Synthesis of IMP-1 S115T and S119G mutants and analysis of catalytic efficiency

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Kellogg Honors College Capstone Project

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Abstract

Background:
Metallo-β-Lactamases (MBLs) work to provide bacteria with resistant characteristics to β-lactam antibiotics. Part of the reason antibiotic resistance continues to be an issue is due to the diversity of these MBLs and newly present mutations that may enhance activity. Within the L7 loop of metallo-β-lactamases IMP-14, 18, and 32, the S115T and S119G mutations are conserved.

Objectives:
Conclude the effect of different amino acid substitutions of S115T and S119G in IMP-type MBL's on the enzymes' ability to hydrolyze and confer resistance toward b-lactam antibiotics. Determine whether the mutations introduced have a relation to catalytic efficiency or are related due to evolutionary origin. Also conclude whether both mutants need to be present at the same time for higher catalytic activity.

Methods:
Using IMP-1, the genes encoding IMP-1-S115T and IMP-1 S119G were produced via PCR-based site directed mutagenesis of the *bla*$_{IMP-1}$ gene in the pET26b vector for overexpression. Protein purification using cation exchange and gel filtration was carried out and protein concentration was determined. Kinetics assays were conducted for both enzymes using penicillin G as the substrate.

Conclusions:
Through analyzing V_{max}, k_{cat}, and K_m, and k_{cat}/k_m values, we were able to determine that both mutants had higher catalytic activity than IMP-1, containing no mutations. From this, we can conclude that the individual mutants were beneficial to the enzyme's activity and did not collectively need to be present for an increase in efficiency.

Introduction

Antibiotic resistance, specifically against β-lactams, continues to be an issue through the production of β-lactamases, separated into 4 classes.[1] The mechanism by which some multi-drug resistant bacteria arise is through the activity of metallo-β-lactamases of Class B, enzymes responsible for hydrolyzing β-lactam antibiotics.[2] MBLs act through the presence of zinc ions to facilitate the hydrolysis and allow for the broad substrate spectrum, including penams, cephems, and carbapenems.[3] MBLs differ from serine-β-lactamases, in that MBLs can hydrolyze carbapenems, which resist hydrolysis from most serine-β-lactamases.[2,3]

Part of the consistent development of antibacterial strains is due to higher catalytic efficiency being associated with mutations within the β-lactamases.[4] The IMP-type enzymes of the subclass B1 are among the most widespread MBLs and contain several mutations associated with catalytic efficiency. IMP-1 was first isolated in Japan in *Pseudomonas aeruginosa* in 1991 and *Serratia marcescens* in 1994.[5] Many variants of IMP exist; however most IMP MBLs present are of IMP-1. In the variants IMP-14, 18, and 32, the S115T and S119G mutations are conserved in the L7 loop of the protein.[6] These mutations may be connected either due to an increasing catalytic efficiency towards substrates or may be the result of a common evolutionary origin.

Methods

Expression and Purification

bla$_{IMP-1-S115T}$ and *bla*$_{IMP-1-S119G}$ were generated through PCR-based site-directed mutagenesis from *bla*$_{IMP-1}$ encoded on a pET26b expression vector. Following sequencing, the *bla*$_{IMP}$ genes were over expressed through transformation in *E. coli* C43 cells with the genes. Two 500 ml cultures were incubated with IPTG inducing expression. The cells were lysed by sonication and supernatant and pellet were separated by centrifugation. Both IMP-1 variants were purified through cation-exchange and gel filtration and concentrated following the purification process. SDS-PAGE analyses were conducted for each to confirm the presence of the protein.

