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Sequence-Structure Based Comparison of Structurally Homologous Thermophilic and Mesophilic Polyethylene Terephthalate (PET) Hydrolases

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ABSTRACT

Protein structure has a direct impact on thermostability. Deviations in the primary sequence can affect structural changes, leading to alterations in thermostability properties. However, the molecular basis of protein thermostability is unspecified; thus, elucidation of key factors that role particular protein thermostability is required when engineering proteins to be thermostable. To address this challenge, the amino acid composition, hydrophobicity/hydrophilicity ratio, cysteine bridges, and intrinsic features of two structurally homologous but different thermostability, poly(ethylene terephthalate) hydrolase (PETase) were compared. According to the findings, thermostable and thermolabile PETases have similar folds, compactness, and disulfide bridges. Interestingly, an abundance gap of aromaticity, hydrophobic cluster area, polar amino acid and hydrogen bond network compositions demonstrated dominant trends of variations for both PET hydrolases, indicating a pivotal role of these features in the thermostability of PET hydrolase. Furthermore, increased hydrophobic amino acid frequency in the inner surface of thermostable proteins contributed significantly to thermostability by forming more internal hydrophobic interactions and a less hydrophobic patch. There are no consistent trends in insertions and deletions between both PETases. Taken together, these observations demonstrate that hydrophobicity and hydrogen bond networks are essential factors in thermostability of thermostable PETase.

1. Introduction

Thermostability is defined as a substance's resistance to irreversible chemical or physical changes caused by temperature rise (Zhou *et al.* 2008). Protein thermostability is thus the ability to maintain of polypeptide chains' unique structure and chemical characteristics under extreme temperatures. Thermostability is a significant feature of enzymes because it increases productivity in the industry. When enzymes can operate at higher temperatures, more reagents and compounds become more soluble (Lasa and Berenguer 1993; Leuschner and Antranikian 1995).

Vieille and Zeikus (2001) published a comprehensive review of the determinant factors of an enzyme's thermostability. Several parameters, for instance, amino acid composition, disulfide bridges, hydrophobic interactions, aromatic amino acid interactions, hydrogen bonds, ion pairs, oligomerization, and metal binding were considered to play the role on thermostability. Thermostable enzymes, on the other hand, are the result of a synergistic interaction of several variables rather than a single main factor, particular for enzymes that are expressed naturally by thermophilic organisms. In thermophilic organisms, several variables are involved in supporting the thermal stability of enzymes. Amino acid preference, the ratio of charged versus uncharged amino acids, ionic interactions, codon use, hydrophobicity, and protein surface area are examples of these (Zhou *et al.* 2008). Even though the enzymes used as examples originate from hyperthermophilic sources (Vieille and Zeikus

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2001), to date, the publication written two decades ago has become guidance for scientists conducting protein engineering (Li *et al.* 2022; Van Wyk *et al.* 2022; Liu *et al.* 2022; Srivastava *et al.* 2023). Since there, numerous engineering methods to obtain thermostable enzymes or other purposes have been documented (Wang *et al.* 2006; Shirke *et al.* 2018; Khersonsky *et al.* 2018; Noda-Garcia *et al.* 2018; Deng *et al.* 2023).

The application of thermostable enzymes is expected in the plastics disposal problem solution. Plastics are polymers composed of monomers linked together by particular and powerful chemical bonds. Poly(ethylene) terephthalate (PET) is a thermoplastic polymer widely utilized in various applications, including packaging, textiles, and consumer goods. PET is a strong, lightweight, adaptable polymer that resists moisture, chemicals, and heat. PET is also a low-cost material to manufacture, making it a popular choice among producers. While PET has many beneficial properties, it is not biodegradable and can last for hundreds of years in the environment. This condition results in a substantial build-up of plastic debris in landfills, oceans, and other natural environments, endangering species and ecosystems. So far, mechanical and chemical recycling are common methods for dealing with PET waste. Overall, PET is a valuable material with many favorable features, yet, its environmental effect and the possibility for long-term accumulation of plastic trash are significant challenges that must be addressed. Biodegradable and compostable PET alternatives are also being developed, which might potentially lessen the environmental impact of plastic trash. These materials, however, are not yet widely available or cost-effective (Bornscheuer 2016; Yoshida *et al.* 2016; Papadopoulou *et al.* 2019; Tournier *et al.* 2020).

PETase or polyethylene terephthalate hydrolase catalyzes the breakdown of PET. Due to its capability to degrade PET, PETase is an attractive enzyme for plastic waste management and processing applications. PETase expression has been reported from various sources of bacterial species that feed on PET, with *Ideonella sakaiensis* and leaf branch compost (LCC) being the most fascinating PET hydrolase (Bornscheuer 2016; Yoshida *et al.* 2016; Son *et al.* 2019; Deng *et al.* 2023).

