AFRL-AFOSR-VA-TR-2018-0385

ENGINEERING ROBUST ENZYME ACTIVITY THROUGH FUNDAMENTAL STUDIES OF EXTREMOPHILE ENZYMES

Mark Blenner
CLEMSON UNIVERSITY

10/05/2018
Final Report

Air Force Research Laboratory
AF Office Of Scientific Research (AFOSR)/ RTB2
Arlington, Virginia 22203
Air Force Materiel Command

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
28-09-2018	Final Report	07/01/15 - 06/30/18

4. TITLE AND SUBTITLE

(YIP) Engineering Robust Enzyme Activity through Fundamental Studies of Extremophile Enzymes

5a. CONTRACT NUMBER

FA9550-15-1-0163

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Blenner, Mark, A.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

CLEMSON UNIVERSITY
OFFICE OF SPONSORED PROGRAMS
201 SIKES HALL

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

USAF, AFRL DUNS 143574726
AF OFFICE OF SCIENTIFIC RESEARCH
875 NORTH RANDOLPH STREET, RM 3112
ARLINGTON VA 22203-1954

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for Public Release; Distribution is Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This is the final report for Grant FA9550-15-1-0163: (YIP) Engineering Robust Enzyme Activity through Fundamental Studies of Extremophile Enzymes. During the final period, During this period, we delved deeper into the flexibility inducing mutations designed into a thermostable lipase on three substrates (p-nitrophenol butyrate, p-nitrophenol laurate, p-nitrophenol octanoate). Different with the previous conclusion, glycine mutations showed decreased specific activities on p-nitrophenol octanoate, p-nitrophenol laurate. At the same time, the glycine mutations result in similarly higher thermostabil

15. SUBJECT TERMS

enzyme engineering, extremophiles, thermal activity profile

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	1	Mark Blenner
					19b. TELEPHONE NUMBER *(Include area code)*
					876-656-0290

INSTRUCTIONS FOR COMPLETING SF 298

1. REPORT DATE. Full publication date, including day, month, if available. Must cite at least the year and be Year 2000 compliant, e.g. 30-06-1998; xx-06-1998; xx-xx-1998.

2. REPORT TYPE. State the type of report, such as final, technical, interim, memorandum, master's thesis, progress, quarterly, research, special, group study, etc.

3. DATE COVERED. Indicate the time during which the work was performed and the report was written, e.g., Jun 1997 - Jun 1998; 1-10 Jun 1996; May - Nov 1998; Nov 1998.

4. TITLE. Enter title and subtitle with volume number and part number, if applicable. On classified documents, enter the title classification in parentheses.

5a. CONTRACT NUMBER. Enter all contract numbers as they appear in the report, e.g. F33315-86-C-5169.

5b. GRANT NUMBER. Enter all grant numbers as they appear in the report. e.g. AFOSR-82-1234.

5c. PROGRAM ELEMENT NUMBER. Enter all program element numbers as they appear in the report, e.g. 61101A.

5e. TASK NUMBER. Enter all task numbers as they appear in the report, e.g. 05; RF0330201; T4112.

5f. WORK UNIT NUMBER. Enter all work unit numbers as they appear in the report, e.g. 001; AFAPL30480105.

6. AUTHOR(S). Enter name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. The form of entry is the last name, first name, middle initial, and additional qualifiers separated by commas, e.g. Smith, Richard, J, Jr.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES). Self-explanatory.

8. PERFORMING ORGANIZATION REPORT NUMBER. Enter all unique alphanumeric report numbers assigned by the performing organization, e.g. BRL-1234; AFWL-TR-85-4017-Vol-21-PT-2.

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES). Enter the name and address of the organization(s) financially responsible for and monitoring the work.

10. SPONSOR/MONITOR'S ACRONYM(S). Enter, if available, e.g. BRL, ARDEC, NADC.

11. SPONSOR/MONITOR'S REPORT NUMBER(S). Enter report number as assigned by the sponsoring/ monitoring agency, if available, e.g. BRL-TR-829; -215.

12. DISTRIBUTION/AVAILABILITY STATEMENT. Use agency-mandated availability statements to indicate the public availability or distribution limitations of the report. If additional limitations/ restrictions or special markings are indicated, follow agency authorization procedures, e.g. RD/FRD, PROPIN, ITAR, etc. Include copyright information.

