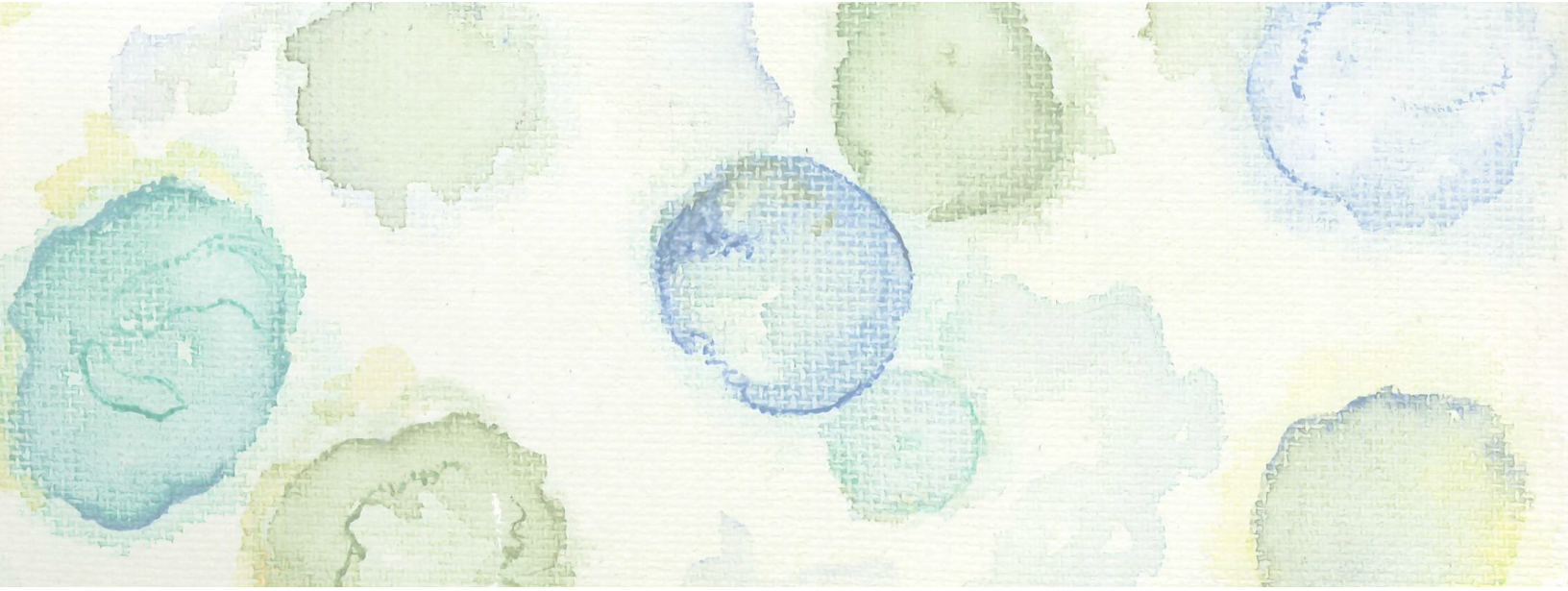

The Impact of PETase's Active Site Disulfide Bond on PET Biodegradation

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Manuscript received 4 July 2021; accepted 24 August 2021

ABSTRACT

Plastic pollution is one of the largest problems globally, with polyethylene terephthalate (PET) plastic as one of the main sources. Effective depolymerization of PET to its monomers for upcycling is a challenge. PETase is reported to be an effective enzyme for biodegradation of PET via C-O bond cleavage of ester linkage. The role of the disulfide bond, present in PETase's active site sequence, is unknown in the cleavage of PET's ester linkage. To understand the role of this bond, two separate versions of PETase – one containing the disulfide bond, and the other without the disulfide bond - were modeled using PyMol™, synthesized, and tested for degradation of PET surrogate compound, bis (2-hydroxyethyl) terephthalate (BHET). Several experiments were performed in the presence and absence of phenylmethylsulfonyl fluoride (PMSF), a serine protease. The results reveal that the role of the disulfide bond in the degradation of BHET's ester linkage is insignificant and the variation in the results (ethylene glycol yields, BHET degradation per microgram of enzyme) are within the experimental uncertainty. This finding is a stepping-stone to further modifying PETase and improving its activity towards commercial adaption of this technology for PET upcycling and creating a circular carbon economy, improving the world's carbon footprint, and mitigating ocean and environmental plastic pollution.

