प्लास्टिक का एंजाइमेटिक अपघटन

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प्लास्टिक अपघटनकारी एंजाइमों की एंजाइमेटिक गतिविधि की निगरानी के लिए स्पेक्ट्रोफोटोमेट्रिक आमापन का विकास

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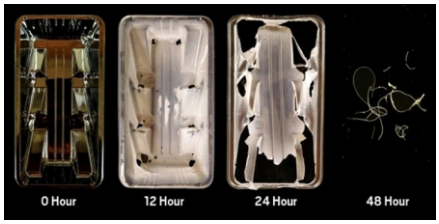

इंजीनियर आई. एस. पी. ई. टी. ए. एस. 48 घंटों में एक प्लास्टिक खाद्य पात्र का क्षरण और घुलनशील करता है।

सारांश

पृथ्वी प्लास्टिक के बोझ से दबी हुई है, जो हमारे पर्यावरण में सदियों तक रह सकती है, और उनके पुनर्चक्रण के लिए आवश्यक नियंत्रण की तत्काल आवश्यकता है। उपभोक्ता के उपयोग के पश्चात प्लास्टिक का एंजाइम-आधारित विकृति प्लास्टिक के बंद-लूप पुनर्चक्रण के लिए एक उभरती हुई दूरगामी हरित कार्यनीति है। एंजाइम, IsPETase पॉलीइथिलीन टेरेफ्थेलेट (PET) को उपयोगी अंतिम उत्पादों में हाइड्रोलाइज करता है जिसका उपयोग वर्जिन PET को संश्लेषित करने के लिए किया जा सकता है। इस प्रकार PETases कम कार्बन फूटप्रिंट के साथ PET के बंद-लूप हरित पुनर्चक्रण में उत्प्रेरक के रूप में कार्य करते हैं। PETases को कुशल PET जैव-अकवर्षक विकसित करने के लिए कृत्रिम बनाया जा रहा है। उनकी उत्प्रेरक क्षमताओं की त्वरित तुलना करने के लिए आमापन विकसित करना आवश्यक है। हमने उत्प्रेरक गतिविधियों और दरों की तेज़ी से निगरानी के लिए पराबैंगनी अवशोषण और प्रतिदीप्ति आधारित सब्सट्रेट का उपयोग करके IsPETase पर स्पेक्ट्रोप्रकाशमिति आमापन विकसित किया गया। इन आमापकों का उपयोग वाइल्ड-टाइप के ISPETase और इसकी उत्परिवर्ती तथा दस गुना अधिक उत्प्रेरक दक्षता प्रदर्शित उत्परिवर्ती की तुलना करने के लिए किया गया।

Enzymatic Degradation of Plastics

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Development of Spectrophotometric Assays to Monitor Enzymatic Activity of Plastic Degrading Enzymes

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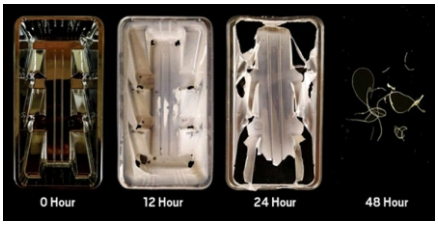

Engineered IsPETase degrading and solubilizing a plastic food container in 48 hours

ABSTRACT

Earth is burdened with plastics, which can stay in our environment for centuries, and necessary controls are required urgently for their recycling. Enzyme-based degradation of post-consumer plastic is an emerging green strategy for sustainable closed-loop recycling of plastics. The enzyme, *Is*PETase hydrolyze polyethylene terephthalate (PET) to useful end products which can be used to synthesize virgin PET. Thus PETases act as catalysts in closed-loop green recycling of PET with a low carbon footprint. PETases are being engineered to develop efficient PET bio-degrader. It is essential to develop assays to quickly compare their catalytic efficiencies. We developed spectrophotometric assays on *Is*PETase using UV absorbance and fluorescence based substrates to quickly monitor the catalytic activities and rates. These assays were used to compare wild-type *Is*PETase and its mutant and mutant exhibiting ten times higher catalytic efficiency.