I. sakaiensis PETase (*Is*PETase) and Leaf Compost Cutinase (LCC) can decompose PET polymer into monohydroxyethyl terephthalate (MHET) monomer. Although the two possess a relatively extensive sequence homology and structure folding, interestingly, both enzymes significantly differ in thermostability (Yoshida *et al.* 2016; Shirke *et al.* 2018; Tournier *et al.* 2020). More specifically, the *Is*PETase has been deeply explored for its potential application in PET depolymerization; nevertheless, its evolution from cutinases is not entirely known, and attempts undertaken to improve *Is*PETase's thermostability and catalytic efficiency have resisted the majority of the accessible sequence space distant from the active site (Son *et al.* 2019; Lu *et al.* 2022; Deng *et al.* 2023). LCC PET hydrolase is, in fact, a cutinase type-enzyme capable of degrading PET that has higher activity and thermostability than *Is*PETase, the naturally occurring form of the enzyme. LCC PET hydrolase might be a viable candidate for application in industrial operations for the degradation of PET polymers due to its thermostability. The employment of LCC PET hydrolase in these processes has the potential to minimize the amount of energy required and the expenses associated with plastic degradation (Sulaiman *et al.* 2012; Bornscheuer 2016; Yoshida *et al.* 2016).

The fundamental question concerning how LCC PET hydrolase has higher thermostability characteristics compared to *Is*PETase and which intrinsic parameters become crucial to LCC PET hydrolase thermostability. In the present study, *Is*PETase, LCC sequence, and structural information were used to identify the detail and main factors governing LCC thermostability. We employ bioinformatic tools, online web servers, and structural modeling to reveal the impact of each parameter. The findings of this study could provide guidance in tailoring thermostable *Is*PETase.

2. Materials and Methods

2.1. Sequence and Structure Database

Sequence of *Is*PETase (accession: A0A0K8P6T7) (Yoshida *et al.* 2016) and LCC (accession: G9BY57) (Sulaiman *et al.* 2012) were extracted from NCBI. Structures of *Is*PETase (PDB: 6ANE) (Fecker *et al.* 2018) and LCC (PDB: 6THT) (Sulaiman *et al.* 2014;l Tournier *et al.* 2020) were derived from Protein Data Bank and visualized by PyMOL (Schrödinger and DeLano 2020).

2.2. Sequence Intrinsic Parameter

Sequence intrinsic parameters (bulkiness, secondary structure, hydrophobicity, polarity, average of buried area and homology/identity) of both PETases were analyzed by EMBOSS Pepstats (Madeira *et al.* 2022) and Web Server EXPASY (https://web.expasy.org/protscale/) (Gasteiger *et al.* 2005). Subsequently, all parameters were compared.

3. Results

3.1. Sequence and Structural Comparison

The thermostability and sequence comparison between *Is*PETase and LCC are summarized in Table 1. Melting temperature of LCC PETase is two-fold higher than *Is*PETase, indicating thermostability of LCC is higher than *Is*PETase. Both enzymes possess similar molecular weights.

The *Is*PETase and LCC comprise 290 and 293 amino acids, respectively. Both sequences have 44.7% of identity and 59.5% of similarity with equivalent isoelectric points, which demonstrates that the two PET hydrolases possess a relatively high degree of sequence homology (Figure 1). Two disulfide bridges and similar salt bridge characteristics reflected by comparable FCR and k values are established by *Is*PETase and LCC (Table 1). Interestingly, the LCC's aromatic, non-polar and

Table 1. Biochemical and structural features comparisons of PETases (Son *et al.* 2019; Tournier *et al.* 2020).

Sequence intrinsic parameters	*Is*PETase	LCC
Melting temperature (°C)	45	84.7
Molecular weight (g.mol-1)	30246.87	30179.21
Amino acid	290	282
Isoelectric point	9.9	9.5
Aromatic residues	25	30
Non polar residues	162	164
Charged residues	128	118
Polar residues	37	43
Hydrophobic cluster area (Å)	2924.5	4662
Hydrogen bonds network	19	24
Salt bridges		
-Fraction of charge residue (FCR)	0.13	0.13
-Kappa value (k)	0.17	0.17
Disulphide bridge	2	2

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IsPETase          1 MNFPRAS------RLMQAAVLGGLMAVSA--------AATAQTNPYARGP     36
                                 |:..||::..|:|::|        :..||:|||.|||
LCC               1 -------MDGVLWRVRTAALMAALLALAAWALVWASPSVEAQSNPYQRGP     43

IsPETase         37 NPTAASLEASAGPFTVRSFTVSR--PSGYGAGTVYYPTNAGGTVGAIAIV     84
                    |||.::|.|. |||:|.::||||  .||:|.|.:||||....|.|.||:.
LCC              44 NPTRSALTAD-GPFSVATYTVSRLSVSGFGGGVIYYPTGTSLTFGGIAMS     92

IsPETase         85 PGYTARQSSIKWWGPRLASHGFVVITIDTNSTLDQPSSRSSQQMAALRQV    134
                    |||||..||:.|.|.|||||||||:.|:|||..|.|.||:||..|||..
LCC              93 PGYTADASSLAWLGRRLASHGFVVLVINTNSRFDYPDSRASQLSAALNY-    141

IsPETase        135 ASLNGTSSSPIYGKVDTARMGVMGWSMGGGGSLISAANNPSLKAAAPQAP    184
                      |..:|.|.:..::|..|:.|.|.||||||:|..|..|||||||.|..|
LCC             142 --LRTSSPSAVRARLDANRLAVAGHSMGGGGTLRIAEQNPSLKAAVPLTP    189

