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A New Type of Thermoalkalophilic Hydrolase of *Paucimonas lemoignei* with High Specificity for Amorphous Polyesters of Short Chain-length Hydroxyalkanoic Acids*

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René Handrick‡, Simone Reinhardt‡, Maria Letizia Focarete§, Mariastella Scandola§, Grazyna Adamus¶, Marek Kowalczuk¶, and Dieter Jendrossek‡

From the ‡Institut für Mikrobiologie, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany, the §Department of Chemistry "G. Ciamician" and Centro di Studio per la Fisica delle Macromolecole del Consiglio Nazionale delle Ricerche, University of Bologna, 40126 Bologna, Italy; and the ¶Polish Academy of Sciences, Centre of Polymer Chemistry, 41-800, Zabrze, Poland

A novel type of hydrolase was purified from culture fluid of *Paucimonas* (formerly *Pseudomonas*) *lemoignei*. Biochemical characterization revealed an unusual substrate specificity of the purified enzyme for amorphous poly((*R*)-3-hydroxyalkanoates) (PHA) such as native granules of natural poly((*R*)-3-hydroxybutyrate) (PHB) or poly((*R*)-3-hydroxyvalerate) (PHV), artificial cholate-coated granules of natural PHB or PHV, atactic poly((*R,S*)-3-hydroxybutyrate), and oligomers of (*R*)-3-hydroxybutyrate (3HB) with six or more 3HB units. The enzyme has the unique property to recognize the physical state of the polymeric substrate by discrimination between amorphous PHA (good substrate) and denatured, partially crystalline PHA (no substrate). The pentamers of 3HB or 3HV were identified as the main products of enzymatic hydrolysis of native PHB or PHV, respectively. No activity was found with any denatured PHA, oligomers of (*R*)-3HB with five or less 3HB units, poly(6-hydroxyhexanoate), substrates of lipases such as tributyrin or triolein, substrates for amidases/nitrilases, DNA, RNA, casein, *N*-α-benzoyl-L-arginine-4-nitranilide, or starch. The purified enzyme (M_r 36,209) was remarkably stable and active at high temperature (60 °C), high pH (up to 12.0), low ionic strength (distilled water), and in solvents (*e.g. n*-propyl alcohol). The depolymerase contained no essential SH groups or essential disulfide bridges and was insensitive to high concentrations of ionic (SDS) and nonionic (Triton and Tween) detergents. Characterization of the cloned structural gene (*phaZ7*) and the DNA-deduced amino acid sequence revealed no homologies to any PHB depolymerase or any other sequence of data banks except for a short sequence related to the active site serine of serine hydrolases. A classification of the enzyme into a new family (family 9) of carboxyesterases (Arpigny, J. L., and Jaeger, K.-E. (1999) *Biochem. J.* 343, 177–183) is suggested.

Poly((*R*)-3-hydroxyalkanoic acids) (PHAs)[1] are a class of storage compounds that are synthesized during unbalanced growth by many bacteria. PHAs are deposited intracellularly in the form of inclusion bodies ("granules") to levels up to 90% of the cellular dry weight. The subject was reviewed recently (1). Poly((*R*)-3-hydroxybutyric acid) (PHB) is the most abundant polyester in bacteria. Bacterial copolymers containing randomly distributed (*R*)-3-hydroxybutyric and (*R*)-3-hydroxyvaleric units (poly(3HB-*co*-3HV)) have been commercialized for over a decade under the trade name Biopol®.

Any research on the biodegradation of PHA should clearly distinguish between (i) extracellular PHA degradation and (ii) intracellular PHA degradation. (i) Extracellular degradation is the utilization of an exogenous carbon/energy source by a not-necessarily-accumulating microorganism. The source of this extracellular polymer is PHA-released by accumulating cells after death. The ability to degrade PHA is widely distributed among bacteria and depends on the secretion of specific PHA depolymerases that are carboxyesterases (EC 3.1.1) and hydrolyze the water-insoluble polymer to water-soluble monomers or oligomers (2, 3). (ii) Intracellular degradation is the active mobilization (hydrolysis) of an endogenous carbon/energy storage reservoir by the accumulating bacterium itself. The differentiation between extra- and intracellular degradation is necessary, because PHA *in vivo* and outside the bacteria are present in two different biophysical states. In intracellular PHA granules the high molecular mass polymer (10^5–10^6 Da) is in the amorphous "rubbery" state (highly mobile chains in disordered conformation), and the granule surface layer consists of proteins and phospholipids (4). Upon extraction from the cell, the granule surface layer is either damaged or lost (5), and the polyester chains tend to adopt ordered helical conformations (6) and develop a crystalline phase. Extracellular PHB, for example, is a partially crystalline polymer (typical degree of crystallinity 50–60%) (7, 8) with an amorphous fraction characterized by the same glass transition temperature as native PHB ($T_g \sim 0$ °C) and a crystalline phase that melts in the range of 170–180 °C (8). For the sake of clarity, in the present paper PHA in the native state (*i.e.* in the intracellular granules) are indicated as nPHA, whereas the same polyesters in the partially crystalline form are denoted as denatured PHA (dPHA). The same notation is used to differentiate PHA depolymerases according to their ability to hydrolyze nPHA (nPHA depolymerases) or dPHA (dPHA depolymerases).

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‖ To whom correspondence should be addressed. Tel.: 49-711-685-5483; Fax: 49-711-685-5725; E-mail: dieter.jendrossek@po.uni-stuttgart.de.

[1] The abbreviations used are: PHA, poly((*R*)-3-hydroxyalkanoic acid); PHB, poly((*R*)-3-hydroxybutyric acid); 3HB, 3-hydroxybutyric acid; n, native state; d, denatured state; PHV, poly(3-hydroxyvalerate); PHO, poly(3-hydroxyoctanoate); PAGE, polyacrylamide gel electrophoresis; MALDI/TOF, matrix-assisted laser desorption ionization/time-of-flight; ESI, electrospray ionization; SCL, short chain length; MCL, medium chain length; a, atactic.

A number of extracellular PHB depolymerases have been isolated to date. Their structure and specificity have been reviewed recently (2, 3). One of the best studied PHA-degrading bacteria is *Paucimonas lemoignei* (formerly *Pseudomonas lemoignei*) (9). It belongs to the β subclass of *Proteobacteria*, and its 16S rRNA sequence is related to *Herbaspirillum* and *Oxalobacter* species. *P. lemoignei* is unique among PHA-degrading bacteria, because it is able to synthesize at least six different extracellular PHA depolymerases (PhaZ1 to PhaZ6 (3)). All extracellular PHA depolymerases from *P. lemoignei* analyzed thus far degrade dPHA but are inactive toward undamaged nPHA. They are therefore dPHA depolymerases, similar to all known extracellular PHA depolymerases secreted by other microorganisms (3).

The inability of the mentioned extracellular dPHA depolymerases to degrade the natural polyester in its native *amorphous* form (such as in nPHB) has been alternatively tested using poly((*R*,*S*)-3-hydroxybutyrate) as a synthetic analog of the natural polyester. This polymer contains 50% monomer units in *S* configuration, randomly distributed along the chain (atactic PHB, aPHB). Crystallization is totally hindered, and aPHB is a completely amorphous polymer (10). Intensive investigations using PHB depolymerase A (PhaZ5) from *P. lemoignei* have shown no significant activity of this enzyme toward aPHB. However, the enzyme hydrolyzed aPHB when this amorphous polymer was blended, or copolymerized, with a variety of crystalline polyesters (10–12). Hydrolytic activity toward aPHB was clearly of an endo type because of the presence of *S* units along the chain, which are known not to be recognized by PHB depolymerases (10, 13). The conclusion was drawn that in aPHB, efficient enzyme binding to the substrate is prevented by exceeding mobility of the polymer chains in the amorphous rubbery state. Conversely, binding is promoted in both dPHB and in the above-mentioned aPHB-containing blends (10–12) by the presence of a stable crystalline phase.

Although all available evidence shows that extracellular PHA depolymerases are inactive toward rubbery amorphous substrates such as nPHA granules and aPHB, recently unexpected high levels of a novel extracellular enzymatic activity of *P. lemoignei* that hydrolyzed both nPHB granules and aPHB was detected. This discovery prompted us to the present investigation on the activity of a newly isolated *extra*cellular PHB depolymerase of *P. lemoignei* with a unique behavior, previously considered typical of *intra*cellular PHB depolymerases.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions

Escherichia coli strains XL1blue and DH5α and *P. lemoignei* (DSMZ7445) were used in this study (9). *E. coli* was grown at 37 °C on Luria Bertani medium with additions as indicated, and *P. lemoignei* was grown at 30 °C in Stinson and Merrick's mineral salts medium (14) with 50 mM sodium succinate or 0.5% 3HB.