KEYWORDS: *Plastic degrading enzyme, IsPETase, Michealis-Menten constant, Lineweaver-Burk, Catalytic efficiency*

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Introduction

The use of plastics in our daily lives is ubiquitous and unavoidable due to convenience and versatility causing a surge in demand for plastics globally. Plastics are resistant to biodegradation, and their natural degradation takes many centuries resulting in accumulation in our environment [1]. Conventional methods of plastic degradation, such as photodegradation and mechano-chemical processes are not environment friendly. Enzymatic bio-degradation of plastics has emerged as a promising green and environment friendly alternative, and closed-loop plastic re-cycling has low carbon footprint [2]. Microorganisms produce plastic degrading enzymes encompassing a diverse array of hydrolases, including lipases, esterases, and cutinases and breaks down bonds present in plastic polymers [3]. Polyethylene terephthalate (PET) is a thermoplastic polymer consisting of the ester-linked monomers of terephthalic acid (TPA) and ethylene glycol (EG). Food packaging industry, water bottles and textile fiber manufacturers use PET. A recently identified bacterium *Ideonella sakaiensis* has two hydrolytic enzymes, PETase (polyethylene terephthalates, *Is*PETase) and MHETase (mono(2-hydroxyethyl) terephthalate hydrolase, *Is*MHETase) capable of depolymerizing PET. *Is*PETase degrades PET into intermediate products, primarily mono (2-hydroxyethyl) terephthalic acid (MHET) and bis (2-hydroxyethyl) terephthalic acid (BHET), TPA and EG as minor products [4] (Fig.1(a)). *Is*MHETase further breaks MHET into TPA and EG (Fig.1(a)) [5]. These enzymes are continuously evolving and are not yet optimized to act on specific plastic substrates. For example, cutinases, known to degrade aliphatic polyester cutin in plants, have evolved to degrade plastics polymers with low substrate specificity. An efficient enzymatic hydrolysis of PET requires a reaction temperature of about 65-70^{0}C. *Is*PETase is active and stable below this temperature. Protein engineering of *Is*PETase using 3D structure-guided and machine learning approaches are being employed to increase the enzymatic activity, thermostability and expand substrate specificity [6-7]. Thus engineered IsPETase can directly recycle post-consumer PET in closed-loop and make virgin PET bottles using TPA and EG produced by enzymatic hydrolysis (Fig.1(b)) [7].

However, comparison of the enzymatic activities of PET hydrolases reported so far are limited to inaccurate weighing methods and cumbersome estimation of complex mixture of end products, BHET, MHET, TPA and EG using HPLC [5]. The development of fast and accurate spectroscopic assays using chromogenic and fluorogenic substrates are necessary to compare these enzymes and their mutants. We report here expression, purification, characterization and assay optimization to develop fast and efficient spectroscopic assays of *Is*PETases using UV absorbance and fluorescence substrates. The catalysis rate, kcat and binding constant, Km were determined for wild-type *Is*PETase and compared with its mutant. Our assays will enable quick comparison of activities of engineered *Is*PETases and other PETases.

Materials and Methods

The plasmids containing genes encoding His6-tagged IsPETase (WT) and its double mutant W159H/S238F (mutant) in pET-21b (+) vector were used to transform *E.coli* BL21(DE3) competent cells using heat shock method. The cells were grown in 600 ml LB media containing ampicillin and induced with 0.1 mM IPTG at 18^{0}C after OD_{600} reached 0.8. Cells were harvested by centrifugation after 18-20 hrs. The cell pellet was lysed in 20 mM Tris-Cl (pH 8), 300 mM NaCl buffer. The his-tagged WT and mutant enzymes were purified by Ni-NTA chromatography and further purified on a size-exclusion column using Akta purifier chromatography system by standard procedures. The purity of protein was checked in 12% SDS-PAGE gel. The UV absorbance substrate, para-nitrophenyl acetate (pNPA) and fluorescence substrate, 4-methylumbelliferyl acetate (4MUA) stocks were prepared in 100% DMSO. The standard calibration curves in triplicates were made using different concentrations of pNP and 4MU. The enzymatic assays were performed in triplicates using 10 mM PBS (pH 7.4) buffer and 1% DMSO in 96-well plates. The reaction progress was optimized by adding enzymes having concentration ranging from 30 nM to 1 nM. The assays were performed by varying the substrate concentrations at an optimized enzyme concentration. The post-cleavage product build-up was measured from the UV absorbance of para-nitrophenol (pNP) at 405 nm and fluorescence emission 4-methylumbelliferone (4MU) at 450 nm after exciting at 320 nm, using Clario Star-multimode plate reader.

