Purification and Characterization of Poly(ethylene succinate) Hydrolase from the Mesophilic Fungus *Aspergillus clavatus* NKCM1003

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Summary: A poly(ethylene succinate) (PESu) hydrolase was purified from the culture supernatant of the PESu-degrading fungus *Aspergillus clavatus* NKCM 1003, which was cultivated on a mineral medium containing PESu as the sole carbon source. The enzyme had a molecular mass of 57,000 Da and an isoelectric point of 5.6, and it was stable in the rage pH5-8; the optimum pH was 7.0. Its PESu hydrolytic activity remained constant below 40 °C after incubation for 30 min. Liquid chromatography electrospray mass spectrometry analysis of enzymatic hydrolysates revealed that the major products are ES, ESE, SES, and ESES(S; succinate unit, E; ethylene glycol unit). This enzyme degraded not only PESu but also poly(3-hydroxybutyrate) (P(3HB)) and its copolymers. Further, it showed high binding affinity to P(3HB). It was concluded that PESu hydrolase belongs to the class of P(3HB) depolymerases.

Keywords: enzymes; mass spectrometry; P(3HB) depolymerase; PESu hydrolase; poly(ethylene succinate)

Introduction

Poly(ethylene succinate) (PESu), a condensate of ethyleneglycol and succinic acid, is a biodegradable polyester that can replace general and non-biodegradable polymers such as polypropylene and polyethylene.[1,2] Thus far, some PESu-degrading microorganisms have been so far isolated from natural environments.[5–7]

We had recently reported [7] that the mesophilic fungus *Aspergillus clavatus* NKCM1003 isolated from a soil shows high PESu-hydrolytic activity. The strain showed both poly(3-hydroxybutyrate) (P(3HB))- and PESu-hydrolytic activities simultaneously in the culture when it was cultivated in a mineral medium containing PESu as the sole carbon source. This suggests that the enzyme responsible for PESu hydrolysis is P(3HB) depolymerase. In this report, we describe the purification and biochemical characterization of the PESu-hydrolytic enzyme.

Materials and Methods

Chemicals

PESu, poly(butylene succinate) (PBSu), poly(butylene succinate-*co*-adipate) (PBSA), poly(lactic acid) (PLA), and poly(ε-caprolactone) (PCL) were donated by Nippon Shokubai Co. Ltd. (Osaka, Japan), Showa High Polymer Inc.(Tokyo, Japan), Shimadzu Co. (Kyoto, Japan), and Daicel Chemical Industries Co. (Osaka, Japan), respectively. P(3HB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-14mol% 3HV)) were produced by *Wautersia eutropha* ATCC17699.[8] Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P(3HB-*co*-5mol% 4HB)) was produced by *Delfitia acidovorans* DS-17.[9] Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (P(3HB-

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co-10mol%3HHx) was produced by *Aeromonas caviae*.[10] Poly(δ-valerolactone) (PVL) was chemically synthesized.[11] To purify the polymers, they were dissolved in dimethylene chloride or chloroform and then reprecipitated by the addition of methanol. Melt-crystallized films of these polymers were prepared under the same conditions as described previously.[12]

Media and Culture Conditions

A. clavatus NKCM1003 was aerobically cultured at 30 °C on either LB medium (pH 7.0, tryptone peptone 10 g/L, yeast extract 5g/L, NaCl 5g/L) supplemented with 2% (wt/vol) glucose, or minimum media (pH 6.0, KH_2PO_4 1.0 g/L, $NaNO_3$ 2.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KCl 0.5 g/L, $FeSO_2 \cdot 7H_2O$ 0.01 g/L, NH_4Cl 1.0 g/L) supplemented with a PESu film as the sole carbon source.

Enzyme Assays

The P(3HB) depolymerase activity in the culture supernatants was determined by a turbidimetric method as described elsewhere.[4] One unit of P(3HB) depolymerase activity was defined as the amount of enzyme that was required to decrease the turbidity by 1.0 at a wavelength of 650 nm of a P(3HB) granule suspension per minute. The activities in the culture supernatants were expressed as that per unit volume (mU/mL). The PESu hydrolytic activity in the supernatant was determined by the weight loss method.[4] A mixture of 800 μL of 100 mM phosphate buffer (pH7.4), 200 μL of the supernatant, and 1cm × 1cm 1cm of the PESu film was incubated at 30 °C. After incubation for 24 h, the film was recovered and dried *in vacuo*. The activity was expressed as the weight loss of the film per unit surface area per hour (μg/cm^2/h).

