Novel Extracellular PHB Depolymerase from \textit{Streptomyces ascomycinicus}: PHB Copolymers Degradation in Acidic Conditions

Javier García-Hidalgo, Daniel Hormigo, Miguel Arroyo, Isabel de la Mata*

Department of Biochemistry and Molecular Biology I. Faculty of Biology, Complutense University of Madrid, Madrid, Spain

Abstract

The ascomycin-producer strain \textit{Streptomyces ascomycinicus} has been proven to be an extracellular poly(R)-3-hydroxybutyrate (PHB) degrader. The \textit{fkbU} gene, encoding a PHB depolymerase (PhaZ\textsubscript{Sa}), has been cloned in \textit{E. coli} and \textit{Rhodococcus} sp. T104 strains for gene expression. Gram-positive host \textit{Rhodococcus} sp. T104 was able to produce and secrete to the extracellular medium an active protein form. PhaZ\textsubscript{Sa} was purified by two hydrophobic interaction chromatographic steps, and afterwards was biochemically as well as structurally characterized. The enzyme was found to be a monomer with a molecular mass of 48.4 kDa, and displayed highest activity at 45°C and pH 6, thus being the first PHB depolymerase from a gram-positive bacterium presenting an acidic pH optimum. The PHB depolymerase activity of PhaZ\textsubscript{Sa} was increased in the presence of divalent cations due to non-essential activation, and also in the presence of methyl-β-cyclodextrin and PEG 3350. Protein structure was analyzed, revealing a globular shape with an alpha-beta hydrolase fold. The amino acids comprising the catalytic triad, Ser\textsuperscript{131}-Asp\textsuperscript{209}-His\textsuperscript{269}, were identified by multiple sequence alignment, chemical modification of amino acids and site-directed mutagenesis. These structural results supported the proposal of a three-dimensional model for this depolymerase. PhaZ\textsubscript{Sa} was able to degrade PHB, but also demonstrated its ability to degrade films made of PHB, PHBV copolymers and a blend of PHB and starch (7:3 proportion wt/wt). The features shown by PhaZ\textsubscript{Sa} make it an interesting candidate for industrial applications involving PHB degradation.

Introduction

Polyhydroxyalkanoates (PHAs) are intracellular polymers accumulated by a wide range of bacteria and archaea as a carbon and energy source when environmental conditions are not optimal for cell growth. Among these biopolymers poly(R)-3-hydroxybutyrate (PHB) is the best known and most common polyhydroxyalkanoate.

PHB has attracted much interest from the industry in the last two decades, since it is a natural thermoplastic that can be produced from renewable sources by different microorganisms or plants; it is biocompatible, nontoxic, biodegradable and presents some interesting chemical and mechanical properties, therefore PHB represents an interesting alternative to petroleum-derived plastics, despite its considerably higher production costs. Another important application of PHAs is related to the monomeric composition of this family of biopolymers, since all PHAs are enantiomerically pure polymers (all the monomers present \textit{R} configuration). Thus, the degradation products of the PHAs are (R)-3-hydroxyalkanoic acids, valuable synthons for the chemical and pharmaceutical industries.

In the producer cell cytoplasm PHAs are stored in complex subcellular structures included under the term carbonosomes [1], which comprise amorphous PHA coated with a phospholipidic monolayer and different proteins involved in PHB production (polymerases), stabilization (phasins) or mobilization (depolymerases). These PHB carbonosomes are also known as native PHB or nPHB granules. When these granules are released into the extracellular medium as a consequence of cell lysis, PHAs become denatured, acquiring a semi-crystalline structure, known as denatured PHB or dPHB.

Insoluble dPHB granules are degraded in virtually every natural environment by extracellular PHB depolymerases produced by a wide variety of microorganisms, mainly bacteria and fungi. PHB depolymerases are specific for dPHB or nPHB, thus they are not able to degrade both types of PHB granules, except for the PHB depolymerase from \textit{Bacillus megaterium}, classified as an intracellular nPHB depolymerase associated to PHB carbonosomes, but exhibiting dPHB depolymerase activity as well [2].

Many of PHB degrading microorganisms present in soil ecosystems are classified into the genus \textit{Streptomyces} [3], which has been one of the most biotechnologically relevant genus during the last decades due to its metabolic versatility and the production of over 7000 natural antibiotics and other important bioactive compounds [4].

\textit{fkbU} gene from \textit{S. ascomycinicus} was described as a part of the FK520 gene cluster [5], responsible for the biosynthesis of ascomycin, a macrolide with immunosuppressive and antifungal activities. \textit{fkbU} was proposed to encode a PHB depolymerase,
no experimental evidence regarding this enzyme was previously provided.

In this work, we demonstrate that *S. ascomycinicus* is able to degrade PHB and the identity of fkbU gene has been confirmed. In this sense, fkbU was cloned in the heterologous host *Rhodococcus* sp. T104, and its gene product, hereafter PhaZ$_{Sa}$, was proven to be an extracellular dPHB depolymerase, which has been expressed in an active and extracellular form. Furthermore this depolymerase was purified as well as biochemically and structurally characterized, and a three-dimensional model was proposed for the tertiary structure of PhaZ$_{Sa}$. Additionally, PhaZ$_{Sa}$ was used to perform film degradation tests employing pure PHB and PHB copolymers containing different monomeric contents of 3-hydroxyvalerate, as well as a blend of PHB and starch that has been reported to confer improved mechanical properties compared to PHB homopolymer, and also would allow industry to reduce the production costs of this kind of biodegradable plastics [6].

### Materials and Methods

#### Chemicals

Cell culture medium reagents were from Difco (Becton Dickinson). All chemical reagents and polymers were purchased from Sigma-Aldrich.