Results and Discussion

Purified *Is*PETase and its mutant were obtained using size exclusion chromatography where single peaks of eluted proteins were collected and used in our experiments (Fig.2(a)). Single bands at ~30 kDa were observed on SDS-PAGE indicating that the proteins were very pure (Figure 2b). The UV absorbance substrate, pNPA and fluorescence substrate, 4MUA were used to first optimize the assays at different enzyme concentrations. The enzymes cleave the ester bond present in the substrates and releases the products which gives UV absorbance (pNP) and fluorescence (4MU) signals. Linear Lineweaver-Burk plots were obtained from initial velocities of Michaelis-Menten curves of hydrolysis reactions (Inset Fig.3(a)-3(d)). Michaelis-Menten constants (Km), maximum velocities (Vmax), turnover numbers (kcat) and

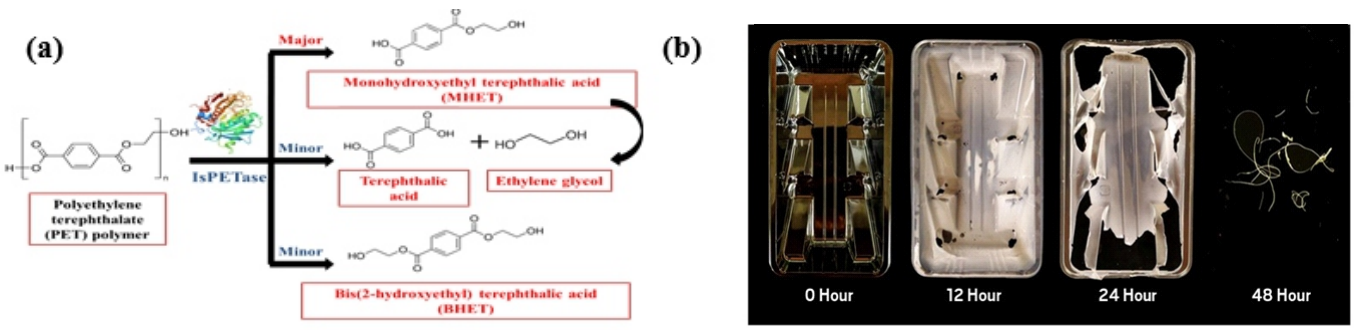

Fig.1: (a) The products released upon PET degradation by IsPETase and IsMHETase. b) Engineered IsPETase degrading and solubilizing a plastic food container in 48 hour [Ref. 7].