Introduction

Polyethylene terephthalate (PET) is one of the most common consumer plastics, which has a variety of uses ranging from plastic water bottles to polyester clothing items.(Paydar & Olfati, 2018) In 2013, the worldwide production of plastic was 299 million metric tons.(Chen *et al*., 2018) By 2015, the worldwide production had reached 322 million metric tons per year, and this amount will continue to increase. Despite plastic's versatile uses, it is also an environmentally hazardous substance because it does not biodegrade in natural ecosystems.(Chen *et al*., 2018)

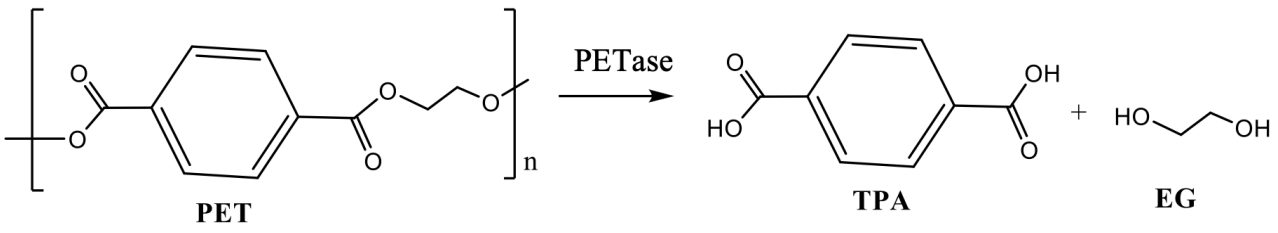
A small fraction of post-consumed plastics is currently recycled, mostly by physical recycling. Physical recycling refers to the process of melting and then re-shaping the plastic into new consumer products. For example, waste PET plastic bottles can be melted to make polyester carpeting.(Tullo, 2019) This method of recycling introduces impurities, ultimately reducing its quality and value. As a result of these challenges, only 18.4% of PET is recycled annually. (Tullo, 2019) All large-scale PET recycling today is physical recycling, and due to the lower quality end product, most manufacturers produce new PET from monomers, derived from crude oil, to meet the market demand.

Chemical recycling of PET plastic is now being explored as an alternative. It involves degradation of PET into its constituent monomers, ethylene glycol (EG) and terephthalic acid (TPA) (Scheme 1). The produced monomers can be chemically re-bonded to form PET plastic of the same caliber as new PET. This advantage makes chemical recycling preferable to physical recycling. Chemical recycling is not used in large-scale facilities due to the lack of an effective technology as well as use of corrosive chemicals such as sodium hydroxide, and harsh reaction conditions (e.g., high pressure requiring expensive reactors).(Khoonkari *et al*., 2015)

Glycolysis is the most efficient method of depolymerizing PET,(Khoonkari *et al*., 2015) which is done at high temperature (180°C to 240 °C).(Khoonkari *et al*., 2015) Ionic liquids such as 1-butyl-3-methylimidazolium bromide ([bmim] Br) have been used as catalysts for PET glycolysis. Wang *et al*. used [bmim] Br to degrade 100% of PET in 8 hours at 180 °C.(Khoonkari *et al*., 2015) This reaction depolymerized long ester chain of PET into short chain ester intermediates and did not fully degrade to produce desired EG and TPA.

Despite glycolysis showing high depolymerization yields, because it does not totally degrade PET, and necessitates high energy (up to 240 °C), more energy efficient and effective alternative methods have been explored for chemical recycling of PET.

One such method is enzymatic degradation of PET.(Kawai *et al*., 2020; Taniguchi *et al*., 2019) This method reduces the activation energy of the reaction, requires no pressure, and involves equipment which is inexpensive and commonly available. Enzymatic degradation of PET film was first accomplished by Muller in 2005.(Kawai *et al*., 2019; Müller *et al*., 2005) Their method depolymerized two kinds of PET films by approximately 40–50% at 55 °C in 3 weeks using cutinase obtained from the bacterium Thermobifida fusca. (Kawai *et al*., 2019; Müller *et al*., 2005) Since then, several thermostable cutinases have been discovered with an ability



Scheme 1. **PET breakdown to terephthalic acid (TPA) and ethylene glycol (EG).**

to break down PET film,(Furukawa *et al*., 2019; Ribitsch *et al*., 2012) but their degradation rates for PET are lower than that with cutin.

Recently, a Gram-negative bacterium, Ideonella sakaiensis 201-F6 was isolated that can consume PET as an energy and carbon source to survive.(Yoshida *et al*., 2016) Research revealed that this bacterium contains the enzyme, PETase, which can biodegrade PET into mono(ethylene terephthalate (MHET) and (bis(2-hydroxyethyl) terephthalate) (BHET). MHET and BHET are then hydrolyzed into EG and TPA. (Yoshida *et al*., 2016) 200 nM of wild type PETase enzyme was found to degrade 3.7 mg/L of PET at 30 °C over the course of 72 hours.(Seo *et al*., 2019) When compared with PET degradation activity of all reported enzymes, PETase demonstrated the best performance (Table 1).