13. SUPPLEMENTARY NOTES. Enter information not included elsewhere such as: prepared in cooperation with; translation of; report supersedes; old edition number, etc.

14. ABSTRACT. A brief (approximately 200 words) factual summary of the most significant information.

15. SUBJECT TERMS. Key words or phrases identifying major concepts in the report.

16. SECURITY CLASSIFICATION. Enter security classification in accordance with security classification regulations, e.g. U, C, S, etc. If this form contains classified information, stamp classification level on the top and bottom of this page.

17. LIMITATION OF ABSTRACT. This block must be completed to assign a distribution limitation to the abstract. Enter UU (Unclassified Unlimited) or SAR (Same as Report). An entry in this block is necessary if the abstract is to be limited.

1) Cover Page

Award Number: FA9550-15-1-0163

PI: Mark Blenner

Dates of Performance: Jul 01 2015- June 30 2018

Report Type: AFOSR YIP Final Report

Title: (YIP) Engineering Robust Enzyme Activity through Fundamental Studies of Extremophile Enzymes

2) Abstract

This is the final report for Grant FA9550-15-1-0163: (YIP) Engineering Robust Enzyme Activity through Fundamental Studies of Extremophile Enzymes. During the final period, During this period, we delved deeper into the flexibility inducing mutations designed into a thermostable lipase on three substrates (p-nitrophenol butyrate, p-nitrophenol laurate, p-nitrophenol octanoate). Different with the previous conclusion, glycine mutations showed decreased specific activities on p-nitrophenol octanoate, p-nitrophenol laurate. At the same time, the glycine mutations result in similarly higher thermostability on all the three substrates mentioned above. Explanation for this observation may be the complexity of the lipase enzyme structure, which includes two metal ions, and mobile lid region. To further test our hypothesis we initiated work using bovine trypsin as a model enzyme. The advantage of using this enzyme is its relative simplicity compare to lipase. By mapping out the allosteric residues using statistical cluster analysis (SCA), we hope changes to these residues can modify the temperature adaptation of the enzyme. We have completed the expression of this enzyme in *Yarrowia lipolytica*, as well as activation and specific activity measurement. These results have been summarized in the report below, have been used in one publication and will be used in two manuscripts to ACS Catalysis and Biochemistry. They have also been presented at regional and national conferences 14 times during the award.

3) Objectives for the period of performance.

Objective 1: Complete the specific activity measurement, kinetics assay and thermal challenge on the selected mutations. As has been shown in the last annual report, *Geobacillus thermocatenulatus* lipase(GTL) is a complex enzyme. Even though we observed an increased specific activity for mutations (E316G, E361G) on the substrate p-nitrophenol butyrate, the mechanism behind this observation is still unclear. More experiments need to be completed to understand these results. Since enzyme activity is not only affected by the structure, but also affected by the substrate due to the substrate specificity. Therefore, we tested orthologous lipase substrates using three different hydrocarbon chain lengths (p-nitrophenol butyrate, p-nitrophenol octanoate, p-nitrophenol laurate). We measured the enzyme kinetics, determined reaction thermodynamic parameters, and measured thermal stability for the deeper comparison. These results were then validated using molecular dynamics simulations.

Objective 2: Determine if flexibility inducing mutations could broaden the thermal activity range for a new model enzyme. Our second model system was bovine trypsin. To choose this new model enzyme, we need to consider several aspects: 1, whether this enzyme is easy to manipulate; 2, whether this enzyme has sufficient literature to provide information to contextualize our results; 3, whether this enzyme has a crystal structure available to facilitate molecular dynamics simulations; 4, whether there are a sufficiently large number of enzyme homologs from different temperature environments to identify functionally conserved regions. Since our mutations in lipase often causes a large conformational change in the lid region, we hope that mapping out all the allosteric residues on the enzyme and avoiding mutating them should be a better option to study the temperature adaptation of enzyme. The target positions will be changed to the residues frequently occurring in the homologous enzymes.