IsPETase        185 WDSSTNFSSVTVPTLIFACENDSIAPVNSSALPIYDSM-SRNAKQFLEIN    233
                    |.:...|:: :||.||...|.|::|||:..|:|.|.:: |...|.::|::
LCC             190 WHTDKTFNT-SVPVLIVGAEADTVAPVSQHAIPFYQNLPSTTPKVYVELD    238

IsPETase        234 GGSHSCANSGNSNQALIGKKGVAWMKRFMDNDTRYSTFACENPNSTRVSD    283
                    ..||.   :.|||.|.|....::|||.::||||||..|.| |.|...:||
LCC             239 NASHF---APNSNNAAISVYTISWMKLWVDNDTRYRQFLC-NVNDPALSD    284

IsPETase        284 FRTAN--CS     290
                    |||.|  |.
LCC             285 FRTNNRHCQ     293
```

Figure 1. Sequence comparison between IsPETase and LCC. The sequences are aligned by EBLOSUM62 matrix with gap and extend penalties are 10 and 0.5, respectively

polar residues are higher than the *Is*PETase. In contrast, charged residues of IsPETase are higher than LCC.

The three-dimensional structure alignment of *Is*PETase and LCC is presented in Figure 2A. Both enzymes share a similar fold and active site. The catalytic triad (Ser-Asp-His) and oxyanion hole (Tyr-Met) are well conserved in terms of position in the protein structure (Figure 2B). Detailed assessment of secondary structure indicated that some additional helix-secondary structures were observed in LCC. The additional helices are located in S63-V68, A244-S247 and N289-Q293. These additional helices may contribute to the thermostability features of LCC.

Furthermore, we compared the hydrophobic clusters area (HCA) to understand better how HCA affects the thermostability of PETases. As displayed in Figure 3, the HCA demonstrated that *Is*PETase (Figure 3A) has less hydrophobic cluster area than LCC (Figure 3B). The HCA of LCC shows almost two-fold wider than *Is*PETase which undoubtedly supports its higher thermostability. This is also reflected by higher aromatic and non-polar residues of LCC PETase (Table 1).

Hydrogen bond network between of both PETases are visualized ini Figure 4. The LCC is supported by 24 hydrogen bonds, while only 19 hydrogen bonds are formed at *Is*PETase (Table 1). Moreover, single amino acids form some hydrogen bonds from both enzymes. In *Is*PETase, Q99, R126, R240, and R258 form more than two hydrogen bonds and R108, Q134, R158, R173, R269, and R286 in LCC form with similar characteristics.

4. Discussion

Further, *Is*PETase and LCC are representatives of two PET hydrolases whose different thermostability and probably different root of enzyme origin. However, for our knowledge, there is no report that summarize explicitly the comparison and analyses between these two enzymes. Therefore, this report has useful information from the mentioned aspect.

Utilizing the distinct feature that determines a certain protein's activity and thermostability offers a possible path toward creating and modifying a highly active thermostable protein. Protein structural similarity is commonly considered to share functional similarity. Moreover, using thermostable enzymes as a comparator also helps us to investigate potential mutation locations in less stable enzymes. An extensive study of amino acid sequences and their interactions within the structure has also demonstrated results in enhancing protein thermostability (Finch and Kim

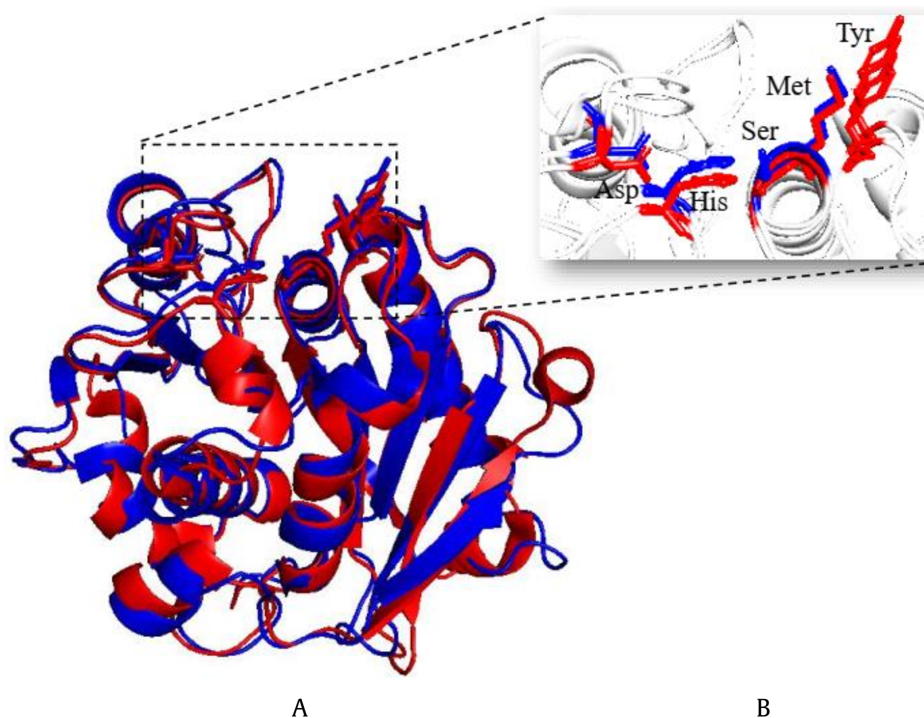

Figure 2. (A) Structure alignment of *Is*PETase (blue) and LCC (red), (B) the position of active site (Ser-Asp-His) and oxyanion hole (Met-Tyr) of two PET hydrolase

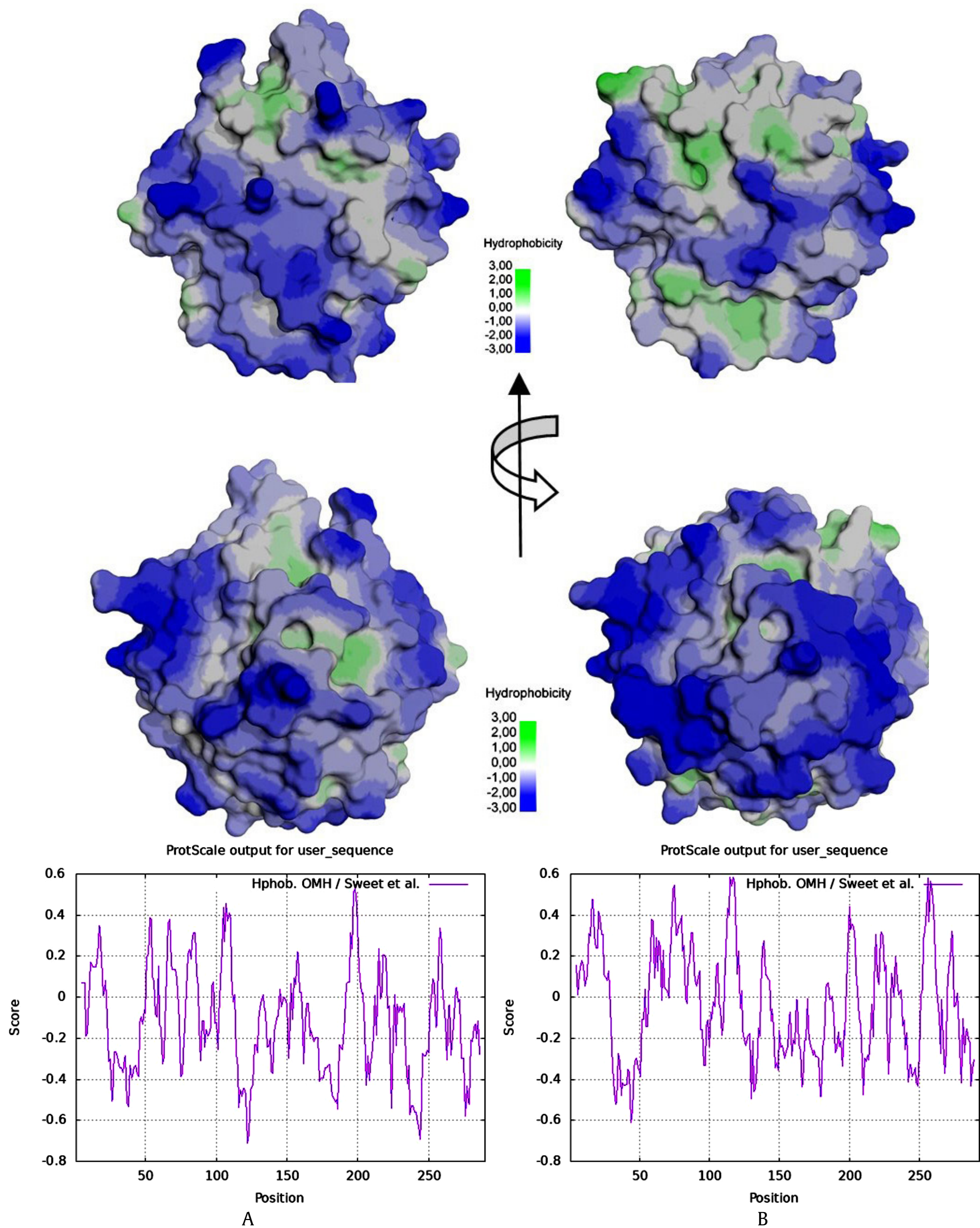

Figure 3. Hydrophobic cluster area (HCA) visualization in hydrophobicity-surface model and analysis of (A) *Is*PETase and (B) LCC

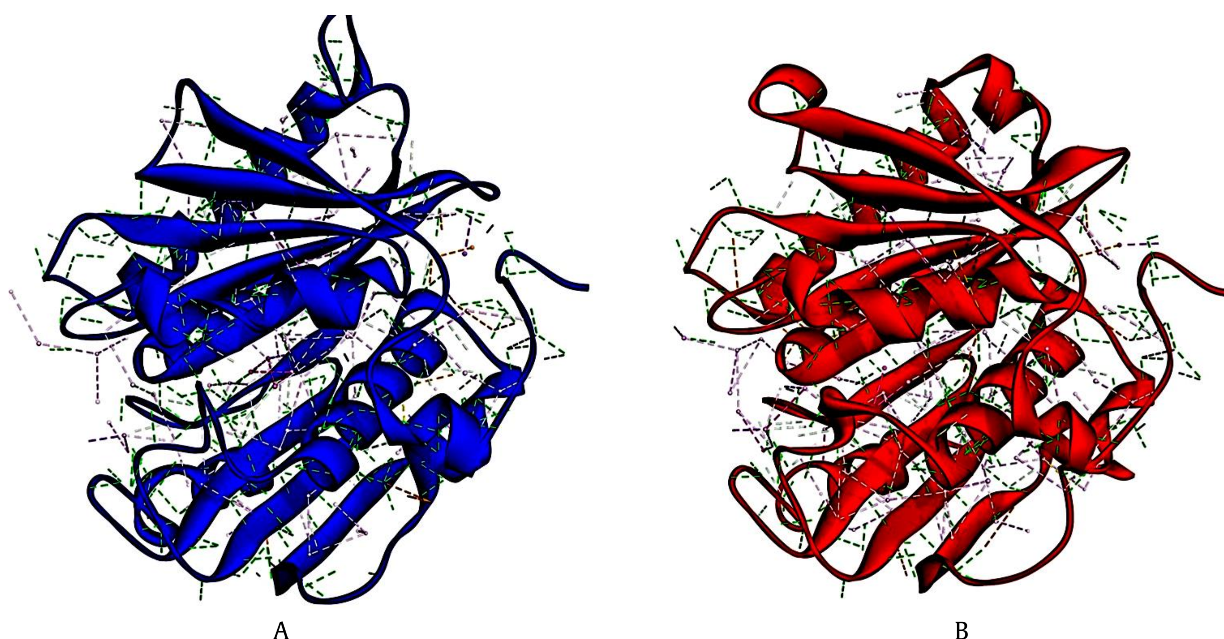

Figure 4. Hydrogen bond network of (A) IsPETase and (B) LCC PETase