PETase's active site is composed of a serine-histidine-asparagine (Ser-His-Asp) triad, which is one of the most commonly studied catalytic triads, and is often found in α/β hydrolases.(Jones & Solomon, 2015; Rauwerdink & Kazlauskas, 2015) It catalyzes a redox reaction to break the ester bond in PET.(Yoshida *et al*., 2016) The redox process is initiated when asparagine removes a hydrogen atom from histidine; thus, effectively removing a proton and an electron. The histidine then replaces its hydrogen by taking a proton and an electron from serine, creating a nucleophile.(Yoshida *et al*., 2016) The Ser-His-Asp triad removes an electron from the oxygen atom of PET's ester linkage and passes the electron to serine.(Yoshida *et al*., 2016) Then this oxygen atom takes an electron from its neighboring atom, making a free radical on the carbon atom, and weakens the C-O bond of the ester linkage. This results in cleavage of the ester linkage and complete the redox cycle. The extra electron gained by asparagine at the beginning of the reaction is given to O2, an electron acceptor, resulting in formation of two molecules of H2O.

PETase does not degrade PET film at a rate high enough to be utilized in recycling facilities. Commercial adaptation of this process would need much higher degradation rate. (O'Brien, 2019) In 2018, Austin *et al*. modified wild type PETase and improved the activity of the modified PETase to 2.5 times the wild type.(Ma *et al*., 2018) In the modification process, width of the active site cleft of the modified PETase was lowered through mutagenesis of two amino acids (phenylalanine and serine). The results indicated that specificity of the active site caused by a narrower cleft allows the substrate (PET) to better interact with the enzyme. (Austin *et al*., 2018) While other enzymes (e.g., cutinases) have already been perfected by nature because of their long existence, PETase, a recently developed enzyme, can be further modified through appropriate protein design.

Ma *et al*. also modified wild type PETase. In their modification, hydrophobicity was increased near the active site of the enzyme through mutagenesis under the hypothesis that hydrophobicity would increase PET degradation. This modification led to a 15-fold increase in degradation of PET as compared to the wild-type PETase.(Ma *et al*., 2018)

Although significant achievements have been made by researchers in improving wild type PETase's activity, more research is necessary in developing enzymes with higher thermostability, and better degradation capability, enabling their utilization for PET recycling by waste management facilities and chemical industries. To develop PETase for effective degradation of ester linkages, the following section summarizes desirable features, e.g. Isoelectric point, amino acid sequence, thermostability etc. of modified PETase.

PETase shares 52% sequence identity with T. fusca cutinase, its closest homolog.(Austin *et al*., 2018; Yoshida *et al*., 2016) This sequence can be used to identify features of PETase which make it an effective degrader of PET. These features include 3-fold wider active site cleft and higher Isoelectric point (caused by dipole; Isoelectric point of PETase is 9.6 while this value of T. fusca cutinase is 6.3) of PETase as compared to T. fusca cutinase.(Austin *et al*., 2018; Yoshida *et al*., 2016) In addition, PETase contains many basic amino acids such as lysine and arginine, which are charged and form salt bridges to make PETase stable.(Yoshida *et al*., 2016) (Austin *et al*., 2018)

Another important feature is thermostability. For example, Leaf Branch Compost cutinase (LCC), another cutinase with similar sequence to PETase, has high thermostability at

70 °C.(Shirke *et al*., 2018) Because glass transition temperature of PET plastic is around 70 °C, and PET becomes more pliable and its bonds weaken, LCC can degrade PET effectively at this temperature. In contrast, PETase is stable and active at much lower temperature (~37 °C) at which PET is not pliable and its bonds are rigid. Modified PETase with high thermostability would be beneficial.

PETase contains a disulfide bond in its active site. LCC and T. fusca cutinases do not have this disulfide bond.(Fecker *et al*., 2018) Simulations predicted that this disulfide bond increases flexibility around the active site.(Fecker *et al*., 2018) Austin *et al*. (Austin *et al*., 2018) have shown that a narrower binding cleft of the active site, arising from flexibility of enzyme, can improve enzyme's affinity to substrates, e.g., PET or BHET.

Table 1

Entry	Enzymes	Reaction Conditions	Degradation amount (methods used)	Ref
1	Modified PETase (mutant I179F)	Modified PETase expressed in *E. coli*, 5 μg PETase reacted with PET (1.5x1 cm2 size pieces) in bicine buffer; pH 8.5, 48 hours, 30°C	Mutant I179F has 2.5 times more degradation than wild-type PET [22.5 mg μmol-1·L−1 PETase per day biodegraded by mutant] (SEM)	(Austin *et al*., 2018)
2	PET-G/TfH*	20-25 mg PET (12 mm diameter disks) reacted with enzyme of concentration 0.1 mg/mL in buffer; 5 mL buffer; pH 7.0; 21 days; 55 °C	49.7 ± 1.0 % mass loss (mass weighed)	(Müller *et al*., 2005)
3	PET-G/rTfH	20-25 mg PET (12 mm diameter disks) reacted with enzyme of concentration 0.1 mg/mL in buffer; 5 mL buffer; pH 7.0; 21 days; 55 °C	54.2%% mass loss (mass weighed)	(Müller *et al*., 2005)
4	AB300432, AB298783, AB300774	7 × 7 cm2 Biomax® films in compost; 55-60 °C; 70-100cm below surface; 3 weeks and 4 weeks	All films fragmented in 4 weeks	(Hu *et al*., 2008)
5	Est119	1 × 1 cm2 PET film reacted with sufficient amount enzyme in the presence of 300 mM Ca2+;50°C; pH 7; 3 h	No weight loss or visible surface change (mass weighed and SEM)	(Thumarat *et al*., 2015)
6	Est1	1 × 1 cm2 PET film reacted with sufficient amount enzyme in the presence of 300 mM Ca2+;50°C; pH 7; 3 h	No weight loss or visible surface change (mass weighed and SEM)	(Thumarat *et al*., 2015)

