Structural analysis of thimet oligopeptidase (EC 3.4.24.15) through surface mutation E107Q E111Q

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Abstract

Thimet oligopeptidase (TOP) is a metalloendopeptidase that has been shown to cleave short structure peptides. Its structural shape is described as a clamshell shape with a cleft. Residues along the cleft of TOP help with conformational change through intermolecular ionic interactions. A recent interest is to monitor the kinetic parameters of the enzyme that is mutated on the cleft, E107Q and E111Q. Quenched fluorescence activity assay were used to measure the change in activity. Assays were done with two different substrates MCA and mca-Bk. Results show a decrease in Kcat for MCA substrate, conversely an increase for the mca-Bk substrate. The Km for both MCA and mca-Bk showed change but slightly, indicating that binding was not affected by the mutation. The inhibition studies show interesting results, the Ki value for wild-type enzyme and MCA substrate was low, indicating a high inhibitor-enzyme complex. Whereas the Ki for wild type with mca-Bk was extremely high, suggesting that the inhibitor-enzyme complex does not form which also indicates that the affinity is better for mca-Bk. Interestingly the Ki for the mutant with mca-Bk did not change significantly. However, the Ki for the mutant with MCA increased dramatically compared to its wild type assays. The results suggest that the conformational change is disturbed with the mutation but does not necessarily indicate that activity will decrease. In other words the change in activity is dependent on the substrate. These results provide insight in how to surface residues on the lip affect the activity of TOP, which are important in many physiological roles.

Introduction

Thimet oligopeptidase (TOP) is in the family of metalloendopeptidase and its structure resembles a clamshell shape. The enzyme contains a binding motif of HEXXH. Were the XX is where the metal ion sits in the enzyme, and for this particular enzyme it contains a zinc atom [1]. The structure of TOP has a cleft that contains a channel where substrate enters [2]. In other words the overall structure of TOP is with two sides connected by a hinge in the middle, like a clam and the substrate goes inside. Because of this narrow channel, substrate specificity is selective for this enzyme [2]. The selectivity is for small peptide chains like MCA, mca-BK, and neurolysin [2]. The surface residues on each side of the lip play an important role in the conformational change. The conformation change occurs when the substrate is bound to the enzyme, which helps with cleavage of peptides.

TOP is an enzyme widely found through out the body. The most notable areas are the brain, pituitary and the testis. TOP is also known to have subcellular functions [2]. This enzyme also contains many other physiological functions, some include metabolism of the central nervous system, the periphery, and also converts angiotensin I. TOP has also been linked to Alzheimer's Disease as being a precursor of Alzheimer amyloid [3] which increases the Aβ degradation [4]. This is a key component of the amyloid plaque in Alzheimer's. Since TOP is found everywhere, it is important to understand the structure and mechanics of this enzyme.

By understanding this enzyme it reveals many other enzymes in the same

family, because function is structure. In other words to understand the function of an enzyme the structure needs to be studied. When the structure is understood than assessment of functions can be made. So enzymes with similar structures can be predicated to function the same way. Previous work has already shown that the binding site residues and surface cysteine residues are important for enzyme activity [5,6]. However, there are not many studies about surface residues on the lip of the cleft where the two sides of the enzyme meet.

In the present study particular attention is put on understanding the effects of two specific mutations at the site of Glutamic acid 107 and Glutamic acid 111. Both of these residues are negatively charged and were mutated to neutral amino acid, Glutamine. These mutated residues are annotated as E107Q and E111Q. Where the first letter designates the original amino acid and the last letter is what it was changed to. The current study will show how the change of surface amino acids will affect enzymatic activity. It has been proposed that the residues will decrease enzyme proficiency because it might alter the conformational change. How drastic the change is dependent on the change of the amino acid. If charges are changed to repulse each other then the activity might go extremely down. However, if the amino acid was changed to neutral, such as this one, it might still have reasonable activity. This study will further increase the understanding of the TOP structure and about its mechanism. It also will provide conformation about certain substrate mechanisms that does not require conformation change of the enzyme for cleavage.

