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Purification and Characterization of Extracellular Poly(3-hydroxybutyrate) Depolymerase from *Penicillium simplicissimum* LAR13

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An extracellular PHB depolymerase was purified from *P. simplicissimum* LAR13 cultural medium by Sepharose CL-6B chromatography. When the fungus was grown in a basal salt medium with poly(3-hydroxybutyrate) (PHB) as the sole carbon source, PHB depolymerase production reached maximum at its stationary phase. The mycelial growth rate was higher at 37°C than at 30°C and even higher than at 25°C, However, the enzyme production was lower at 37°C than 30°C or 25°C. The isolated enzyme is composed of a single polypeptide chain with a molecular mass of about 36 kDa as determined by SDS-PAGE. The optimum conditions for the enzyme activity are pH 5.0 and 45°C. The enzyme was stable for 30 min at a temperature lower than 50°C, and stable at pH higher than 2.0 but it was unstable at pH 1.0. 1 mM Fe^{2+} reduced the enzyme activity by 56% and the enzyme was inhibited almost completely by 4 mM Fe^{2+}. The enzyme was partially inhibited by phenylmethylsulfonyl fluoride and was very sensitive to diazo-DL-norleucine methyl ester, dithiothreitol and mercuric ion. However, *N-p*-tosyl-L-lysinechloromethyl ketone, *p*-hydroxymercuricbenzoate and *N*-acetylimidazole had no influence upon its activity.

Key words: depolymerase, *Penicillium simplicissimum*, poly(3-hydroxybutyrate)

With the development of the petrochemical industry, about one hundred million tons of chemically synthesized plastics are produced per year, and environmental contamination is rising because the chemically synthesized plastics are not biodegradable. Accordingly, there is a growing interest in the disposal of used plastic materials and in the development of alternative materials for the nondegradable plastics (Doi, 1990).

Poly(3-hydroxybutyrate) (PHB) has been synthesized by more than 75 microbial species. The microorganisms accumulate PHB as a polymeric material for preserving energy in their cells (Anderson and Dawes, 1990; Doi, 1990). Many studies have been done on the degradation of PHB in soil (Mergaert *et al.*, 1993), the ocean (Mergaert *et al.*, 1995) and compost (Scherer, 1996) and on the mechanism of PHB degradation (Laemmli, 1970). The primary decomposer of the biodegradable polymer is usually microorganisms which secrete a PHB depolymerase in an extracellular manner. *Agrobacterium* sp. K-03 (Nojima *et al.*, 1996), *Comamonas* sp. (Jendrossek *et al.*, 1995B), *Alcaligenes faecalis* (Zacharius *et al.*, 1969), and *Pseudomonas lemoignei* ATCC 17989 (Mergaert *et al.*, 1995), have been reported to be PHB degrading microorganisms. Some eukaryotes such as *Aspergillus fumigatus* (Scherer, 1996), *Paecilomyces lilacinus* D218 (Oda *et al.*, 1997) and *Penicillium funiculosum* ATCC 9644 (Brucato and Wong, 1991) are also known to be capable of PHB degradation.

Some PHB depolymerases have serine, histidine and aspartate residues in their active sites (Jendrossek *et al.*, 1995B; Shinomiya *et al.*, 1997) and most PHB depolymerases are deactivated by diisopropylfluorophosphate, phenylmethylsulfonyl fluoride and dithiothreitol, indicating the importance of serine residues and disulfide bonds in the activation of the enzymes (Jeong, 1996; Sadocco *et al.*, 1997). In addition, some PHB depolymerases are found to be serine esterases because they contain lipase boxes composed of a series of amino acids such as Gly-X-Ser-X-Gly (Schirmer and Jendrossek, 1994; Shinohe *et al.*, 1996).

Many studies have been carried out on extracellular PHB depolymerase from bacteria, while few studies have been reported on the extracellular PHB depolymerase from eukaryotes. Scherer (1996) reported that the extracellular PHB depolymerase of *A. fumigatus* M2A isolated from compost had a molecular weight of 57 kDa whose activity was maximum at pH 8.0 and 70°C. Oda *et al.* (1997) revealed that the extracellular PHB depolymerase of *P. lilacinus* D218 isolated from soil had a molecular weight of 48 kDa and its optimum activation conditions were pH 7.0 and 45°C.

The used PHB is normally buried under the ground and

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can be decomposed by the extracellular PHB depolymerase secreted from the soil microbes. In this study, the extracellular PHB depolymerase from *P. simplicissimum* LAR13 (Kim *et al.*, 2000) which was isolated from soil, was purified in order to examine the capability for PHB decomposition, biochemical characteristics and the active sites of the enzyme.