Entry	Enzymes	Reaction Conditions	Degradation amount (methods used)	Ref
7	Thc_Cut1	10 × 100 mm2 PET film reacted with 6.75 μM enzyme in 13 mL buffer; 50 °C; pH 7.0; 2 hours	WCA decreased from 74.2° ± 1.6° to 66.3° ± 2.7° (WCA analyzer)	(Herrero Acero *et al*., 2011)
8	Thc_Cut2*	10 × 100 mm2 PET film reacted with 6.75 μM enzyme in 13 mL buffer; 50 °C; pH 7.0; 2 hours	WCA decreased from 74.2° ± 1.6° to 71.2° ± 0.9° (WCA analyzer)	(Herrero Acero *et al*., 2011)
9	Thf42_Cut1	10 × 100 mm2 PET film reacted with 6.75 μM enzyme in 13 mL buffer; 50 °C; pH 7.0; 120 hours	Crystallinity loss (FTIR-ATR)	(Herrero Acero *et al*., 2011)
10	Tha_Cut1	10 × 100 mm2 PET film reacted with 6.75 μM enzyme in 13 mL buffer; 50°C; pH 7.0; 2 hours	WCA decreased from 87.7° ± 4.8° to 45.0° ± 6.0° (WCA analyzer)	(Ribitsch *et al*., 2012)

*Tfu = T. Fusca; **TfCu = T. Fusca Cutinase; SEM = Scanning Electron Microscopy; FTRI = Fourier-transform Infrared spectroscopy; WCA = Water contact angle

It is hypothesized that the flexibility provided by the disulfide bond attributed to PETase's degradation performance.

The role of the disulfide bond, which is unique to PETase, has not been experimentally tested in the degradation of PET, despite it predicted positive traits from simulation. The present work experimentally demonstrates the effect of disulfide bond of PETase to the degradation of ester linkage of PET. First, amino acid sequence of a PETase protein without disulfide bond was modeled. Then PETases with and without disulfide bond were synthesized following their sequences and their activities in the degradation of ester linkage of PET have been experimentally tested using BHET as a model compound. BHET, a building block oligomeric unit of PET which contains EG and TPA monomeric units in 2:1 molar ratio, is used as a surrogate substate in this study because (1) it is expected to have faster degradation rate than PET and (2) it can completely degrade to EG and TPA, which can be quantitatively measured to accurately determine the role of the disulfide bond. Controlled experiments were performed without PETase and in the presence of phenylmethylsulfonyl fluoride (PMSF) to evaluate if PMSF disrupts PETase's activity by interacting with their catalytic triad serine. The results indicate that the disulfide bond does not significantly influence the degradation of BHET's ester linkage.

Experimental Section

Materials

Luria Bertani (LB) broth, calcium chloride, ampicillin, lysogeny broth, Tris buffer, HCl, phenylmethylsulfonyl fluoride (PMSF), Hi-Trap columns, and NaCl were procured from Sigma-Aldrich. Isopropyl β- d-1-thiogalactopyranoside (IPTG) and bichionic acid assay were obtained from Fisher Scientific. A refractometer was purchased from Hanna

Instruments. BL21 *E. coli* cells were obtained from Dr. Clark Gedney's lab in Biological Science department at Purdue University. PETase recombinant plasmid containing the gene to produce PETase with a disulfide bond (referred hereto as A2) was purchased from Integrated DNA Technologies (IDT). Amino acid sequence of the modified PETase without a disulfide bond (referred here to as A1) was modeled using PyMol™ software (Schrödinger, Inc. Version 3).
The sequence was sent to IDT to synthesize the modified PETase. Both the organism containing/expressing the plasmid were cultured for extracting proteins by following similar procedures as described below.

PETase Sequence Modification

The software Pymol™ was utilized in order to model A1 and A2. A2, which contains a disulfide bond, was produced by isolating the active site of PETase. Its sequence is shown in Figure 1c. To produce the sequence of A1 (Figure 1b), disulfide bond (red colored portion) of the sequence in Figure 1c was removed. Rather than using the entire PETase sequence, the active site was isolated to enable a focused investigation into the disulfide bond's effect on PETase's active site.