2018; Sternke *et al.* 2019). The structure-sequence relationship has been assessed on several enzymes using bioinformatic tools to identify the important features in the enzyme that play a pivotal role in activity or thermostability (Swillens *et al.* 1986; Gan *et al.* 2002; Mazumder and Vasudevan 2008; He *et al.* 2017; Guzzi *et al.* 2023). Because each enzyme is unique, there is no precise consensus that a particular interaction within the enzyme is the primary key to enzyme thermostability (Suplatov *et al.* 2015).

This study compares sequence-structure-based thermostability of two fascinating structurally homologous PET hydrolases with different thermal stability. The *Is*PETase belongs to thermolabile enzymes, while LCC is a thermostable one. Because of the substantial level of sequence homology, this protein folding similarity is achievable.

However, a closer look at the structures reveals attractive differences in structural features that LCC have evolved to function at high temperatures. The interactions of amino acid residues along the polypeptide chain and the surrounding media determine PET hydrolase's unique three-dimensional and stable secondary structures. In fact, the total number and strength of covalent and noncovalent connections vary amongst *Is*PETase and LCC, and all of these interactions are critical in achieving the observable properties, especially the stability and function of these proteins.

Thermostable LCC has more unique amino acid features, particularly aromaticity, hydrophobic interaction, polarity, HCA, hydrogen bond networks, and tight structural packing. Even though the *Is*PETase has significantly higher charged residues than LCC, they do not support the protein stability of the protein structure.

The presence and distribution of aromatic amino acids in a protein, for instance, phenylalanine, tyrosine, and tryptophan, is referred to as protein aromaticity. Because these amino acids have an aromatic ring structure, they have distinct chemical and physical properties when compared to other amino acids. Aromatic amino acids are essential for protein structure and function. They can participate in hydrophobic interactions, which are critical for protein structural stabilization, as well as ligand binding and catalytic activity in enzymes. The aromaticity of a protein varies based on its amino acid sequence and structure. Proteins with a high aromaticity may have more stable structures due to an increase in the number of hydrophobic contacts, whereas proteins with a low aromaticity may be more unstable (Baker and Grant 2007; Martin and Holehouse 2020; Dudek *et al.* 2022).

In line with aromaticity, the hydrophobic cluster area (HCA) of a protein is a measure of the amount of clustering of hydrophobic residues in the protein structure. Hydrophobic residues, such as alanine,

valine, leucine, isoleucine, phenylalanine, and tryptophan, have a low affinity for water and tend to cluster together in the protein interior, away from the watery environment. The HCA is measured by locating clusters of hydrophobic residues in the protein structure and measuring the area of the protein surface hidden by these clusters. The HCA is often stated as a percentage of the total protein surface area. HCA can provide insights into the stability and function of a protein. Proteins with a high degree of hydrophobic clustering are often more stable, as the clustered hydrophobic residues form a hydrophobic core that stabilizes the protein structure. In addition, hydrophobic residues can be important for protein-protein interactions, and proteins with high HCA values may have a greater propensity for forming stable protein complexes (Lemesle-Varloot *et al.* 1990; Callebaut *et al.* 1997; Bitard-Feildel *et al.* 2018).

