Production of PHA depolymerase A (PhaZ5) from Paucimonas lemoignei in Bacillus subtilis

Reinhard Braaz a, Sui-Lam Wong b, Dieter Jendrossek a,*

a Institut für Mikrobiologie, Universität Stuttgart, Allmandring 31, 70550 Stuttgart, Germany
b Department of Biological Sciences, Division of Cellular, Molecular and Microbial Biology, University of Calgary, Calgary, AB, Canada

Received 22 January 2002; received in revised form 5 February 2002; accepted 6 February 2002
First published online 6 March 2002

Abstract

Purification of poly(3-hydroxybutyrate) depolymerase (EC 3.1.1.75) from Paucimonas lemoignei is complicated because the bacterium produces several isoenzymes which are difficult to separate from each other. The phaZ5 gene of P. lemoignei encoding extracellular poly-(3-hydroxybutyrate) depolymerase A was functionally expressed from the constitutive P43 promoter of pWB980 in a multiple protease-negative mutant of Bacillus subtilis (strain WB800) and secreted to the culture medium. The depolymerase (apparent \( M_r \), 42 kDa; 1.9 mg purified protein per liter culture) was purified from cell-free culture fluid to homogeneity by applying only one chromatography step in comparison to at least two necessary steps if poly(3-hydroxybutyrate) depolymerases are purified from P. lemoignei. The recombinant depolymerase lacked any carbohydrate content in contrast to the glycosylated depolymerase of the wild-type. Glycosylation was not essential for activity but enhanced the thermal stability of the enzyme at high temperature. Overexpression of poly(3-hydroxybutyrate) depolymerase in B. subtilis is more efficient than in Escherichia coli. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Poly(3-hydroxyalkanoate) depolymerase; Poly(3-hydroxybutyrate) depolymerase; Polyhydroxybutyrate; Bacterial glycoprotein

1. Introduction

The ability to degrade extracellular poly(3-hydroxybutyrate) (PHB) and related polyhydroxyalkanoates (PHA) is widely distributed among bacteria and depends on the secretion of specific polyester depolymerases which hydrolyze the water-insoluble polymer to water-soluble monomers or oligomers [1]. Paucimonas (formerly Pseudomonas) lemoignei [2] is unique among PHA-degrading bacteria because it is able to synthesize at least seven different extracellular PHA depolymerases (PhaZ1–PhaZ7 [3]). Other PHB-degrading bacteria such as Alcaligenes faealalis T1 [4] synthesize only one PHB depolymerase.

Biochemical analysis of PHA depolymerases (e.g. crystallization studies) requires the availability of a high amount of purified depolymerase protein. Most PHA depolymerases can be purified by one or two chromatography steps using ion exchange chromatography and/or hydrophobic interaction chromatography. However, purification of PHA depolymerases from P. lemoignei can be difficult due to the presence of at least seven isoenzymes, which have similar properties and which are therefore difficult to separate from each other. Indeed, many purified PHA depolymerase fractions from P. lemoignei actually contain traces of other PHB depolymerase isoenzymes, e.g. inspection of ‘purified’ PHB depolymerase B (PhaZ2) in our laboratory by silver-staining of SDS–PAGE gels revealed the presence of traces of a new, yet undiscovered PHB depolymerase (PhaZ7) [3]. The contamination of PhaZ2 by PhaZ7 was responsible for some unexpected properties of the putative purified PHB depolymerase, e.g. the hydrolysis of atactic PHB and of native PHB granules [3].

In order to avoid misinterpretation of the biochemical characteristics of P. lemoignei PHA depolymerases it is necessary to clone and express the respective structural depolymerase genes in a PHB depolymerase-free host. An easy way to obtain this goal could be the overexpression of the respective gene in Escherichia coli. However, cloning of the PHB depolymerase A gene (phaZ5) and several other depolymerase genes in E. coli resulted in
only poor yields [5]. We decided to express the major PHB depolymerase isoenzyme PhaZ5 of P. lemoignei in Gram-positive hosts which lack an outer membrane and should be more suited for overexpression of extracellular proteins. One of the best-studied Gram-positive bacteria, whose ability to produce high levels of recombinant extracellular proteins is already well established, is Bacillus subtilis [6]. Moreover, several mutant strains of B. subtilis with multiple knock-outs of protease genes are available [7]. Extracellular protease-negative strains are necessary for efficient production of recombinant depolymerase protein in order to avoid a (partial) degradation of the expressed recombinant protein by host proteases. In this contribution we describe the cloning, heterologous expression in B. subtilis, purification and some properties of purified PHB depolymerase PhaZ5.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

B. subtilis WB800, an eightfold protease-negative mutant (trpC2, ΔaprE, ΔaprF, Δapr, Δprf, Δmpr::ble, ΔaprB::bsr, ΔaprG::hyg) and plasmid pWB980 (Km, Blę, sacB signal peptide, P43 promoter) were used in this study [7,8]. Bacteria were grown on tryptose-blood-agar base (TBAB, 33 g/l) and were subsequently stained for carbohydrate content.

