Biological basis of enzyme-catalyzed polyester degradation: 59 C-terminal amino acids of poly(3-hydroxybutyrate) (PHB) depolymerase A from *Pseudomonas lemoignei* are sufficient for PHB binding

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SUMMARY: Biodegradable polyesters such as biologically produced poly[(*R*)-3-hydroxybutyric acid], (PHB) other polyhydroxyalkanoic acids and related chemosynthetic polyesters have attracted industrial interest, and bacterial produced PHB is commercially available since 1990. A large variety of polyester degrading microorganisms have been found to be present in environment. The microorganisms decompose the polymers by secretion of extracellular polyester depolymerases and utilize low molecular weight degradation products for growth. Microbial polyester depolymerases have the unique property to be water soluble and to be able to bind specifically to polyester surfaces. The objective of this contribution is a functional analysis of a bacterial PHB depolymerase polyester binding domain. In addition, a detailed summary of the present knowledge on the biochemistry of enzymatic polyester hydrolysis is provided.

Introduction

Polyhydroxyalkanoic acids (PHA) are bacterial storage compounds of variable monomeric composition that are synthesized from renewable resources and are deposited intracellularly in form of inclusion bodies ("granules") which might amount up to 90% of the cellular dry weight. For reviews see [1-7]. Poly[(*R*)-3-hydroxybutyric acid] (PHB) was the first of the PHA discovered by Lemoigne in 1925 and is the most abundant polyester in bacteria. Because PHA have thermoplastic properties and are biodegradable to water and carbon dioxide they have attracted industrial interest. Bacteriologically procduced PHB and its copolymers with 3-hydroxyvaleric acid are commercially available since 1990.

Although most PHA are water-insoluble, hydrophobic and partially crystalline polymers they can be degraded by a large variety of microorganisms. PHA-degrading microorganisms can be identified and isolated on solid agar media which contain the polymer as a sole source of carbon and energy. Usually the polymer is applied as a milky suspension of

 CCC 1022-1360/98/$ 10.00

granules in a top agar layer onto a bottom agar that provides the other nutrients required for growth. Microorganisms secrete specific PHA depolymerases which hydrolyze the polymer extracellularly to water-soluble products thus creating a clearing zone around the depolymerase-producing microbial colony.

The first PHA-degrading microorganisms were isolated in 1963 and included bacteria of the genera *Bacillus*, *Pseudomonas* and *Streptomyces* [8]. In this early study the extracellular location of the hydrolyzing enzyme (PHB depolymerase) and the identification of 3-hydroxybutyric acid as product of enzymatic hydrolysis was already shown. Two years later Delafield et al. [9] isolated and characterized 16 PHB-degrading bacteria most of which were identified to belong to a new species named *Pseudomonas lemoignei* (in honor to Maurice Lemoigne, see above). During the last 15 years many PHA degrading bacteria and fungi have been isolated and many of their extracellular PHA depolymerases have been characterized (recently reviewed [10, 11]). As far as it has been tested PHA depolymerases share several characteristics: (i) stability at various conditions such as pH, temperature, ionic strength etc. is very high, (ii) M_r is relatively small (< 100 kDa), and most depolymerases consist of only one polypeptide; (iii) PHA depolymerases do not bind to anion exchangers such as DEAE (at neutral pH) but have a pronounced affinity to hydrophobic materials; (iv) the pH optimum is in the alkaline range (7.5 to 9.8). Only the depolymerases of *Ralstonia pickettii* and of *Penicillium funiculosum* have pH optima at 5.5 or 6.0, respectively. (v) Most PHA depolymerases are inhibited by serine hydrolase inhibitors such as diisopropylfluoryl-phosphate (DFP) or acylsulfonylcompounds which bind covalently to the active site serine of serine hydrolases [12]. (vi) The substrate specificity of PHA depolymerases is high: all currently known PHA depolymerases are specific for either short-chain-length PHA (PHA_{SCL}, 3 to 5 carbon atoms per monomer) or medium-chain-length PHA (PHA_{MCL}, 6 to 14 carbon atoms per monomer), and none of the depolymerases has any lipase or protease activity [10, 13 - 15]. While most PHA-degrading bacteria apparently contain only one depolymerase, *P. lemoignei* has at least six which differ slightly in their biochemical properties [10, 11, 16].