#### Bacterial Strains, Media, and Growth Conditions

All strains used in this study are summarized in table 1. *Streptomyces ascomycinicus* nov. DSMZ 40822 [8], (formerly known as *S. hygroscopicus* subsp. *hygroscopicus* or *S. hygroscopicus* subsp. *ascomyceticus* ATCC 14891), described as a putative extracellular PHB depolymerase producer, was used as chromosomal DNA source. *Streptomyces exfoliatus* DSMZ 41695 [9,10], was used as positive control and *Streptomyces coelicolor* CECT 3243 as negative control for degradation of PHB. *Escherichia coli* KACC 21099 was used as host for subcloning experiments, *E. coli* BL21(DE3) and wild type strain *Rhodococcus* sp. T104 KACC 21099 were used as hosts for gene expression [9,11]. *E. coli* cells were grown in Luria–Bertani (LB) medium at 37°C, supplemented, when necessary, with 1 mM IPTG to induce overexpression of the cloned genes. For DNA purification, *S. ascomycinicus* cells were sporulated in solid SFM (Soya Flour Mannitol) medium and cultured aerobically under submerged conditions in S-YEME liquid medium (yeast extract/malt extract/0.5% glycine to allow dispersed growth) at 30°C and 250 rpm [12]. For PHB depolymerase extracellular activity detection, *S. ascomycinicus* spores previously collected and washed with 0.9% (wt/vol) NaCl were grown in solid basal mineral medium [13] supplemented with 1 mg/ml PHB as sole carbon source; plates were incubated for 120 hours at 30°C. *Rhodococcus* sp. T104 cells were grown in 2YT (yeast extract/bactotriptone/NaCl) medium supplemented with glucose (5 g/l) [12].

#### Plasmids, DNA Manipulation and Sequencing

All plasmids used in this study are summarized in table 1. pET28a(+) (Km$^R$, T7 promoter, lacI$^-$ (Novagen)) was used for gene expression in *E. coli* BL21(DE3). Bifunctional pEM4 (Ap$^R$, Tsr$^R$, pemE$^R$) [14] and pNV19 (Km$^R$, lacZ$^+$) [13] plasmids were used to obtain the recombinant vectors for gene expression in *Rhodococcus* sp. T104. Chromosomal DNA from *S. ascomycinicus* DSMZ 40822 was purified according to the method described elsewhere [12]. Plasmid DNA preparations, restriction endonuclease digestions, ligations, and other DNA manipulations were carried out according to standard procedures for *E. coli* [16] and *Streptomyces* [12]. DNA sequences were determined by the dideoxy-chain-termination method [17] with an automated sequencer, DNA Analyzer 3730 (Applied Biosystems).

#### Construction of Strains Expressing the fkbU Gene

The putative PHB depolymerase encoding DNA sequence fkbU, (GenBank accession number: AF235504.1) was amplified by PCR using chromosomal DNA from *S. ascomycinicus* DSMZ 40822 as template. The PCR primers were designed according to the DNA sequence of fkbU [5]. Restriction sites XhoI, XbaI, and EcoRI were included in the primers to facilitate subcloning of PCR fragments. A *Streptomyces* RBS consensus sequence (GGAGG) was included in HPEM primer. PCR amplifications were performed in a Mastercycler Personal thermocycler (Eppendorf), employing *Pfu* DNA polymerase (Promega). The PCR products were purified by High Pure PCR Product Purification Kit (Roche), digested with endonucleases XhoI and XbaI and EcoRI, and cloned into the XhoI–EcoRI site of pET28 vector, resulting to recombinant plasmid pHPEM, and into the XbaI–EcoRI site of pEM4 vector, resulting to recombinant plasmid pHPEM. Recombinant plasmid pHPEM was used to transform competent *E. coli* BL21(DE3) cells by heat shock. Recombinant pHPEM plasmid was digested with HindIII and EcoRI in order to obtain the fragments containing the ORF along with the strong *ermE* promoter of the erythromycin resistance gene from Saccharopolyspora erythraea [18,19] and transferred into the pNV19 vector, obtaining the recombinant plasmid pHPVM which was used to transform electrocompetent *Rhodococcus* sp. T104 cells, as previously described [9]. All resulting recombinant plasmids were purified by the High Pure Plasmid Isolation Kit (Roche) and sequenced to confirm the absence of mutations and the correct orientation.

#### Production and Purification of PhaZ$_{Sa}$

Recombinant *Rhodococcus* sp. T104 (pHPVM) cells were cultured aerobically under submersed conditions in 1 liter 2 x YTGY with 100 µg/ml kanamycin at 30°C for 72 h at 250 rpm orbital shaking. Ammonium sulfate was added to the cell-free culture broth at a final concentration of 0.5 M. Then, the solution was centrifuged at 10,000 g for 10 minutes and loaded onto a 100 ml Octyl FF sepharose column (GE Healthcare) equilibrated with 20 mM potassium phosphate buffer pH 7.0, 0.5 M ammonium sulfate (Buffer A) using a BioLogic LP chromatographic system (Bio-Rad). The column was extensively washed with 100 ml of the same buffer and the retained proteins were eluted with a linear decreasing gradient of 0.5 to 0 M ammonium sulfate in buffer A. The protein fractions containing PhaZ$_{Sa}$ were pooled and ammonium sulfate was added until final concentration of 0.5 M. After centrifugation at 10,000 g for 10 minutes, the supernatant was loaded onto a HiTrap Phenyl HP sepharose cartridge (GE Healthcare) (1 ml bed volume) equilibrated with buffer A. The cartridge was washed with 7 ml of the same buffer and a decreasing linear gradient from 0.5 to 0 M ammonium sulfate allowed elution of the bound proteins (puration table is shown in table S2). Purity of the fractions showing PHB depolymerase activity was analyzed by SDS-PAGE [20]. The amount of protein in the enzyme solutions was routinely determined by the Coomassie Blue method [21].