Polymer Substrates

PHA Granules—Denatured semicrystalline PHB (dPHB) and denatured poly(3-hydroxyvalerate) (dPHV) were isolated from gluconate- or valerate-grown cells of *Ralstonia eutropha* H16 (DSMZ428) or *Chromobacterium violaceum* (DSMZ30191), respectively, by sodium hypochlorite digestion and subsequent solvent extraction with acetone/diethylether as described elsewhere (15). Denatured poly(3-hydroxyoctanoate) (dPHO) was purified from octanoate-grown cells of *Pseudomonas oleovorans* (ATCC 29347) by chloroform extraction and repeated methanol precipitation. nPHB and nPHV granules with intact surface layers were prepared from crude extracts (French press) of accumulated cells by two subsequent glycerol density gradient centrifugations as described recently (16). nPHO granules were purified by sodium phosphate-buffered glycerol density gradient centrifugation with or without 50 mM dithiothreitol + 1% (w/v) SDS (pH 8.0) to prevent self-hydrolysis by granule-bound depolymerases. Artificial cholate-coated PHA granules were prepared by emulsifying 3 ml of a PHA solution in chloroform (12.5% (w/v)) with an aqueous solution of sodium cholate or SDS (30 ml, 50 mM), respectively, by ultrasonication and subsequent evaporation of the solvent as described in Ref. 17. The granules obtained were purified by glycerol density gradient centrifugation. The amorphous state of the granules was verified by x-ray diffraction (18).

PHA Films—Films of bacterial dPHB (PHB GO8 granules from ICI, average molecular weight (M_n) = 5.39×10^5, polydispersity index = 4.11) were obtained by granule compression molding in a Carver C12 laboratory press (T = 195 °C, 1 min, 2 ton/m^2 pressure) between Teflon plates. The obtained dPHB films (thickness ~ 0.2 mm) were aged for 3 weeks at room temperature to obtain a stable degree of crystallinity. Films of atactic PHB (aPHB, M_n = 31000, weight average M_r/M_n = 1.1), synthesized as reported previously (19, 20), were prepared by casting a dichloromethane solution of aPHB on thin polypropylene sheets as described earlier (10).

PHA Oligomers—Linear and cyclic oligomers of (*R*)-3HB or (*S*)-3HB with defined number of units were prepared according to Ref. 21 and were kindly provided by P. Waser and D. Seebach (ETH Zürich, Switzerland).

Enzymes

Purification of Extracellular dPHB Depolymerase A (PhaZ5), dPHB Depolymerase B (PhaZ2), and new nPHB Depolymerase (PhaZ7)—10 liters of a succinate-grown (80 h) culture of *P. lemoignei* were harvested by centrifugation. The cell-free culture fluid was concentrated by ultrafiltration (cut off size 10 kDa) and subsequent ammonium sulfate precipitation (30–85%). The proteins of the 85% precipitation step were dissolved in 20 mM Tris-HCl, pH 8.0, and diafiltrated against 10 mM succinate NaOH, pH 4.7, containing 1 mM $CaCl_2$ and 5% (v/v) glycerol. After centrifugation (10 min, 3,800 × *g*), the sample (250 ml, 153 mg) was applied to a CM-Sepharose CL-6B column (diameter, 26 mm; bed volume, 100 ml; flow rate, 0.75 ml/min) that had been equilibrated with the same buffer. After washing with equilibration buffer, the proteins were eluted by a linear gradient of NaCl (0–200 mM in equilibration buffer, 750 ml). Protein fractions were tested for activity toward both dPHB and nPHB. Succinate-grown cells contained high levels of dPHB depolymerase A (PhaZ5) and dPHB depolymerase B (PhaZ2) (2, 3) in addition to a previously undetected activity toward nPHB. dPHB depolymerase A (PhaZ5), dPHB depolymerase B (PhaZ2), and a new nPHB depolymerase were eluted around 17, 50–55, and 55–65 mM NaCl, respectively. dPHB depolymerase A (PhaZ5) turned out to be homogeneous and was stored at −20 °C. Peaks of dPHB depolymerase B (PhaZ2) and of the new nPHB depolymerase activity overlapped partially. Separation of dPHB depolymerase B from nPHB depolymerase was performed by applying the diafiltrated (25 mM 1,3-diaminopropane-HCl, pH 10.5) and concentrated pool (97 ml, 58 mg) onto a Mono-P column (diameter, 5 mm; bed volume, 4 ml; flow rate, 0.5 ml/min) equilibrated with the same buffer. After washing with 5 bed volumes of equilibration buffer, the proteins were eluted by 46 ml of diluted (1:60) Amersham Pharmacia Biotech Pharmalyte® polybuffer, pH range 8.0–10.0, pH (HCl) 8.0. The new nPHB depolymerase activity appeared between pH 9.3 and 8.8 with a maximum at pH 9.2. dPHB depolymerase B was separated completely and appeared between pH 9.4 and 9.6. Fractions containing high amounts of nPHB depolymerase or dPHB depolymerase activity were pooled separately, dialyzed against 25 mM diaminopropane buffer, pH 9.0, and stored at −20 °C. 10 mg of purified nPHB depolymerase PhaZ7 (38,000 units, 7.8% yield) and 1.1 mg of purified dPHB depolymerase B were obtained. All enzyme preparations were homogeneous (>98%) as judged by SDS-PAGE analysis and subsequent silver-staining.

Lipases and Esterases—Lipases (from pig and *Candida antarctica* (Lip II)) were obtained from Sigma. *Pseudomonas aeruginosa* lipase and recombinant esterases of *Pseudomonas fluorescens* (22) or *Streptomyces diastachochromogenes* (23) were gifts from K.-E. Jaeger and U. Bornscheuer, respectively.

PHB Depolymerase Activity Assays

nPHB and dPHB Granules—The activity of PHB depolymerases was assayed photospectroscopically at 650 nm in a microtiterplate reader (KC4, Bio-tek© Instruments, Inc.) using 5–20-μl samples in a 200-μl assay mixture at 40 °C. For determination of dPHB depolymerase activity the assay mixture contained 100 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, and 500 μg/ml sodium hypochlorite-purified dPHB granules. For the assay of nPHB depolymerase activity, the assay mixture contained 100 mM Tris-HCl, pH 9.0, 1 mM $CaCl_2$, and 500 μg/ml nPHB granules purified from *R. eutropha* as described earlier (16). 1 unit of activity is defined as the decrease of one A_{650} unit in 1 min. Alternatively, PHB depolymerase activity was assayed by titration of the released acid

using a recording titristat consisting of a pH micro electrode, pH meter 620, impulsomat 614, dosimat 665, and a 10-ml syringe burett (Meterohm, Herisau, Switzerland). The reaction mixture (4–10 ml) contained 250 μg/ml purified nPHA granules in distilled water at 40 °C. The pH of the assay mixture was maintained at pH 9.0, 8.0, or 7.5 (depending on the experiment) by the addition of 10-μl pulses of 10 or 2 mM NaOH. The reaction was started with 10–100 μl (0.5–5 μg) of the depolymerase. When necessary, the enzyme was diluted with water.

3HB Oligomers—For the assay of 3HB oligomer hydrolysis, 2 mg of the oligomer dissolved in 0.5 ml of dichloromethane were used to coat the bottom and ~5 mm of the inside walls of the reaction vessel by evaporation resulting in a thin film of the oligomer. 2 ml of distilled water were added, and the pH was adjusted to 7.5 or 9.0 using the titristat at 40 °C as described above. The reaction was started by the addition of the enzyme. 1 unit of activity is the release of 1 μmol acid/min.

dPHB and aPHB Films—The activity of PHB depolymerases was assayed by incubating film samples (12.5 × 7 mm^2, initial weight 15–25 mg) in duplicate at 37 ± 0.1 °C in separate vials containing 1.0 ml of 50 mM Tris-HCl buffer, pH 8.0, 1 mM $CaCl_2$, and 2.8 μg/ml enzyme. The films were removed from the enzymatic solution after 20 h, washed with distilled water, and dried under vacuum over P_2O_5 at room temperature to a constant weight (Sartorius RC210D electronic balance, reproducibility ± 0.02 mg). After each incubation interval, enzymatic hydrolysis was quantified as weight loss normalized to the exposed film surface area ($\Delta m/S$). Over the time scale of the activity assay no appreciable weight losses were found in control tests run without enzyme.

