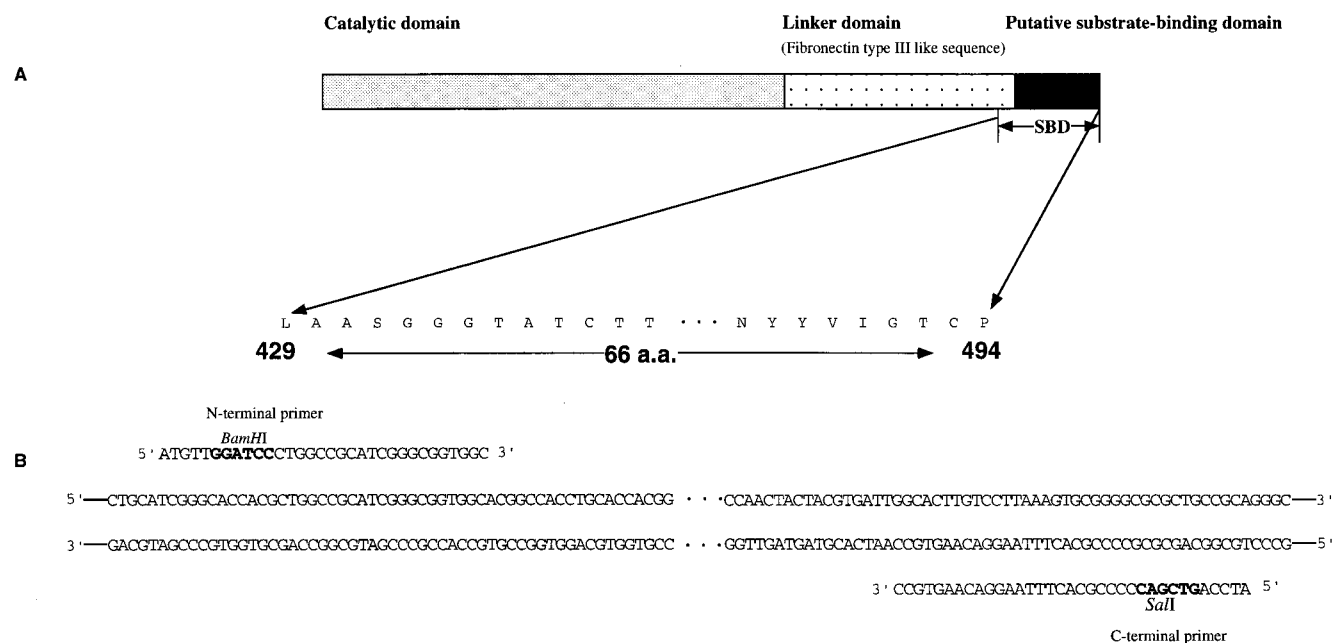


FIG. 5. Subcloning and expression of the putative SBD gene. (A) Schematic description of the domains of *C. acidovorans* PHB depolymerase. a.a., amino acids. (B) Primers used for PCR.

sequence of *C. acidovorans* PHB depolymerase against those of other PHB depolymerases suggest that the PHB depolymerase is composed of two discrete domains connected by a linker region. The N-terminal catalytic domain contained conserved amino acids of the catalytic triad Ser45, Asp132, and His166, which functions as an active center in many known serine hydrolases, and its amino acid sequence exhibited a high degree of similarity (47 to 53%) to the sequences in the catalytic domains of PHB depolymerases from *Comamonas* sp. (20), *C. testosteroni* (42), and *S. exfoliatus* (28), indicating that the PHB depolymerase from *C. acidovorans* is type II (24, 28). On the other hand, the C-terminal domain of *C. acidovorans* PHB depolymerase showed significant homologies (60 to 73%) to those of other PHB depolymerases (24, 28). The C-terminal region of *A. faecalis* PHB depolymerase was previously suggested to function as an SBD for water-insoluble P(3HB) substrate (14). In this study, to elucidate the function of C-terminal putative binding domain, the putative SBD of *C. acidovorans* PHB depolymerase was expressed in *E. coli* as a fusion protein with GST, and then adsorption of the resulting fusion protein to P(3HB) single crystals was visualized by immunogold labeling and TEM. The results of TEM (Fig. 6) demonstrate that the fusion protein adsorbed strongly and homogeneously to the surface of P(3HB) single crystals, whereas GST alone was incapable of binding there, indicating that the C-terminal domain has the capability of binding on the hydrophobic surfaces of P(3HB) crystals.

Recently, Nobes et al. (39) reported that *P. lemoignei* PHB depolymerase A worked like a wedge along the long axes of P(3HB) single crystals to produce splinters and then hydrolyzed preferentially from the edges of the crystals. This conclusion was reached on the basis of observations by TEM of enzymatically degraded P(3HB) crystals (39). On the other hand, our TEM results demonstrated a homogeneous distribution of the GST-SBD fusion protein on the surfaces of the crystals, suggesting that enzymatic hydrolysis takes place preferentially at the edges of P(3HB) single crystals, though a

depolymerase adsorbs uniformly on the surfaces of single crystals.

The hydrolysis activity of depolymerase was inhibited by the addition of diisopropyl fluorophosphate, which is known to bind to the hydroxy group of active serine residues selectively and irreversibly, suggesting that the PHB depolymerase from *C. acidovorans* belongs to a serine hydrolase. This result supports the presence of the active site (Ser45 in a lipase box) deduced by a homology search (20, 28). The presence of a reducing agent, dithiothreitol, also reduced the activity of depolymerase, suggesting that disulfide bonds are present in the enzyme. In addition, the enzyme activity was not inhibited by EDTA, which is a chelator for cations, suggesting that cations are not essential for the formation of active conformation. The *C. acidovorans* PHB depolymerase as well as those from *Comamonas* sp. and *C. testosteroni* belonging to type II (the lipase boxes are located adjacent to N termini) showed relatively low sensitivity toward PMSF, which is an inhibitor for serine hydrolase (22, 35), while the depolymerases from *A. faecalis* and *P. lemoignei* (PhaZ5 and PhaZ2) that are type I or III (the lipase boxes are located at the center of catalytic domain) were very sensitive to PMSF (40, 44).