Materials and Methods

PHB

PHB was purchased from ICI, UK. The molecular weight of PHB was 470,000 g/mol. All experiments were performed using PHB powder.

PHB depolymerase

P. simplicissimum LAR13 was isolated in our laboratory from an activated sludge soil (Kim *et al.*, 2000). It was cultivated in malt extract agar slant medium at 30°C for 7 days.

Spore suspension of *P. simplicissimum* LAR13 (1.1×10^7 spores/l) was placed into mineral PHB media (0.15% PHB, 0.7 g K_2HPO_4, 0.7 g KH_2PO_4, 0.7 g $MgSO_4$, 1.0 g NH_4Cl, 1.0 g $NaNO_3$, 5 mg NaCl, 2 mg $FeSO_4$, 7 mg $ZnSO_4$ in 1L of D.W.) and cultivated at 25°C, 30°C or 37°C. The culture solution was centrifuged for 15 min at 8,000×g (Beckman J2-21) and the supernatant was collected. Dry cell weight was determined according to Oda *et al.* (1995) after extraction of the PHB in the cells with chloroform/water (1:1) mixture followed by drying at 105°C for 18 h.

Enzyme assay

The PHB depolymerase assay was carried out by adding the enzyme to 0.1 M sodium acetate buffer (pH 5.0) containing 100 μg of PHB at 40°C for 1 h. The declining absorption rate was measured at 650 nm using a spectrophotometer (Shimadzu, UV-1201). One unit of enzyme was defined as the amount of enzyme required to decrease 1 OD of the reaction mixture per hour.

Enzyme purification

PHB depolymerase was purified from the supernatant of the fungal culture solution. The supernatant was loaded on a Sepharose CL-6B column (1×40 cm) equilibrated with a 10 mM sodium acetate buffer (pH 5.0) containing 1.5 M ammonium sulfate. Ammonium sulfate was then slowly diluted (1.5 M~0.0 M) and the protein was eluted by reverse phase linear gradient. The fractions were collected and used for the enzyme assay.

Molecular weight determination

Electrophoresis (12.5% solution dodecyl sulfate polyacrylamide gel electrophoresis) was carried out to measure the molecular weight according to Laemmli (1970). Phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.5 kDa) were used as standard molecular weight markers. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. The protein concentration was measured with the Bio-Rad protein assay (Bradford, 1976) using bovine serum albumin as the standard protein.

Carbohydrate determination

Glycoprotein carbohydrate content was estimated using sodium meta-periodate oxidation (Scherer, 1996) by dialysis in 50 mM $NaIO_4$/50 mM Tricine (pH 5.5), for 24 h at 4°C, and by redialysis in 5.0 mM Tricine-NaOH (pH 8.0). Carbohydrate staining of glycoprotein in SDS-PAGE gel was carried out with fuchsin-sulfate after periodate oxidation according to Zacharius *et al.* (1969).

Effects of temperature and pH on the enzyme activity

To find out the effect of temperature on PHB depolymerase activity, enzyme activity was measured at different temperatures in the range of 25°C to 70°C at optimum pH. The ability of the enzyme to withstand heat was measured by incubating the enzyme in standard reaction solution without PHB for 30 min at 25~70°C.

The effect of pH on enzyme activity was determined by using buffers of pH 1.0~20. (0.1M sodium lactate), pH 2.0~3.0 (0.1 M Glycine-HCl), pH 3.0~6.0 (0.1 M sodium acetate), pH 6.0~8.0 (0.1 M phosphate), pH 8.0~9.0 (0.1 M Tris-HCl), and pH 9.0~10.0 (0.1 M Glycine-NaOH). The optimum pH for enzyme activity was determined by measuring the activity in each of the buffers containing PHB and enzyme. To examine the pH stability of the enzyme, buffers containing enzyme without substrate were incubated at 25°C for 2 h, and then controlled to have the optimum pH in order to measure the residual activity of the enzyme.

Results and Discussion

Production of PHB depolymerase

Production of enzyme depends on the growth period of microorganisms (Han *et al.*, 1998; Scherer, 1996). For example, *P. funiculosum* (Brucato and Wong, 1991) and *A. faecalis* (Tanio *et al.*, 1982) cultured at 30°C produces the most PHB depolymerase in the stationary phase.