Other Activity Assays—The test for lipolytic activity using Rhodamine B-triolein plates and tributyrin were performed as described elsewhere (24). The assay of lipases LipA and LipB for nPHB depolymerase activity and assay for esterase activity using *p*-nitrophenyl-hydroxyalkanoates (10 mM dissolved in ethanol, final concentration of 0.25 mM) with a chain length of the fatty acid moiety of 2–16 carbon atoms was performed by K.-E. Jaeger (Bochum, Germany) according to the procedure described in Ref. 25. Alternatively, gum arabicum and deoxycholate of the reaction mixture were omitted, and the reaction was performed in the absence of any detergents in 100 mM Tris-HCl buffer, pH 8.0, containing 1 mM $CaCl_2$ at 40 °C. Low but significant autohydrolysis rates of the esters were taken into account for each assay.

PHB-binding Assay—The assay mixture contained 100 mM Tris-HCl, pH 8.0 (dPHB depolymerase) or 9.0 (nPHB depolymerase), 1 mM $CaCl_2$, and 500 μg/ml dPHB granules (or nPHB granules) purified from *R. eutropha*. The reaction was started by the addition of 1.25 μg of purified PhaZ7. After incubation at 40 °C for 5 min the granules were centrifuged, and the activity of the soluble depolymerase in the supernatant was assayed as described above. The pellet was washed once with assay buffer (1 ml) at room temperature and centrifuged again. The washed pellet was resuspended with SDS-denaturing buffer (10 μl), heated to 95 °C for 3 min, and centrifuged again. The supernatant (~10 μl) was analyzed by SDS-PAGE. Alternatively, the washed pellet was resuspended in 2-propanol and centrifuged. The activity of the solubilized depolymerase was assayed as described above after removal of the solvent by dialysis.

Genetic Characterization of PhaZ7 Depolymerase

Generation of Internal Peptides of nPHB Depolymerase PhaZ7—1 mg of purified PhaZ7 was electroeluted from 12% SDS-PAGE, desalted, and concentrated by ultrafiltration. 200 μg of purified PhaZ7 were digested with BrCN at room temperature in the dark. The reaction mixture contained 200 μl of formic acid (70%) and two crystals of BrCN under nitrogen. After incubation for 19.5 h the sample was diluted 1:10 with water and frozen in liquid nitrogen before evaporation of formic acid. BrCN-generated fragments were separated by 15% Tricine SDS-PAGE (26) or 12% SDS-PAGE and Western blotted onto a polyvinylidene difluoride membrane. Coomassie Blue-stained bands of interest were cut out and subjected to N-terminal amino acid sequencing. Alternatively, 500 μg of purified PhaZ7 (Pharmalyte® polybuffer removed by 1.25 M NaCl and subsequent diafiltration on YM03 including desalting of the sample) were digested by trypsin (sequencing grade, Sigma) for 24 h at 37 °C. The assay mixture for reduction (12 h at 45 °C) contained 25 μl of urea (8 M in 0.4 M NH_4HCO_3 purified by Amberlite MB ion exchanger) and 5 μl of dithiothreitol (dithiothreitol, 45 mM). 5 μl of 100 mM iodoacetamide were added for alkylation (15 min). After dilution with water (*ad* 100 μl) 16.6 μg of trypsin were added. PhaZ7 was digested completely within 12 h at room temperature. Trypsin-generated fragments were separated by high pressure liquid chromatography using an Amersham Pharmacia Biotech LKB μ-separation unit with a Hyposil ODS column (150 × 2.1 mm; eluent A, 0.1% trifluoric acid in water; eluent B, 0.085% trifluoric acid in acetonitrile; linear gradient 7–50% B at 100 μl/min). Peaks containing internal fragments of trypsin were recognized by MALDI/TOF analysis and discarded. The remaining peaks were subjected directly to N-terminal amino acid sequencing.

Cloning of the nPHB Depolymerase Structural Gene phaZ7—DNA manipulation was done by using standard procedures (27). Chromosomal DNA of *P. lemoignei* was polymerase chain reaction-amplified using the oligonucleotides 5′-AARGGNACNCARACICARTAYGC-3′ and 5′-YTGDATDATNGCNCCIGTRTT-3′, which were derived from internal peptide fragments 1 (KGTQTQYA) and 6 (NTGAIIQ) of PhaZ7. The resulting 921-base pair amplification product was cloned into the *Eco*RV site of pBluescriptII SK(−). Additional primers (5′-GCTCCGGGCGTGGGTTAC-3′ and 5′-GTCTTGGTGCGGAAGTGG-3′) deduced from the primary polymerase chain reaction product were used for the construction of a DNA probe (866 base pairs) to detect recombinant *E. coli* DH5α clones harboring a genomic library of chromosomal DNA in pBluescriptII KS(−). Positive clones were used for DNA sequencing of both strands.

Other Instrumental Techniques

Electrophoretic and Immunological Techniques—Routinely, reducing and denaturing SDS-PAGE analysis was performed with 12% polyacrylamide gels (cross-linking degree, 0.8%). The gels were silver-stained. Alternatively, the proteins were electroblotted onto polyvinylidene difluoride membranes for the detection of glycoproteins using a DIG glycan detection kit from Roche Molecular Biochemicals according to manufacturer instructions and with purified dPHB depolymerase A as a positive control (28). Polyclonal mouse antibodies, raised against dPHB depolymerase PhaZ5, were obtained using SDS-PAGE-separated depolymerase material as the antigen.

Mass Spectrometry—Electrospray ionization (ESI) mass spectrometric analyses were performed using a Finnigan LCQ ion trap mass spectrometer (Finnigan, San Jose, CA). The freeze-dried supernatants from the enzymatic degradation of PHB and PHV were dissolved in chloroform/methanol (10:1 (v/v)), and such solutions were introduced continuously into the ESI source by means of the instrument syringe pump at a rate of 3 μl/min. Dry nitrogen was used for sample nebulization. The ESI source was operated at 4.25 kV, and the capillary heater was set to 200 °C. For multistage ESI MS^n experiments, mass-selected monoisotopic parent ions were isolated in the trap and collisionally activated with 33% ejection radio-frequence amplitude at standard helium pressure. The experiments were performed in the negative-ion mode. MALDI/TOF mass spectrometric analysis was performed on GSG*future* MS with time lag focusing and a 337-nm UV laser (GSG Mess-und Analysengeräte Vertriebsgesellschaft mbH). Samples (1 μl, desalted) were embedded in a 1-volume mixture of 3.5-dimethoxy-4-hydroxycinnamic acid and acetonitrile/0.1% trifluoroacetic acid (40:60 (v/v)) and dried at 21 °C. The system was calibrated with carbonic anhydrase B, and the measurement was performed at 20 kV.

RESULTS

Purification of Extracellular nPHB Depolymerase PhaZ7—Extracellular nPHB depolymerase activity was followed during growth of *P. lemoignei* on acetate, succinate, 3HB, and dPHB. Samples were taken 9.5 (exponential growth phase) and 22 h (beginning of stationary phase) after inoculation. Low (<1 unit/ml) or medium (1–2.5 units/ml) activities of nPHB depolymerase were found during exponential growth on acetate and 3HB or on succinate and dPHB, respectively. nPHB depolymerase activity was almost unchanged in the 3HB and PHB cultures at the beginning of the stationary phase (1–2.5 units/ml), but the activities on succinate and acetate were increased significantly (>6 units/ml). Prolonged incubation of the cells for 2–4 days in succinate cultures resulted in the ncreased formation of nPHB depolymerase activity up to 50–60 units/ml. Apparently, the expression of nPHB depolymerase is derepressed after the exhaustion of the carbon source. Purification of the corresponding nPHB depolymerase protein (PhaZ7) was performed from 10 liters of culture fluid of an 80-h succinate culture as described under "Experimental Procedures." 10 mg of purified nPHB depolymerase protein (yield 7.8%) were obtained (Table I). The purified nPHB depolymerase was free from dPHB depolymerase activity or any other proteins as

TABLE I
Purification of nPHB depolymerase PhaZ7

All measurements by turbidometric assay.

Step	Volume	Protein	Protein total	Activity	Activity total	Sp. activity
	ml	*mg/ml*	*mg*	*units/ml*	*10^3 units*	*units/mg*
Supernatant	798	0.50	436	617	492	1130
Ammoniumsulfate precipitation after dialysis (30–85%)	250	0.61	153	1140	285	1860
Pool CM-Sepharose	97	0.60	58	1520	147	2540
Pool Mono-P	2	5.06	10.1	19200	38.4	3800

confirmed by (i) activity test with dPHB granules, (ii) silver staining of SDS-PAGE analysis, and (iii) Western blot analysis using antibodies raised against dPHB depolymerase (Fig. 1). In contrast to dPHB depolymerases of *P. lemoignei*, the purified nPHB depolymerase was not glycosylated (data not shown). In addition to nPHB depolymerase, two dPHB depolymerases, namely dPHB depolymerase A (PhaZ5) and dPHB depolymerase B (PhaZ2), also were purified (Fig. 1).