#### PHB Depolymerase Assays

Extracellular PHB depolymerase activity was measured by spot test or using the turbidimetric method, as described [9,22] but using 50 mM MES buffer pH 6. One unit (U) of PHB depolymerase activity is the amount of enzyme needed to catalyze the decrease of 0.01 absorbance units (at 600 nm) per minute in the assay conditions described.
Effects of pH on PhaZ stability were assessed by incubating 2 mg of pure PhaZ for 45 minutes at 40°C and pH values from 4 to 9 in 20 mM phosphate/citrate/borate buffer with constant ionic strength of 120 mM, adjusted by addition of different amounts of NaCl.

Effects of temperature on PhaZ stability were assessed by incubating 2 mg of pure PhaZ for 45 minutes at temperatures ranging from 25 to 70°C in water bath with gentle shaking.

After the incubations, aliquots of enzyme were drawn at different times and placed on ice bath for five minutes. Remaining activity was immediately measured by the standard turbidimetric method. All assays were performed in triplicate.

PHB depolymerase activity of pure PhaZ aliquots dialyzed against 50 mM MES buffer pH 6 was measured in the presence of different concentrations of divalent (MgCl₂, CaCl₂ and MnCl₂) and monovalent (NaCl and KCl) cation chlorides by the standard turbidimetric assay. PhaZ activity was also assessed by the standard turbidimetric assay in the presence of several concentrations of EDTA (with 2 mM MgCl₂), methyl-β-cyclodextrin, polyethylenglycol 3350, reducing agents (DTT and 2-mercaptoethanol), corn starch and detergents (SDS, Tween 20 and Triton X-100), as well as in presence of twelve different organic solvents with 10% (vol/vol) concentration.

The apparent $K_m$ and $V_{max}$ values of PhaZ for PHB hydrolysis were calculated by non-linear hyperbolic regression, using the starting values obtained by linear regression fitting of a Hanes-Woolf plot, [23,24] with the Hyper32 software (freely available at http://homepage.ntlworld.com/john.easterby/hyper32.html). These parameters were calculated using the turbidimetric activity assay with PHB, the natural substrate of PhaZ, and considering a PHB weight average molecular mass ($M_W$) of 437 kDa, provided by the manufacturer.

The release of (R)-3-hydroxybutyrate by PhaZ was measured using a spectrophotometric activity assay employing the β-

### Table 1. Bacterial strains, plasmids and constructs used in this study.

| Strain or plasmid | Relevant genotype or description | Reference |
|-------------------|---------------------------------|-----------|
| **Strains**       |                                 |           |
| *Escherichia coli* DH5α | F⁻ φ80lacZΔM15 endA1 recA1 hsdR17 (rK− mK') supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 λ− | [7]       |
| *Escherichia coli* BL21(DE3) | F− ampT gal dcm lon hsdS30 (rK− mK') (DE3 [lacU5-V5-T7 gene 1 ind1 sam7 nim5]) | Invitrogen |
| *Streptomyces ascozymicus* DSMZ 40822 | PHB depolymerase producer | [5,8]     |
| *Streptomyces exfoliatus* DSMZ 41693 | PHB and PHO depolymerase producer, used as positive control | [10]      |
| Rhodococcus sp. T104 KACC 21099 | Wild strain, suitable for gene cloning/expression with pHV19 vector | [11]      |
| **Plasmids**      |                                 |           |
| pET28             | Cloning/expression vector for *E. coli* strains. 3.4 kb | Novagen   |
| pHET              | pET28 derivative containing fkbU gene | This work |
| pEM4              | Shuttle vector E. coli/Streptomyces. Ap φ Tsn permE pUCori pWHMori 7.9 kb | [14]      |
| pHPEM             | pEM4 derivative containing fkbU gene | This work |
| pHV19             | Shuttle vector E. coli/Rhodococcus. Km φ pAL5000ori lacZ CoE1ori. 4.4 kb | [9,15]    |
| pHPNV             | pHV19 derivative containing fkbU gene under control of ermE* promoter | This work |
| pHPNV S131A/S131C/D209N/H269E/H269Q | pHPNV derivatives with mutated codons encoding catalytic amino acids | This work |

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**Figure 1.** Multiple sequence alignment of PhaZ with other PHB depolymerase sequences. Only regions with conserved amino acids are shown. All shown sequences present an identity below 58% among each other to avoid redundancy. Main catalytic amino acids are marked with black boxes. Conservation level of each position can be observed in the yellow bars below. Vertical blue lines represent gaps in the complete sequence.

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hydroxybutyrate dehydrogenase from Pseudomonas lemoignei (Sigma) [22,25]. Production of NADH as a result of (R)-3-hydroxybutyrate oxidation was measured at 340 nm after incubation of 100 μl samples for 30 minutes at 37°C with 9.5 mU of β-hydroxybutyrate dehydrogenase and 1 mM NAD+, in 75 mM Tris-HCl buffer pH 8, in a total volume of 500 μl. Reaction was stopped on ice and absorbance at 340 nm was immediately measured. Concentration of (R)-3-hydroxybutyrate was calculated by interpolating the absorbance values in a standard curve.

Degradation of PHB and PHBV films by PhaZ\textsubscript{Sa}

Thin solvent-cast films of pure PHB, PHBV with 5 or 12% (wt) 3-hydroxyvalerate and PHB-starch 7:3 (wt) proportion were prepared by dissolving 100 mg of the polymer or blend in 20 ml of chloroform with heating and vigorous stirring. The solutions were poured on glass Petri dishes and then chloroform was evaporated at room temperature overnight. The films were subsequently submerged in 20 ml of 150 mM MES buffer pH 6 with 5 mM MgCl\textsubscript{2} and 4 mM M\textsubscript{B}CD. Finally 250 μl of enzyme solution containing 30 μg of PhaZ\textsubscript{Sa} or 20 mM potassium phosphate buffer pH 7 (in the case of controls) were added to each plate. Plates were incubated at 37°C for 40 hours. Aliquots and pictures were taken at different times in order to monitor the degradation of the films; (R)-3-hydroxybutyrate concentration in the aliquots was quantified by the β-hydroxybutyrate dehydrogenase assay.

