# *POLY-β-HYDROXYBUTYRATE DEPOLYMERASES OF PSEUDOMONAS LEMOIGNEI**

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The polymeric ester of D(−)-β-hydroxybutyric acid, poly-β-hydroxybutyrate (PHB), is a major cellular constituent of many bacteria which accumulate it as a reserve of carbon and reducing power.[1] Certain soil bacteria are also capable of using this extremely insoluble substance as an exogenous source of carbon by excreting extracellular enzymes that depolymerize it. One such organism, *Pseudomonas lemoignei*, has been shown to produce an extracellular depolymerase system which hydrolyzes PHB to hydroxybutyrate and the dimeric ester of the acid, as well as an intracellular hydrolase that further degrades the dimer.[2, 3] The depolymerase system, which has some intriguing properties, has been fractionated, and the properties of the two fractions will be described here.

*Materials and Methods.*—The methods used for the cultivation of *P. lemoignei*, the assays of depolymerase activity, of monomeric and polymerized D-β-hydroxybutyrate, and of protein have already been described, as has the preparation of the polymer granules used as substrate, of the dimeric ester of hydroxybutyrate, and of the β-hydroxybutyric dehydrogenase and dimer esterase used in the assay procedures.[2, 3]

The trimeric ester of D-β-hydroxybutyrate was obtained by the incomplete hydrolysis of poly-β-hydroxybutyrate granules with fraction *B* of the depolymerase, the preparation of which will be described further. When about half of the polymer had been solubilized, the reaction was stopped by acidification and the soluble products were extracted and chromatographed on a silicic acid column.[3] The trimer was eluted with water-saturated chloroform. It was identified by the following properties: per 3.0 equivalents of total β-hydroxybutyric residues (by chemical assay) it had 0.94 equivalents of titrable acidity and yielded 2.9 moles of D(−)-β-hydroxybutyrate (by enzymatic assay). Treatment with hydroxylamine gave 1.9 moles of hydroxamic acid. Hydrolysis with depolymerase yielded 1.0 mole of β-hydroxybutyrate, and the further hydrolysis with dimer esterase gave an additional 2.0 moles.

Assays for monomer, dimer, and trimer in mixtures were carried out spectrophotometrically. All three compounds can be measured by determining the equilibrium NADH levels attained after the successive additions of hydroxybutyric dehydrogenase, depolymerase, and dimer esterase. In practice, it is simpler and more accurate to measure only monomer and trimer enzymatically and to calculate the dimer by difference between the sum of these and the total hydroxybutyric residue content of the sample as determined by chemical means. After NAD reduction by dehydrogenase (*ca.* 0.5 units/ml) ceases, depolymerase (*ca.* 2 units/ml) is added and the final NADH level is measured. Monomer is calculated from the first and trimer from the second equilibrium (1 mole of trimer yielding 1 of monomer). The importance of rigorous pH control throughout such assays should be emphasized.

Many methods of concentrating and fractionating the depolymerase were tested. All of these were tedious and resulted in losses of enzyme activity at some stage. The following method was finally adopted. The crude enzyme in the culture medium was concentrated in dialysis sacs with sucrose as previously described.[2] The enzyme was transferred to $1 \times 10^{-3}$ *M* trishydroxymethylaminomethane (tris)-succinate buffer, pH 6.5, containing $1 \times 10^{-3}$ *M* $CaCl_2$ by passage through large Sephadex G-50 columns equilibrated with this buffer. All buffers used in the further steps contained $10^{-3}$ *M* $CaCl_2$. The column eluate was passed through a diethylaminoethyl cellulose (DEAE) column equilibrated with the same buffer and lyophilized. For fractionation, the lyophilized enzyme was taken up in water and transferred to $10^{-3}$ *M* tris-succinate buffer, pH 5.0, by passage through a Sephadex column equilibrated with this buffer. Units of the eluted enzyme (60,000) were adsorbed on a carboxymethyl cellulose column (85-ml bed volume) thoroughly

equilibrated with the same buffer. The column was washed with 2.5 bed volumes of the same buffer. Depolymerase fraction *A* was then eluted with 0.01 *M* tris-succinate buffer, pH 5.0. The column was further washed with two bed volumes of the same buffer, followed by 3.5 bed volumes of 0.03 *M* tris-sulfate buffer, pH 8.0 (the pH of the eluate changed to 8.0 after approximately two bed volumes of this buffer had been passed through). Depolymerase fraction *B* was then eluted with 0.1 *M* tris-sulfate buffer, pH 8.0. The eluted enzyme fractions were concentrated to 1–5 mg protein/ml in dialysis sacs surrounded by dry Sephadex, and transferred to $10^{-2}$ *M* phosphate buffer, pH 6.8, by passage through Sephadex G-50 columns equilibrated with this buffer. The total recovery of enzyme varied from experiment to experiment, the greatest losses occurring in the lyophilization and the final concentration steps. At best, about 50% recovery was obtained. The enzymes were unstable in dilute solutions, but retained their activity for many months at 0° after the final concentration. In the fractionation step, approximately 80% of the enzyme was recovered as fraction *A* and 20% as fraction *B*.