Biochemical Characterization of Purified nPHB Depolymerase PhaZ7—The purified depolymerase PhaZ7 was highly active with $nPHA_{SCL}$ such as nPHB and nPHV but was completely inactive with medium chain-length PHA (PHA_{MCL}) such as native poly(3-hydroxyoctanoate) (nPHO) granules, artificial amorphous PHO, or other PHA_{MCL} (Table II). This lack of activity with nPHO granules was astonishing, because the enzyme had low but significant activity with *p*-nitrophenylalkanoates with a relative maximum for the C-8 ester (see below) and relatively low activity with the corresponding C-4 ester (Table II). The purified protein had an apparent molecular mass of 36 ± 3 kDa as determined by SDS-PAGE (Fig. 1). This value was confirmed by MALDI/TOF MS analysis (36,199 ± 45 Da). The enzyme was specific for nPHB and did not hydrolyze dPHB (Table II). The enzyme had no detectable activity with *p*-nitrophenylacetate and had extreme low activity with *p*-nitrophenylbutyrate, *p*-nitrophenyloctanoate, *p*-nitrophenyldecanoate, or *p*-nitrophenylhexadecanoate as a substrate when assay conditions were used that have been established for determination of esterase activity of lipases (*i.e.* in the presence of detergents and gum arabicum). This activity was not linearly dependent on the enzyme concentration. However, when the detergent (Tween 80) and gum arabicum of the assay mixture were omitted, low but significant enzyme-dependent esterase activities were detected (0.019–0.38 units/mg with a maximum for *p*-nitrophenyloctanoate) (Table II). This low activity could be abolished by incubation of the enzyme at 100 °C for 30 min. The purified enzyme exhibited no amidase/nitrilase activity (valeronitrile, benzonitrile, and 2-phenylpropionitrile tested), no lipase activity (triolein and tributyrin), no protease activity (casein and *N*-α-benzoyl-L-arginine-nitranilide), no DNase activity (chromosomal bacterial DNA), and no significant RNase activity (16 S rRNA). For all substrates mentioned above, positive controls with appropriate enzymes confirmed the experimental setup. To our knowledge, an esterase with such an unusually restricted substrate specificity has not yet been described.

The activity and stability of the purified depolymerase strongly depended on temperature, pH, and composition of the storage buffer. The optimum temperature of the enzymatic reaction was at 65 °C; the enzyme was unstable above 60 °C (Fig. 2). However, complete thermal inactivation required the incubation of the depolymerase in distilled water at 100 °C for at least 30 min. The pH optimum with nPHB granules as a substrate was at pH 9.5–10.0 (Fig. 3). The purified depolymerase was stable between pH values of 7.0 and 12.0 for at least 6.5 h. Interestingly, the higher the pH of the storage buffer (up to pH 12.0) the higher the activity was (measured in Tris-HCl buffer, pH 9.0). During storage at pH below 7.0, a partial reduction of the remaining activity was observed within 6.5 h. At pH 13.0 or 1.0, the enzyme was inactivated completely (Fig. 3). A significant rate of chemical (spontaneous) hydrolysis of the substrate (nPHB granules) could not be detected at any of the pH values tested. Apparently the surface layer of nPHB granules (4) protected the polymer from chemical hydrolysis.

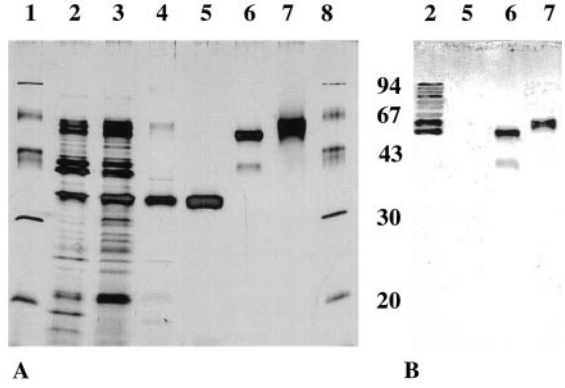

FIG. 1. **Silver-stained SDS-PAGE (*A*) and Western blot analysis with anti-PhaZ5 mouse antibodies (*B*) of nPHB depolymerase and dPHB depolymerase-containing fractions at various stages of purification.** *Lanes 1* and *8*, marker proteins; *lane 2*, concentrated supernatant of succinate-grown *P. lemoignei* cells; *lane 3*, nPHB depolymerase (PhaZ7) after ammonium sulfate precipitation; *lane 4*, PhaZ7 after CM-Sepharose; *lane 5*, purified PhaZ7 after MonoP; *lane 6*, purified dPHB depolymerase A (PhaZ5) (note that the faint band at 40 kDa is a degradation product of PhaZ5 appearing during denaturation at high temperature); *lane 7*, purified dPHB depolymerase B (PhaZ2).

The activity of the purified depolymerase did not strictly depend on the presence of cations. However, the addition of low concentrations (1–25 mM) of NaCl, KCl, $CaCl_2$, or $MgCl_2$ slightly increased the activity (Table III). High concentrations of ions, particularly $MgCl_2$, partially inhibited the reaction. EDTA partially but never completely inhibited the reaction; this inhibition was reversible by the addition of $CaCl_2$ acting better in reactivation than $MgCl_2$ (Table III). Purified nPHB depolymerase was hardly inhibited (<10% inhibition) by 1 or 10 mM of the serine esterase inhibitors phenylmethylsulfonyl fluoride or dodecylsulfonylchloride, but another serine esterase inhibitor, diisopropylfluorylphosphate, inhibited the depolymerase significantly (40 and 90% inhibition at 1 and 10 mM, respectively). Reducing agents (1 mM) such as dithiothreitol, 2-mercaptoethanol, alkylating agents (1–10 mM), cyanide, azide, or nonionic detergents (Triton X-100, Tween 20, and Tween 80) did not affect the enzyme activity significantly even at high concentrations (10%) of the detergent (Table III). Ionic detergents (SDS) had a stimulatory effect on the reaction (up to 100% increase, Table III). The higher activity in the presence of SDS was determined both in the turbidimetric assay and in the titration assay and therefore corresponds with a real increase of the hydrolysis reaction. However, the increase of the depolymerization rate apparently resulted from the interaction of SDS with the nPHB granules but not from the direct activation

TABLE II
Substrate specificity of purified nPHB depolymerase

Substrate	Relative activity[a]
nPHB granules isolated from:	
R. eutropha	++ (9000)[b]
B. megaterium	++
E. coli (*phaCAB*)	++
E. coli (*phaCAB* + *phaP*)	++
nPHV granules lated from:	
C. violaceum	+
nPHO granules lated from:	
P. oleovorans	−
aPHB	25 mg cm^{-2} h^{-1} mg protein^{-1}[c]
Denatured PHA	
dPHB, dPHV, dPHO, dPHO/HD	−
Artificial PHA granules	
PHB, SDS- or cholate-coated	+
PHV, SDS- or cholate-coated	+
PHO, PHO/HD[d]	−
Other compounds	
poly(6-hydroxyhexanoate)	−
triolein, butyrin	−
valeronitrile, benzonitrile, 2-phenylpropionitrile	−
DNA, RNA	−
starch	−
caseine	−
N-α-benzoyl-L-arginine-nitranilide	−
p-Nitrophenylalkanoates[e]	
C_2	0.019
C_4	0.048
C_6	0.18
C_8	0.38
C_{10}	0.12
C_{16}	0.055

[a] Measured at pH 9.0, activity > 1,000 units/mg (++), activity > 100 units/mg (+), activity < 10 units/mg (−).
[b] Measured at pH 9.0, value in units/mg protein, measured by titristat method.
[c] Measured by weight-loss analysis on aPHB films as described under "Experimental Procedures."
[d] Copolymer of mainly 3-hydroxyoctanoate and 3-hydroxydecanoate.
[e] Values in units/mg, in the absence of gum arabicum and deoxycholate at pH 8.0, ε, 15,000 M^{-1} cm^{-1}.

of the depolymerase, because the activation was higher (up to 100%) if the nPHB granules had been preincubated with SDS. If the depolymerase had been preincubated with SDS, no or only a slight stimulatory effect of SDS was found (0–20%). This finding was in agreement with the observation that SDS did not stimulate the hydrolysis of *p*-nitrophenylesters by PhaZ7. Solvents such as *n*-propyl alcohol partially inhibited hydrolysis of nPHB in a concentration-dependent manner. This inhibition was reversible by evaporation of the solvent.