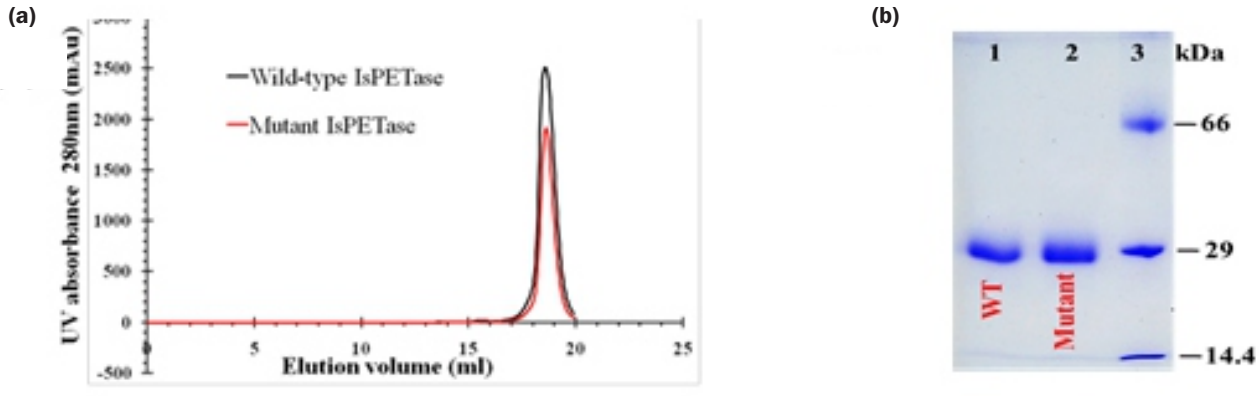

Fig.2: (a) Chromatogram of eluted enzymes from size exclusion column. (b) SDS-PAGE of purified WT IsPETase (lane 1) and its mutant (lane 2). Molecular weight markers are shown in lane 3.

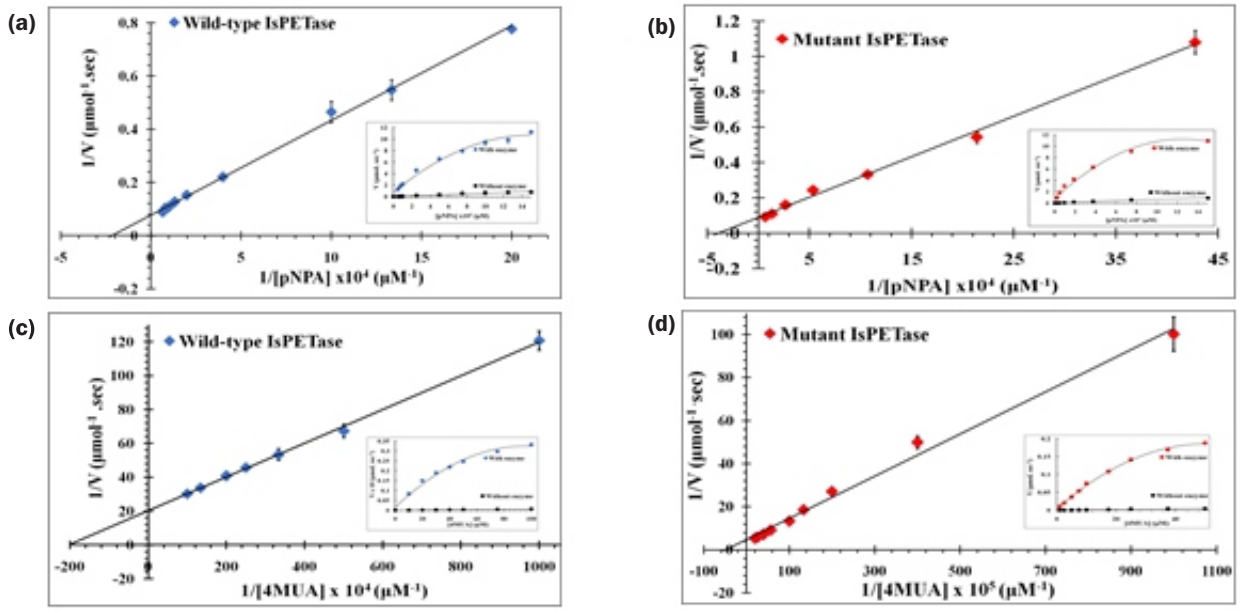

Fig.3: Lineweaver-Burk plot using (a) pNPA substrate with WT IsPETase, (b) pNPA substrate with mutant IsPETase, (c) 4MUA substrate with WT IsPETase, (d) 4MUA substrate with mutant IsPETase. Inset: Michaelis-Menten plots with and without enzyme.

catalytic efficiency (kcat/Km) from the intercepts (Table 1).