Substrate Specificity of PESu Hydrolase

Substrate specificity of PESu hydrolase was evaluated using the weight loss method with the polymer films as the substrate. Melt-crystallized films of PESu, PBSu, PBSA, PCL, PLA, PVL, P(3HB), and P(3HB) copolymers were prepared as described previously,[5] cut into strips (1 × 1 × 0.15 cm, *ca.* 25 mg), and sterilized by soaking in ethanol followed by drying. After incubation for 24 h at 37 °C, the residual films were recovered, washed with distilled water, and dried to a constant weight. The weight loss of each film was calculated by subtracting the weight of the film after degradation from its initial weight.

Protein Analysis

The proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using the method of Laemmli[13] with a molecular weight marker. After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250 (Kanto Chemical, Tokyo, Japan). Protein concentrations were determined by the method of Bradford[14] by using protein assay kit II (Bio-Rad Lab., Tokyo, Japan) with bovine serum albumin as the standard.

Enzyme Purification

A. clavatus was aerobically cultivated for 3 days at 30 °C in 3000 mL of minimum medium containing 0.2% PESu pellets. The liquid culture of *A. clavatus* was centrifuged in a Kubota AG-5006 rotor at 10 000×*g* for 30 min, and the supernatant was used for enzyme preparation. The supernatant was filtered through a mixed cellulose membrane filter with a pore size of 0.45 μm (Toyo Roshi Kaisha. Ltd., Tokyo, Japan) to remove any contaminating mycelium. The supernatant supplemented with 0.3M $(NH_4)_2SO_4$ was applied to a Butyl-Toyopearl column equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.3M $(NH_4)_2SO_4$. The column was washed with 2 bed volumes of the buffer, and the enzyme was eluted with a linear gradient of 0.3 to 0 M $(NH_4)_2SO_4$ and 0 to 40% ethanol in the same buffer (Figure 2). The fractions with high activity were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.0). These fractions were concentrated to 1 mL with AQUA KEEP™ (Sumitomo Seika Chemicals Co. Ltd., Osaka, Japan) and dialyzed against 10 mM

sodium phosphate buffer (pH 7.0) for 24 h. The concentrated solution was stored at 4 °C.

Liquid Chromatography Electrospray Mass Spectrometry Analysis

The water-soluble fraction obtained after the enzymatic degradation of the PESu film was analyzed using the Micromass ZQ system (Nihon Waters K.K., Tokyo, Japan). Separation of the water-soluble products by liquid chromatography (LC) was carried out as described previously.[15]

Results and Discussion

P(3HB) Depolymerase and PESu Hydrolase Activities in the Culture Supernatant of *A. Clavatus* NKCM1003

Both P(3HB) depolymerase and PESu hydrolase activities in the culture supernatant of *A. clavatus* NKCM1003 were assayed when it was cultivated on the minimum medium containing the PESu film as the sole carbon source.

Figure 1 shows that both the activities reached a peak after incubation for 3 days. The sequential pattern of the PESu hydrolase activity was analogous to that of the P(3HB) depolymerase activity. For enzyme purification, the 3-day-old culture supernatant was used as the source of PESu hydrolase.

Purification of PESu Hydrolase from *A. Clavatus* NKCM1003

The PESu hydrolase that was obtained from the culture supernatant of *A. clavatus* NKCM1003, which was grown in a mineral medium containing the PESu film as the sole carbon source, was purified on a hydrophobic interaction column.[3] As shown in Figure 2, the elution volume of the PESu-hydrolytic activity peak was almost identical to that of the P(3HB)-hydrolytic activity peak. The enzyme gave a single protein band on the SDS-PAGE gel with a molecular mass of approximately 57,000 Da (Figure 3). Isoelectric focusing analysis indicated that the isoelectric point of the enzyme was 5.6. Table 1 lists the purification step of the enzyme.