Products of Enzymatic nPHB Hydrolysis—The following experiments were performed with nPHB granules purified from *R. eutropha* using the titristat method and measuring the A_{650} changes with time (Fig. 4). Within 1 min the milky granule suspension became clear, indicating that insoluble nPHB granules had been hydrolyzed to water-soluble products. A high specific activity of 9,000 units × mg^{-1} was obtained. Analysis of the reaction products by NAD-dependent 3HB dehydrogenase showed that monomeric 3HB had been released. However, the amount of 3HB was low and corresponded only 0.5–2.5% of total 3HB equivalents present in the nPHB granules of the assay. Interestingly (Fig. 4), a second phase of slow hydrolysis followed, indicating that oligomers of 3HB might have been formed in addition to the 3HB monomer, which were hydrolyzed in this second phase. The observed slow hydrolysis rate could be attributed at least partially to spontaneous chemical hydrolysis of the putative oligomers at pH 9.0, because the second slow hydrolysis rate was (i) independent from the concentration of the depolymerase, (ii) dependent on the pH (the higher the pH the higher the enzyme-independent hydrolysis rate), and (iii) also observed using synthetic oligomers without depolymerase (see below). When dPHB depolymerase A, which has 3HB oligomer hydrolase activity in addition to the depolymerase activity (29), was added during the second hydrolysis rate, a concentration-dependent increase of the hydrolysis rate was obtained and confirmed the presence of 3HB oligomers as additional degradation products of PhaZ7-catalyzed hydrolysis of nPHB. Almost the same results were obtained when nPHB granules were used as substrate that had been isolated from other PHB-accumulating bacteria (*Bacillus megaterium*) or from recombinant *E. coli* harboring the PHB biosynthetic genes *phaCAB* of *R. eutropha* with or without the phasin gene *phaP*. The phasin proteins are small structural polypeptides that have been detected in the surface layer of PHA granules and are suspected to provide a proper link between the hydrophobic core of the polymer and the hydrophilic cytoplasm and to influence the number and size of the granules (4, 30). Apparently, the microbial origin of the polymer and the absence or presence of the phasin protein does not influence the hydrolysis of the polymer by nPHB depolymerase.

To identify hydrolysis products other than the 3HB monomer, nPHB granules from recombinant *E. coli* were subjected to enzymatic hydrolysis by nPHB depolymerase at pH 8.0, and the degradation products were analyzed by ESI MS. As shown as an example in Fig. 5*A*, a series of singly charged negative ions with *m/z* values starting from 103 and a constant peak to peak mass increment of 86 was detected. These *m/z* values correspond to the molecular masses of the deprotonated 3HB monomer R1 (*m/z* 103), the 3HB dimer R2 (*m/z* 189), the 3HB trimer R3 (*m/z* 275), the 3HB tetramer R4 (*m/z* 361), the pentamer R5 (*m/z* 447), the hexamer R6 (*m/z* 533), the heptamer R7 (*m/z* 619), and the octamer R8 (*m/z* 705). The most intense peak of this series corresponds to the anion of pentamer R5. The identity of the putative 3HB pentamer was confirmed by ESI MS^n analysis, giving the fragmentation pattern typical for deprotonated 3HB oligomer anions (31). The ESI MS spectrum (Fig. 5*A*) contained an additional series of peaks with the most pronounced ions at *m/z* values of 419, 505, and 591. The difference between these values is again 86, and each value is larger by 58 compared with the *m/z* values of the tetra-, penta-, and hexamer anions of 3HB. The ESI MS^n analysis of these parent ions yielded the fragmentation pattern typical for 3HB oligomers. It is concluded that the peaks differing in *m/z* by 58 from the values of respective 3HB oligomer anions represent singly charged chlorine adduct ions of the sodium salt of tetra-, penta-, and hexamers of 3HB. This assumption was additionally confirmed by the agreement of the acquired and calculated isotopic patterns for the observed ion at *m/z* 505 of the general formula $C_{20}H_{31}O_{11}NaCl$.

The relative composition of the 3HB oligomers R_n, estimated from the intensities of both deprotonated and chlorine adduct ions, slightly changed with time of enzymatic hydrolysis, with the maximum remaining on the pentamer R5 (Fig. 5*B*). The main change observed is the disappearance of the octamer R8 between 5 and 10 min; after 10 min the oligomer distribution did not change significantly, confirming that the 3HB pentamer is the major hydrolysis product. Analogous degradation products were obtained when other substrates were used (nPHB granules of *R. eutropha* and artificial cholate-coated PHB granules). When artificial cholate-coated PHV granules were hydrolyzed, a comparable pattern of (*R*)-3-hydroxyvaleric acid oligomers with a maximum for the (*R*)-3-hydroxyvaleric acid pentamer was obtained (data not shown).

Hydrolysis of 3HB Oligomers—To verify that the 3HB pentamer and lower oligomers of nPHB are not hydrolyzed by

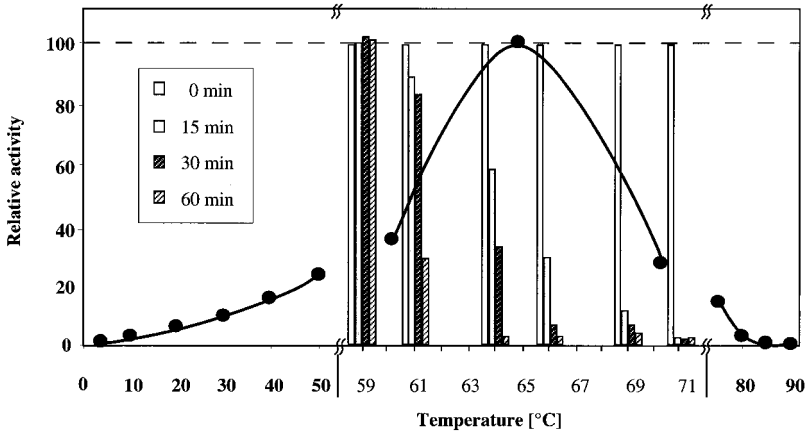

FIG. 2. **Effect of temperature on activity and stability of purified nPHB depolymerase PhaZ7.** *Black circles*, activity: the assay was performed at temperatures as indicated by the addition of purified water-diluted (1:1000) nPHB depolymerase. For the stability assay (*bar diagram*), the depolymerase was incubated in diaminopropane buffer, pH 9.0, at temperatures and for time intervals as indicated before the remaining activity was determined.

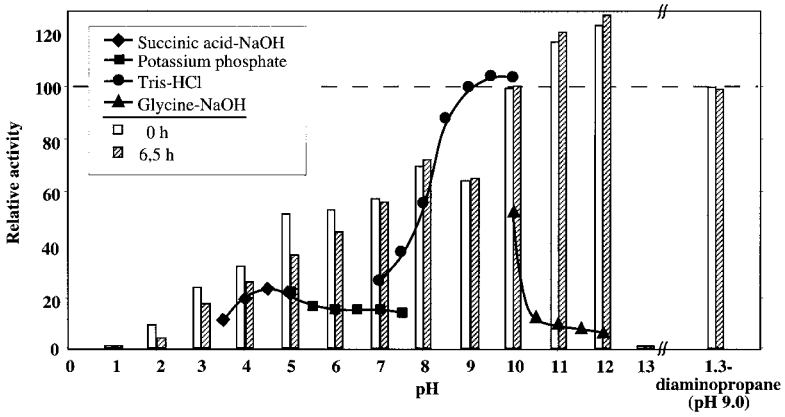

FIG. 3. **Effect of pH and composition of buffer on activity and stability of purified nPHB depolymerase PhaZ7.** *Black symbols*, activity: the assay mixture with different buffers and pH as indicated was preincubated (40 °C) for 5–10 min before the reaction was started by the addition of purified water-diluted (1:1000) nPHB depolymerase. *Bar diagram*, stability: purified nPHB depolymerase (stored in diaminopropane buffer, pH 9.0) was diluted (1:1000) in buffers as indicated below. After incubation on ice for 0 and 6.5 h, the samples (5 μl) were assayed for remaining nPHB depolymerase activity in 250 mM Tris-HCl buffer, pH 9.0, and 1 mM $CaCl_2$ (40 °C). The following buffers were used for stability analysis (values refer to 1 liter): 3.7 g of KCl and 134 ml of 1 N HCl, pH 1.0; 6.43 g of citric acid-monohydrate, 3.58 g of NaCl, and 8.2 ml of 1 N HCl, pH 2.0; 8.47 g of citric acid-monohydrate, 3.49 g of NaCl, and 20.6 ml of 1 N NaOH, pH 3.0; 11.76 g of citric acid-monohydrate, 2.57 g of NaCl, and 68 ml of 1 N NaOH, pH 4.0; 100 mM potassium phosphate buffer, pH 5.0–7.0; 100 mM Tris-HCl buffer, pH 7.0–9.0; and 100 mM glycine-NaOH, pH 10.0–13.0.