Materials

Tris(hydroxymethyl)aminomethane (Tris) was obtained from Fischer Scientific (Fair

Lawn, New Jersey). The quenched fluorescent substrates 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-Lys-dinitrophenol (MCA), and 7-methoxycoumarin-4-acetyl-[Ala^{7}, Lys(DNP)^{9}]-bradykinin (mca-Bk), and inhibitors Angiotensin I/II (1-7) (Ang 1-7), and Bradykinin 1-5 (Bk 1-5) were obtained from Bachem (King of Prussia, PA, U.S.A). Potassium Chloride (KCl) was obtained from Sigma Aldrich (St. Louis, MO), and [tris-(2-carboxy-ethyl)phosphine] (TCEP) was obtained from Invitrogen (Carlsbad, CA, U.S.A.). Marc J. Glucksman provided both wild type and mutant thimet oligopeptidase from Rosalind Franklin University of Medicine and Science.

PROCEDURES

Molecular Modeling

Molecular modeling was done on SWISS PDBviewer version 4.0.1. The x-ray crystal structure of TOP was obtained from Protein Data Bank and was used to analyze residues on the lip of the cleft. Four residues E107, E111, K165 and K168 were isolated to view potential salt bridge interactions.

Blast Comparison

The blast search was done with SWISS ExPASy server for a comparison of TOP across different species and to its closest homolog neurolysin (EC 3.4.24.16) [7]. Other enzymes were also compared to see conservation.

TOP Preparation

Preparation of high concentration of TOP, both mutant and wild type, were mixed with buffer containing KCl (125 mM) and Tris (25mM). TCEP (1 mM or 5 mM) was added to buffer to reduce disulfide bond formation between cysteine residues [6]. The pH was

maintained at 7.8 for all stocks [9]. Enzyme concentration was determined by UV-vis spectroscopy with Abs_{280nm} and the molar absorptivity coefficient ε_{280} = 73.11 mM^{-1} cm^{-1} determined previously [1]. Aliquots were made and frozen immediately after preparation in -80 °C freezer. Enzyme for assays were made fresh daily to minimize loss of enzyme activity. Once buffer with TCEP (5 mM) was added the enzyme was incubated on ice for 30 minuets for full reduction.

Kinetic Assays

A PerkinElmer luminescence spectrometer LS 50 B was used to perform all the kinetic assays. Hydrolysis of fluorogenic substrates, MCA and mca-Bk, was monitored at emission 400 nm and excitation at 350 nm over 150 seconds [8]. A series of six to seven different substrate concentrations were used for a full set of kinetic analysis. The concentrations of substrate were determined by UV-vis with Abs_{366} and molar absorptivity ε_{366} = 17.3 mM^{-1} cm^{-1} [8]. The conditions of the samples were all the same with the exception of the salt study, which contained different concentrations of KCl, mentioned in Salt Studies. The cuvette containing 10 μL of substrate and 1.980 mL of buffer is incubated at 23 °C in 25 mM Tris HCl and KCl at pH of 7.8 prior to addition of 10 μL of enzyme. Temperature and pH was determined previously to minimize degradation of the enzyme and substrate, and also maximize activity of the enzyme [9]**.** TCEP (1 mM) was added in buffer solution to prevent dimerization of sulfides on the enzyme. All assays were ran in duplicates for reproducibility. The concentration of peptide product formed was determined by conversion of the slope in change of fluorescence over time using a standard curve with inner filter effect corrected [10].