Protein polarity refers to the distribution of electric charge across the amino acid residues in a protein. Amino acids can be classified as polar or nonpolar based on the presence or absence of charged or electronegative groups in their side chains. Polar amino acids have charged or electronegative groups that can form hydrogen bonds with water or other polar molecules. In contrast, nonpolar amino acids have hydrophobic side chains that tend to exclude water. A protein's polarity can influence its solubility, stability, and function. Proteins with a high proportion of polar amino acids tend to be more soluble in water, as the charged and polar groups can form favourable interactions with the surrounding water molecules. In contrast, proteins with a high proportion of nonpolar amino acids tend to be more hydrophobic and less soluble in water. The distribution of polar and nonpolar amino acids in a protein can also affect its stability (Kamtekar *et al.* 1993; Panja *et al.* 2015; Qiao *et al.* 2019).

Hydrophobic interactions between nonpolar amino acids can stabilize the protein structure, while polar interactions between charged or polar amino acids can also contribute to stability through the formation of hydrogen bonds or salt bridges. The polarity of a protein can also affects its function. Polar amino acids are often involved in binding to other polar molecules, such as nucleic acids, ions, or other proteins, through hydrogen bonding or electrostatic interactions. Nonpolar amino acids are often found in the hydrophobic core of proteins, where they can stabilize the protein structure or interact with nonpolar ligands (Gromiha *et al.* 1999; Kumar *et al.* 2000).

The intricate network of hydrogen bonds that can form between the backbone and side chain atoms of amino acid residues in a protein is referred to as the protein hydrogen bond network. Hydrogen bonds are electrostatic interactions between electronegative atoms like oxygen or nitrogen and a hydrogen atom that is covalently bound to another electronegative atom. In a protein, hydrogen bonds can occur between neighbouring amino acid backbone amide and carbonyl groups, as well as between side chain groups and the backbone or other side chains. Hydrogen bonding can lead to the creation of secondary structures such as alpha helices and beta sheets, which can help to stabilize the protein structure. A protein's hydrogen bond network can also influence protein activity. Hydrogen bonding, for example, can be involved in ligand binding or recognising other proteins or nucleic acids. Protein conformational changes and protein-protein interactions can benefit from changes in the hydrogen bond network (Vogt and Argos 1997; Khechinashvili *et al.* 2006; Vieira and Degreve 2009).

Together, thermostability characteristics revealed that structure packing plays a pivotal role in the thermostable PET hydrolase. This pivotal role is backed up by aromaticity, hydrophobic cluster area and hydrogen bond network of the amino acid within the protein structure. As a result, these features contribute to the maintenance of strong connections with other residues and the stability of the native state. It could be due to the substantial packing impact, as packing is considered to be one of the essential features for thermophilic protein stability. It is worth to note that, of course, to validate these findings, the next in vitro experiments is necessary, such as optimizing IsPETase to enhance its thermostability based on the data in this report.