P. simplicissimum LAR13 was cultured at 25°C, 30°C or 37°C in a medium containing PHB as the sole carbon source. The growth of *P. simplicissimum* LAR 13 and the change in the activity of extracellular PHB depolymerase were examined and the results are shown in Fig. 1. The fungus grew most rapidly at 37°C. In the early stage of the growth, the enzyme production was the highest at 30°C.

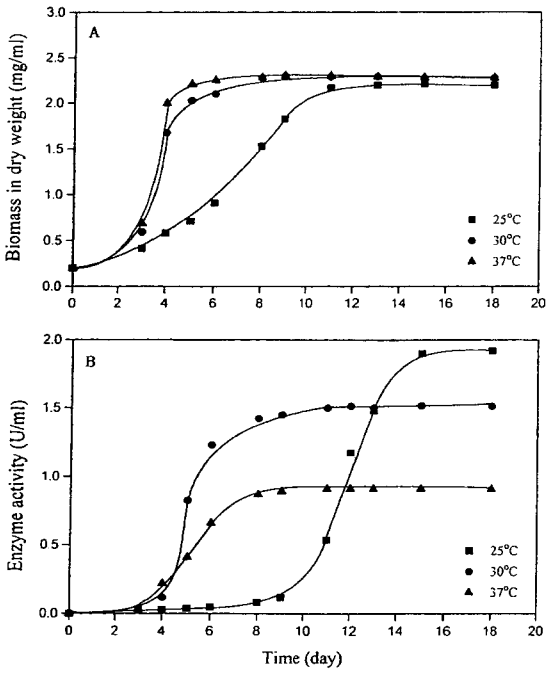

Fig. 1. Effect of temperature on fungal growth (A) and PHB depolymerase production (B) from *P. simplicissimum* LAR 13.

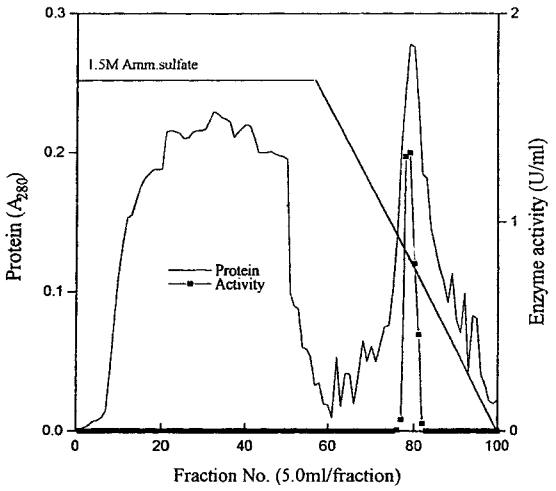

Fig. 2. Chromatogram of PHB depolymerase from *P. simplicissimum* LAR 13. The supernatant (containing 1.5 M ammonium sulfate) from the culture medium was applied to a Sepharose CL-6B column (1×40 cm) equilibrated with 1.5 M $(NH_4)_2SO_4$ in 10 mM sodium acetate buffer, pH 5.0. After washing, the column was developed with a 200 ml decreasing linear salt gradient.

However, in the later part of the culture period, the enzyme production was maximal at 25°C. The enzyme production increased with the culture time and then leveled off at the stationary phase.

Purification of PHB depolymerase

The PHB depolymerase of *P. simplicissimum* LAR13 was purified as shown in Fig. 2. Crude enzyme solution was loaded on a Sepharose CL-6B column (140 cm), and eluted with 1.5 M ammonium sulfate. The concentration of ammonium sulfate was diluted from 1.5 M. The PHB depolymerase fraction (fraction 7582) was eluted with a reverse phase ammonium sulfate gradient.

In Table 1, the purification results of PHB depolymerase from *P. simplicissimum* LAR13 are summarized. The enzyme activity increased 2.4 fold after the purification, and the yield was 61%. According to the literature, the yield of PHB depolymerase after purification was 66% from *P. funiculosum* (Brucato and Wong, 1991); 27% from *A. faecalis* (Kita *et al.*, 1995); 42% from *Pseudomonas* sp. (Jeong, 1996); 66% from *A. fumigatus* (Scherer, 1996); and 73.5% from *Aureobacterium saperdae* (Sadocco *et al.*, 1997). The increases of the enzyme activity were 4.5 fold for *P. funiculosum* (Brucato and Wong, 1991); 1.5 fold for *A. faecalis* (Kita *et al.*, 1995); and 5.67 fold for *Pseudomonas* sp. (Jeong, 1996).