Chemical Modification of Recombinant PhaZ\textsubscript{Sa}

Modification by Phenylmethylsulfonyl fluoride (PMSF), diethyl-pyrocarbonate (DEPC), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were performed incubating 5 μg of recombinant PhaZ\textsubscript{Sa} with the appropriate amount of the suitable group-specific reagent as described [9]. PMSF solution was prepared in DMSO, DEPC in ethanol, and EDC was dissolved in 30 mM MES buffer pH 5. The concentration of DMSO or ethanol in the enzymatic assay did not exceed 0.1% (vol/vol) and was found to have no noticeable effect on the stability or activity of the enzyme. The remaining enzyme activity was determined by the standard turbidimetric assay.

Sulphhydryl groups were quantified spectrophotometrically with DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) by Ellman’s method [26]. All experiments were carried out in triplicate, and mean values are shown in the tables. Controls for enzyme activity were carried out in all experiments.

Secondary Structure Elucidation

PhaZ\textsubscript{Sa} secondary structure was predicted using several bioinformatic tools or servers: PSIPRED (freely available at bioinf.cs.ucl.ac.uk/psipred) [27], Jpred 3 (freely available at www.

Figure 2. Detection of extracellular PHB depolymerase activity in Streptomyces ascomycinicus. Basal mineral medium plates supplemented with PHB were inoculated with fresh spores of Streptomyces ascomycinicus (A), Streptomyces exfoliatus (positive control) (B) or Streptomyces coelicolor (negative control) (C), and incubated for 120 hours at 30°C. The clear halos surrounding the microbial growth indicate the degradation of PHB. doi:10.1371/journal.pone.0071699.g002

Figure 3. Analysis of recombinant PhaZ\textsubscript{Sa} purification steps. A) SDS-PAGE analysis: Lane 1: Rhodococcus T104 pHPNV culture broth; lane 2: 1 μg protein after octyl FF sepharose purification step; lane 3: 0.89 μg of purified protein after phenyl HP sepharose purification step; lane 4: Bio-Rad broad range molecular weight standards. Bands corresponding to PhaZ\textsubscript{Sa} are marked with an arrow. B) Spot test activity assay: Rhodococcus T104 pNV19 culture broth (negative control), Rhodococcus T104 pHPNV culture broth, pooled fractions after octyl FF sepharose purification step and pooled fractions after phenyl HP sepharose. doi:10.1371/journal.pone.0071699.g003
compbio.dundee.ac.uk/www-jpred) [28] and PredictProtein, a collection of prediction tools available at www.predictprotein.org. Secondary structure content of PhaZ$_{Sa}$ was also experimentally obtained by circular dichroism spectrum deconvolution, using pure PhaZ$_{Sa}$ aliquots. Spectra were recorded with 102 µg/ml PhaZ$_{Sa}$ in 5 mM potassium phosphate buffer pH 7 at 25°C between 190 and 260 nm under thermostated conditions by using a JASCO J-715 spectropolarimeter. The CD readings were expressed as the mean residue molar ellipticity (degrees $ \cdot $ cm$ ^2 $ $ \cdot $ dmol$ ^{-1} $), assuming a residue molecular mass of 104 Da according to the average amino acid molecular mass of PhaZ$_{Sa}$. Secondary structure data was obtained from CD spectra deconvolution, using the CDNN V2.1 program [29]. Thermal unfolding of PhaZ$_{Sa}$ was analyzed by CD variation at 209 nm in 25–80°C range scanned at 20°C/h.

### Identification of the PhaZ$_{Sa}$ Reaction Products

After 18 hours reaction with 1 µg PhaZ$_{Sa}$ and 300 µg/ml PHB in 20 mM MES buffer pH 6 at 40°C and 300 rpm orbital shaking, reaction products were derivatized with bromophenacyl bromide (BPB), and subsequently detected and identified by HPLC-MS as described [9,30].

### Site-directed Mutagenesis Studies

In order to ascertain the identity of the amino acids which constitute the catalytic triad of PhaZ$_{Sa}$, the candidates chosen according to multiple sequence alignment were mutated using the Quikchange II XL site-directed mutagenesis kit (Stratagene). pHPNV plasmid was used as template for mutagenic PCR, using the primers listed in table S1. The resulting mutant constructions were sequenced to confirm the mutations and then transferred to Rhodococcus sp. T104 for protein expression. Serine 131 was exchanged for Alanine (mutant S131A) or Cystein (S131C); Aspartic acid 209 was exchanged for Asparagine (D209N), and Histidine 269 was exchanged for Glutamic acid (H269E) or Glutamine (H269Q).

### Analytical Ultracentrifugation Analysis

Aliquots of pure PhaZ$_{Sa}$ with three different concentrations (65, 129 and 259 µg/ml) in 25 mM potassium phosphate pH 7 with 100 mM NaCl were subjected to sedimentation velocity experiments with a Beckman Coulter XL-I analytical ultracentrifuge equipped with absorbance optics, at 48,000 $ \times $g and 20°C, using an An-60Ti rotor and standard (12 mm optical path) double sector centerpieces of Epon-charcoal. Baseline offsets were measured afterwards at 200,000 $ \times $g. The apparent sedimentation coefficient of distribution, c(d), and sedimentation coefficient s were calculated from the sedimentation velocity data using the SEDFTT software [31].