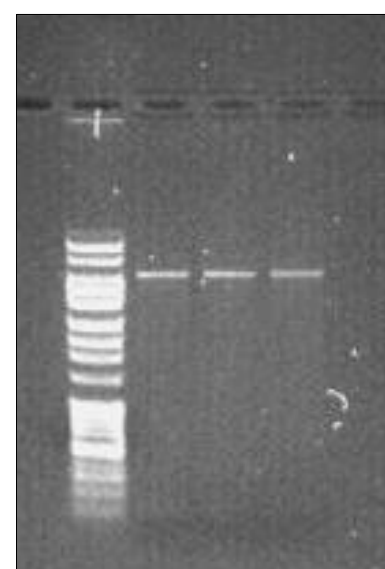
Biophysical Characterization

Protein quantification was calculated following concentration using centrifugation. 1:50, 1:100, and 1:200 dilutions were prepared with the 50 mM MOPS (no salt) buffer and absorbance was measured at 280 nm using spectrophotometer. The exctinction coefficient of IMP-1 (49,000 M^{-1} cm^{-1}) was used along with Beer's law to determine the protein concentrations.

Kinetic Assays

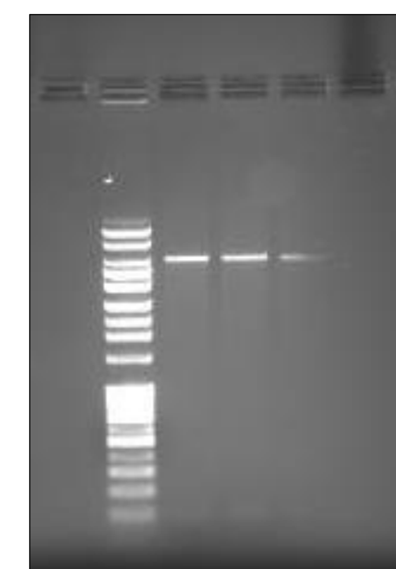
Purified enzymes were prepared (2 nM final concentration) in 50 mM MOPS - 100 µM $ZnSO_4$ and 10 µg/ml BSA. Following the addition of substrate at different concentrations initial velocities were determined by measuring degradation of the substrate. The substrate used was Penicillin G. The enzymes were prepared three times per substrate concentration resulting in three sets of data per antibiotic. The absorbance was measured using a SQ-2802 UV/Vis Spectrophotometer and the values were determined using the GraphPad Prism software.

Experimental Results



IMP-1 S115T Gel post Dpn-1 digestion

Lane Assignments:
1. 1 kb DNA ladder
2. PCR Sample #1 (54.0°C)
3. PCR Sample #2 (58.1°C)
4. PCR Sample #3 (62.9°C)



IMP-1 S119G Gel post Dpn-1 digestion

Lane Assignments:
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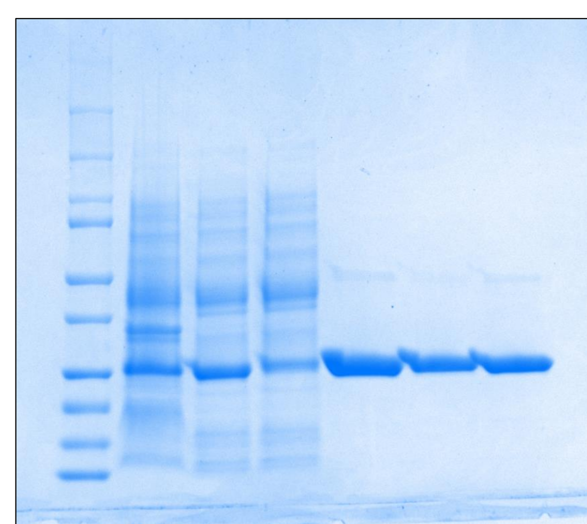
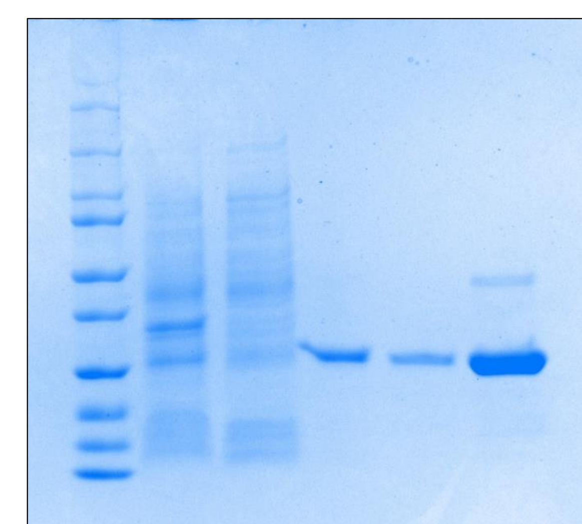
2. PCR Sample #1 (62.0°C)
3. PCR Sample #2 (66.0°C)
4. PCR Sample #3 (70.9°C)