Characterization of PESu Hydrolase

This enzyme maintained high PESu hydrolytic activities in the pH range of 5–8; the optimum pH for the enzyme activity was

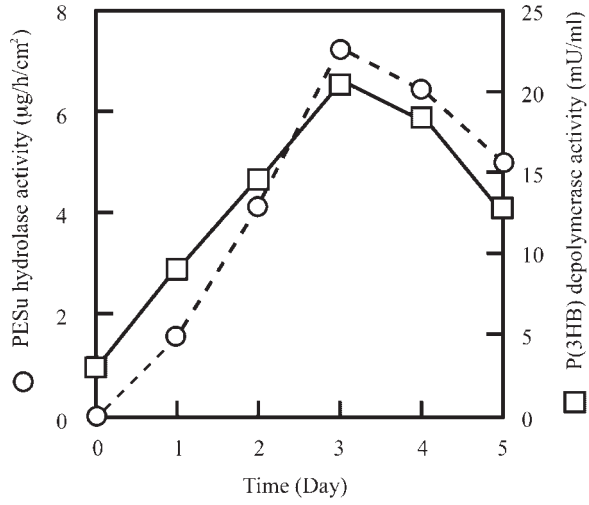

Figure 1.
PESu hydrolase and P(3HB) depolymerase activities in the culture supernatant of *A. clavatus* NKCM1003. The strain was aerobically cultivated in the minimum medium at 30 °C for 0–5 days.

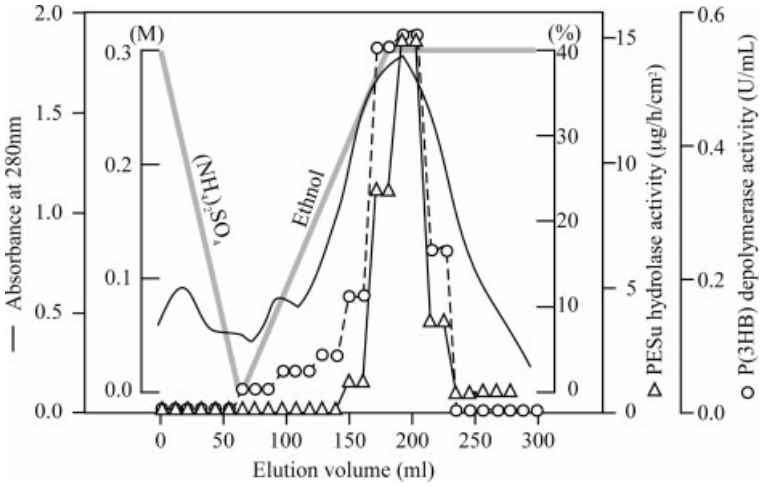

Figure 2.
Elution profile of PESu hydrolase. Three liters of the supernatant of *A. clavatus* NKCM1003 culture supernatant was applied to a Butyl-Toyopearl column. PESu hydrolase was eluted from the column with concentration gradients of $(NH_4)_2SO_4$ and ethanol. PESu hydrolase activity was determined by the weight loss method by using the PESu film. The P(3HB) depolymerase activity was determined by a turbidimetric method. After recovering the active fractions, they were concentrated with a water-absorption gel.

7.0. The enzyme was sensitive to the serine hydrolase inhibitor diisopropyl fluorophosphonate, indicating that it belongs to a class of serine hydrolases.

Enzymatic degradation products of PESu were investigated using liquid chromatography electrospray mass spectrometry (LC-MS). The products were composed of S (observed molecular weight: 118.1), ES (162.1), ESE (206.2), SES (262.2), ESES (306.3), ESESE (350.3), SESES (406.3), ESESES (450.4), ESESESE (494.4), SESESES (550.5), ESESESES (594.5), ESESESESE (638.5), SESESESES (694.6), ESESESESES (722.7), ESESESESESE (782.7), and SESESESESES (838.7) (S; succinate unit, E; ethylene glycol unit). From among them, ES, ESE, SES, and ESES were the major components.

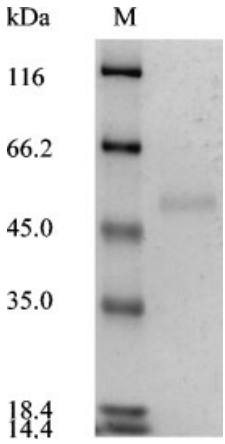

Figure 3.
SDS-PAGE analysis of PESu hydrolase. Lane M: molecular mass markers.

Figure 4 shows the substrate specificity of the enzyme. The enzyme-degraded polyesters had some common chemical structures; the number of carbon and oxygen atoms between 2 carbonyl groups of the backbone is 3 or 4. This substrate specificity is similar to that of P(3HB) depolymerase that has been studied so far, suggesting that the PESu hydrolase belongs to the class of P(3HB) depolymerase.

Kinetic Analysis

A turbidimetric method was used to investigate the kinetic parameters of the enzyme were by using the P(3HB) suspension substrate. The rate R (U) of P(3HB) enzymatic hydrolysis could be expressed by

Table 1.
Purification of PESu hydrolase from the *A. clavatus* NKCM1003 supernatant.