Transformation of *E. coli* cells

Frozen BL21 cells were grown on petri dishes of Luria-Bertani broth media until 20 colonies were visible on each plate. Approximately 20 colonies of *E. coli* were transferred to 250 µl of 0.1M calcium chloride solution in a sterile microcentrifuge tube and mixed. To another microcentrifuge tube, 20 colonies of *E. coli* were added to 250 µl of calcium chloride solution and mixed. 10 µl of PETase plasmid (e.g. A1) was added to one of these two tubes (Tube 1) and vortexed for 5 seconds. The second tube without plasmids is referred here to as Tube-2. Then both tubes were placed in an ice bath for 10 minutes, followed by 90 seconds in a heating block preset at 42 °C, and finally submerged in an ice bath for an additional 2 minutes. 500 µl of Super Optimal (SO) broth was added to both tubes and vortexed, and then the tubes were placed in a heating block at 37 °C for 30 minutes.

The contents of Tube-1 and Tube-2 were added to two separate flasks, each of which contained 1 L of lysogeny broth medium and 200 mg ampicillin, and they were cultured in a shaker at 37 °C until an optical density of 0.6 at 600 nm was reached. To confirm transformation, the cells from flask-1 were checked against the cells from flask-2. Flask-1 was cloudy due to the addition of the plasmids, whereas flask 2 was the negative control and remained clear. After this control test, the content of flask 2 was discarded. 0.1 mM IPTG was added to flask-1 at this stage, and the cells of flask-1 were further incubated for 24 h at 37 °C in the shaker at rpm of 250.(Yoshida *et al*., 2016)

The content of flask-1 was transferred to several 15 mL centrifuge tubes and they were centrifuged at 4000 rpm for 20 minutes on an Allegra centrifuge instrument. Supernatant from all of the centrifuged tubes was stored in a glass bottle. The cell pellets from all centrifuged tubes were collected and resuspended in 50 mL of 40 mM pH 7.4 Tris-HCl buffer containing 0.5M NaCl (Buffer 1). The resuspended cells were vortexed and divided equally into two centrifuge tubes of size 30 mL. To one of the tubes, 0.1M PMSF was added to test the effect of this protease inhibitor on enzyme activity. It is used for protein purification during cell lysis to prevent the proteases from degrading the protein.

Lysis

The cells from both 30 mL size tubes were separately added to a mortar and pestle containing liquid nitrogen in it to allow the content of each tube to solidify and ground until fine powders were formed, which were then stored at -20 °C. 5 grams of these lysed cells were resuspended in 20 mL of 20 mM phosphate buffer at pH 7.4 (Buffer 2) containing 20 mM imidazole. They were centrifuged at 4300 rpm for 30 minutes. The pellet was discarded and the supernatant, which contained protein expressed from the transformed cells, was syringe-filtered through a 0.45 micrometer syringe.

Protein Purification

A histidine column (1-mL HiTrap syringe) was used to purify the protein in the supernatant. The elution buffer consisted of 500 mM imidazole in Buffer 1, and the binding buffer consisted of 20 mM imidazole in Buffer 1. The Hi-Trap column was first prepared by eluting the binding buffer twice through the column at a rate of approximately 1 mL/min.

The supernatant containing the expressed protein was then eluted through the column. The elution buffer was run through the column in batches of 4 mL each time to elute the expressed protein which was collected separately in 15 mL centrifuge tubes. A total of 5 samples of each containing 4 mL eluant was collected. These 5 aliquots or samples were used for studying degradation experiments of PET model compound written below. Plasmid containing PETase enzyme with disulfide bond (A2) was similarly processed to obtain expressed protein for degradation study written below.

A bichionic (BCA) assay of each aliquot was done to measure total protein concentration. For this assay, each sample was mixed with bichionic acid obtained from Sigma and the mixture was kept on a microplate for 30 minute at 37 °C before measuring its color change at 526 nm using a Tecan microplate reader.

BHET Degradation

1M of Bis(2-Hydroxyethyl) terephthalate (BHET) was added to each eluted protein samples and the mixtures were allowed to continuously shake at 250 rpm and 37 °C for 72 hours. After 72 hours of reaction, the tubes were centrifuged, and 1 mL supernatant from each tube was collected for analysis by a refractometer and a UPLC instrument. The reaction was terminated by heating the solution in tubes at 85 °C for 15 minutes .(Austin *et al*., 2018) A total of 5 A1 samples, 5 A2 samples and 2 buffer solutions (control) were tested for BHET degradation.

Product Analysis.

Analysis of EG production in each sample collected at 72 hours was done using a UPLC equipped with a C18 column. A mixture of water and acetonitrile (45/55 wt/wt) was used as a mobile phase at a flow rate of 50 μL/min. The sample injection volume was 1 μL. The yield of EG was calculated from UPLC peak area using a pre-calibrated plot of peak area versus EG concentrations ranging between 10 μg/mL to 1000 μg/mL.

A refractometer was also used to measure EG production. For refractometer, a standard calibration plot for EG solutions of known concentrations was prepared to assess the accuracy in measurements of EG percentage for known solutions. Then, the percentage of EG yields in the BHET degraded solutions were measured. A good correlation in the yield of EG from UPLC and refractometry techniques was observed. The conversion of BHET from the yield of EG was calculated using the following equation:

BHET conversion

BHET (g) = ((% EG)(1097 g/L EG)(254.238 g/mol BHET)(0.5))/((100)(62.07 g/mol EG))

The yield of terephthalic acid was not measured because it precipitates in water.