Table 2. Structural composition percentages of PhaZ$_{Sa}$.

| Structure type | CD   | PSIPRED | Jpred 3 | PredictProtein |
|---------------|------|---------|---------|----------------|
| α-helix       | 16.8 | 12.4    | 11.5    | 12.8           |
| β-sheet       | 32.2 | 27.1    | 29.9    | 36.5           |
| Other         | 51.2 | 60.5    | 58.6    | 60.7           |

Comparison between the values deduced by CD spectrum deconvolution and those obtained by different secondary structure prediction servers. doi:10.1371/journal.pone.0071699.t002

Table 3. PHB depolymerase activity of PhaZ$_{Sa}$ in the presence of 10% (vol/vol) of different organic solvents.

| Solvent     | Relative activity (%) |
|-------------|-----------------------|
| Control     | 100                   |
| Glycerol    | 97                    |
| Ethylene glycol | 72                |
| THF         | 49                    |
| Acetonitrile | 30                    |
| Diethylene glycol | 29              |
| 2-propanol  | 29                    |
| Triethylene glycol | 24              |
| Acetone     | 18                    |
| Ethanol     | 16                    |
| DMF         | 15                    |
| DMSO        | 14                    |
| Methanol    | 9                     |

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### Results

#### Multiple Sequence Alignment of PhaZ$_{Sa}$ Amino Acid Sequence

Complete amino acid sequence of the putative PHB depolymerase PhaZ$_{Sa}$ (519 amino acids, accession number AAF86381.1) deduced from fbbU gene from Streptomyces ascomycinicus sp. nov. DSMZ 40822 [5], was analyzed and aligned with a selection of homologous PHB depolymerase sequences from different Gram-positive (Catenulispora acidiphila, Streptomyces flavogriseus, Nocardiosis dassonvillei, Thermobifida fusca and Janibacter sp.) and Gram-negative (Halangium ochraceum, Pseudomonas ldeoae and Raistoma picketii) microorganisms (Figure 1). All chosen sequences present a maximum identity below 58% among each other, in order to minimize redundancy; sequence similarity/identity matrix was calculated using MatGAT v 2.01 [32]. Multiple sequence alignment was performed with T-Coffee software, freely available at www.tcoffee.org [33]. In this alignment some highly conserved regions can be found, including the putative oxyanion hole and

**Figure 4.** Far UV circular dichroism spectrum of pure recombinant PhaZ$_{Sa}$ Inset: thermal unfolding of PhaZ$_{Sa}$ studied by CD variation at 209.0 nm in 25–80°C range. doi:10.1371/journal.pone.0071699.g004
lipase box (Gly-Leu-Ser-Ala-Gly). Putative catalytic amino acids (Ser^{131}-Asp^{209}-His^{269}) are strictly conserved, as well as the putative histidine in the oxyanion hole (His^{48}). PhaZ<sub>Sa</sub> shows the typical arrangement of extracellular dPHB depolymerases with secretion signal peptide, catalytic domain, fibronectin type III linking domain and a C-terminal substrate binding domain [34]. According to the PHA Depolymerase Engineering Database [35] available at http://www.ded.uni-stuttgart.de, PhaZ<sub>Sa</sub> belongs to the dPHAscl homologous family 11.

**Detection of PHB Depolymerase Activity in S. ascomycinicus**

The putative gene fkbU encoding PHB depolymerase from S. ascomycinicus was previously described [5], however no activity has been reported so far. In order to determine the extracellular PHB depolymerase activity of S. ascomycinicus, fresh spores were grown on solid basal mineral medium with PHB as sole carbon source, as described in materials and methods section. After 120 hours of incubation at 30°C a clear zone around the streak could be observed (Figure 2) demonstrating that S. ascomycinicus is able to degrade extracellular denatured PHB. This result shows the ability of this microorganism to produce an extracellular dPHB depolymerase.

**Table 4. Effect of different concentrations of several reagents on the PHB depolymerase activity of PhaZ<sub>Sa</sub>.**

| Reagent                  | Concentration | Relative activity (%) |
|--------------------------|---------------|-----------------------|
| Control                  | ~             | 100                   |
| EDTA (+2 mM MgCl<sub>2</sub>) | 1 mM         | 88                    |
|                          | 10 mM         | 18                    |
|                          | 20 mM         | 14                    |
| Methyl-β-cyclodextrin    | 1 mM          | 126                   |
|                          | 5 mM          | 146                   |
|                          | 10 mM         | 146                   |
| PEG 3350                 | 1 mM          | 112                   |
|                          | 5 mM          | 106                   |
|                          | 10 mM         | 98                    |
| DTT                      | 1 mM          | 90                    |
|                          | 5 mM          | 44                    |
|                          | 10 mM         | 27                    |
| 2-Mercaptoethanol        | 1 mM          | 100                   |
|                          | 5 mM          | 97                    |
|                          | 10 mM         | 91                    |
| Corn starch              | 50 µg/ml      | 103                   |
|                          | 200 µg/ml     | 99                    |
|                          | 400 µg/ml     | 98                    |
|                          | 1000 µg/ml    | 104                   |
| SDS                      | 0.1%          | Not detected          |
|                          | 1%            |                       |
| Tween-20                 | 0.1%          | 1%                    |
|                          | 1%            |                       |
| Triton X-100             | 0.1%          | 1%                    |

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**Figure 5. Activity of dialyzed PhaZ<sub>Sa</sub> in presence of different concentrations of Magnesium (▲), Calcium (●), Manganese (■), Sodium (←) or Potassium (→) chlorides.**

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**Construction of Strains Expressing the fkbU Gene and Detection of PhaZ<sub>Sa</sub>**

The putative PHB depolymerase-coding gene fkbU was amplified by PCR and cloned into different vectors with the aim of expressing an active form of this enzyme. The pET28 derivative plasmid pHPET was used to clone and overexpress fkbU in E. coli BL21(DE3), but after inducing its expression with IPTG this recombinant strain was not able to produce an active form of PhaZ<sub>Sa</sub> leading to the formation of insoluble inclusion bodies of PhaZ<sub>Sa</sub>.