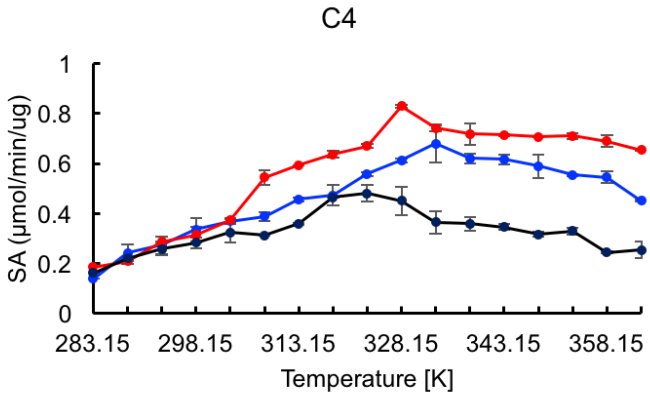
Objective 1 was completed and 2 was initiated during this period of performance. The second is scheduled to be completed shortly. Due to delays in initiating the project, we expected to request

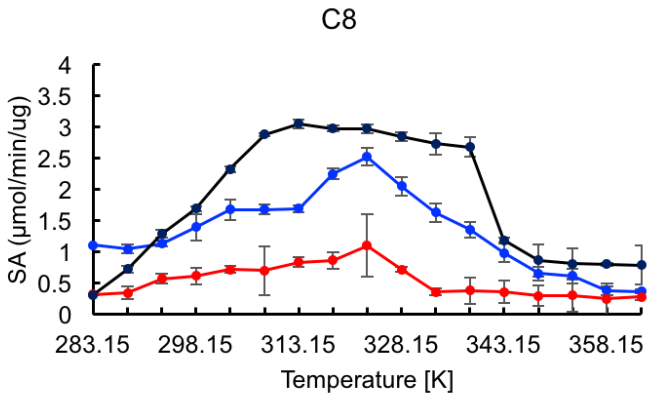
a no cost extension to complete the third objective after the next performance period. The request was denied and the project ended before we could complete objective 2 and 3.

4) Findings for each objective including any changes these findings compelled you to make and all major accomplishments for the period of performance.

Measurement of Activity and Broad Temperature Range Activity

Since we want to study if the specific activity changes of flexibility inducing mutations are substrate specific, we measured the specific activity of GTL WT and mutants on another two substrates p-nitrophenol octanoate(C8) and p-nitrophenol laurate(C12). The results showed that the E361G and E316G all had similarly increased optimal temperatures, from 40°C to either 50°C or 60°C. Other noteworthy observations include the decrease in activity of the E361G and E316G mutants at all temperatures, which is in contrary with the specific activity change on p-nitrophenol butyrate(C4).





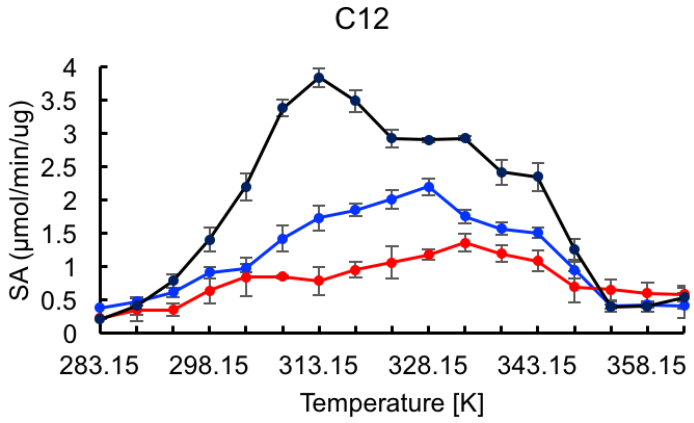


Figure 1. The specific activity of WT(black line), E361G(red line) and E316G (blue line) on different substrates(A, p-nitrophenol butyrate, B, p-nitrophenol octanoate, C, p-nitrophenol laurate). The test temperature ranges from 283.15 to 358.15 K with 5 K as the increment. The specific activity was measured at 405nm in 50 mM pH 7.4 sodium phosphate buffer.