2.2. Assay of PHB depolymerase activity

Activity of PHB depolymerase was assayed photospectroscopically at 650 nm as the decrease of optical density of suspensions of denatured PHB granules in 100 mM Tris–HCl, pH 8 containing 1 mM CaCl2. One unit is the amount of enzyme necessary to hydrolyze 1 µg of PHB granules to soluble products per min. For details see [3].

2.3. Construction of a PHB depolymerase expression vector

The plasmid pWB980 [8] harbors a polylinker downstream of a constitutive P43 promoter and the DNA sequence of a sacB signal peptide. The DNA encoding the mature PHB depolymerase PhaZ5 (1256 bp) was cleaved with HindIII and BamHI and ligated in frame of the sacB signal peptide sequence into the HindIII- and BamHI-opened vector. The resulting construct encoded a hybrid peptide consisting of the 29-amino acid SacB signal peptide including a signal peptide cleavage site after Ala29 and the 407-amino acid sequence of mature PHB depolymerase PhaZ5 protein beginning with Ala30. In order to minimize PCR-induced mutations an internal 1171-bp KpnI–EagI DNA fragment was cut out and was replaced by the corresponding KpnI–EagI fragment obtained from the parent plasmid. The DNA sequence of the resulting construct (pWB980::phaZ5) was confirmed by DNA sequencing.

2.4. Purification of PHB depolymerase

Purification of extracellular PHB depolymerase isoenzymes from P. lemoignei was performed from a succinate-grown 10-l culture. The proteins of the cell-free culture fluid were purified by ultrafiltration (30 kDa cut-off), ammonium sulfate precipitation and subsequent column chromatography on CM-Sepharose CL-6B and MonoP (for details see [3]). Two chromatography steps were necessary due to the presence of isoenzymes. Purification of PHB depolymerase expressed by recombinant B. subtilis was performed from 1.3 l of a culture on SR medium (15 h, 37°C). The proteins of the cell-free culture fluid were precipitated by ammonium sulfate (70%), dialyzed against 10 mM succinic acid–NaOH, pH 4.7 and separated by ion exchange chromatography on CM-Sepharose CL-6B as described elsewhere [3]. A second purification step by MonoP chromatofocusing was not necessary. Purified proteins were stored at −20°C without significant loss of activity.

2.5. Immunological techniques

Polyclonal antibodies against PHB depolymerase A (PhaZ5) were obtained by immunization (two times) of mice with purified PHB depolymerase A. Serum obtained after 2 months was used without purification for Western blot analysis. Commercial preparations of anti-antibodies conjugated with peroxidase were used for visualization of the antigen. A serum sample obtained before the beginning of the immunization served as a control.

2.6. Staining for carbohydrate

Glycosylated proteins were separated by reducing SDS-PAGE blotted onto a polyvinylidene difluoride membrane and were subsequently stained for carbohydrate content using the Dig–Glycan detection kit obtained from Boehringer-Mannheim according to the manufacturer’s instructions. Fetuin served as a positive control.

3. Results

3.1. Expression of PHB depolymerase from recombinant B. subtilis

The plasmid pWB980::phaZ5 was constructed as de-
scribed in Section 2 and transformed to _B. subtilis_ WB800. Resulting transformants were grown on opaque PHB-containing TBAB agar. Large clearing zones became visible within 12 h of incubation at 37°C and indicated functional expression and secretion of PHB depolymerase activity. Control strains harboring only the empty vector pWB980 did not produce any clearing zone. When cell-free culture fluid (2Wl) of a liquid culture of _B. subtilis_ (pWB980::phaZ5) was assayed on opaque PHB indicator plates (0.3% PHB in 100 mM Tris-HCl, 1 mM CaCl₂ and 1.5% agar), clearing zones appeared within 1 h and confirmed expression of PhaZ5 and secretion of active PHB depolymerase activity into the culture fluid.