Hydrolysis of end-labeled 3-hydroxybutyrate oligomers by purified *Alcaligenes faecalis* PHB depolymerase showed that the enzyme mainly cleaved the second and third ester linkage from the hydroxyl terminus [17]. However, since the enzyme also hydrolyzes cyclic oligomers, the *A. faecalis* depolymerase has *endo*-hydrolase activity in addition to *exo*-hydrolase activity [18]. Experiments using culture fluid of *Acidovorax delafieldii* are in agreement with the presence of *endo*-hydrolase activity of *A. delafieldii* PHB depolymerase [19]. Depending on the depolymerase the hydrolysis products are monomers (*Comamonas* sp. PHB depolymerase; [14]) or monomers and dimers (PHO hydrolysis by *P. fluorescens*; [15]) or a mixture of oligomers (mono- to trimers) as in the case of the *A. faecalis* and *P. lemoignei* [17,] depolymerases. In a second step the oligomers are hydrolyzed to monomers by oligomer hydrolases [20 - 22].

All PHA depolymerases analyzed so far are specific for polymers consisting of monomers in the (*R*)-configuration. P[(*S*)-3HB)] is not degraded by PHB depolymerases [23]. However, P([(*R,S*) 3HB] with isotactic diad fractions between 0.68 - 0.92 showed increased erosion when compared with biological produced P([(*R*) 3HB]. In that case a larger fraction of oligomers (dimers, trimers and tetramers) was observed. The degradation rate of atactic P([(*R,S*) 3HB] was moderately slower [23] or drastically slower [24-26] than that of P([(*R*) 3HB]. Apparently, bacterial PHA depolymerases are not able to hydrolyze ester bonds between monomers of the (*S*)-configuration.

Besides the substrate specificity of PHA depolymerases the polymer itself highly influences the biodegradability. The most important factors are: (i) stereoregularity of the polymer (see above), (ii) crystallinity of the polymer: the degradability of a polyester decreases as the overall crystallinity or its crystallinity phase perfection increases [27 - 30]. However, even a 100% crystallinity did not prevent degradation. Degradation of single crystals by purified *P. lemoignei* PHB depolymerase A started at the edges of the crystals and resulted in needlelike, splintered intermediates [31]. Recently, these results were confirmed for *Pseudomonas stutzeri* PHB depolymerase by Doi and coworkers [32], (iii) molecular weight of the polymer: low molecular weight polymers generally are faster degraded than high molecular weight polyesters, (iv) monomeric composition of PHA: e. g. the rate of degradation by purified *A. faecalis* PHB depolymerase was slower for PHB than for copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate [33]. However, similar experiments with other copolymers (41% 3HV content) and results obtained with two of the PHA depolymerases of *P. lemoignei* as well as *in situ* studies with compost soils showed the reverse order [34 - 36]. Therefore, degradation of PHA in complex ecosystems can not be predicted from laboratory experiments using pure cultures and/or purified enzymes alone. In addition, the position of the hydroxy-function and thus the length of the side chain highly influences the rate of degradation: polymers of ω-hydroxyfatty acids, e. g. polycaprolactone or poly(4-hydroxybutyrate) are good substrates for PHA depolymerases and lipases and thus are likely to be more susceptible to biodegradation in complex ecosystems [13, 37, 38].

Besides PHB and other well known PHA synthesized by bacteria additional biodegradable polyesters have been described such as: (i) cutin: a mixture of long-chain-length ω-hydroxyfatty acids as the major component of the cuticle of plant leaves [39 - 41], (ii) chemically produced polycaprolactone (PCL) [42, 43]. (iii) poly(malate): a water-soluble polyanion produced by slime molds such as *Physarum polycephalum* [44, 45], and (iv) polyesters of 2-hydroxyacids such as polylactides and copolymers of lactic acid and glycolic acid. [13, 46, 47].