We next calculated Michaelis-Menten kinetic parameters for the E361G and E316G mutants, and compared these to the wild type. Kinetic parameters were determined from 35-65°C, at 5°C intervals. The temperature dependence of k_{cat} was then used to calculate the activation enthalpy and entropy for the reaction using the lower temperature data that could be linearly fit to an Arrhenius plot. We found that indeed, the mutations caused a decrease in the activation enthalpy as well as a decrease in the activation entropy, consistent wtih our postulated central hypothesis. However, flexibility increases were not observed in the molecular dynamics simulations or acrylamide quenching assays, indicating that enthalpy and entropy of activation can be altered by mutation with increasing flexibility per se.

	Mutants	$\Delta H^{\#}$(kJ/mol)	$\Delta S^{\#}$(J/mol/K)	$\Delta\Delta H^{\#}$(kJ/mol)	$\Delta\Delta S^{\#}$(J/mol/K)
PNPB	E361G	3.37 ± 0.84	-194.93 ± 7.04	37.55 ± 0.19	-97.83 ± 0.11
	E316G	35.03 ± 0.85	-100.28 ± 7.08	-5.89 ± 0.15	-23.18 ± 0.04
	WT	40.92 ± 0.11	-77.10 ± 0.95	-	-
PNPO	E361G	24.51 ± 0.62	-126.32 ± 0.91	-38.80 ± 1.05	-129.67 ± 2.45
	E316G	60.97 ± 0.68	-7.59 ± 1.64	-2.34 ± 1.11	-10.94 ± 3.18
	WT	63.31 ± 0.31	3.35 ± 1.54	-	-
PNPL	E361G	14.41 ± 4.24	-154.36 ± 3.12	-35.65 ± 4.89	-151.42 ± 4.09
	E316G	39.51 ± 1.71	-38.41 ± 2.63	-10.55 ± 2.36	-34.57 ± 4.60
	WT	50.06 ± 0.65	-3.84 ± 1.97	-	-

Table 1. The ΔH# and ΔS# changes between mutations and wild type GTL. The activation enthalpy and entropy values ΔH# and ΔS# were calculated from the Eyring plots (Supplementary Figures 1,2 and 3). The kinetics experiments were completed over temperature range 30-65°C with 5°C as an increment. PNPB(C4), PNPO(C8) and PNPL(C12) were used as the substrates. $\Delta\Delta H^{\#}$ and $\Delta\Delta S^{\#}$ represent activation enthalpy and

entropy difference between mutants and wild type separately.

We sought to determine if the mutations showed similar increase in the thermostability on the two new substrates, the same thermal challenge experiments were conducted where the enzyme was held at 70°C for 3 hours and then returned to 25°C where it was assayed and compared to the enzyme without heat treatment. Similarly, all mutations led to an increase in thermostability compared to the WT, which retains on 20% of its initial activity. Interestingly, the E361G mutants both retain, if not gain activity by the heat treatment. It is possible that heat treatment allows these enzymes to fluctuate enough to form a more stable structure or perhaps to induce lid opening.

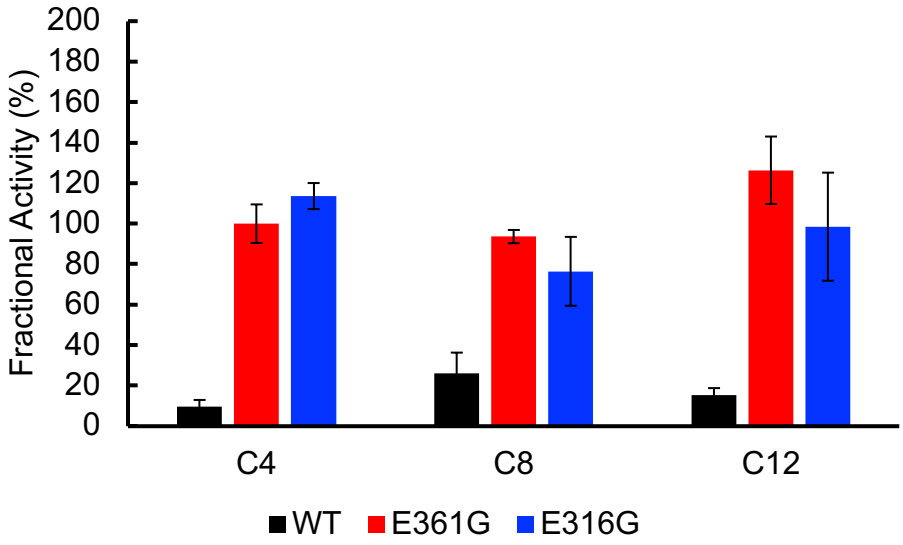


Figure 2. The retained activities of WT and GTL mutants. The fractional activities were calculated based on the ratio of the specific activities between 0 and 3 hours incubation at 70°C. PNPB(C4), PNPO(C8) and PNPL(C12) were used as the substrate separately.