In order to express fkbU gene in a homologous host, pHPEM plasmid was digested, this pEM4 derivative plasmid contains the strong and constitutive ermE* promoter upstream the fkbU ORF, enabling the expression of this gene in gram positive hosts.

pHPEM was digested, and the HindIII-EcoRI fragment was cloned into pNV19, giving rise to the expression plasmid pHNV, which was used to transform electrocompetent Rhodococcus sp. T104 cells. The recombinant Rhodococcus sp. T104 pHPNV cells were grown in 2xYTG medium and the PHB depolymerase activity of intracellular cell extract, insoluble cell debris and cell-free culture broth was assayed by the spot test method (Figure S1–B). Most of the activity was located in the fermentation broth, and a protein band of around 48 kDa corresponding to PhaZ<sub>Sa</sub> was detected by SDS-PAGE analysis of the fermentation broth, demonstrating that this enzyme was being secreted to the extracellular medium (Figure S1–A).

The N-terminal sequence of this extracellular protein obtained by Edman sequential degradation [36] was Ala-Ala-Gly-Leu-Ala-Lys-Pro-Gly-Leu-Thr-Lys-Ala-Asp-Leu-Thr-Glu-Val. Therefore, mature PhaZ<sub>Sa</sub> consists of 461 amino acids, with a theoretical mass of 48 kDa. It is noteworthy that the signal peptide is correctly recognized and cleaved by Rhodococcus sp. T104.

**Purification and Analysis of Recombinant PhaZ<sub>Sa</sub>**

Recombinant PhaZ<sub>Sa</sub> produced by Rhodococcus sp. T104 pHPNV was purified by only two consecutive hydrophobic interaction chromatographic (HIC) steps (Figure 3) MALDI-TOF analysis of pure enzyme showed a main peak of 48.4 kDa which fits the theoretical value deduced from the sequence (48.0 kDa), and several minor peaks corresponding to different protein aggregation states (Figure S2), the peak of 24.2 kDa...
corresponds to the same form with double electric charge. In addition, aliquots of pure PhaZ\textsubscript{Sa} with three different concentrations (65, 129 and 269 mg/ml) were subjected to sedimentation velocity analysis to ascertain the expected monomeric nature of this enzyme. The experiments with all these preparations showed a single peak with an apparent molecular mass between 40 and 46 kDa, corresponding to the active monomeric form of PhaZ\textsubscript{Sa}, and also showed characteristic values of a globular shaped protein.

The secondary structure content of PhaZ\textsubscript{Sa} was experimentally calculated by CD spectrum deconvolution (Figure 4), and was also predicted according to the amino acid sequence with the online servers Jpred 3, PSIPRED and PredictProtein. Experimentally obtained structural percentages differ slightly from the predicted values. However, all the programs employed for the prediction of secondary structure provide similar values, pointing to an alpha-beta structure.

Thermal denaturation of PhaZ\textsubscript{Sa} was also monitored by CD, measuring the change of ellipticity at 209 nm in a temperature range from 25 to 80°C (Figure 4 inset), showing a single melting temperature \(T_m\) of 58.4°C.

**Biochemical Characterization of Recombinant PhaZ\textsubscript{Sa}**

Recombinant PhaZ\textsubscript{Sa} was functionally characterized, showing highest activity at pH 6 and 45°C. It showed full stability at the entire range of pH values (4 to 9) and temperatures up to 50°C.

In addition, the effect of different cations on activity of dialyzed PhaZ\textsubscript{Sa} was also studied, (Figure 5), revealing a strong dependence on the presence of divalent cations such as magnesium, calcium or manganese. Monovalent cations did not exert evident effects on activity of PhaZ\textsubscript{Sa} at concentrations up to 30 mM, although the increase of ionic strength gradually inhibited the PHB depolymerase activity leading to its complete inactivation at 2.5 M NaCl.

The activity of PhaZ\textsubscript{Sa} was also assayed in the presence of several organic solvents at 10% (vol/vol) (Table 3), as well as in the presence of different additives (Table 4). It is remarkable the slight effect of reducing agents on PhaZ\textsubscript{Sa} activity compared to other PHB depolymerases, [10,37,38,39] and the notable inhibitory effect of reducing agents on PhaZ\textsubscript{Sa} activity compared to other PHB depolymerases, [10,37,38,39] and the notable inhibitory

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**Figure 6. Identification of PhaZ\textsubscript{Sa} reaction products by HPLC-MS.** The insets on the spectrum show the structure of the BPB derivatized compounds and their corresponding molecular weights. Double picks with similar intensity and a mass difference of 2 Da correspond to bromine-containing molecules, due to the isotopic abundance of this element (50.69% for Br\textsuperscript{79} and 49.31% for Br\textsuperscript{81}).

![HPLC-MS spectrum](image-url)

\(\text{Mw} = 301.14\)

\(\text{Mw} = 387.23\)

![Derivatized monomer](image-url)

![Derivatized dimer](image-url)
Figure 7. Degradation of different PHB films by PhaZ$_{Sa}$. Solvent cast films of PHB, PHBV 5%, PHBV 12% and PHB-starch 7:3 were incubated with 30 µg of PhaZ$_{Sa}$ at 37°C without shaking. Control films were incubated in the same conditions without enzyme. Pictures were taken at different times.