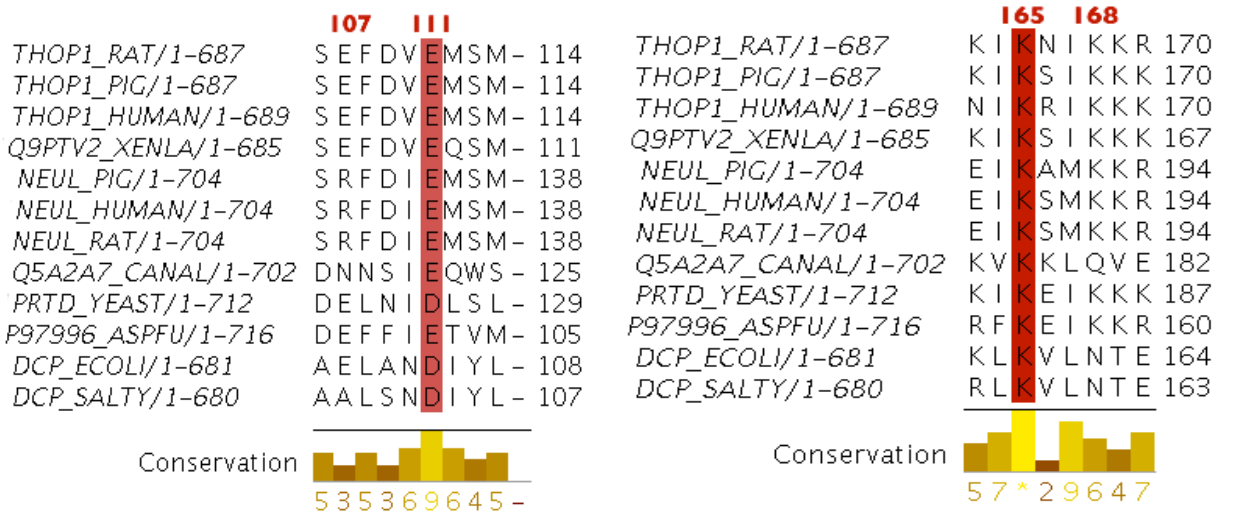

Figure 1. **Conservation comparison among different species and different enzymes at the same site.**

Inhibition Study

For the inhibition studies two different inhibitors were used. However, Ang 1-7 was primarily used for this study. Both the inhibitors were prepared from dry powder that was mixed in Tris buffer with KCl (125 mM). Buffer was filtered through syringe filter to prevent bacteria growth. Final stock concentration of inhibitor was 100 mM. The concentration of inhibitor used for the assays was determined by gauging the lost of activity through trial and error. A low concentration of inhibitor would be used to start with but if there was not enough decrease in activity more inhibitor was added to the next sample. This was done several times before finding where the inhibitor concentration decreased the activity about half way. The finally concentrations of inhibitors used are roughly little less than half the no inhibitor activity. The K_i values were derived by empirical equations [11,12].

Salt Study

The salt study was done using KCl at different concentrations in buffer solution. Each buffer contained Tris HCl (25 mM). Concentrations of stock solutions were 0 mM, 50 mM, 125 mM, and 500 mM KCl. Other concentrations were made by dilution between 0 mM and 500 mM KCl. All buffer solutions were filtered daily through syringe filter prior to use and pH adjusted to maintain at 7.8.

Determination of Kinetic Parameters

The determination of kinetic parameters K_m and V_{max} was done using nonlinear curve fitting {rate=V_{max}[S]/(K_m+[S])}. The program Plot (Wesemann, M., V0.997) was used to help determine the curve fit. Other kinetic parameters K_{cat} and K_{cat}/K_m were found based on the data obtained from Plot.

Results

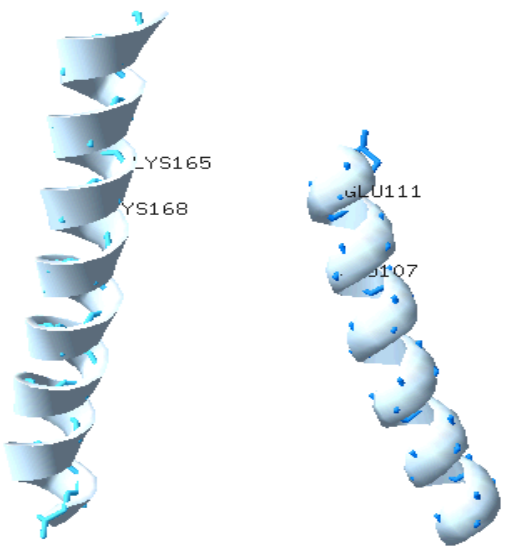

Figure 2. Shows potential salt bridge formation between residues during conformational change

Blast and Molecular Modeling

The blast search reveals the conservation of the mutation sites for rat, human and the closest homolog neurolysin but only for one pair Figure 1. The conserved pair is the 111 and 165 pair. This conservation is based on amino acid charges. This indicates that these pair of residues are important in maintaining the closed form of the enzyme, more so than the 107 and 168 pair. Molecular modeling was further used to see potential salt bridge