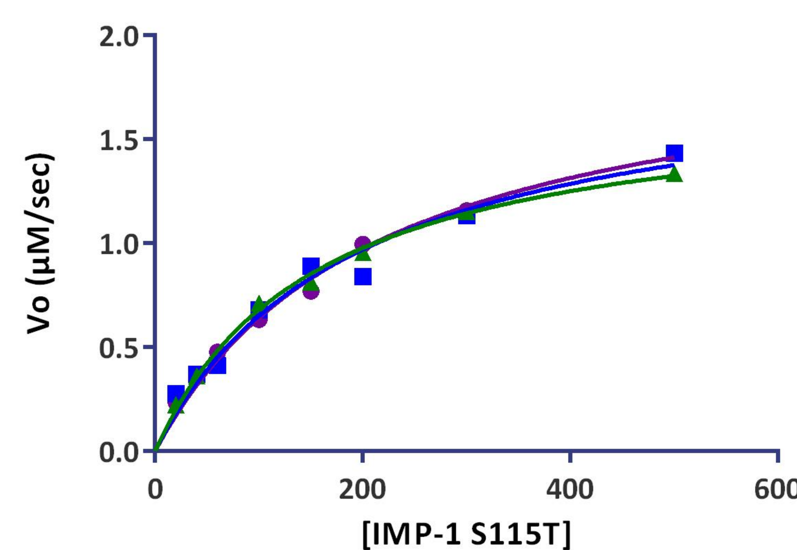
IMP-1 S115T SDS Page

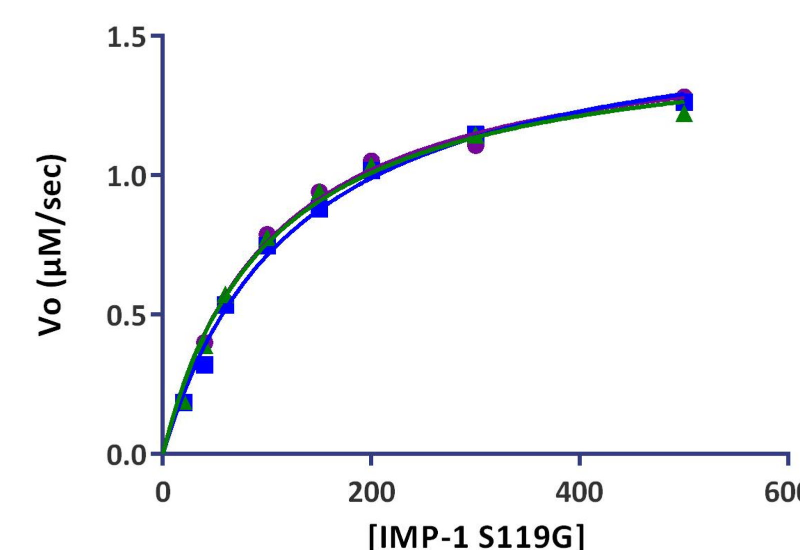
Lane Assignments:
1. 25 kDa Molecular Weight Marker (4 µL)
2. Soluble Fraction (2 µL)
3. Insoluble Fraction (2 µL)
4. Flow Through (2 µL)
5. After Cation Exchange (10 µL)
6. After Gel Filtration (10 µL)
7. After Concentration (2 µL)