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References

Baker, C.M., Grant, G.H., 2007. Role of aromatic amino acids in protein–nucleic acid recognition. *Biopolymers.* 85, 456-470. https://doi.org/10.1002/bip.20682

Bitard-Feildel, T., Lamiable, A., Mornon, J.P., Callebaut, I., 2018. Order in disorder as observed by the "hydrophobic cluster analysis" of protein sequences. *Proteomics.* 18, 1800054. https://doi.org/10.1002%2Fpmic.201800054

Bornscheuer, U.T., 2016. Feeding on plastic. *Science.* 351, 1154-1155. https://doi.org/10.1126/science.aaf2853

Callebaut, I., Labesse, G., Durand, P., Poupon, A., Canard, L., Chomilier, J., Henrissat, B., Mornon, J.P., 1997. Deciphering protein sequence information through hydrophobic cluster analysis (HCA): current status and perspectives. *Cell. Mol. Life Sci.* 53, 621–645. https://doi.org/10.1007/s000180050082

Deng, B., Yue, Y., Yang, J., Yang, M., Xing, Q., Peng, H., Wang, F., Li, M., Ma, L., Zhai, C., 2023. Improving the activity and thermostability of PETase from *Ideonella sakaiensis* through modulating its post-translational glycan modification. *Commun. Biol.* 6, 39. https://doi.org/10.1038/s42003-023-04413-0

Dudek, W.M., Ostrowski, S., Dobrowolski, J.C., 2022. On Aromaticity of the aromatic α-amino acids and tuning of the NICS indices to find the aromaticity order. *J. Phys. Chem. A.* 126, 3433-3444. https://doi.org/10.1021/acs.jpca.2c00346

Fecker, T., Galaz-Davison, P., Engelberger, F., Narui, Y., Sotomayor, M., Parra, L.P., Ramírez-Sarmiento, C.A., 2018. Active site flexibility as a hallmark for efficient PET degradation by *I. sakaiensis* PETase. *Biophys J.* 114, 1302-1312. https://doi.org/10.1016/j.bpj.2018.02.005

Finch, A.J., Kim, J.R., 2018. Thermophilic proteins as versatile scaffolds for protein engineering. *Microorganisms*. 6, 97. https://doi.org/10.3390/microorganisms6040097

Gan, H.H., Perlow, R.A., Roy, S., Ko, J., Wu, M., Huang, J., Yan, S., Nicoletta, A., Vafai, J., Sun, D., Wang, L., Noah, J.E., Pasquali, S., Schlick, T., 2002. Analysis of protein sequence/structure similarity relationships. *Biophys J.* 83, 2781-91. https://doi.org/10.1016/s0006-3495(02)75287-9

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., Bairoch, A., 2005. Protein identification and analysis tools on the expasy server, in: Walker, J.M. (Eds.), *The Proteomics Protocols Handbook*. Humana Press: Totowa, NJ, pp. 571-607.

Gromiha, M.M., Oobatake, M., Sarai, A., 1999. Important amino acid properties for enhanced thermostability from mesophilic to thermophilic proteins. *J. Protein. Chem.* 82, 51-67. https://doi.org/10.1016/s0301-4622(99)00103-9

Guzzi, P.H., di Paola, L., Puccio, B., Giuliani, A., Veltri, P., 2023. Computational analysis of the sequence-structure relation in SARS-CoV-2 spike protein using protein contact networks. *Sci. Rep.* 13, 2837. https://doi.org/10.1038/s41598-023-30052-w

He, Y., Maisuradze, G.G., Yin, Y., Kachlishvili, K., Rackovsky, S., Scheraga, H.A., 2017. Sequence-, structure-, and dynamics-based comparisons of structurally homologous CheY-like proteins. *Proc. Natl. Acad. Sci.* 114, 1578-1583. https://doi.org/10.1073/pnas.1621344114

Kamtekar, S., Schiffer, J.M., Xiong, H., Babik, J.M., Hecht, M.H., 1993. Protein design by binary patterning of polar and nonpolar amino acids. *Science*. 262, 1680-1685. https://doi.org/10.1126/science.8259512

Khechinashvili, N.N., Fedorov, M.V., Kabanov, A.V., Monti, S., Ghio, C., Soda, K., 2006. Side chain dynamics and alternative hydrogen bonding in the mechanism of protein thermostabilization. *J. Biomol. Struct. Dyn.* 24, 255-262. https://doi.org/10.1080/07391102.2006.10507117