### 3.2. Purification and properties of PHB depolymerase from recombinant _B. subtilis_ (pWB980::phaZ5)

Cells were grown in SR medium at 37°C for 13 h. PHB depolymerase was purified from the cell-free supernatant by ammonium sulfate precipitation (0–70%), dialysis and ion exchange chromatography on CM-Sepharose CL-6B (Table 1). 2.5 mg of purified PHB depolymerase were obtained from 1.3 l of cell-free culture fluid. The protein was highly pure and homogeneous as revealed by SDS-PAGE analysis and subsequent silver staining (Fig. 1A). The yield of purified PhaZ5 (1.9 mg l⁻¹ culture fluid) and specific activity (42 × 10³ U mg⁻¹) were almost fourfold or double as high as had been determined for the wild-type depolymerase earlier (≈0.5 mg l⁻¹ and 19 × 10³ U mg⁻¹), respectively [9]. For details of the purification procedure see Table 1. Purification of PHB depolymerase from recombinant _E. coli_ harboring _phaZ5_ on a multicopy vector such as pBluescript resulted in only poor yield (<0.1 mg l⁻¹) even if the periplasmic fraction of the cells was added to the cell-free culture fluid before the beginning of the purification procedure. PHB depolymerase was found both in the periplasmic fraction and in the cell-free culture fluid at low concentration (data not shown).

Interestingly, the SDS-PAGE-determined apparent molecular mass of the recombinant (_B. subtilis_) purified PHB depolymerase amounted to 42 kDa and corresponded well with the DNA-deduced value of 42.2 kDa but was significantly lower than the values that had been determined for the PhaZ5 depolymerase purified from the wild-type (53 kDa). The identity of the recombinant depolymerase was confirmed by Western blot analysis using polyclonal antibodies raised against the wild-type PHB depolymerase _PhaZ5_ (Fig. 1B). A strong and specific signal appeared at 53 and 42 kDa for the wild-type (_P. lemoignei_) and for the recombinant (_B. subtilis_) depolymerase, respectively. Since PHB depolymerase PhaZ5 is glycosylated in _P. lemoignei_ [5,10] the difference in the apparent molecular mass (53 kDa versus 42 kDa) is probably due to the absence of glycosylation in _B. subtilis_. A staining for carbohydrates confirmed the glycosylation for the _P. lemoignei_ enzyme and the absence of glycosylation in the _B. subtilis_-expressed depolymerase (data not shown). Due to the high activity of the _B. subtilis_-expressed depolymerase glycosyl-

#### Table 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg ml⁻¹)</th>
<th>Protein total (µg ml⁻¹)</th>
<th>Activity (10³ U l⁻¹)</th>
<th>Activity total (10⁵ U)</th>
<th>Specific activity (10³ U mg⁻¹)</th>
<th>Purification (x-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free culture fluid</td>
<td>1320</td>
<td>0.190</td>
<td>251</td>
<td>1.0</td>
<td>1320</td>
<td>5.0</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Concentration of supernatant</td>
<td>710</td>
<td>0.217</td>
<td>154</td>
<td>1.7</td>
<td>1210</td>
<td>8.0</td>
<td>1.5</td>
<td>92</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation (0–70%)</td>
<td>42</td>
<td>0.392</td>
<td>16</td>
<td>10.9</td>
<td>460</td>
<td>28</td>
<td>5.3</td>
<td>35</td>
</tr>
<tr>
<td>CM-Sepharose pool</td>
<td>50</td>
<td>0.050</td>
<td>2.5</td>
<td>2.1</td>
<td>105</td>
<td>42</td>
<td>8.0</td>
<td>8</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** PHB depolymerase PhaZ5 of recombinant _B. subtilis_ at various stages of purification. Proteins were separated by denaturing 12% SDS-PAGE and stained with silver (A) or Western-blotted (B). Concentrated cell-free culture fluid (lane 1), ammonium sulfate precipitation (lane 2), protein CM-Sepharose CL-6B pool (lanes 3 and 6), PhaZ5 purified from _P. lemoignei_ (lanes 4 and 7), standard (lane 5). Lanes 1–5 were silver-stained; lanes 6 and 7 were Western-blotted and immunostained with polyclonal antibodies against PhaZ5.

---


FEMSLE 10386 3-5-02

Downloaded from https://academic.oup.com/femsle/article-abstract/209/2/237/543642 by guest on 01 January 2019
4. Discussion

Biochemical characterization of proteins requires the availability of sufficient amounts of purified protein. Especially for crystallization studies highly purified protein is a prerequisite. In addition, biochemical data obtained with crude proteins are often questionable if the organism synthesizes more than one polypeptide with the same catalytic function. Therefore, yield and specific activity of the purified enzyme were significantly higher than those obtained with the depolymerase purified from the wild-type P. lemoignei. Another difference of the wild-type and B. subtilis-expressed depolymerases was the absence of carbohydrate in the B. subtilis preparation which resulted in different mobilities during SDS–PAGE. Glycosylation was not essential for activity but slightly enhanced the thermal stability of the enzyme at elevated temperature (50°C). In conclusion, expression of extracellular proteins in B. subtilis as a host can be highly recommended.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft to D.J. and by the Natural Sciences and Engineering Research Council of Canada (NSERC) to S.-L.W.

References