We performed molecular docking study to probe the differences in the specific activity of the mutants and the WT with different substrates. The above slide shows the fraction of structures obtained with the molecular dynamics simulations that bind appropriately with different substrates at two temperatures. Molecular docking (Autodock Vina) was used to first screen the substrate-enzyme complexes that have a high docking score, which is based on a crude estimate of the free energy of enzyme-substrate binding. We then calculated the number of complexes that have the substrate reaction sites closer to the active site residues of the enzyme. The criteria used for finding reaction sites closer to the active site residues are shown in the reaction mechanism (i) distance between oxygen of active site residue Ser and carbonyl carbon of the substrate and ii) distance between carbonyl oxygen of the substrate and the hydrogens of the amide nitrogen of the oxyanion hole residues. We used 0.36 nm and 1.0 nm cutoff distances for (i) and (ii), respectively. This additional filter was used to identify the complexes that have the substrate oriented appropriately within the active site of the enzyme.

A greater fraction of WT structures can accommodate the C4 substrate than C8/C12 substrates at 320 K. The mutants have a higher binding preference with the longer substrates (C8 and C12) at 320 K than WT. The results at 340 K indicate that the WT has a better ability to bind

with all three substrates than the two mutants. These results do not correlate with the trends of specific activity of the enzymes with the three substrates obtained using experiments. This indicates that either the substrate binding may not be a rate limiting factor in the specific activity of the enzyme or possible limitations of the applied docking strategy. For the latter reason, we are looking into Boltzmann docking score-based method to check if the method used to screen the substrate—enzyme complexes based on docking score has any influence in our results.

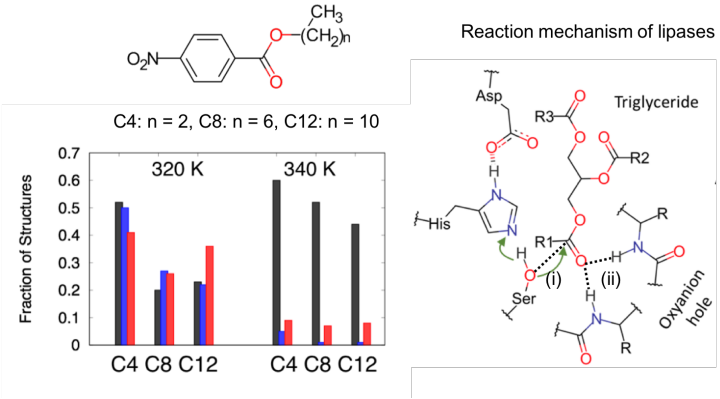


Figure 3. Molecular docking simulations based on the binding of substrate to the active site.

Last, we calculated the correlations between residue-residue fluctuations in the enzyme to understand the underlying reason for the changes in the dynamics of the enzyme with mutations. How did a single mutation near active site residue influence fluctuations of lid domain? We used distance correlation coefficient to capture any nonlinear correlations between the residue-residue fluctuations. The above slide shows the resulting correlations as a heat map for the E361G mutant and the WT at 340 K. If the correlations are similar between the mutant and the WT, then the upper and lower diagonals in the figure should be mirror images. Increase in fluctuation of a residue increases the fluctuations of the other residues if the other residues are in the red regions. Similarly, blue regions indicate less correlations between residue-residue fluctuations.