Khersonsky, O., Lipsh, R., Avizemer, Z., Ashani, Y., Goldsmith, M., Leader, H., Dym, O., Rogotner, S., Trudeau, D.L., Prilusky, J., Amengual-Rigo, P., Guallar, V., Tawfik, D.S., Fleishman, S.J., 2018. Automated design of efficient and functionally diverse enzyme repertoires. *Mol. Cell.* 72, 178-186.e5. https://doi.org/10.1016/j.molcel.2018.08.033

Kumar, S., Tsai, C.J., Nussinov, R., 2000. Factors enhancing protein thermostability. *Protein Eng.* 13, 179-191. https://doi.org/10.1093/protein/13.3.179

Lasa, I., Berenguer, J., 1993. Thermophilic enzymes and their biotechnological potential. *Microbiologia*. 9, 77-89.

Lemesle-Varloot, L., Henrissat, B., Gaboriaud, C., Bissery, V., Morgat, A., Mornon, J.P., 1990. Hydrophobic cluster analysis: procedures to derive structural and functional information from 2-D-representation of protein sequences. *Biochimie*. 72, 555-574. https://doi.org/10.1016/0300-9084(90)90120-6

Leuschner, C., Antranikian, G., 1995. Heat-stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. *World J. Microbiol. Biotechnol.* 11, 95-114. https://doi.org/10.1007/BF00339139

Li, Y., Zhang, R., Wang, C., Forouhar, F., Clarke, O.B., Vorobiev, S., Singh, S., Montelione, G.T., Szyperski, T., Xu, Y., Hunt, J.F., 2022. Oligomeric interactions maintain active-site structure in a noncooperative enzyme family. *EMBO J.* 41, e108368. https://doi.org/10.15252/embj.2021108368

Liu, S., Rao, S., Chen, X., Li, J., 2022. Enhanced salt-tolerance of *Bacillus subtilis* glutaminase by fusing self-assembling amphipathic peptides at its N-terminus. *Front. Bioeng. Biotechnol.* 10, 996138. https://doi.org/10.3389/fbioe.2022.996138

Lu, H., Diaz, D.J., Czarnecki, N.J., Zhu, C., Kim, W., Shroff, R., Acosta, D.J., Alexander, B.R., Cole, H.O., Zhang, Y., Lynd, N.A., Ellington, A.D., Alper, H.S., 2022. Machine learning-aided engineering of hydrolases for PET depolymerization. *Nature*. 604, 662–667. https://doi.org/10.1038/s41586-022-04599-z

Madeira, F., Pearce, M., Tivey, A.R.N., Basutkar, P., Lee, J., Edbali, O., Madhusoodanan, N., Kolesnikov, A., Lopez, R., 2022. Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic Acids Res.* 50, 276–279. https://doi.org/10.1093/nar/gkac240

Martin, E.W., Holehouse, A.S., 2020. Intrinsically disordered protein regions and phase separation: sequence determinants of assembly or lack thereof. *Emerg. Top. Life Sci.* 4, 307-329. https://doi.org/10.1042/ETLS20190164

Mazumder, R., Vasudevan, S., 2008. Structure-guided comparative analysis of proteins: principles, tools, and applications for predicting function. *PLoS Comput. Biol.* 4, e1000151. https://doi.org/10.1371/journal.pcbi.1000151

Noda-Garcia, L., Liebermeister, W., Tawfik, D.S., 2018. Metabolite-enzyme coevolution: from single enzymes to metabolic pathways and networks. *Annu. Rev. Biochem.* 87, 187-216. https://doi.org/10.1146/annurev-biochem-062917-012023

Panja, A.S., Bandopadhyay, B., Maiti, S., 2015. Protein thermostability is owing to their preferences to non-polar smaller volume amino acids, variations in residual physico-chemical properties and more salt-bridges. *PloS One.* 10, e0131495. https://doi.org/10.1371/journal.pone.0131495

Papadopoulou, A., Hecht, K., Buller, R., 2019. Enzymatic PET degradation. *Chimia (Aarau).* 73, 743-749. https://doi.org/10.2533/chimia.2019.743

Qiao, B., Jiménez-Ángeles, F., Nguyen, T.D., Olvera de la Cruz, M., 2019. Water follows polar and nonpolar protein surface domains. *Proc. Natl. Acad. Sci.* 116, 19274-19281. https://doi.org/10.1073/pnas.1910225116

Schrödinger, L., DeLano, W., 2020. PyMOL. Available at: http://www.pymol.org/pymol. [Date accessed: 1 March 2023]