In the better preparations, the specific activities (sp. act.) of fractions *A* and *B* were about 220 and 160 units/mg protein, respectively. In some instances, however, enzymes of considerably lower sp. act. were obtained. Furthermore, much higher sp. act. were occasionally recorded for preparations immediately after fractionation and before the final concentration step (up to 400 and 250 units/mg for fractions *A* and *B*, respectively). Such dilute preparations, however, lost part of their activity on standing at 0° or in the concentration procedure.

*Results.—Physical properties of fractions A and B:* Each fraction was homogenous by ultracentrifugal and electrophoretic analysis. In $10^{-2}$ *M* pH 6.5 phosphate buffer containing $10^{-3}$ *M* $CaCl_2$, the sedimentation coefficients ($S_{20,w}$) were 3.45 and 3.32 for fractions *A* and *B*, respectively, at a concentration of 0.9 mg protein/ml. The $S_{20}$ values observed at 5 mg protein per ml were not significantly different. The apparent molecular weights determined with the short-column equilibrium technique of Van Holde and Baldwin[4] at 0.9 mg protein/ml were 38,700 and 37,500 for fractions *A* and *B*, respectively ($\bar{v}$ used for calculation, 0.74). Both fractions migrated toward the cathode in acrylamide gel at pH 9.5. In 3.75 per cent gel with 0.02 *M* β-alanine-acetate buffer, pH 4.3, fraction *B* migrated at a rate 3.5-fold greater than that for fraction *A*.

*Specificity and products of hydrolysis:* Both fractions were highly specific for poly-β-hydroxybutyrate, but also hydrolyzed the trimeric ester of D(−)-hydroxybutyric acid. The end products in all cases were monomer and dimer. Neither fraction hydrolyzed the dimer, nor ethyl acetate, ethyl propionate, butyl butyrate, or triolein at an appreciable rate. Ethyl acetoacetate and ethyl lactate were not hydrolyzed by fraction *B*, but were slowly attacked by fraction *A*. At $5 \times 10^{-3}$ *M*, these compounds were hydrolyzed by fraction *A* at a rate of the order of 1 per cent or less (on the basis of ester bonds cleaved) of the rate of polymer digestion under standard assay conditions.

The $K_M$ values for trimer hydrolysis were calculated to be $2.5 \times 10^{-3}$ *M* and $1.5 \times 10^{-3}$ *M* for fractions *A* and *B*, respectively. With $5.6 \times 10^{-4}$ *M* trimer, the respective rates of bond cleavage were approximately 0.6 and 0.3 of the rates for polymer hydrolysis in the standard assay.

Both fractions hydrolyzed the purified PHB granules prepared from either *Bacillus megaterium* or *Pseudomonas multivorans* at approximately the same rate. Extracted and precipitated polymers of *B. megaterium* (mol wt, *ca.* 9,000) and of *P. saccharophila* (mol wt, >100,000) were hydrolyzed at somewhat lower rates, but no conclusions could be drawn from the relative rates because of the very different physical states of the precipitated materials. The nature and relative abundance of the products of hydrolysis was quite constant for each fraction, regardless of the polymer used.

TABLE 1

PRODUCTS OF INCOMPLETE AND COMPLETE PHB HYDROLYSIS

| Extent of PHB hydrolysis | Enzyme fraction | Solubilized Hydroxybutyryl Residues* (%) Recovered as: Monomer | Dimer | Trimer |
|---|---|---|---|---|
| *Ca.* 50% | *A* | *10* (9–13) | *78* (72–82) | *12* (9–15) |
| | *B* | *19* (16–20) | *16* (4–25) | *65* (59–82) |
| 100% Hydrolysis of PHB and accumulated trimer | *A* | *15* (13–17) | *85* (83–87) | *0* |
| | *B* | *43* (37–46) | *57* (54–63) | *0* |

* Italicized figures are mean values; figures in parentheses show ranges of values for a number of separate experiments with different preparations.

Fractions *A* and *B* differed significantly with respect to the course of PHB digestion and to the quantitative yields of the end products. Fraction *A* produced small amounts of trimer during polymer digestion and, at the end, when both polymer and trimer had been hydrolyzed, the average ratio of monomer to dimer (expressed as hydroxybutyric residues) was less than 0.2. With fraction *B*, on the other hand, trimer accumulated as the principal product until the polymer had virtually disappeared. Subsequently, when the trimer had been hydrolyzed, the average ratio of monomer to dimer was about 0.75. These differences can be seen in Table 1, which summarizes the results of a large number of experiments with different enzyme and polymer preparations. The range of the calculated concentrations of the products in different experiments may be attributable not only to slight variations in the experimental materials and procedure, but also to inaccuracies inherent in the assay of the products in mixtures.