12 bacterial PHA depolymerase genes (*phaZ*) have been cloned and analyzed since 1989 [10, 11, 48]. All PHB depolymerase proteins have a composite domain structure (Fig. 1) and consist of: (i) a 25 to 37 amino acid long signal peptide, which is cleaved off during the passage across the cytoplasmic membrane, (ii) a large catalytic domain at the N-terminus, (iii)

a putative C-terminal substrate-binding domain, and (iv) a linking domain, which linkes the catalytic domain and the substrate-binding domain. Three strictly conserved amino acids, namely serine, aspartate, and histidine, constitute the active center of the catalytic domain. The serine is part of the lipase-box pentapeptide Gly-Xaa$_1$-Ser-Xaa$_2$-Gly which has been found in all known serine hydrolases such as lipases, esterases, and serine-proteases [49]: the free electron pair of the hydroxyl oxygen atom of the serine side chain is the nucleophil that attacks the ester bond. The nucleophilic capacity of the hydroxyl oxygen is enhanced by a hydrogen bond to the imidazol ring of the histidine. The positive charge of the latter is stabilized by the carboxyl group of the aspartate. In contrast to most bacterial lipases, in which Xaa$_1$ of the lipase-box is a histidine, a leucine is found in all PHA$_{SCL}$ depolymerases. It is likely that the hydrophobic side-chain of leucine is more compatible with the hydrophobic polymer than a positively charged imidazol ring. A transient tetrahedral intermediate is formed and is stabilized by a main chain peptide bond around a second strictly conserved histidine (oxyanion hole). The intermediate is subsequently hydrolyzed by a water molecule resulting in the release of the alcohol and the fatty acid as products.

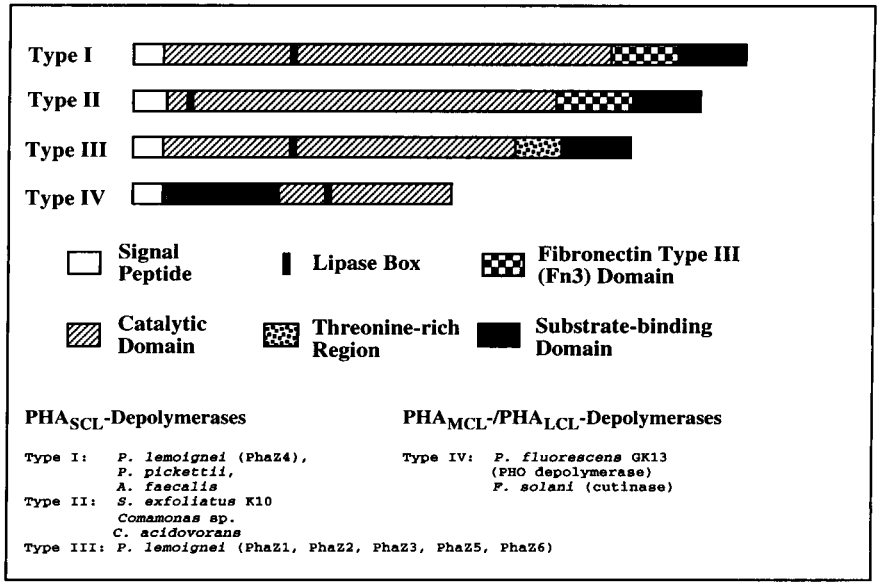

Fig. 1: Domain structure and subclasses of bacterial PHA depolymerases
An interpretation of the amino acid sequences of microbial PHA depolymerases is shown (from [10, 53], modified).

PHB depolymerases specifically bind to PHB-granules. This ability is lost in truncated proteins which lack a 6 kDa peptide fragment obtained after partial digestion with trypsin [50], and these truncated proteins do not hydrolyze PHB. However, the catalytic domain is unaffected since the activity with oligomers or artificial water-soluble substrates such as paranitrophenylesters is unchanged. These results are in agreement with findings of Doi et al. [38, 51] who proposed a two step reaction for enzymatic polyester hydrolysis, namely adsorption of the depolymerase to the polymer and subsequent hydrolysis. Analysis of PHA depolymerase genes suggested rather the C-terminal part (≈50 amino acids) to constitute the PHB binding domain than the N-terminal part. This assumption was confirmed by construction of deletion proteins which lack 55 C-terminal amino acids of a PHB depolymerasé [52]. The truncated protein had lost the PHB binding ability but was still able to hydrolyse artificial water-soluble *p*-nitrophenylbutyrate. However, these experiments could not exclude the possibility of additional essential parts of the protein necessary for PHB binding. Therefore, we constructed a fusion protein consisting of the soluble maltose-binding protein MalE of *Escherichia coli* and 59 C-terminal amino acids of the PHB depolymerase PhaZ5 and analyzed the PHB binding abililty of the purified fusion protein.