Molecular weight and carbohydrate content

The purified PHB depolymerase from *P. simplicissimum* LAR13 was subjected to electrophoresis using an SDS-PAGE and was found to be a single polypeptide chain with a molecular weight of 36 kDa (Fig. 3) The molecular weight is similar to that of the PHB depolymerase obtained from *P. funculosum* (Brucato and Wong, 1991) and *P. pinophilum* (Han *et al.*, 1998), but lower than that of the PHB depolymerase from other bacteria (Nakayama, *et al.*, 1985; Kita *et al.*, 1995; Jeong, 1996; Scherer, 1996; Oda *et al.*, 1997; Sadocco *et al.*, 1997).

The carbohydrate content of the enzyme by sodium meta-periodate oxidation and subsequent SDS-PAGE was estimated to be less than 5%, because only a small change was observed in the electrophoretic mobility of the protein (Fig. 4). This can be confirmed by the fact that the purified PHB depolymerase was difficult to dye with fuchsin-sulfate. Carbohydrates were not detected in the PHB depolymerase either from *A. fumigatus* (Scherer, 1996) or from *A. saperdae* (Sadocco *et al.*, 1997). However, the PHB depolymerase from *P. funiculosum* (Brucato and Wong,

Table 1. Purification of PHB depolymerase from *P. simplicissimum* LAR 13

Purification step	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture medium	109	9.6	11.4	1.0	100
Sepharose CL-6B	66	2.4	27.5	2.4	61

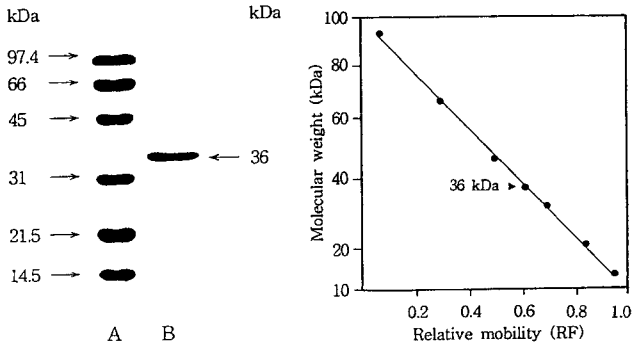

Fig. 3. SDS-PAGE of PHB depolymerase from *P. simplicissimum* LAR 13. Lane A: Molecular standard containing phosphorylase b (MW 97.4 kDa), BSA (MW 66 kDa), egg albumin (MW 45 kDa), carbonic anhydrase (MW 31 kDa), trypsin inhibitor (MW 21.5 kDa), lysozyme (MW 14.5 kDa) Lane B : Purified enzyme.

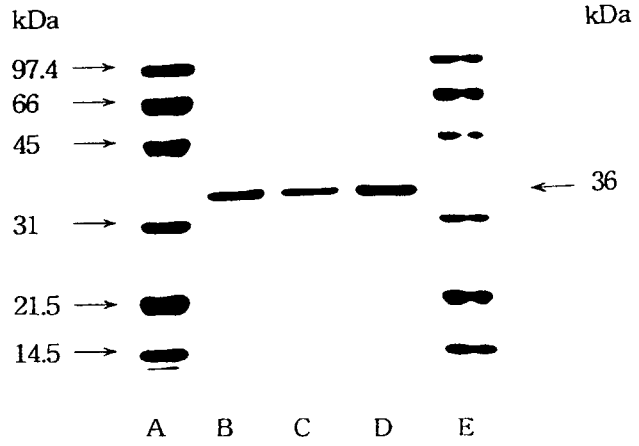

Fig. 4. SDS-PAGE determination of PHB depolymerase glycosylation extent. Lane A, E: Molecular standard containing phosphorylase b (MW 97.4 kDa), BSA (MW 66 kDa), egg albumin (MW 45 kDa), carbonic anhydrase (MW 31 kDa), trypsin inhibitor (MW 21.5 kDa), lysozyme (MW 14.5 kDa). Lane B, D: PHB depolymerase untreated with $NaIO_4$. Lane C: PHB depolymerase treated with $NaIO_4$.

1991) contained about 12% (w/w) carbohydrate.