The HPLC analysis of water-soluble products demonstrated that monomer and dimer of 3-hydroxybutyric acid were produced during the enzymatic hydrolysis of P(3HB) film, as shown in Fig. 1. At low enzyme concentrations of 0.2 and 2.0 $\mu\text{g/ml}$, dimer was a major product, while at a high enzyme concentration of 20 $\mu\text{g/ml}$, only monomer was observed in the solution. A similar dependency of enzyme concentration on the composition of water-soluble products was observed in other PHB depolymerases from *A. faecalis* and *C. testosteroni* (25, 26). These results indicate that the primary product arising from the surface hydrolysis of P(3HB) film by a PHB depolymerase is dimer of 3-hydroxybutyric acid and that the dimer is subsequently hydrolyzed into monomer by PHB depolymerase in the reaction solution.

In previous studies (25, 36), we proposed a kinetic model in

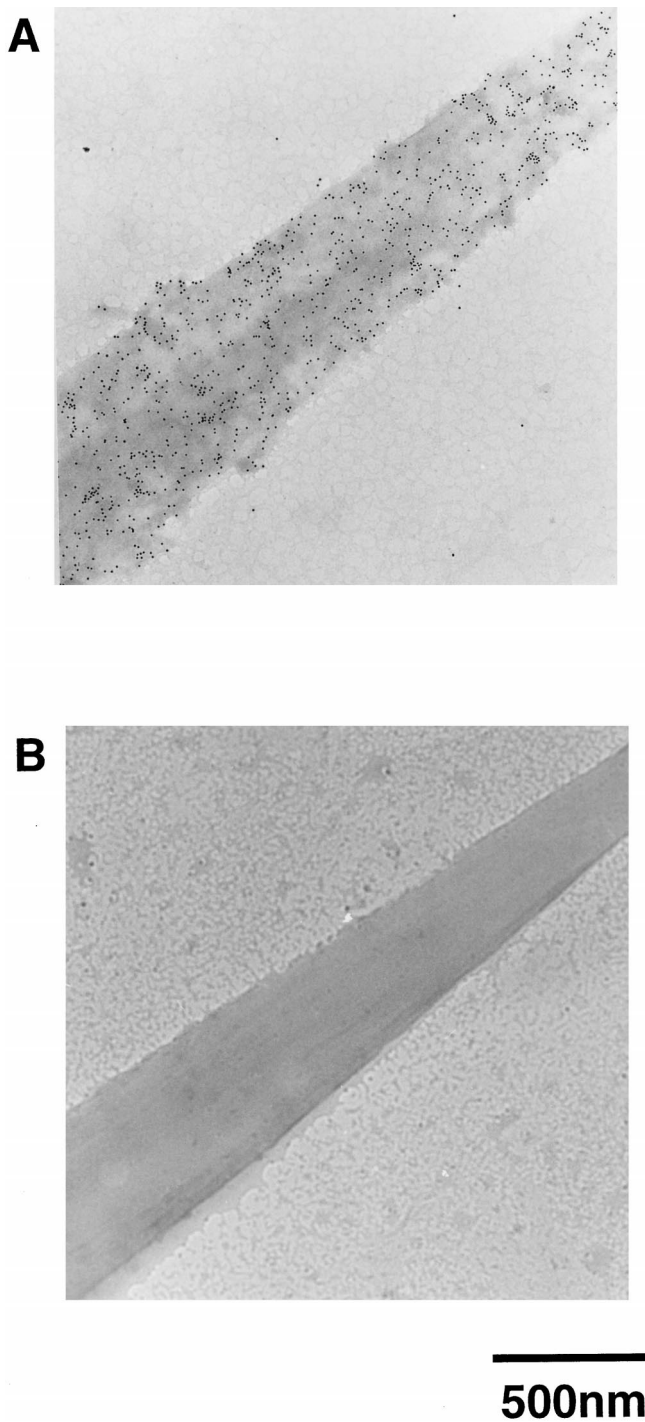


FIG. 6. Electron microscopic visualization of adsorption of (A) a fusion protein (GST-SBD) and (B) GST to P(3HB) single crystals.

which the surface hydrolysis of P(3HB) film by a PHB depolymerase takes place via two reaction steps of adsorption and hydrolysis. In this study we applied this kinetic model for the enzymatic degradation of P(3HB) film by *C. acidovorans* PHB depolymerase and determined the kinetic parameters with equation 1. The value of adsorption equilibrium constant K , corresponding to the affinity between an SBD and the surface of P(3HB) film, was almost consistent with the values deter-

mined for the PHB depolymerases from *C. acidovorans* and *A. faecalis*. In contrast, the value of hydrolysis rate constant k_s , implying the hydrolysis rate of a P(3HB) chain by catalytic domain, for *C. acidovorans* PHB depolymerase was approximately twice as large as the value for *A. faecalis* PHB depolymerase (type I). This result is in agreement with those of the sequential structure of enzymes showing that the primary structures of binding domains of both PHB depolymerases from *C. acidovorans* and *A. faecalis* are similar (level of identity, 60%), while the result for the amino acid sequence of the catalytic domain of *C. acidovorans* PHB depolymerase shows a low homology (level of identity, 16%) to that of *A. faecalis* PHB depolymerase.

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