Results and Discussion

Modeling modified PETase plasmid sequence

It has been reported that *E. coli* is a compatible host of PETase.(Seo *et al*., 2019) BL21 *E. coli* cells were utilized as vectors of the plasmids. Using PyMol™ software (Schrödinger, Inc. Version 3), amino acid sequence of PETase was modeled. Figure 1a shows the complete PETase structure, with the active site highlighted in yellow and red. In Figure 1b, the active site of PETase was isolated and modified, and the region which contains disulfide bond sequence was removed. This was done to test the effect of this disulfide bond on the activity of PETase. Removal of the disulfide bond sequence produced a much shorter protein with two helices and three beta strands (Figure 1b). The sequence of the modified active site (A1) was GVMGWSMGGGGSLISAANNPSLKAA APQAPWDSSTNFSSVTVPTLIFACENDSIAPVN. The sequence of the modified PETase was sent to IDT to synthesize it. The unmodified PETase active site sequence (A2) consisted of three alpha helices and four beta strands (Figure 1c). The portion highlighted in red displays the section of protein which was not included in A1. The sequence of A2 was GVMGWSMGGGG SLISAANNPSLKAAAPQAPWDSSTNFSSVTVPT LIFACENDSIAPVNSSALPIYDSMSRNAKQFLEI

NGGSHSC. BUSCA software,(Savojardo *et al*., 2018) which is a server through which a protein sequence can be analyzed, was used to predict expression of the PETase active site. This program determined that unmodified PETase

is an extracellular protein while the modified PETase is intracellularly expressed in the cytoplasm. 6-histidine tags were attached to the ends of the modified PETase to enable purification with a histidine syringe column.

BHET degradation efficiency using A1 and A2 PETase active sites

Figure 2 shows protein concentrations, which is equivalent to enzyme concentrations of A1 and A2 PETase, in the absence and presence of PMSF. The error bars show standard deviation from five measurements. Protein concentrations of A1 PETase in the absence and presence of PMSF are 31.57 ± 3.09 μg/mL and 37.52 ±2. 59 μg/mL, respectively, while these values for A2 PETase are 33.82 ± 3.52 μg/mL and 33.82 ± 3.48 μg/mL respectively.

The results indicate that protein concentration of A2 PETase, which contains disulfide bond, did not change significantly in the presence of PMSF. There is a significant increase in protein concentration of A1 PETase, which does not contain disulfide bond, in the presence of PMSF. PMSF is a serine protease inhibitor, which blocks the activity of proteases such as elastase that degrade PETase. A1 PETase is less stable in comparison to the A2 due to its lack of the disulfide bond. The disulfide bond makes proteins more globular, less likely to denature, and increases protein durability. Removal of this disulfide bond from A1 made PETase more susceptible to degradation by protease inhibitors, and the addition of PMSF blocked proteases from degrading A1. Total protein concentration of A1 PETase increases in the presence of PMSF. A2 is more stable due to its disulfide bond, so it was not impacted by proteases to the same extent as A1, and the addition of PMSF did not create a significant difference in protein concentration in it. BCA assay of a control experiment without addition of PETase shows no protein.

BHET degradation by A1 and A2 PETase demonstrate that both can efficiently degrade BHET to EG and TPA within 72 hours (Figure 3). A control experiment containing only buffer solution showed no BHET degradation in terms of the yield of EG. A1 and A2 PETase produced similar amount of EG (14 – 15%). The error bars in Figure 3 represent standard deviation from 5 replicates. The presence of PMSF with A1 or A2 PETase showed insignificant changes in the yield of EG.