The residue-residue fluctuations in the WT are more correlated for many residue pairs than the mutant. This indicates that the fluctuations of residues in the enzyme are tightly coupled with each other in the WT. Though the mutation sites (316 and 361) itself is less correlated with certain residues in the WT, its coupling to a residue that is strongly coupled to all the other residues in the enzyme is sufficient to change the entire residue-residue fluctuation correlations within the enzyme. Indeed, the resulting correlation network in the mutant is quite different than the WT. This provides some explanation to the large changes in the fluctuations of the lid domain in the mutants, even though the mutations are both sequentially away from the lid domain. This heat map also serves as a starting point to identify mutations that can be less detrimental to the overall fluctuations of the enzyme. We are currently working to investigate the mechanism of the changes in the protein dynamics using this heat map and identify potential residues for mutations in wet-laboratory experiments.

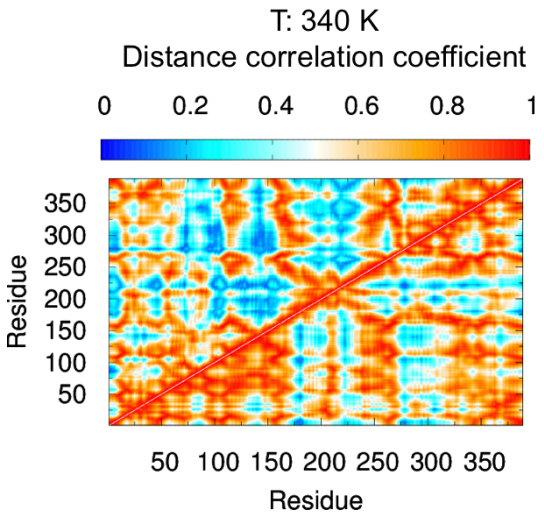


Figure 4. A map of the residue-residue correlations representing network connectivity.

Objective 2

Model Enzyme Selection and SCA Analysis

Trypsin was selected as our model enzyme based on the selection rules mentioned above. We mapped out the allosteric residues in the enzyme and color them differently as shown below. The target mutation sites were selected and shown in the spheres. It is easily found that most of the sphered residues in same color has direct or indirect contacts within the same sector, which is in accordance with the published features of SCA-identified sectors.

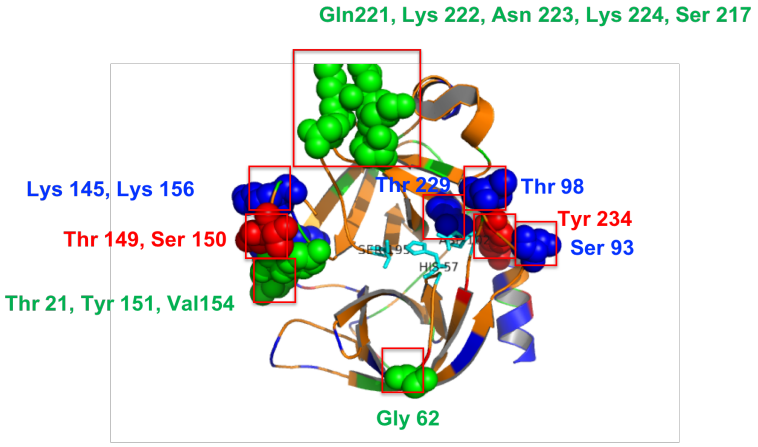


Figure 5. SCA-identified amino acids used to design mutations. The crystal structure of the bovine trypsinogen (PDB entry 3PTB) is shown by orange ribbon. Amino acids colored and shown in spheres are targeted mutation sites. Positions were picked based on the distance(12Å) to the active sites. Sectors are shown in different colors.

High Frequency of Amino Acids on the Target Sites in Cold Trypsin Analog and Designed Mutations

We target the matching positions from psychrophilic salmon trypsin by aligning the protein sequences between bovine and salmon trypsin. After selecting the matched positions, multiple sequence alignment was completed by using salmon trypsin sequence as the template. We believe the higher frequency of amino acid (highlighted in red) is necessary for the cold adaptation.