An experiment was devised to test whether the enzymes could hydrolyze trimer in the presence of polymer under the usual assay conditions. With fraction *B*, the rate of PHB disappearance and of monomer production was unaffected by the addition of $2.4 \times 10^{-4}$ *M* or $4.8 \times 10^{-4}$ *M* trimer, and the amount of trimer at all times was equal to the amount produced from polymer alone plus the amount initially added. With fraction *A*, the rate of polymer disappearance was also unchanged by the addition of $2.8 \times 10^{-4}$ *M* trimer, but the rate of monomer production was 60 per cent greater than the rate with polymer alone and 119 per cent greater than that with trimer alone. From this, it could be calculated that trimer hydrolysis by fraction *A* was inhibited by 65 per cent in the presence of polymer. Thus, it appears that both fractions have a high affinity for polymer and that fraction *B* is so firmly bound that it does not attack dissolved trimer, while fraction *A* remains partly in solution during polymer digestion. Other types of experiments, not to be reported here, support this interpretation. No higher oligomers than the trimer could be detected during the course of PHB digestion.

*Effect of activators and inhibitors:* The activity and stability of both fractions were influenced by the ionic composition of the environment and by chelating agents, but in somewhat different ways. In the absence of divalent metals, fraction *A* was relatively stable when diluted to 60$\gamma$ protein or less per ml in $2 \times 10^{-3}$ *M* ammonium phosphate buffer, pH 6.9; under the same conditions, the activity of fraction *B* decreased to 50 per cent or less within an hour, unless $10^{-3}$ *M* Ca or Mg or $10^{-1}$ *M* NaCl was added to the diluent. In the assay, fraction *A* was not activated by the express addition of Ca, Mg, or of monovalent cations at high concentrations. The activity of fraction *B*, on the other hand, was decreased 20–40 per cent by the omission of Ca or Mg from the assay mixture and was increased up to twofold when the standard assay medium containing $10^{-3}$ *M* Ca was

supplemented with 0.1 $M$ $CaCl_2$, $MgSO_4$, $Na_2SO_4$, $Na_2SO_3$, KCl, $K_2SO_4$, $(NH_4)_2SO_4$, or LiCl. Neither sucrose nor glycerol at high concentrations increased the activity.

Sodium ethylenediaminetetraacetate (EDTA) at concentrations of $1–5 \times 10^{-3}$ $M$ inhibited the activity of fraction $A$ by 40–60 per cent, and this inhibition could be completely reversed by the addition of excess Ca or Mg during PHB hydrolysis. The activity of fraction $B$ deteriorated rapidly to 10 per cent or less in the presence of PHB and $10^{-3}$ $M$ EDTA and could be restored only to a small extent by the subsequent addition of Ca, Mg, or of any other metal tested. At high concentrations of monovalent cations, the inhibition of this fraction by EDTA was less pronounced. In fact, $3 \times 10^{-3}$ $M$ EDTA was less inhibitory (50–70%) than $10^{-3}$ $M$ EDTA, presumably because of the contribution of sodium ions. In the absence of substrate, concentrated solutions of either fraction could be treated with EDTA, and their initial activity could be recovered upon removal of the chelating agent on a Sephadex column. Pyrophosphate ($2 \times 10^{-3}$ $M$), $\alpha,\alpha$-dipyridyl, 8-hydroxyquinoline, and $o$-phenathroline inhibited both fractions to varying degrees. In contrast with inhibition by EDTA, the inhibition of fraction $A$ by dipyridyl, hydroxyquinoline, and phenanthroline was enhanced by the addition of excess Ca or Mg, while the activity of fraction $B$ was greatly increased by the addition of these metals during PHB hydrolysis. Unfortunately, interpretation of these observations is difficult in view of the general response of fraction $B$ to the ionic environment and the possibility that the assay mixtures were contaminated with other metals.

In an attempt to demonstrate the specific participation of various metals in the structure and function of the enzymes, fraction $A$ (4 mg/ml) was exhaustively dialyzed against $10^{-3}$ EDTA in tris buffer, and fraction $B$ (2 mg/ml) was briefly treated with $10^{-2}$ $M$ EDTA. Each enzyme was then transferred to metal-free $2 \times 10^{-3}$ $M$ pH 6.9 ammonium phosphate buffer containing no EDTA, by passage through a Sephadex column, and each was analyzed for various metals by X-ray fluorimetry. Neither enzyme contained more than 0.1 $\mu$g of Mn, Fe, Co, or Zn per mg protein (less than 0.1 atom per mole). The determination of calcium was not entirely satisfactory with the method used, but maximal values could be tentatively assigned at 0.1 and 0.5 $\mu$g per mg of fractions $A$ and $B$, respectively. After the removal of EDTA, both fractions possessed their full initial specific activities and could be inhibited with EDTA in the assay.