The Km value indicate the binding affinity of substrate to the enzyme. Both substrates show lower Km values with mutant enzyme and the values are half than that of WT enzyme, indicating tighter binding of substrates in the mutant active site. The Km values of the fluorescence substrate, 4MUA are about hundred-fold lower than that of pNPA indicating much higher affinity of 4MUA to IsPETase enzymes. The mutant enzyme binds 4MUA more tightly than WT. The kcat values of WT and mutant enzymes are similar for pNPA. The kcat values of mutant enzyme is 4.5 times higher than the WT enzyme for 4MUA indicating faster cleavage of the fluorescence substrate by the mutant. The catalytic efficiency (kcat / Km) of mutant is ten times higher than that of WT enzyme in case of 4MUA cleavage, and in case of pNPA mutant, it is 1.5 times more efficient. Thus, our studies indicate that mutant IsPETase is more efficient than the WT enzyme. Absorbance assays with PETases using pNPA have been used routinely by researchers worldwide. The catalytic efficiencies and Km values of 4MUA are higher than pNPA. The kinetic and binding parameters obtained experimentally indicate 4MUA is better substrate than pNPA and mutant enzyme. Our results indicate that fluorescence assay with 4MUA is more sensitive than

Table 1: Comparison of kinetic parameters of wild-type IsPETase and its mutant.

Substrate / Enzyme	pNPA		4MUA	
	WT	Mutant	WT	Mutant
K_m (µM)	4545 ± 344	2634 ± 235	48.47 ± 2.9	21.51 ± 6.2
V_m (µmol s^{-1})	14.00 ± 0.12	12.00 ± 0.07	0.05 ± 0.0016	0.22 ± 0.004
k_{cat} (s^{-1})	466.67 ± 39	400.00 ± 23	48.94 ± 1.6	217.01 ± 47.5
k_{cat} / K_m ($\mu M^{-1} s^{-1}$)	0.103 ± 0.014	0.152 ± 0.026	1.01 ± 0.034	10.09 ± 0.22

absorbance assay. The assay can be used to compare the relative catalytic efficiencies of engineered PETases and other PET degrading enzymes.

Conclusion

The IsPETase enzyme and its mutant were recombinantly expressed and obtained to high purity in our lab. The enzymatic assays using two different UV absorbance and fluorescence-based substrates were optimized. The kinetic and binding parameters obtained experimentally indicate 4MUA is better substrate than pNPA and mutant enzyme has ten times higher activity than WT enzyme. In future, these assays will be developed further using such substrates to compare engineered PETases and other PET degrading enzymes.

Acknowledgment

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References

[1] https://plasticseurope.org/knowledge-hub/plastics-the-fast-facts-2023/

[2] I. Taniguchi, S. Yoshida, K. Hiraga, et. al., Biodegradation of PET: Current Status and Application Aspects, ACS Catalysis, 2019, 9, 4089–105.

[3] M. Dimarogona, E. Nikolaivits, M. Kanelli, et. al., Structural and Functional Studies of a Fusarium Oxysporum Cutinase with Polyethylene Terephthalate Modification Potential, Biochimica et Biophysica Acta (BBA) - General Subjects, 2015, 1850, 2308–17.

[4] S. Yoshida, K. Hiraga, T. Takehana, et. al., A Bacterium That Degrades and Assimilates Poly (Ethylene Terephthalate), Science, 2016, 351, 1196–99.

[5] B.C. Knott, E. Erickson, M.D. Allen and J.E. McGeehan, Characterization and Engineering of a Two-Enzyme System for Plastics Depolymerization, PNAS (USA), 2020, 117, 25476–85.

[6] H. F. Son, I. J. Cho, S. Joo, et. al., Rational Protein Engineering of Thermo-Stable PETase from Ideonella Sakaiensis for Highly Efficient PET Degradation, ACS Catalysis, 2019, 9, 3519–26.

[7] H. Lu, D.J. Diaz, N.J. Czarnecki, et. al., Machine learning-aided engineering of hydrolases for PET depolymerization, 2022, Nature, 604, 662–667.