Effects of temperature and pH

The enzyme exhibited the highest activity at 45°C and was stable at temperatures below 50°C (Fig. 5), indicating that the enzyme was thermally as stable as other PHB depolymerases reported (Brucato and Wong, 1991; Kita *et al.*, 1995; Nojima *et al.*, 1996; Scherer, 1996; Han *et al.*, 1998). Mergaert *et al.* (1995) reported that PHB showed a high degradation rate in summer in their PHB decomposition experiments and explained that such a result was due to high activation of the PHB decomposing organisms. The PHB depolymerase produced by PHB decomposing organisms has high activity and stability at high temperatures.

The enzyme was the most active at pH 5.0 as shown in Fig. 6. The optimum pH of the PHB depolymerase from

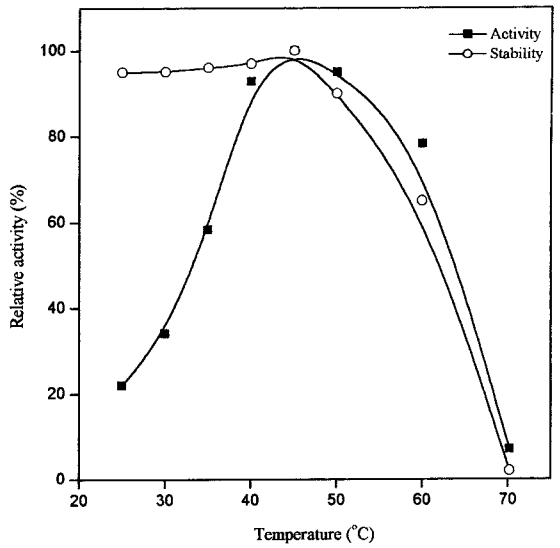

Fig. 5. Effect of temperature on PHB depolymerase activity and stability from *P. simplicissimum* LAR 13.

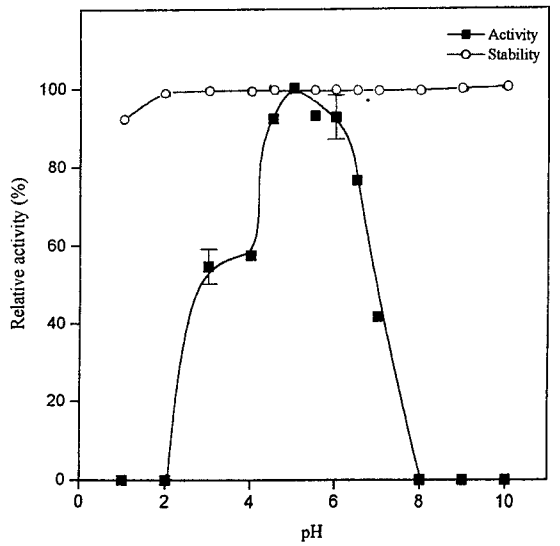

Fig. 6. Effect of pH on PHB depolymerase activity and stability from *P. simplicissimum* LAR 13.

bacteria (Jeong, 1996; Kita *et al.*, 1995; Nakayama *et al.*, 1985; Sadocco *et al.*, 1997) was in the range of 7.5~9.5 depending on the strain. The enzyme was stable in the range of pH 2.0 to 10.0 other than pH 1.0 (Fig. 6). The PHB depolymerase of *A. saperdae* (Sadocco *et al.*, 1997) was completely inactivated at pH 1.5 in 2 hours and that of *P. lilacinus* (Oda *et al.*, 1997) maintains the activity only in the range of pH 4.0 to 6.0. Accordingly, it was concluded that the PHB depolymerase of *P. simplicissimum* LAR13 was very stable under acidic conditions and maintains activity with variations of pH.

Effect of ions

Metal ions were added at a concentration of 1 mM and the

Table 2. Effect of metal ions on PHB depolymerase activity from *P. simplicissimum* LAR 13

Ion (1 mM)	Relative activity (%)
Ca^{2+}	97
Mg^{2+}	97
Mn^{2+}	98
Cu^{2+}	97
Fe^{2+}	44
Zn^{2+}	95
Co^{2+}	97
Cd^{2+}	100

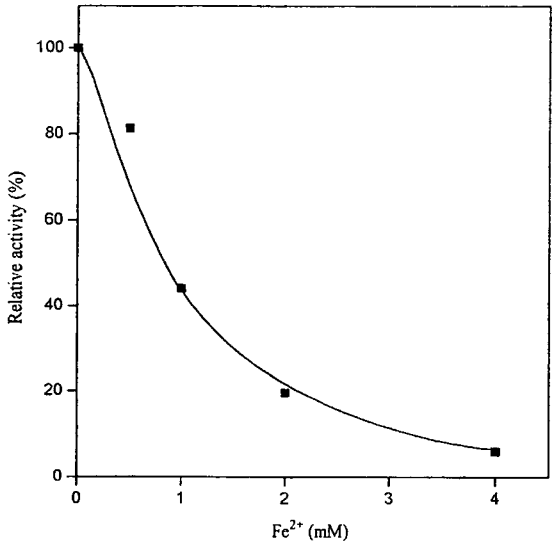

Fig. 7. Inhibition of *P. simplicissimum* LAR 13 PHB depolymerase activity as a function of Fe^{2+} concentration.