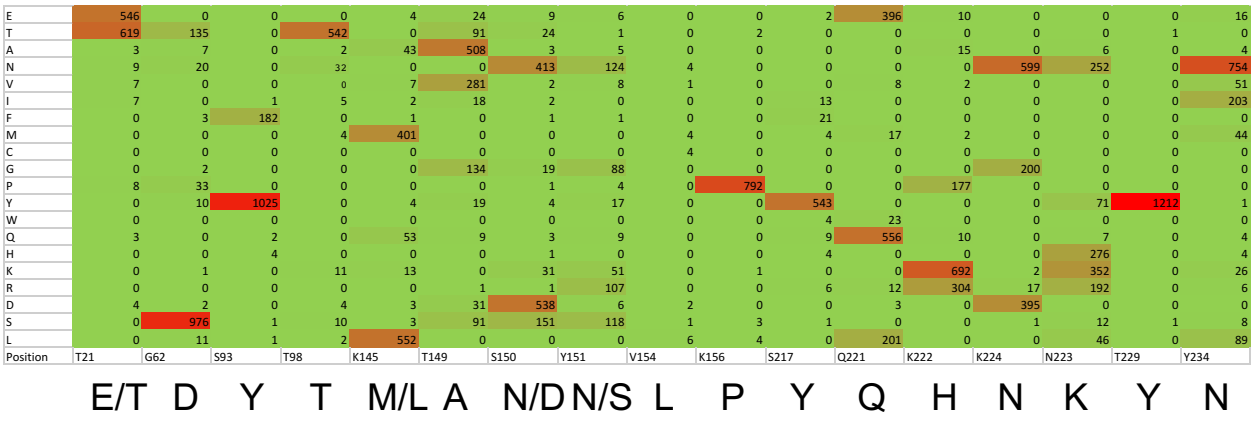


Figure 6. The occurrence frequency of amino acids on the target sites in Salmon Trypsin (PDB entry: 1BIT). The alignment was completed by PSI-BLAST. The threshold is 0.005, and sequences downloaded are 1230.

Sectors	Designed Mutations
1	T21E, G62D, Y151S, Y151N, V154L, S217Y, Q221E, K222H, N223K, K224N
2	S93Y, K145M, K145L, K156P, T229Y
3	T149A, S150D, S150N, Y234N

Table 2. The designed mutations for bovine trypsin using conserved amino acids.

Specific Activity Measurement of Wild Type Bovine Trypsin

To compare the activity change of bovine trypsin after mutation, we spent a lot of time to routine the activation and enzymatic assay method. The purified bovine trypsinogen (data not shown) was activated by his-tagged enterokinase and then purified by his-tag spin column. The activity of the pure and activated trypsin was measured over different temperatures ranging from 10 to 90°C, with 5°C as an increment. We found bovine trypsin retained high activity at low temperatures(10-45°C), but decreased sharply after 50°C.

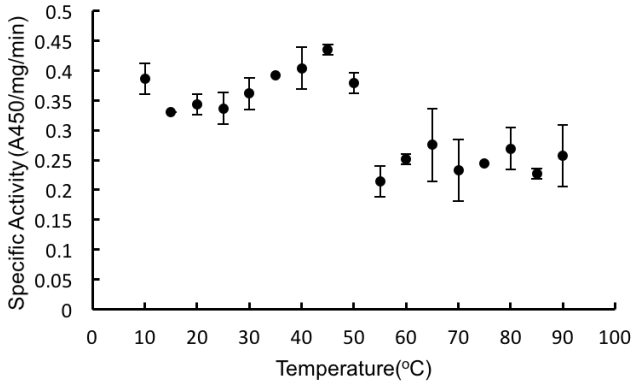


Figure 7. The specific activity of bovine trypsin. The test temperature ranges from 283.15 to 358.15 K with 5 K as the increment. The specific activity was measured at 450nm in 50 mM pH 8.5 borate buffer.

5) Supported Personnel.

The personnel supported by this award include: Mark Blenner (PI), Weigao Wang, a PhD student in Chemical & Biomolecular Engineering (Blenner Lab), and Siva Dasetty, a PhD student in Chemical & Biomolecular Engineering (Sarupria Lab). Additionally, four total undergraduate student – Tiffany Yu, Mary Kate Rumph, Tanner Karp, Calvin Martin. Undergraduates have not been directly supported by this funding, however, research materials are supported by this funding. These undergraduates enroll in a Creative Inquiry course and receive academic credit for their research.

6) Collaborations.