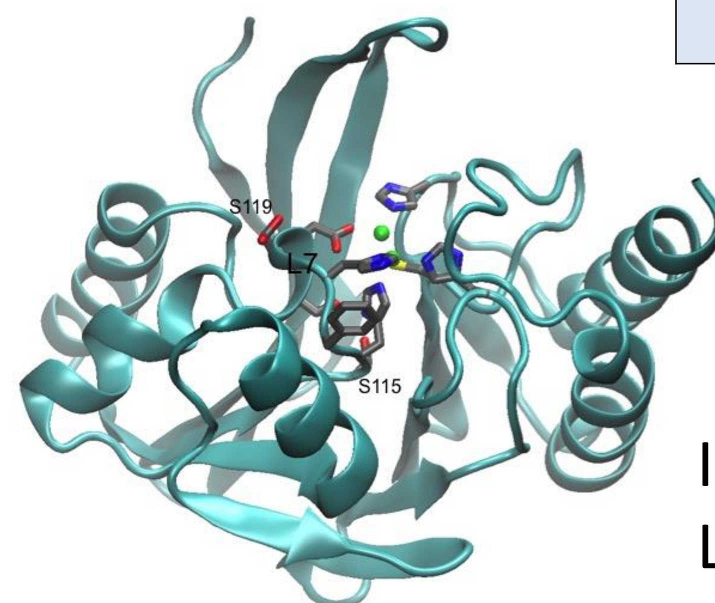
IMP-1 S119G SDS Page

Lane Assignments:
1. 25 kDa Molecular Weight Marker (4 µL)
2. Soluble Fraction (2 µL)
3. Insoluble Fraction (2 µL)
4. After Cation Exchange (10 µL)
5. After Gel Filtration (10 µL)
6. After Concentration (10 µL)

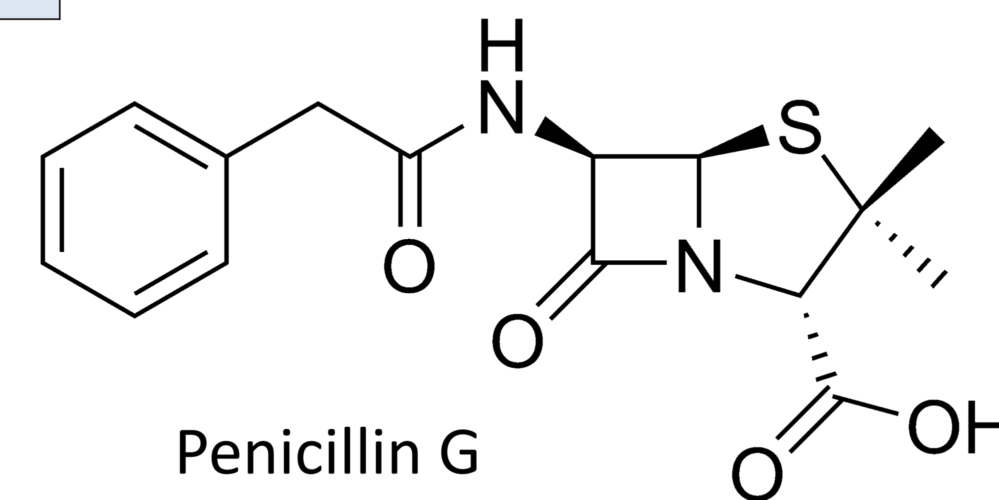




	k_{cat}/K_m (µM^{-1} s^{-1})
IMP-1	3.8 ± 0.2
IMP-1 S115T	5.1 ± 0.5
IMP-1 S119G	7.1 ± 0.7



IMP-14, 18, 32 L7 loop



Penicillin G

Conclusions

- IMP-1 S115T and IMP-1 S119G mutants were successfully expressed and purified using the methods indicated, with the S115T mutation resulting in higher yield of protein, over 3 fold
- Both mutants were more active than the wild type IMP-1 with degrading penicillin G
- Since both mutants are individually more active than IMP-1, we can conclude that there is no penalty to the enzyme of having either mutation present individually
- It is possible that both mutations are present in combination in clinically observed variants as a result of common evolutionary ancestry, but having both mutations in the same enzyme variant does not seem to be a requirement for increased catalytic activity

Acknowledgements

I would like to sincerely thank Dr. Peter Oelschlaeger for allowing me to perform research in his lab, providing me with this project, and mentoring me throughout the process. This research project would not have been possible without the immense guidance and teaching from Antonia Zhang, the master's student present in the research lab. Lastly, I would like to thank the Western University of Health Sciences Department of Pharmaceutical Sciences and the Kellogg Honors College for all their support and funding for the completion of this capstone project.

References

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