PhaZ7, as suggested by the above results (Fig. 5), hydrolysis of chemosynthetic oligomers (dimer to octamer) of (*R*)-3HB was investigated. A relatively high rate of spontaneous chemical hydrolysis (0.044 units (dimer) up to 0.17 units (octamer)) was detected at pH 9.0. The experiments were repeated at pH 7.5. At this pH the spontaneous hydrolysis rates of the oligomers were reduced to 0.01 and 0.11 units, respectively. Under these conditions no or only very low enzymatic hydrolysis rates were determined for the dimer and trimer or the tetramer and pentamer, respectively. The specific activities increased with the number of 3HB units from values below 7 units/mg for tetra- and pentamer and to 23 and 500 units/mg for hexamer and octamer, respectively. These results confirmed that the purified enzyme is a true polymer hydrolase and are in agreement with the finding that the pentamer is the main hydrolysis product. Cyclic oligomers of (*R*)-3HB such as the hexamer were hydrolyzed at initial rates (170 units/mg) that were much higher than the spontaneous chemical hydrolysis rate of the circular oligomer (0.8 units). This result confirmed that the purified depolymerase has *endo*-hydrolase activity (see above, results with aPHB). Oligomers of (*S*)-3HB were not hydrolyzed at all and confirmed the specificity of the enzyme for the *R* isomers as it has been found for dPHB depolymerase of *Alcaligenes faecalis* (13).

Cloning of the nPHB Depolymerase Structural Gene phaZ7—The N-terminal amino acid sequences of purified undigested nPHB depolymerase and three BrCN-digested and of two trypsin-digested internal peptides were determined: undigested protein peptide 1, N-LTXGTNSGFVXKGTQTQYAGGFAPGVGYGGFGGGS; BrCN-digested protein peptide 2, N-MPPGNVSGYGTPA; BrCN-digested protein peptide 3, N-GNVSGYGTPA; BrCN-digested protein peptide 4, N-ATLQYYNN; trypsin-digested protein peptide 5, N-FASTTSNVK; and trypsin-digested protein peptide 6, N-TKTNTGAIIQ. Using synthetic oligonucleotides derived from peptides 1 and 6, a 921-base pair fragment was polymerase chain reaction-amplified from chromosomal DNA of *P. lemoignei* and used as a hybridization probe for the cloning of the total structural gene. The DNA sequence of both strands of the complete structural gene, *phaZ7*, was determined (GenBank™ accession number AY026355).

Characterization of PhaZ7—The DNA-deduced sequence of the mature nPHB depolymerase PhaZ7 (GenBank™ accession number AY026355) contained the amino acid sequences of all 6 peptide fragments and confirmed that the correct gene had been cloned. The DNA sequence encoded a 380-amino acid polypeptide (40,019 Da) with the characteristic features of a secretory protein including an Ala^{37}-Leu^{38} signal peptidase cleavage site. The predicted molecular mass of the mature protein (36,209 Da) corresponded well with the SDS-PAGE- and MALDI/TOF MS-determined values of 36 ± 3 kDa and 36,199 ± 45 Da, respectively. The sequence contained the mo-

TABLE III
Dependence of purified nPHB depolymerase from ions and inhibitor studies

Addition	Concentration	Activity
	mM	%
None		100
NaCl	1	130
	10	130
	25	120
	50	100
	100	80
	150	70
KCl	1	120
	10	130
	25	130
	50	100
	100	65
	150	65
$MgCl_2$	0.1	120
	1	100
	5	100
	10	80
	25	10
	50[a]	15
	100[a]	15
$CaCl_2$	0.1	140
	1	140
	5	160
	10	100
	25	90
	50	70
	100[a]	70
EDTA	0.1	40
	1	30
	10	30
EDTA + $CaCl_2$	2 + 5	100
EDTA + $MgCl_2$	2 + 5	40
SDS	0.02	130
	0.2	150
	2	200[b]
Triton X-100	0.02	105
	0.2	90
	2	90
	10	100
Tween 20	0.02	110
	0.2	130
	2	110
	10	120
Tween 80	0.02	110
	0.2	120
	2	100
	10	100
Ethanol	5 % (v/v)	100
n-Propanol	0.1 % (v/v)	80
	1	65
	10	50
DTT[c]	1	100
Mercaptoethanol[c]	1	100
Dithiodinitrobenzoate[c]	1	100
KCN	1	100
	10	100
NaN_3	1	100
	10	100
Iodoacetamide	1	100
	10	100
p-Hydroxymercurybenzoate	1	100
	10	100
PMSF[d]	1	100
	10	90
DDSC[d]	1	100
	10	90
DFP	1	60
	10	10

[a] Precipitation at high salt concentrations observed.
[b] Titration method.
[c] Enzyme was preincubated at room temperature in the presence of 10 mM inhibitor. After 10 min the depolymerase and the inhibitor were added to the assay system; the final concentration of the inhibitor was 1 mM.
[d] Dissolved in isopropanol; value has been corrected for inhibition by the solvent. The activity was determined at pH 9.0, 40 °C, by the turbidimetric method using nPHB granules as a substrate.

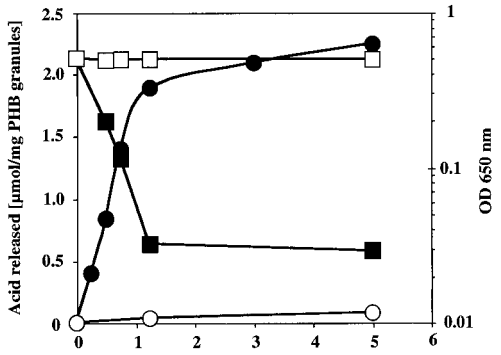

FIG. 4. **Degradation of nPHB granules by nPHB depolymerase PhaZ7: $A_{650\ nm}$ (OD_{650}) (■) and acid released (●) as a function of time.** Control experiments in the absence of enzyme (*white symbols*) are also shown.

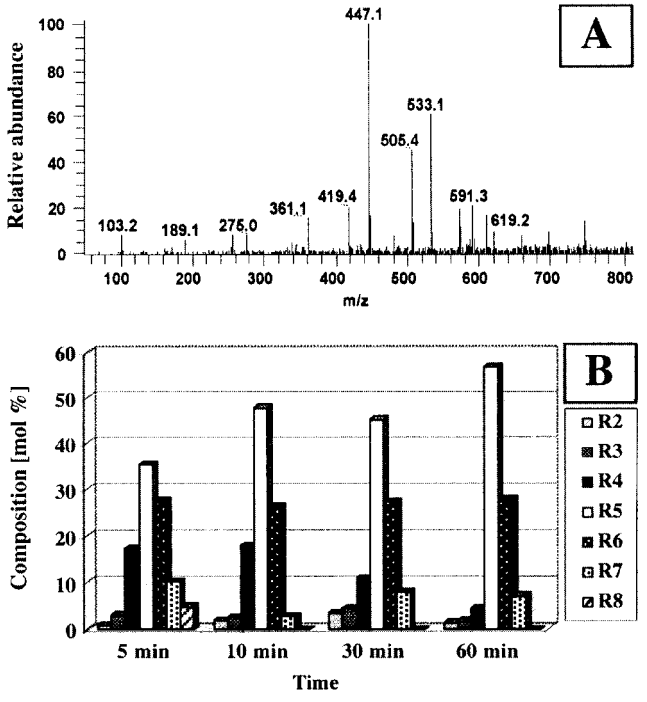

FIG. 5. **Degradation products of nPHB granules from recombinant *E. coli* by PhaZ7.** *A*, ESI MS spectrum acquired after 10 min of degradation. *B*, composition of 3HB oligomers after specified degradation intervals.

tifs P*XXXX*HG and AHSMG, which are related to the oxyanion pocket and the lipase box pentapeptide (G*X*S*X*G) of many lipases and other serine hydrolases, respectively (32–34). The highest degree of homology was found around both motifs to (i) sequences, which have been found in the genome sequence of the nematode *Caenorhabditis elegans* that were related to lipases LipB and LipA of *Bacillus subtilis*, and (ii) the same region of other Bacilli lipases (Fig. 6). However, purified LipA and LipB of *B. subtilis* were not able to hydrolyze nPHB granules, and purified nPHB depolymerase PhaZ7 clearly is different from LipA, LipB, and other lipases because of the lack of lipolytic activity with both tributyrin and triolein. Furthermore, homologies beyond those mentioned above to any of the ~20 known PHB depolymerase sequences, to any lipase or esterase or to any other proteins of data bases, could not be detected and confirmed that PhaZ7 is a member of a new type of hydrolase.