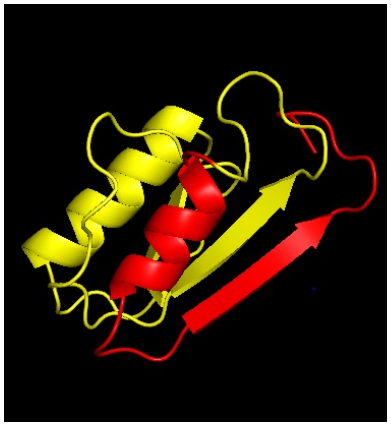


Figure 1a. **Structure of complete PETase.**

Figure 1b. **Modified PETase sequence without a disulfide bond (A1).**

Figure 1c. **Unmodified PETase sequence with a disulfide bond (A2).**

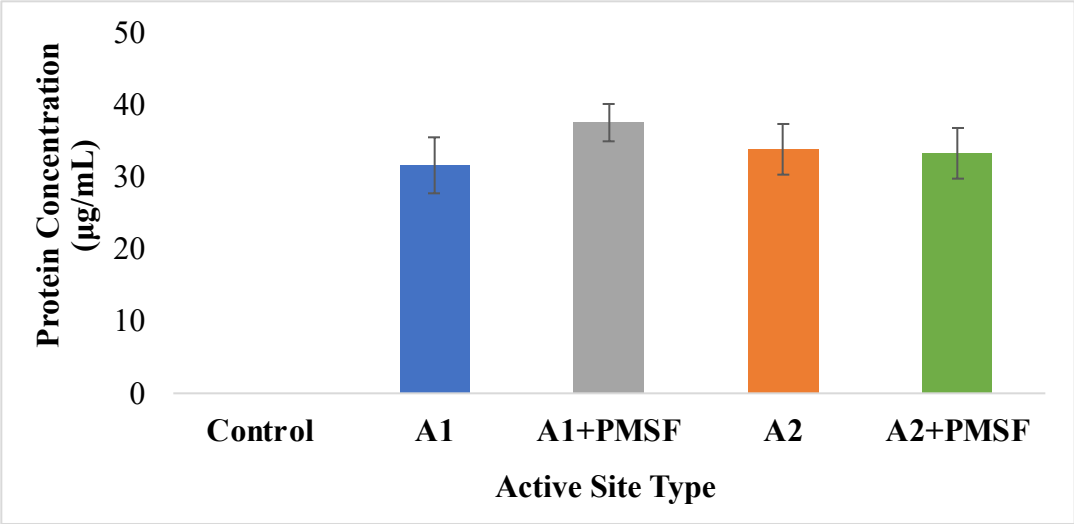


Figure 2. **Experimentally measured protein concentration of A1 and A2 PETase in the presence and absence of PMSF.**

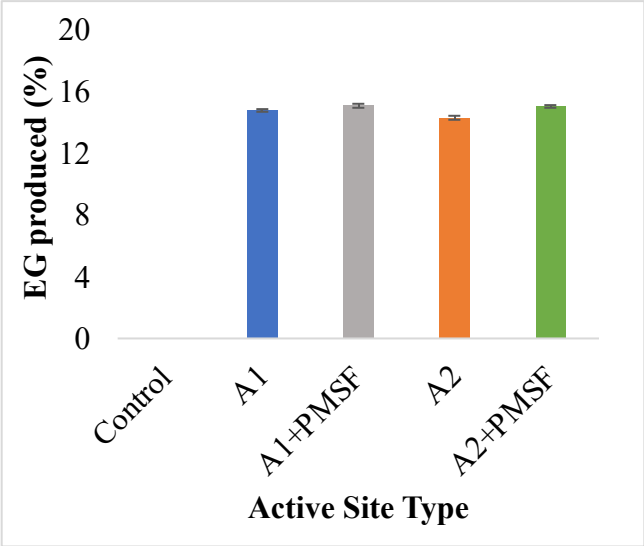


Figure 3. **Percentage of ethylene glycol produced by A1 and A2 PETase with and without PMSF in 72 hours.**

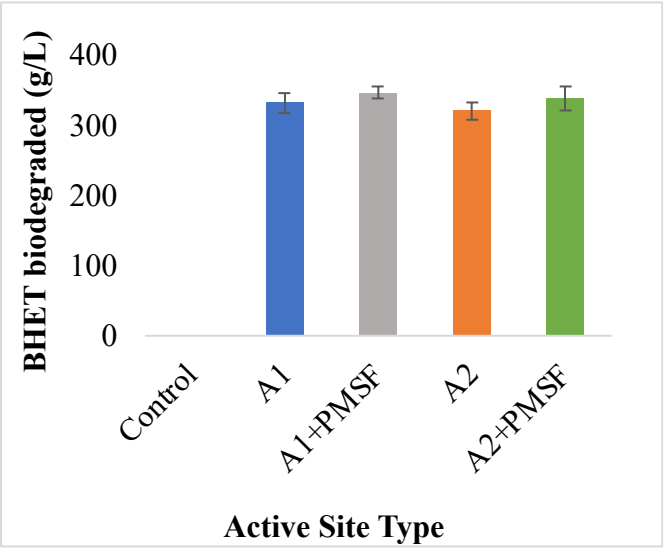


Figure 4. **Total BHET degraded by A1 and A2 PETase with and without PMSF in 72 hours.**

Figure 4 shows total amount of BHET degraded in 72 hours. A1 and A2 PETase resulted in 332.05 ± 14.44 g/L and 320.85 ± 12.58 g/L BHET conversion, respectively, in the absence of PMSF, which is quite high. BHET conversion by A1 PETase is a little higher in the presence of PMSF, which is likely because of a slightly higher amount of protein in the solution as seen in Figure 2.

The activity of both proteins was further determined in terms of BHET degradation per unit (microgram) of each protein (Figure 5). The error bars in Figure 5 display standard deviation from 5 replicates. It shows that the activity of A1 PETase (0.1073 ± 0.0168 g/µg enzyme) is slightly higher than that of A2 (0.0962 ± 0.0113 g/µg). The activity of A1 in the presence of PMSF is slightly lower (0.0843 ± 0.0191 g/µg) because of total measured protein concentration in the presence of PMSF was higher as discussed above. Upon consideration of standard deviation, the activity of A1 and A2 PETase appears to be similar, which ranges between

0.08-0.1 g BHET degradation/μg of protein. While absence of the disulfide bond in A1 structural sequence makes it less stable, owing to the lack of globular structure, and its activity was expected to be lower with reference to the activity of A2, the similar activity of A1 and A2 indicates that the active site disulfide bond does not have an impact on PETase's activity.