Shirke, A.N., White, C., Englaender, J.A., Zwarycz, A., Butterfoss, G.L., Linhardt, R.J., Gross, R.A., 2018. Stabilizing leaf and branch compost cutinase (LCC) with glycosylation: mechanism and effect on PET hydrolysis. *Biochem*. 57, 1190-1200. https://doi.org/10.1021/acs.biochem.7b01189

Son, H.F., Cho, I.J., Joo, S., Seo, H., Sagong, H.Y., Choi, S.Y., Lee, S.Y., Kim, K.J., 2019. Rational protein engineering of thermo-stable PETase from *Ideonella sakaiensis* for highly efficient PET degradation. *ACS Catal.* 9, 3519-3526. https://pubs.acs.org/doi/10.1021/acscatal.9b00568

Srivastava, A.K., Scalcione, L.J., Arosio, P., Bou-Abdallah, F., 2023. Hyperthermostable recombinant human heteropolymer ferritin derived from a novel plasmid design. *Protein Sci.* 32, e4543. https://doi.org/10.1002/pro.4543

Sternke, M., Tripp, K.W., Barrick, D., 2019. Consensus sequence design as a general strategy to create hyperstable, biologically active proteins. *Proc. Natl. Acad. Sci.* 116, 11275-11284. https://doi.org/10.1073/pnas.1816707116

Sulaiman, S., Yamato, S., Kanaya, E., Kim, J.J., Koga, Y., Takano, K., Kanaya, S., 2012. Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach. *Appl Environ Microbiol.* 78, 1556-62. https://doi.org/10.1128/AEM.06725-11

Sulaiman, S., You, D.J., Kanaya, E., Koga, Y., Kanaya, S., 2014. Crystal structure and thermodynamic and kinetic stability of metagenome-derived LC-cutinase. *Biochem*. 53, 1858-69. https://doi.org/10.1021/bi401561p

Suplatov, D., Voevodin, V., Švedas, V., 2015. Robust enzyme design: bioinformatic tools for improved protein stability. *J. Biotechnol.* 10, 344-355. https://doi.org/10.1002/biot.201400150

Swillens, S., Ludgate, M., Marcken, L., Dumont, J.E., Vassart, G., 1986. Analysis of sequence and structure homologies between thyroglobulin and acetylcholinesterase: possible functional and clinical significance. *Biochem. Biophys. Res. Comm.* 137, 142-148. https://doi.org/10.1016/0006-291x(86)91187-3

Tournier, V., Topham, C.M., Gilles, A., David, B., Folgoas, C., Moya-Leclair, E., Kamionka, E., Desrousseaux, M.L., Texier, H., Gavalda, S., Cot, M., Guémard, E., Dalibey, M., Nomme, J., Cioci, G., Barbe, S., Chateau, M., André, I., Duquesne, S., Marty, A., 2020. An engineered PET depolymerase to break down and recycle plastic bottles. *Nature*. 580, 216-219. https://doi.org/10.1038/s41586-020-2149-4

Van Wyk, J.C., Sewell, B.T., Danson, M.J., Tsekoa, T.L., Sayed, M.F., Cowan, D.A., 2022. Engineering enhanced thermostability into the *Geobacillus pallidus* nitrile hydratase. *Curr. Res. Struct. Biol.* 4, 256-270. https://doi.org/10.1016/j.crstbi.2022.07.002

Vieille, C., Zeikus, G.J., 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiol. Mol. Biol. Rev.* 65, 1-43. https://doi.org/10.1128/mmbr.65.1.1-43.2001

Vieira, D.S., Degreve, L., 2009. An insight into the thermostability of a pair of xylanases: the role of hydrogen bonds. *Mol. Phys.* 107, 59-69. http://dx.doi.org/10.1080/00268970902717959

Vogt, G., Argos, P., 1997. Protein thermal stability: hydrogen bonds or internal packing? *Fold. Des.* 2, S40-S46. https://doi.org/10.1016/s1359-0278(97)00062-x

Wang, L., Xie, J., Schultz, P.G., 2006. Expanding the genetic code. *Annu. Rev. Biophys. Biomol Struct.* 35, 225-49. https://doi.org/10.1146/annurev.biophys.35.101105.121507

Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., Toyohara, K., Miyamoto, K., Kimura, Y., Oda, K., 2016. A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science*. 351, 1196-9. https://doi.org/10.1126/science.aad6359

Zhou, X.X., Wang, Y.B., Pan, Y.J., Li, W.F., 2008. Differences in amino acids composition and coupling patterns between mesophilic and thermophilic proteins. *Amino Acids.* 34, 25-33. https://doi.org/10.1007/s00726-007-0589-x