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PHB Depolymerase from Streptomyces ascomycinicus

Figure 8. Release of (R)-3-hydroxybutyrate from different PHB films catalyzed by PhaZ 

Table 5. Residual PHB depolymerase activity after chemical modification of PhaZ 

Discussion

PHB depolymerases from many Gram-negative bacteria have been purified and are well-characterized in contrast to the limited knowledge of PHB depolymerases from Gram-positive bacteria. With this work, Streptomyces ascomycinicus has been proven to produce an extracellular PHB depolymerase (PhaZ 

Identification of Catalytic Amino Acids of PhaZ 

The presence of a catalytic triad involving a serine, a histidine and an aspartic acid was clearly suggested by previously described PHB depolymerases with known tertiary structure or catalytic mechanisms [34]. This fact is also supported by the clearly conserved amino acids found when performing a multiple sequence alignment (Figure 1). With the purpose of identifying the essential catalytic residues of PhaZ 

Degradation of PHB and PHBV Films by PhaZ 

The degradation by recombinant PhaZ 

Identification of Catalytic Amino Acids of PhaZ 

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Effect of EDTA, which was expected due to the high dependence of PhaZ 

Furthermore, the kinetic parameters of recombinant PhaZ 

Finally, the products released after PHB hydrolysis catalyzed by recombinant PhaZ 

Table 5. Residual PHB depolymerase activity after chemical modification of PhaZ 

Discussion

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denatured PHB, in contrast to the hypothesis proposed by Wu et al. (5); in that work \textit{fkbU} gene was proposed to encode a PHB depolymerase that could be responsible for maintaining the intracellular levels of butyryl-CoA during the stationary phase of growth, using the accumulated PHB as the carbon storage for this biosynthetic pathway. Since \textit{fkbU} encodes an extracellular depolymerase, it cannot be directly related to the FK520 synthetic pathway, and despite its adjacent location, this gene should not be considered as a member of this cluster.

In addition, \textit{fkbU} gene, encoding PhaZ\textsubscript{Sa}, has been cloned into pNV19 vector, expressed under control of the strong and constitutive \textit{ermE}\textsuperscript{p} promoter and secreted to extracellular medium.

**Figure 9.** Three-dimensional structure model of PhaZ\textsubscript{Sa}. **A** Complete model. This structure was modeled by the Phyre2 server (Protein Homology/analogY Recognition Engine V 2.0) [43] available at www.sbg.bio.ic.ac.uk/phyre2, which combines homology and \textit{ab initio} modeling algorithms. These figures were rendered using Discovery Studio 3.1 software (Accelrys software Inc.) **B** Detail of the active site, catalytic amino acids (Ser\textsuperscript{131}, Asp\textsuperscript{209}, His\textsuperscript{269}) and oxyanion hole histidine (His\textsuperscript{48}).

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In this work, the identity of the catalytic triad has been confirmed according to the predicted amino acid sequence of PhaZ<sub>sa</sub> genes, such as the <i>myces</i> the PHB depolymerase from "secretory signal peptide, same length as reported for binding site (GGACG) located seven bp upstream, which would the predicted sequence) [12], preceded by a potential ribosome alternative translation start codon (GTG, residue number 33 in the catalytic triad (Ser131-Asp209-His48, the putative oxyanion hole histidine that stabilizes the catalytic amino acids can be observed, as well as the proximity of substrates. In this model the spatial arrangement of the domain type 1, which might play an important role in the establishment of hydrophilic, hydrophobic or electrostatic interactions with the polymeric substrate [41,42]. The catalytic triad (Ser<sup>131</sup>-Asp<sup>209</sup>-His<sup>260</sup>) which was previously postulated by multiple sequence alignment [34], is also present, as well as a lipase box pentapeptide (Gly-Leu-Ser-Ala-Gly), which includes the catalytic serine (Ser<sup>131</sup>). In this work, the identity of the catalytic triad has been confirmed by chemical modification and site-directed mutagenesis of specific amino acid residues.

The homology modeling of the tertiary structure of PhaZ<sub>sa</sub> using Phyre2 server [43] (Figure 9), suggested that this enzyme lacks a lid domain similarly to other described extracellular PHB depolymerases [44]. In this model the spatial arrangement of the catalytic amino acids can be observed, as well as the proximity of His<sup>48</sup>, the putative oxyanion hole histidine that stabilizes the tetrahedral transition state. The structure of the substrate binding domain has not been reliably established yet, due to the lack of homologous proteins with known three-dimensional folding, however, <i>ab initio</i> modeling of this domain provides an outline of its structure, which would comprise coiled regions and several β-sheets.

The extracellular expression of PhaZ<sub>sa</sub> has facilitated its purification from the fermentation broth by only two hydrophobic interaction chromatography (HIC) steps. A good level of separation is achieved in the first HIC step (octyl sepharose), nevertheless a second HIC step (butyl HP sepharose) is required to reach a higher degree of purity and concentration, in order to characterize this enzyme.

Regarding the optimum reaction conditions, PhaZ<sub>sa</sub> shows highest activity at 45°C, which is a very common optimum temperature among PHB depolymerases from soil microorganisms. However, the optimum pH of this enzyme is 6, this acidic pH is quite unusual among fungal PHB depolymerases, but it is very uncommon in bacterial PHB depolymerases. Only two enzymes of this group, both from the Gram-negative <i>Ralstonia picketti</i> have been reported to show acidic optimum pH values of 5.5 and 6 [45]. Hence, to the best of our knowledge PhaZ<sub>sa</sub> is the first reported PHB depolymerase from a Gram-positive bacterium with an acidic pH optimum. This singular feature makes PhaZ<sub>sa</sub> an interesting biocatalyst, suitable for PHB-derived residues degradation in acidic media.

Hydrolytic activity of PhaZ<sub>sa</sub> is strongly enhanced (over 300%) in presence of low concentrations of divalent cations such as calcium, magnesium or zinc. However, PhaZ<sub>sa</sub> remains slightly active even when it is thoroughly dialyzed in the presence of EDTA. These results indicate that PhaZ<sub>sa</sub> shows a non-essential activation by divalent cations. Likewise, methyl-β-cyclodextrin (MBCD) exerts an important activating effect (up to 146%) at concentrations as low as 5 mM. This effect was previously described for PHB depolymerase from <i>Streptomyces exfoliatus</i> [9], but it remains unclear whether the effect of MBCD is produced directly on the enzyme, on the polymeric substrate (e.g. facilitating the access to PHB particles), or even associating with the reaction products, enhancing its solubility in the medium or shifting the equilibrium of the reaction towards the products.