Neither enzyme was inhibited by $10^{-3}$ $M$ $p$-hydroxymercuribenzoate, arsenite, or iodoacetamide. Both fractions were completely inhibited by $10^{-3}$ $M$ dithiothreitol (DTT), but were unaffected by $10^{-2}$ $M$ mercaptoethanol. Enzyme preparations incubated with $10^{-3}$ $M$ DTT and diluted 20-fold before assay regained full activity. The activity of both fractions that had been partially inhibited by $10^{-4}$ $M$ DTT could be further drastically reduced by incubation with $10^{-3}$ $M$ iodoacetamide.

Neither fraction was inhibited by $10^{-2}$ $M$ D- or DL-$\beta$-hydroxybutyrate or the dimeric ester of D-hydroxybutyrate. Under the usual conditions of assay, fraction $A$ was completely inactivated by a 10-min exposure to $1.25 \times 10^{-7}$ $M$ diisopropylfluorophosphate (DIPFP), while fraction $B$ required a 20-min exposure to $10^{-4}$ $M$ DIPFP to achieve the same result.

*Thermal stability:* In dilute solution, both fractions were quite labile to heat, but fraction $B$ was more stable than fraction $A$ in $5 \times 10^{-2}$ $M$ tris-sulfate buffer, pH

8.0, containing $5 \times 10^{-3}$ $M$ $CaCl_2$. At 60°, fraction $A$ was inactivated exponentially with a half life of about 2 min, while for fraction $B$ the rate of inactivation decreased with time and the kinetics were not identical in different experiments. One half to two thirds of the activity disappeared with an initial half life of 10–12 min; subsequently, the half life increased to 30–35 min. No significant change in the characteristic ratio of hydrolysis products was observed when PHB was hydrolyzed with either enzyme that had been partially inactivated by heat.

*Immunological reactions:* The immunological purity of the fractions and any cross-reactions were tested by the immunodiffusion technique on Ouchterlony plates. Immune serum against each fraction was prepared by the intradermal injection of separate rabbits with 0.1 mg of protein in complete Freund's adjuvant containing 3 mg BCG preparation. Serum samples were taken 1 month later. Six months thereafter, the rabbits were reinjected with the same vaccines (0.3 mg fraction $A$ and 0.15 mg fraction $B$). Serum samples were taken 10 and 28 days later. The immune sera obtained 30 days after the initial injections reacted with the homologous antigens, and no visible reaction was obtained with heterologous ones. Later samples of anti-fraction $A$ serum still reacted only with this fraction; the later sera against fraction $B$, on the other hand, reacted with both antigens. This indicates that the two fractions are immunologically distinct, but that the fraction $B$ preparation used for immunization was contaminated with fraction $A$ in a quantity too small to be detected with anti-fraction $A$ serum.

*Discussion and Summary.*—The two fractions of extracellular PHB depolymerase of *P. lemoignei* show many similarities in their physical properties, substrate specificity, products of PHB digestion, and susceptibility to chelating agents. They are, however, distinct with respect to the course of PHB digestion and the ratio of end products, their response to divalent and monovalent cations in the presence and absence of chelating agents, their susceptibility to diisopropylfluorophosphate, and their immunological properties. Neither fraction has sensitive sulfhydryl groups, but, unlike many extracellular hydrolases, they both contain cystine (the indirect evidence obtained in experiments with dithiothreitol has been corroborated by the chemical demonstration of hemicystine residues by Dr. W. A. Rombauts). Although calcium and magnesium appear to be implicated in the stability and activity of fraction $B$, the role of metals in the activity of fraction $A$ remains obscure. Nor is it clear whether the effect of high concentrations of monovalent cations on fraction $B$ has the same basis as the effect of low concentrations of divalent metals. Although cobalt, iron, zinc, manganese, and, probably, calcium do not appear to be firmly bound constituents of either enzyme, the presence of these or other metals as contaminants in assay mixtures cannot be ruled out.

A number of enzymes that have a high specificity for hydrolyzing hydroxybutyryl-hydroxybutyrate ester bonds is now available for study. These include the two extracellular enzymes described above, the intracellular dimer esterase of the same organism, a variety of extracellular enzymes of other pseudomonads,[2] and the intracellular depolymerase and dimer esterase of *Rhodospirillum rubrum*.[5] Detailed comparative biochemical studies of such enzymes might be very useful in the elucidation of some basic problems of enzyme specificity and of the structural relationships among different enzymes in a given organism and among isofunctional and related enzymes in different species.

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