Material and methods

Bacterial strains and plasmids

The bacterial strains and the plasmids used in this study as well as their relevant characteristics are listed in Table 1.

Tab. 1: Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic	Reference
Escherichia coli TB1	F⁻ *ara D(lac pro AB) rpsL* (Str^r) [*f80* d*lacD(lacZ)M15*] *thi* *hsdR* (r_K^- m_K^+)	New England Biolabs, Beverly, MA, USA
pMAL-p2	expression of MalE in periplasm	New England Biolabs, Beverly, MA, USA
pSN1286	pMAL-p2::*phaZ5*$_{Ple}$ (coding for amino acid 349-407 of PhaZ5) fusion I: MalE-substrate-binding domain	this study
pSN1284	pMAL-p2::*phaZ5*$_{Ple}$ (coding for amino acid 308-407) fusion II: MalE-threonine-rich region + substrate-binding domain	this study

Construction of MalE-fusion proteins

In order to purify MalE-fusion proteins from recombinant *Escherichia coli*, different regions of the PHA depolymerase structural gene *phaZ5* from *P. lemoignei* (coding for C-terminal parts of PHB depolymerase A) were fused to *malE* of the expression vector pMAL-p2 using a protein fusion and purification kit (version 3.02, New England Biolabs, Beverly, MA, USA). Three synthetic oligonucleotides (P1: 5'-AGA ATT CAA AGC GCT GGC AGC GGC GGT GGA ACG A- 3', P2: 5'-AGA ATT CAA AGC GCT GCG ACT TGC TAC ACC TCC-3' and P3: 5'-CGC TAA GCT TGA GCT CCT TAG TTA CTT AG-3') served as primers for PCR-mediated fusion (*Thermus aquaticus* DNA polymerase, MBI Fermentas) of *phaZ5*-coding regions to *malE* of pMAL-p2. The nucleotide sequence of all PCR-amplified DNA was confirmed by DNA sequencing. The resulting constructs (see Table 1) were transformed to *E. coli* TB1. Due to the signal sequence at the 5'-end of *malE* the MalE-fusion proteins were directed to the periplasmic space of recombinant *E. coli*. Resulting MalE-fusion protein I included the last 59 amino acids of PhaZ5 (49,380 Da) and MBP-fusion protein II (52,959 Da) included the substrate-binding domain plus linker region of PhaZ5 (100 amino acids), respectively.

Expression and purification of MalE-fusion proteins

Recombinant *E. coli* strains harboring (i) pSN1284, (ii) pSN1286, and (iii) pMAL-p2 without insert (control) were grown in 80 ml medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 2 g/l glucose, 100 µg/ml ampicillin) after inoculation with 0.01 vol. of a seed culture on the same medium at 30°C. At an OD_{600} of 0.4, IPTG (0.3 mM) was added, and growth was allowed for additional 2 h. The cells were harvested by centrifugation, and the fusion proteins were purified from the periplasm fraction by affinity chromatography on amylose columns (1.5 ml bed volume) using the Protein Fusion & Purification System (New England Biolabs, Beverly, MA, USA) under the conditions described by the manufacturer.

Binding studies

About 1 µg of the protein of interest was incubated in 3 ml 50 mM Tris-HCl, pH 8.0 containing 1 mM $CaCl_2$ and 180 µg sodium hypochlorite-purified denatured PHB granules in glass tubes for 10 min at room temperature. The granules were collected by centrifugation (5 min. 3,500 *g*) and washed once. The pellet was resuspended in sodium-dodecylsulfate (SDS)-denaturation solution and solubilized proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The first supernatant after about 15-fold concentration (Centricon, Pall-Filtron, Germany) was also analyzed by SDS-PAGE.

Results and discussion

The C-terminal amino acid sequences (about 55 amino acids) of PHB depolymerases have significant homology to each other but no detectable homologies to any other amino acid sequences [52]. The identity values of the C-termini for 9 PHB depolymerases are in the range of 51 to 92%. Only the PHB depolymerases PhaZ1 and PhaZ4 of *P. lemoignei* have lower values (16-33% and 16-26%, respectively but 50% identity to each other; Table 2 and Fig. 2).