Regarding kinetic parameters of PhaZ<sub>sa</sub>, it is a difficult task to compare our data with those previously reported from other PHB depolymerases, since different measurement methods were employed in literature; moreover, the polymeric nature of the substrate is a problem when substrate concentration should be expressed in molarity terms. In this work, this problem was overcome by using the weight average molecular mass/ <i>M<sub>W</sub></i> of the PHB, estimated by the manufacturer (437,000 g/mol), to calculate the moles of polymer degraded in activity assays. Using this approach, PhaZ<sub>sa</sub> parameters ( <i>K<sub>m</sub></i> 0.61 μM or 268 μg/ml and <i>V<sub>max</sub></i> 9,797 U/mg) can only be compared to those of PhaZSex from <i>S. exfoliatus</i> [9], which were obtained by the same procedure.

In this sense, PhaZ<sub>sa</sub> shows a 2-fold higher <i>K<sub>m</sub></i> Value and 3-fold higher <i>V<sub>max</sub></i> value than PhaZSex, what means a better catalytic efficiency (1.75-fold higher). In general terms, when comparing <i>K<sub>m</sub></i> Value of PhaZ<sub>sa</sub> with those previously reported for other PHB depolymerases, several enzymes with higher affinity (lower <i>K<sub>m</sub></i>) for PHB were found, such as PHB depolymerases from <i>Leptothrix sp.</i> HS [46], <i>Thermus thermophilus</i> HB8 [47] or <i>Pseudomonas</i> <i>lilacinus</i> D218 [48]. However PHB depolymerases with acidic pH optima (predominantly fungal) generally had a higher <i>K<sub>m</sub></i> Value than PhaZ<sub>sa</sub> ranging from 0.69 to 14 mg/ml [49,50,51,52]. This result supports PhaZ<sub>sa</sub> as a suitable biocatalyst for PHB degradation in slightly acidic conditions.

Finally, PhaZ<sub>sa</sub> has been successfully employed for degradation of PHB solvent cast films. Two PHBV copolymers with 5 or 12% hydroxyvalerate have been tested, as well as a blend of PHB and starch in 7:3 (wt/wt) proportion. PHBV polymers containing both hydroxybutyrate and hydroxyvalerate monomers have many important advantages regarding their thermomechanical properties in comparison to the PHB homopolymer: they are more ductile, flexible, less crystalline and present better tensile strength and lower melting points [53,54]. Likewise, blends of PHB with starch has been reported to confer improved mechanical properties as well, giving these films more tensile strength and increasing the extension needed to break them. Additionally, blending virgin PHB with starch would reduce considerably the high production costs of PHB without affecting its biodegradability [6]. In this work, PhaZ<sub>sa</sub> has been proven to degrade all types of PHB films tested (Figure 7); the presence of starch or a moderate amount of incubation. Production of (R)-3-hydroxybutyrate by PhaZ<sub>sa</sub> under mild conditions is an interesting feature, since this
enantiomerically pure hydroxyalkanoic acid has a wide range of industrial and medical applications, serving as building block for synthesis of many fine chemicals or tailor-made plastics with controlled properties [35,36].

To conclude, PhaZSa has very interesting properties for its industrial implementation, since it is an extracellular hydrolytic enzyme, stable up to 50°C and within a broad pH range (from 4 to 9), and is able to degrade different PHB copolymers. Moreover, PhaZSa can resist freezing and hofpiliation with no need to add any protective additive, and its PHB depolymerase activity can be recovered even after denaturation with 6 M guanidinium chloride. These features make PhaZSa a promising candidate for the development of a robust biocatalyst able to degrade PHB-derived residues present in urban solid wastes.

Supporting Information

Figure S1 Production of recombinant PhaZSa in Rhodococcus T104. A) SDS-PAGE analysis; Lanes 1–3: Rhodococcus T104 pNV19 (control strain). Lanes 5–7: Rhodococcus T104 pHPNV (fkbU clone). Lanes 1 and 5: culture broths, lanes 2 and 6: cell extracts, lanes 3 and 7: cellular debris, lane 4: Sigma wide range molecular weight standards. A protein band of about 50 kDa not present in the control strain is marked with an arrow in the culture broth of pHPNV clone. B) Spot test assay activity of different fractions of these strains. Wells are marked with their corresponding lane number in SDS-PAGE analysis from panel A.

Figure S2 MALDI-TOF mass spectrum of pure recombinant PhaZSa expressed by Rhodococcus T104 pHPNV.

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Figure S3 Site-directed mutagenesis of the catalytic triad residues of PhaZSa. A) SDS-PAGE of the fermentation broths from the Rhodococcus T104 strains carrying the mutant pHPNV plasmids. Lane 1: S131A; lane 2: S131C; lane 3: D209N; lane 4: H269E; lane 5: H269Q; lane 6: pNV19 negative control; lane 7: pHPNV positive control; lane 8: Bio-Rad broad range molecular weight standards. Band corresponding to PhaZSa or its mutant forms is marked with an arrow. B) Spot test PHB depolymerase activity assay of the fermentation broths containing the mutant forms of PhaZSa, the native PhaZSa and the negative control pNV19.

Table S1 PCR primers used in this work for cloning of fkbU and site-directed mutagenesis of PhaZSa.

Table S2 Purification table of PhaZSa.

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Author Contributions

Conceived and designed the experiments: JGH DH MA IM. Performed the experiments: JGH DH. Analyzed the data: JGH MA IM. Wrote the paper: JGH MA IM.
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