relative activity of the enzyme was measured (Table 2). The enzyme activity was decreased by 56% in the presence of Fe^{2+}, while Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+} and Cd^{2+} had a negligible influence on the enzyme activity. The sensitivity of the enzyme to Fe^{2+} is demonstrated in Fig. 7. The enzyme was almost completely inactivated by 4 mM Fe^{2+}. Nojima *et al.* (1996) observed that the activity of the PHB depolymerase from *Agrobacterium* sp. was nearly independent of Fe^{2+} as well as Co^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+}. However, the PHB depolymerase from *P. lilacinus* (Oda *et. al.*, 1997) was 80% deactivated by Cu^{2+}, Zn^{2+}, and Mn^{2+}, and 88% by Fe^{2+} at the same concentration. The PHB depolymerase of *Agrobacterium* sp. (Nojima *et al.*, 1996), *P. pinophilum* (Han *et al.*, 1998) and *P. simplicissimum* LAR13 are relatively stable in the presence of ions.

Effect of inhibitors

Effect of inhibitors on the activity of the enzyme was investigated in order to identify the active sites in the PHB depolymerase of *P. simplicissimum* LAR13 (Table 3). Phenylmethylsulfonyl fluoride (PMSF) is known as an

Table 3. Relative activity of the PHB depolymerase from *P. simplicissimum* LAR 13 in the presence of various inhibitors

Reagent	Concentration (mM)	Relative activity (%)
PMSF	10	58
DAN	25	3
TLCK	10	98
PHMB	5	93
DTT	5	0
Mercuric chloride	10	0
N-Acetylimidazole	10	100

Phenylmethylsulfonyl fluoride (PMSF); diazo-$_{DL}$-norleucine methyl ester (DAN); *N*-*p*-tosyl-$_{L}$-lysinechloromethyl ketone (TLCK); *p*-hydroxymercuricbenzoate (PHMB); dithiothreitol (DTT)

inhibitor of serine residues which has something to do with the activity of the PHB depolymerase (Brucato and Wong, 1991; Nakayama *et al.*, 1985; Yamada *et al.*, 1993). The PHB depolymerase of *A. saperdae* (Sadocco *et al.*, 1997) was partially inactivated by 10 mM PMSF, and that of *P. lemoignei* (Nakayama *et al.*, 1985) and *Agrobacterium* sp. (Nojima *et al.*, 1996) was completely inhibited by 1 mM PMSF. However, the PHB depolymerase of *P. simplicissimum* LAR 13 showed 58% activity in the presence of 10 mM PMSF. The enzyme was inhibited by a carboxyl group-directed inhibitor such as diazo-DL-norleucine methyl ester (DAN), indicating that the enzyme had carboxyl groups at the active site (Sadocco *et al.*, 1997). Jendrossek *et al.* (1995A) evidenced the existence of the histidine groups in the enzyme. However, Table 3 shows that the enzyme activity was nearly unaffected by 10 mM of *N*-*p*-tosyl-$_{L}$-lysinechloromethyl ketone (TLCK), which should transform the histidine groups. The PHB depolymerases of *P. funiculosum* (Brucato and Wong, 1991) and *A. saperdae* (Sadocco *et al.*, 1997) also exhibited 100 % activity in the presence of 10 mM TLCK. Dithiothreitol (DTT) and mercuric chloride inhibited the enzyme activity strongly, indicating that the sulfide bonds were critical for the enzyme activity. The enzyme was not inhibited in the presence of *p*-hydroxymercuricbenzoate (PHMB), a sulfhydryl regent. Thus the free sulfhydryl group may not be located at the active site. This result is in conformity with the report that the PHB depolymerases of *P. funiculosum* (Brucato and Wong, 1991) and *A. saperdae* (Sadocco *et al.*, 1997) maintained 80% and 100% activities, respectively with 5 mM PHMB. *N*-acetylimidazole did not affect the enzyme activity, suggesting that tyrosine is not involved in the active site of the enzyme.

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