The results shown in Figure 5 demonstrate that the activity of A1 PETase is significantly affected by the presence of PMSF. It indicates that the short structure of A1 is unstable in the presence of PMSF as the serine is blocked in the catalytic triad. In contrast, A2 exhibits a slight increase in activity upon the addition of PMSF because of the higher stability given by the disulfide bond, but it could be within the experimental uncertainty. Thus, PMSF did not inhibit the activity of A2 PETase's active site as hypothesized from the fact that PMSF would block the serine at the catalytic triad and disrupt enzyme activity. The addition of PMSF prevents protease activity throughout the reaction, resulting in preserving the activity of enzyme A2.

This work advances the understanding on the role of the disulfide bond of PETase in the degradation of BHET as a model compound, which will enable future development of more active PETase without this bond for degradation of PET to EG and TPA.

Conclusions

This work described the cleavage of ester linkages, present in PET plastic, with PETase enzymes in the presence and absence of a disulfide bond to elucidate the role of the disulfide bond on the enzyme's activity. A modified PETase enzyme without disulfide bond sequence was first modeled and synthesized. Then, PETase enzymes with and without the disulfide bond were cultured, purified, assayed, and used for cleavage of ester linkages of a PET surrogate substrate, BHET. Typically the reaction between PET and PETase must be run for several days, and the results are analyzed with cumbersome microscopic techniques to measure crystallinity loss of PET, which does not give a direct measurement of ester linkage cleavage. In contrast, BHET degradation forms EG and TPA monomers to quantitively measure the degree of ester linkage degradation and allows evaluation of enzyme's activity more effectively. Controlled experiments were also conducted in the presence and absence of PMSF as well as without an enzyme. The results showed that the total BHET degradation to EG and TPA was not significantly influenced by the disulfide bond of PETase, which strongly conflicted prior works that predicted the disulfide bond increased protein activity.

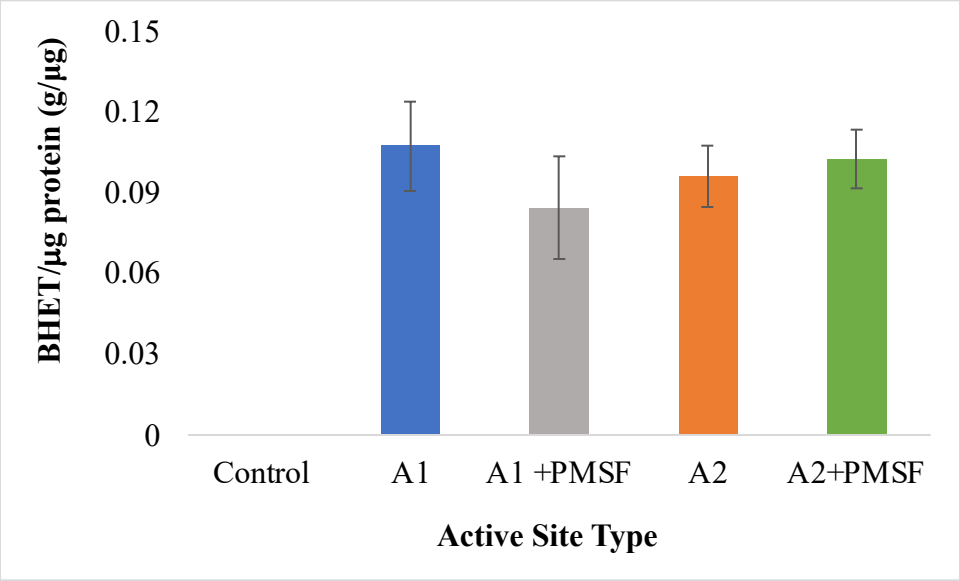


Figure 5. **Comparison of BHET degradation per microgram of enzymes in the presence and absence of PMSF. Control experiment showed no degradation of BHET.**

Further research is necessary to determine the cause of this phenomenon. It is likely that A1's short structure facilitates easy accessibility of its active site to BHET in the degradation process. Although A2 may be more stable, A1 has the ability to degrade a greater amount of BHET in the same time period. The finding of this work - that removal of the active site disulfide bond in PETase does not have an impact on its activity - is a stepping stone in designing a more effective version of the PETase enzyme.

Acknowledgements

K.S. greatly acknowledge Dr. Clark Gedney for allowing to conduct this project in his laboratory, acting as a mentor, and providing necessary resources. Dr. Lavanya Reddivari at Purdue University is acknowledged for giving access to her UPLC instrumentation facility for the purpose of analyzing samples. Ms. Brittany Croy at West Lafayette Jr/Sr. High School is acknowledged for acting as a mentor of K.S. in this project, which was submitted to the International Science and Engineering Fair (ISEF).

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