Tab. 2: Similarity and identity values of PHB depolymerase substrate-binding domains

	PhaZ1	PhaZ2	PhaZ3	PhaZ4	PhaZ5	PhaZ6	Afa	Cac	Csp	Rpi	Sex
PhaZ1		35.2	34.6	63.8	37.7	34.6	32.1	40.4	46.7	34.0	41.3
PhaZ2	**24.1**		71.7	34.0	79.3	63.8	64.4	72.7	64.4	62.7	66.0
PhaZ3	**20.0**	**68.3**		26.8	77.6	69.0	61.0	64.4	64.4	59.3	67.9
PhaZ4	**50.0**	**16.1**	**19.6**		38.2	37.0	43.1	37.5	39.3	42.0	29.8
PhaZ5	**26.4**	**69.0**	**67.2**	**23.6**		75.4	78.0	71.2	74.6	78.0	70.7
PhaZ6	**21.8**	**60.3**	**62.1**	**20.4**	**72.0**		69.0	69.0	74.1	69.0	65.5
Afa	**16.1**	**56.0**	**50.9**	**25.5**	**67.8**	**63.8**		68.3	73.3	96.7	72.0
Cac	**25.5**	**56.0**	**54.2**	**19.6**	**69.5**	**63.8**	**63.3**		80.0	68.3	70.2
Csp	**28.9**	**57.6**	**54.2**	**19.6**	**67.8**	**69.0**	**63.3**	**75.0**		75.0	70.2
Rpi	**14.3**	**57.6**	**50.9**	**26.0**	**71.2**	**67.2**	**91.7**	**68.3**	**70.0**		72.0
Sex	**32.6**	**51.8**	**51.8**	**22.8**	**60.3**	**52.7**	**56.1**	**71.4**	**56.1**	**59.7**	

The last 60 amino acids of PHB depolymerases are compared to each other.
Similarity and identity scores (%) of C-terminal amino acids of PHB depolymerases are given in normal and bold type letters, respectively. The origins of the depolymerase substrate binding domains are from *Pseudomonas lemoignei* (PhaZ1 - PhaZ6), *Alcaligenes faecalis* (Afa), *Comamonas acidovorans* (Cac), *Comamonas* sp. (Csp), *Ralstonia pickettii* (Rpi), *Streptomyces exfoliatus* (Sex).

In order to analyze the function of these carboxyterminal sequences two fusion proteins of (soluble) MalE and (i) 59 C-terminal amino acids (fusion I, 49,380 Da) or (ii) 100 C-terminal amino acids (fusion II, 52,959 Da) of *P. lemoignei* PHB depolymerase A (PhaZ5) were constructed and purified as described in materials and methods (Fig. 3A). The identity of purified fusion protein was confirmed by Western Blot analysis using MalE-specific antibodies (data not shown). Both the wild-type MalE and the purified MalE-fusion proteins I and II were subjected to a binding assay with PHB granules (Fig. 3B). Almost all MalE-fusion I protein was present in the granule fraction (lane 2 in fig. 3B) and almost all of the MalE was present in the supernatant (lane 5 in fig. 3B).