We have collaborated with Dr. Sapna Sarupria, an Assistant Professor of Chemical & Biomolecular Engineering at Clemson University. Her expertise is in MD simulations. She and her graduate student have done molecular dynamics simulations of the M37 Lipase and the GTL. We have one publication together and anticipate two additional publications submitted together by 2019 resulting from this work.

7) Publications.

At this time, we have one publication to report.

> Dasetty, S., Blenner, M.*, Sarupria, S.* "Engineering Lipases: Walking the Fine Line Between Activity and Stability" *Materials Research Express* – Emerging Investigators Awards Series **4**:114008 (2017) DOI: 10.1088/2053-1591/aa9946

There are two journal article to ACS Catalysis and Biochemistry in preparation. There were 14 presentations on work resulting from this project: These talks are

> Wang, W.*, Dassetty, S., Sarupria, S., Blenner, M. "Improving the Activity of a Thermophilic Lipase By Increasing the Flexibility Proximal to the Active Site" American Institute of Chemical Engineers Annual Meeting, San Francisco, CA (November 2016).

Dasetty, S.*, Wang, W., Blenner, M., Sarupria, S. "Understanding the Structural Differences Between Psychrophilic and Thermophilic Enzymes: A Molecular Dynamics Study". American Institute of Chemical Engineers Annual Meeting, San Francisco, CA (November 2016).

Dasetty, S.*, Wang, W., Blenner, M., Sarupria, S. "Engineering Robust Activity in Extremophilic Enzymes: A Molecular Dynamics Study". Clemson Biological Sciences Student Symposium, Clemson, SC (February 2017).

Dasetty, S.*, Wang, W., Blenner, M., Sarupria, S. "Engineering Robust Activity in Extremophilic Enzymes", Graduate Symposium, Chemical Engineering, Clemson University, Clemson, SC (April 2017).

Wang, W.*, Dasetty, S.*, Sarupria, S., Blenner, M., "Improving the Activity of a Thermophilic Lipase by Increasing the Flexibility Proximal to the Active Site", Graduate Symposium, Chemical Engineering, Clemson University, Clemson, SC (April 2017).

Dasetty, S.*, Wang, W., Blenner, M., Sarupria, S., "Engineering Robust Activity in Extremophilic Enzymes", The Future of Integrative Structural Biology, Physics, Clemson University, Clemson, SC (April 2017).

Wang, W., Dassetty, S., Sarupria, S., Blenner, M.* "Engineering Enzymes For Broad Temperature Range Applications Through Active Site Flexibility" American Chemical Society Meeting, San Francisco, CA (April 2017).

*Sarupria, S. "Using molecular simulations and experiments to engineer robust enzymes", Soft matter: Workshop, Telluride Science Research Center, Telluride, CO (June 2017)

Dassetty, S.*, Wang, W., Blenner, M., Sarupria, S. "Engineering Robust Activity in Extremophilic Enzymes" American Institute of Chemical Engineers Annual Meeting, Minneapolis, MN (November 2017).

Wang, W.*, Dassetty, S., Sarupria, S., Blenner, M. "Engineering Lipase for Broad Temperature Range Applications Through Active Site Flexibility" Southeastern Regional American Chemical Society Fall Meeting, Charlotte, NC (November 2017).

*Sarupria, S, "Bridging experiments and molecular simulations to elucidate heterogeneous ice nucleation", Session: Atmospheric Surface Science, EGU General Assembly, Vienna, 8-13 April, 2018

Dasetty S.*, Wang, W., Blenner, M., Sarupria, S. "Controlling Working Temperatures of Enzymes using Rational Approaches". Graduate Research and Discovery Symposium, Clemson, SC (April 2018)

Dasetty S.*, Wang, W., Blenner, M., Sarupria, S. "Towards Computer Aided Engineering of Proteins and Protein—Surface Complexes in Aqueous Media". Graduate Symposium, Clemson, SC (April 2018)

Wang, W.,* Blenner, M. "Improving the cold adaptation of bovine trypsin through protein coevolution analysis" Annual Meeting of the Society of Industrial Microbiology and Biotechnology, Chicago, IL (August 2018).

8) Interactions/Transitions

At this time, we have no transitions to report. The initial press releases generated coverage from local news media, included Fox, NBC, and the CW.