Step	Total activity[a)] (mg/h/cm^2)	Total protein (mg)	Specific activity[a)] (mg/h/cm^2/protein mg)	Recovery (%)
Supernatant	36.75	17.00	2.16	100
Butyl Toyopearl	3.35	0.15	22.3	9.1

[a)] The PESu hydrolase activity was determined by the weight loss method[4] by using the PESu film.

the following equation:

$$R = ksK[E]/[(1 + K[E])(1 + K[E])] \qquad (1)$$

where *ks* (U) is the rate constant of surface hydrolysis of P(3HB) chains by the enzyme, *K* (mL/μg) is an apparent adsorption equilibrium constant of enzyme, and [*E*] (μg/mL) is the apparent concentration of the added enzyme.[4]

Table 2 lists the kinetic parameters of this enzyme together with those of the fungus *Penicillium funiculosum* P(3HB) depolymerase (PhaZ$_{Pfu}$) and bacterium *Pseudomonas stutzeri* P(3HB) depolymerase (PhaZ$_{Pst}$). On comparing with the *K* values of these values, we found that the PESu hydrolase had a higher P(3HB)-binding affinity than PhaZ$_{Pfu}$. Considering that the molecular mass of PESu hydrolase is greater than that of PhaZ$_{Pfu}$ without the substrate-binding domain (SBD), the relatively high binding affinity of PESu hydrolase might be attributed to the presence of SBD. On the other hand, the P(3HB)-binding affinity of bacterial PhaZ$_{Pst}$ was higher than the PESu hydrolase, suggesting that bacterial SBD has a higher affinity than that of fungal SBD. The rate constant *ks* of the PESu hydrolase was also greater than that of PhaZ$_{Pfu}$, indicating that this enzyme

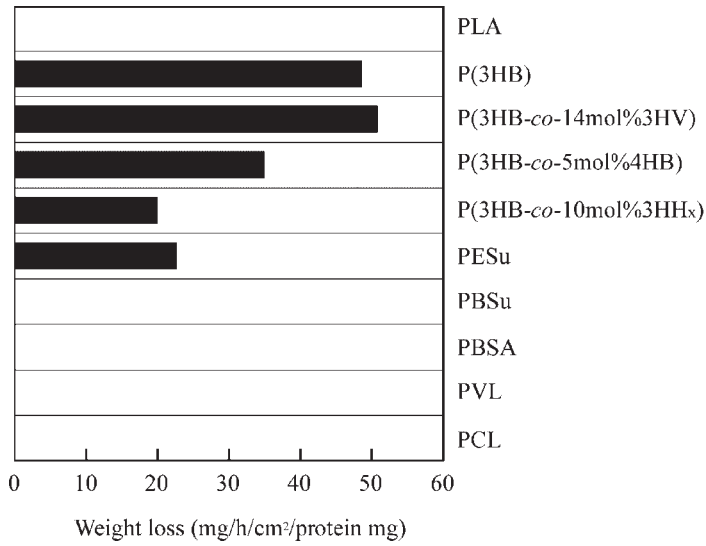

Figure 4.
Substrate specificity of PESu hydrolase. Ten polyester films (1 × 1 × 0.15 cm) were used as the substrate. The reaction mixture contained a film, enzyme, and 1 mL of phosphate buffer (pH 7.4) in a small bottle. Enzymatic degradation was performed at 37 °C for 24 h. The activity was expressed as the weight loss of the film per unit surface area per hour (mg/cm^2/h).

Table 2.
Kinetic parameters of enzymes.

Strain	Enzyme	*K* (mL/μg)	*ks* (U)
A. clavatus NKCM1003	PESu hydrolase	0.11	0.50
*P. funiculosum*IFO6354[3]	$PhaZ_{Pfu}$	0.06	0.20
Pseudomonas stutzeri JCM10168[3]	$PhaZ_{Pst}$	0.17	0.52

can degrade P(3HB) faster than $PhaZ_{Pfu}$. Taken together, these observations suggest that PESu hydrolase is a multi-domain type P(3HB) depolymerase.

Conclusion

A PESu hydrolase was purified to its electrophoretic homogeneity from a culture supernatant grown in a mineral medium containing the PESu film as the sole carbon source. This enzyme not only showed PESu- but also P(3HB)-hydrolytic activities. Kinetic analysis revealed that PESu hydrolase has a high binding affinity to P(3HB). It was concluded that PESu hydrolase belongs to the class of P(3HB) depolymerases.

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