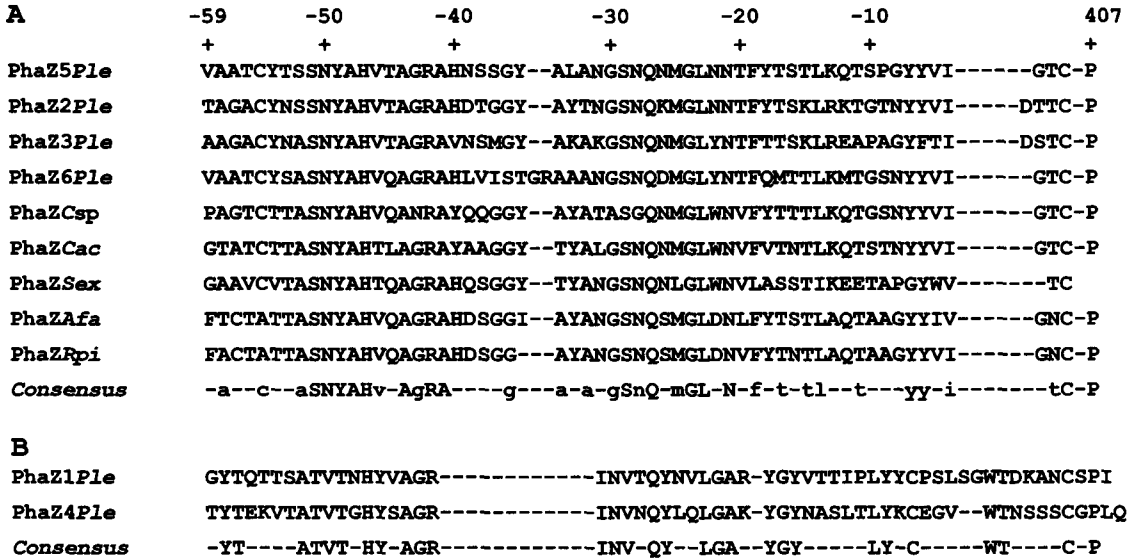

Fig. 2: Alignment of PHB depolymerase substrate-binding domains. An alignment of 60 C-terminal amino acids of 11 bacterial PHB depolymerases is shown. The last three letters of each depolymerase refer to the bacterial origin: *Pseudomonas lemoignei* (Ple), *Comamonas* sp. (Csp), *Comamonas acidovorans* (Cac) (accession no, AB003186), *Streptomyces exfoliatus* (Sex), *Alcaligenes faecalis* (Afa), and *Ralstonia pickettii* (Rpi). For references see [10, 11]. The numbering is according to 407 amino acid long PHB depolymerase A (PhaZ5) of *P. lemoignei*. The sequences under A form a separate cluster compared to both sequences of cluster B (for details, see text). Consensus sequences are provided below each cluster. Capital letters in the consensus sequence A indicate amino acids present in all 9 sequences of cluster A, and low letters were used if at least 7 amino acids were identical.

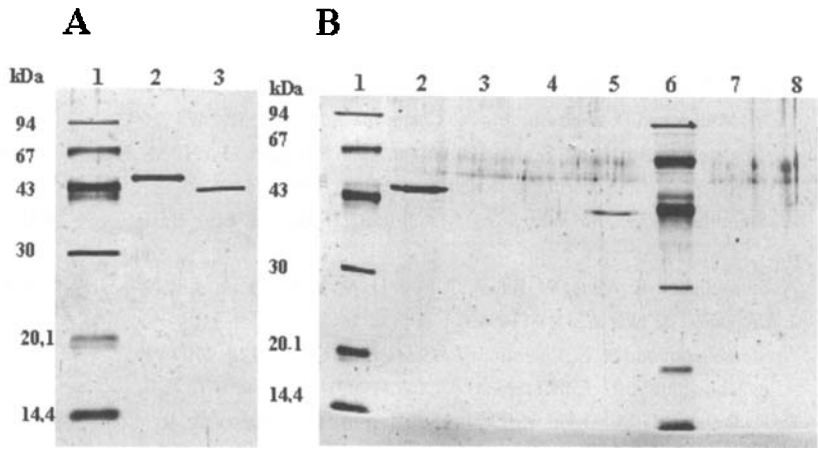

Fig. 3: SDS-PAGE analysis of wild-type and MalE-fusion protein I before and after PHB binding assay. A: SDS-PAGE of purified MalE-fusion protein I (lane 2) and of MalE (lane 3). B: SDS-PAGE of purified PHB granules (pellet, lane 7; supernatant, lane 8); purified MalE-fusion protein I incubated with PHB granules (pellet, lane 2; supernatant, lane 3); MalE incubated with PHB granules (pellet, lane 4; supernatant, lane 5).

This indicated that fusion protein I but not MalE was able bind to PHB granules. Similar results were obtained for fusion protein II (data not shown). We conclude that the presence of the 59 C-terminal amino acids of PHB depolymerase A (PhaZ5) are sufficient to confer PHB-binding ability to water soluble MalE and that no other parts of the depolymerase protein are necessary for polymer binding.

This is in agreement with results obtained with a truncated PHB depolymerase which lacked 55 C-terminal amino acids. The purified truncated protein was not able to bind to PHB and could not hydrolyze PHB [52]. However, the catalytic domain of the truncated protein was uneffected because it hydrolzed water soluble *p*-nitrophenylbutyrate. Substrate-binding domains have also been described for other exoenzymes like cellulases, chitinases or cell wall hydrolases and apparently are a characteristic of polymer hydrolases [54-59]. Future experiments will focus on the identification of amino acids which participate in the specific interaction between the enzyme and its polymeric substrate.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft and the Studienstiftung des Deutschen Volkes.

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