Biophysical Requirements of the Polymer Substrate for Efficient Hydrolysis by PhaZ7—The purified depolymerase PhaZ7 was highly specific for (amorphous) $nPHA_{SCL}$ such as nPHB

PhaZ7	VDIV**AHSMG**V	AY026355
B. subtilis LipB	VDIV**AHSMG**G	C69652
B. subtilis LipA	VDIV**AHSMG**G	S23934
B. pumilis		A34992
B. thermooleovorans	IHII**AHSQG**G	AF134840
B. licheniformis	VDIV**AHSMG**G	CAB95850
B. stearothermophilus	VHII**AHSQG**G	U78785
B. thermocatenulatus	VHII**AHSQG**G	X95309
B. licheniformis (esterase)	VQLV**AHSMG**G	U35855
C. elegans[a)]	INIV**AHSMG**V	T22227
consensus	IxAHSxG	
lipase box consensus	**GxSxG**	
consensus family 1	VNLI**GHSQG**	(44)
consensus family 2	**GDSLG**	(44)
consensus family 4	**GDSAGG**	(44)
consensus family 5	**GxSMGG**	(44)
consensus family 6	**GFSQG**	(44)
consensus dPHB dep.	**GLSxG**	(2, 3)

[a)], only one of 10 related hypothetical proteins of the genomic sequence of *C. elegans* is shown.

FIG. 6. **Alignment of regions around the lipase box-like sequence of PhaZ7 and selected lipases or related enzymes.** Consensus sequences of Bacilli lipases, carboxyesterase families according to Ref. 47, and dPHB depolymerases (3) are indicated.

and nPHV but was completely inactive with (partially crystalline) dPHB or any other dPHA. Apparently PhaZ7 has an unusual preference for nPHA granules. The polymers of nPHB and of dPHB granules have the same composition, chemical structure, and molecular mass. A reason for the inability of PhaZ7 to hydrolyze semicrystalline dPHB could be the lack of a particular substrate-binding domain in the amino acid sequence of PhaZ7 (see above). An ~50-amino acid-long characteristic amino acid sequence at the C-terminal end of dPHB depolymerases is known to be responsible for efficient and specific binding of the water-soluble enzyme to the insoluble partially crystalline polymer and to constitute a dPHB-specific substrate-binding domain in all thus-far-known dPHB depolymerases (35). To test whether PhaZ7 was able to bind to nPHB and dPHB, binding studies with purified PhaZ7 and purified nPHB or dPHB were performed. After incubation of PhaZ7 in the presence of nPHB or dPHB granules for 5 min, the granules were removed by centrifugation, and the activity of the supernatant was determined. In case of nPHB the polymer was hydrolyzed completely to water-soluble products and indicated efficient binding and complete hydrolysis of nPHB to water-soluble products by PhaZ7. With dPHB no significant hydrolysis of the polymer was detected either by turbidity measurements or by the titration method and confirmed the inactivity of PhaZ7 with dPHB. Analysis of the supernatant of the PhaZ7-dPHB experiment showed no nPHB depolymerase activity in the supernatant. In a control experiment (PhaZ7 without any polymer), more than 95% of the activity was detected in the supernatant after centrifugation. The (washed) dPHB pellet of the binding assay was analyzed for bound PhaZ7 by resuspending the pellet in SDS-PAGE denaturing buffer, subsequent removal of the polymer by centrifugation, and analysis of solubilized depolymerase protein by SDS-PAGE analysis (see "Experimental Procedures" for details). High amounts of PhaZ7 protein were detected and confirmed that PhaZ7 was able to bind to dPHB. Therefore, the inactivity of PhaZ7 with dPHB can not be explained by insufficient binding to the polymer. When PhaZ7-bound dPHB granules were washed with isopropanol, the enzyme could be removed from the polymer surface, and the resolublized enzyme was still highly active at appropriate assay conditions after the removal of isopropanol. Therefore, the presence of potential inhibitory compounds associated with dPHB is unlikely. In conclusion, the inability of PhaZ7 to degrade dPHB can not be explained by a different binding behavior of PhaZ7 compared with dPHB depolymerases.

It is well established that nPHA granules have a particular surface layer consisting of proteins and phospholipids (4). Electron microscopy of this surface layer revealed that the proteins of the surface layer are highly organized and apparently constitute a partial crystalline lattice embedded in the phospholipids (36, 37). To investigate whether the ordered surface layer of nPHB granules is necessary for hydrolysis by nPHB depolymerase PhaZ7, experiments were designed to prepare amorphous PHB granules that lack the ordered structure and were completely protein-free (called artificial PHB granules). Such artificial PHB granules were prepared by the emulsification of solutions of purified PHB in chloroform with water to which a surfactant (*e.g.* SDS or cholate) had been added and evaporation of the solvent afterward. The resulting artificial PHB granules resemble nPHB granules with respect to the amorphous state of the polymer but lack the ordered structure provided by the phasin and other proteins. When such SDS- or cholate-coated artificial PHB granules were used in an assay with purified PhaZ7 the polymers were hydrolyzed within minutes. The same result was obtained with artificial amorphous PHV granules (SDS- or cholate-coated) that had been prepared by the same method. We concluded that PhaZ7 has a high affinity to the artificial polymers resulting in immediate hydrolysis of the polymer. Apparently, the presence of proteins or a potential paracrystalline structure of proteins in the surface layer of nPHB granules is not necessary for the efficient binding and hydrolysis. Interestingly, the temperature optimum of the hydrolysis of artificial PHB granules was ~5 °C lower (~60 °C) compared with nPHB (65 °C). When SDS or oleic acid was added at various concentrations (0.02–2%) to suspensions of partially crystalline dPHB granules, PhaZ7 was not able to hydrolyze dPHB. Therefore, the presence of lipids in nPHB granules or the presence of SDS in artificial PHB granules cannot be the main reason for the high activity of PhaZ7 on nPHB.

The main difference between nPHB and dPHB lays in the chain conformation of the polyester, that is totally disordered in nPHB (amorphous material), whereas in dPHB a crystalline phase with ordered helical conformation coexists with disordered chain segments. If PhaZ7 is able to discriminate between an amorphous and crystalline polyester, the inactivity of PhaZ7 with dPHB could be explained by the physical state of dPHB (presence of a crystalline phase). To test this hypothesis we prepared a completely amorphous surfactant- and protein-free PHB by polymerization of racemic β-butyrolactone. Because of the random distribution of both enantiomers in the polymer chain crystallization of the material is totally hindered. Such a polymer is called atactic (a)PHB (19, 20). The only difference between aPHB and dPHB is the presence of (*S*)-3-hydroxybutyrate units in atactic PHB and the presence of a crystalline phase in dPHB (dPHB and nPHB consist of only (*R*)-3HB units).

aPHB and dPHB in film form were used as substrates for nPHB depolymerase PhaZ7, and the results of such activity tests are reported in Fig. 7 together with those obtained using dPHB depolymerases PhaZ5 and PhaZ2, for the sake of comparison. Both dPHB depolymerases hydrolyzed dPHB, PhaZ2 at a higher rate than PhaZ5 (21 mg of polymer cm^{-2} h^{-1} mg of $protein^{-1}$ *versus* 13 mg of polymer cm^{-2} h^{-1} mg of $protein^{-1}$), whereas nPHB depolymerase PhaZ7 was inactive toward the partially crystalline dPHB substrate. On the other hand, when amorphous aPHB was used as substrate, the activity of nPHB depolymerase PhaZ7 was high (25 mg of polymer cm^{-2} h^{-1} mg

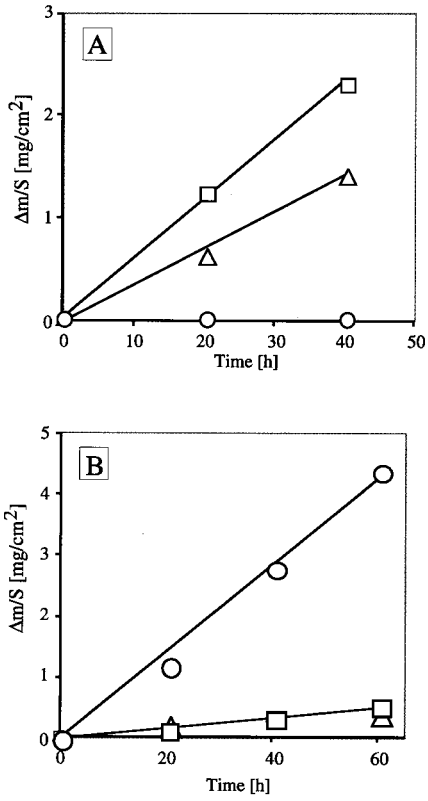

FIG. 7. **Normalized weight loss of dPHB (*A*) and aPHB (*B*) films as a function of exposure time to dPHB depolymerase PhaZ2 (□), dPHB depolymerase PhaZ5 (△), and nPHB depolymerase Phaz7 (○).** Average value of two replicate tests is shown.

protein^{-1}), whereas both dPHB depolymerase PhaZ5 and PhaZ2 showed only very low activity (<3 mg of polymer cm^{-2} h^{-1} mg of protein^{-1}). Therefore the activity of the nPHB depolymerase PhaZ7 changed from high to very low values with the changing physical state of the substrate from amorphous to partially crystalline. The activity of dPHB depolymerases PhaZ5 and PhaZ2 changed in the opposite way. Both dPHB depolymerases behaved identically toward PHB granules in the corresponding physical state (Table II). These results clearly identify the amorphous state of the polymer as the factor responsible for the different behavior of PhaZ7 toward nPHB and dPHB.

Because aPHB contains monomer units both in the "natural" *R* and in the *S* configurations and because it was shown already that only *R*--*R* bonds are cleaved (see above and Refs. 10 and 13), the results additionally show that nPHB depolymerase PhaZ7 must have an endo-type activity toward aPHB. This *endo*-hydrolase activity is responsible for the cleavage of randomly distributed *R*–*R* linkages of atactic PHB, confirming the findings described with cyclic 3HB oligomers (see above).

Other esterases such as the esterase of *P. fluorescens* (22) or *S. diastachochromogenes* (23) or a variety of lipases did not hydrolyze aPHB, nPHB, amorphous artificial PHB, or other PHA_{SCL} and confirmed that nPHB depolymerase PhaZ7 is a unique yet undescribed type of a hydrolytic enzyme.

DISCUSSION

In this contribution a new type of extracellular carboxyesterase (nPHB depolymerase PhaZ7) that is specific for amorphous short chain-length (*R*)-hydroxyalkanoic acids has been purified and characterized. The amorphous rubbery state of the polymer substrate is an essential requirement for hydrolysis by PhaZ7 and has been demonstrated independently by the use of $nPHA_{SCL}$ granules, artificial SDS- or cholate-coated $nPHA_{SCL}$ granules, and chemosynthetic aPHB. On the other hand, the enzyme is totally inactive toward semicrystalline dPHB, and therefore nPHB depolymerase PhaZ7 behaves just opposite to the conventional dPHB depolymerases. The inability of PhaZ7 to hydrolyze dPHB cannot be explained by insufficient binding of the depolymerase to the polymer, because the purified enzyme was able to bind to dPHB.

The difference of hydrolytic behavior between the new depolymerase PhaZ7 and dPHB depolymerases is also supported by the lack of amino acid homology and by different biochemical characteristics (*e.g.* resistance to reducing agents and detergents). PhaZ7 shares also no amino acid homology with the intracellular PHB depolymerase of *R. eutropha* (38) and the putative intracellular PHA depolymerases of *P. oleovorans* (MM48445) and related bacteria. The enzyme is a true depolymerase, because the activity with nPHB (9000 units/mg) or 3HB octamer (500 units/mg) as substrates is higher by more than 2–4 orders of magnitude compared with 3HB oligomers with less than six 3HB units or to *p*-nitrophenylalkanoates (<0.5 units/mg). The 3HB pentamer was identified as a main product of enzymatic hydrolysis. This is in agreement with results obtained with linear chemosynthetic 3HB oligomers, in which efficient hydrolysis was only obtained with the 3HB hexamer or higher oligomers. It should be mentioned that all dPHB depolymerases analyzed thus far produced either mainly 3HB monomer (*e.g.* the *Comamonas* sp. PHB depolymerase (2, 3)) or a mixture of monomer, dimer, and trimer (*e.g.* dPHB depolymerases PhaZ5 and PhaZ2 of *P. lemoignei*). A PHB depolymerase with the 3HB pentamer as the main degradation product of bacterial PHB has not been described thus far.

A BLAST search revealed that PhaZ7 showed significant homologies to lipase LipB of *B. subtilis* and to other lipases of Bacilli as well as to hypothetical proteins of *C. elegans* (Fig. 6). However, the homology was restricted to the lipase box pentapeptide sequence G*X*S*X*G of serine hydrolases and to short regions around a putative oxyanion pocket of LipA and related proteins. This result in combination with the observed sensitivity of the purified enzyme to the serine esterase inhibitor diisopropyfluorylphosphate suggests that PhaZ7 is a member of the serine hydrolase family having a catalytic triad of Ser^{136}-Asp^{242}-His^{306} (32, 33). Interestingly, in PhaZ7 and Bacilli lipases, the first glycine residue of the lipase box is replaced by an alanine (AHSMG). Similar to some of the Bacilli lipases (25, 39–41), purified PhaZ7 has a comparable or even a higher degree of stability at high pH (up to pH 12.0) and at high temperatures (Figs. 2 and 3). This is astonishing, because *P. lemoignei* cannot survive at high pH and at temperatures above 41 °C. However, PhaZ7 cannot be considered as a lipase because it has no lipase activity either with triolein or with tributyrin. PhaZ7 is also distinguished from most other lipases by its insensitivity against low and high concentrations (up to 10%) of ionic and nonionic detergents such as SDS, Triton X-100, Tween 20, and Tween 80. As far as it has been tested, most lipases including those of Bacilli are very sensitive at least to ionic detergents (41–45), and some are also sensitive to nonionic detergents (*e.g.* the lipase of *Bacillus thermocatenulatus* is inactivated completely by the presence of 1% Triton X-100 or Tween 80 (40)). The purified enzyme was resistant to low (1 mM) and high (10 mM) concentrations of reducing agents (2-mercaptoethanol and dithiothreitol), oxidizing agents (dithiodinitrobenzoate), alkylating agents, and other potential enzyme inhibitors (iodoacetamide, *p*-hydroxymercurybenzoate, cyanide, and azide). Therefore the enzyme apparently has no essential disulfide bridges or essential SH groups. This finding was astonishing, because 10 cysteine residues were found in the amino acid sequence of PhaZ7 and confirmed the difference

of nPHB depolymerase PhaZ7 to dPHB depolymerases, all of which are sensitive to reducing agents (2, 3). The depolymerase is not a member of conventional carboxyesterases because of its extreme low activity with *p*-nitrophenylalkanoates (<0.5 units/mg) and its high substrate specificity for water-insoluble PHA_{SCL}. Esterases and lipases usually have high specific activities with *p*-nitrophenylalkanoates in the order of 10^2 to $>10^4$ units/mg (25, 46).

The large group of known lipolytic enzymes has been classified into six, and more recently, into eight families (34, 47). Family 1 consists of the true lipases and is subgrouped into six subfamilies (1.1, *P. aeruginosa* lipase subfamily; 1.2, *Burkholderia glumae* subfamily; 1.3, *P. fluorescens* subfamily; 1.4, *B. subtilis* subfamily; 1.5, *B. stearothermophilus* subfamily; and 1.6, *Pseudomonas acnes* subfamily). Families 2–8 consist of esterases with different substrate specificities (for details see Ref. 47). Regions of PhaZ7 around the catalytic triad amino acids resemble those of Bacilli lipases (family 1.4 and 1.5) or family 5 esterases. However, PhaZ7 cannot be grouped to any of these families because of the (i) absence of significant amino acid homologies within the major part of the polypeptide, (ii) lack of lipase activity and the low degree of esterase activity with *p*-nitrophenylalkanoates, (iii) insensitivity to inhibitors and detergents, and (iv) high preference of the enzyme for nPHB granules or 3HB oligomers of six 3HB or more units. From the properties described above it is evident that PhaZ7 is a member of the serine hydrolase family, but it cannot be classified in any of the described lipase/esterase families. We therefore suggest that a new family of serine esterases be created (family 9, extracellular PHA depolymerases) that consists of two subfamilies: subfamily 9.1 (extracellular nPHA depolymerases) currently consists of only one enzyme (PhaZ7 of *P. lemoignei*). Because extracellular dPHA depolymerases do not fit into any of the families mentioned above and are clearly different from nPHB depolymerase, these enzymes are grouped into subfamily 9.2 (extracellular dPHA depolymerases (2, 3)).

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A New Type of Thermoalkalophilic Hydrolase of *Paucimonas lemoignei* with High Specificity for Amorphous Polyesters of Short Chain-length Hydroxyalkanoic Acids

René Handrick, Simone Reinhardt, Maria Letizia Focarete, Mariastella Scandola, Grazyna Adamus, Marek Kowalczuk and Dieter Jendrossek

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