MCA	Kcat (s-1)	Km (μM)	Kcat/Km (μM-1 s-1)	Ki (μM)
wt 0μM Ang1-7	1.37E+00	3.77E+01	3.64E-02	-------
wt 25μM Ang1-7	1.37E+00	8.85E+01	1.55E-02	1.85E+01
E107QE111Q 0μM Ang1-7	3.37E-01	3.28E+01	1.03E-02	-------
E107QE111Q 125μM Ang1-7	3.37E-01	5.56E+01	6.07E-03	1.80E+02

Table 1. Kinetic parameters for MCA substrate with both wild type (wt) and double mutant.

formation between the two pairs. It was thought that the salt bride formations between the two residues were responsible, in someway, with the mechanism of the enzyme. Figure 2 shows a potential salt bridge formation along with the distance in the open form of the enzyme.

Kinetic Assays

Kinetic parameters, K_{cat}, Km, K_{cat}/K_m were derived from Michaelis-Menten plot, which was converted from the intensity over time plot. **Table 1 and 2** shows summarized kinetic data for the substrates MCA and mca-Bk, respectively for both wild type and the double mutant. Activity of TOP with the MCA substrate (**Table 1**) shows a significant decrease of K_{cat}, about 4 folds, from the wild type to the mutant. The K_m however had minimal change compare to K_{cat}. The over all efficiency, K_{cat}/K_m, of the enzyme was decreased about 3 folds from the wild type to the mutant. This follows the trend of decrease and increase in K_{cat} and K_m, respectively.

Conversely, the mca-Bk substrate (**Table 2**), which functions under a different mechanism, shows an increase of both K_{cat}

mca-Bk	Kcat (s-1)	Km (μM)	Kcat/Km (μM-1 s-1)	Ki (μM)
wt 0uM Ang1-7	7.91E-01	1.60E-01	4.95E+00	------
wt 125uM Ang 1-7	7.74E-01	3.28E-01	2.36E+00	1.19E+02
E107QE111Q 0uM Ang 1-7	5.07E+00	5.48E-01	9.26E+00	------
E107QE111Q 125uM Ang 1-7	5.18E+00	1.19E+00	4.34E+00	1.06E+02
E107KE111K/K165E K168E 0uM Ang1-7	4.73E+00	8.98E-01	5.26E+00	------
E107KE111K/K165E K168E 125uM Ang1-7	4.73E+00	2.52E+00	1.87E+00	6.91E+01

Table 2. Kinetic parameters for mca-Bk with wild type (wt), double and quadruple mutant.

and K_m from in the mutant. The increase is about 6.5 and 3.5 folds with K_{cat} and K_m, respectively. Both K_{cat} and K_m increase, which increased the K_{cat}/K_m to almost double for the mutant. For further comparison a quadruple mutant, E107KE111K/K165EK168E, with pairs of amino acids charges were switched to opposite sides. This mutation was thought to

MCA	Kcat/Km (μM-1 s-1)	Kcat/Km_{obs} Bk1-5 (μM-1 s-1)	Ki (μM)
wild-type	3.32E-02	5.00E-06	8.23E+01
E107QE111Q	4.50E-03	1.30E-04	4.43E+02

Table 3. Kinetic parameters for MCA substrate, with Bk-15 inhibitor for both wild-type and double mutant.

have similar behavior as the wild type since ionic interactions were still possible.

However, the results present an increase in both K_{cat} and K_m, similar to the double mutant. Interestingly the K_{cat}/K_m did not show a dramatic change between the wild type.

Inhibition Studies

Inhibition studies were done primarily with Ang 1-7 (**Table 1 and 2**) with a short study using Bk 1-5 (**Table 3**). The K_i with Ang 1-7 and the MCA substrate shows a significant increase, almost 10 fold, with the mutant compared with the wild type. Interestingly the K_i for Ang 1-7 with mca-Bk substrate does not increase between the mutant and wild type; rather it has no real significant change. The K_m, on the other hand, for the MCA substrate shows decease in the mutant compare to the wild type, whereas, the mca-Bk substrate shows an increase. This data shows interesting results regarding the mechanism of the enzyme with substrates that operate on different mechanisms. Further data was obtained using the quadruple mutant. The quadruple mutant showed an increase in the K_m with a decrease in the K_i (**Table 3**). Both support the decrease of K_{cat}/K_m.

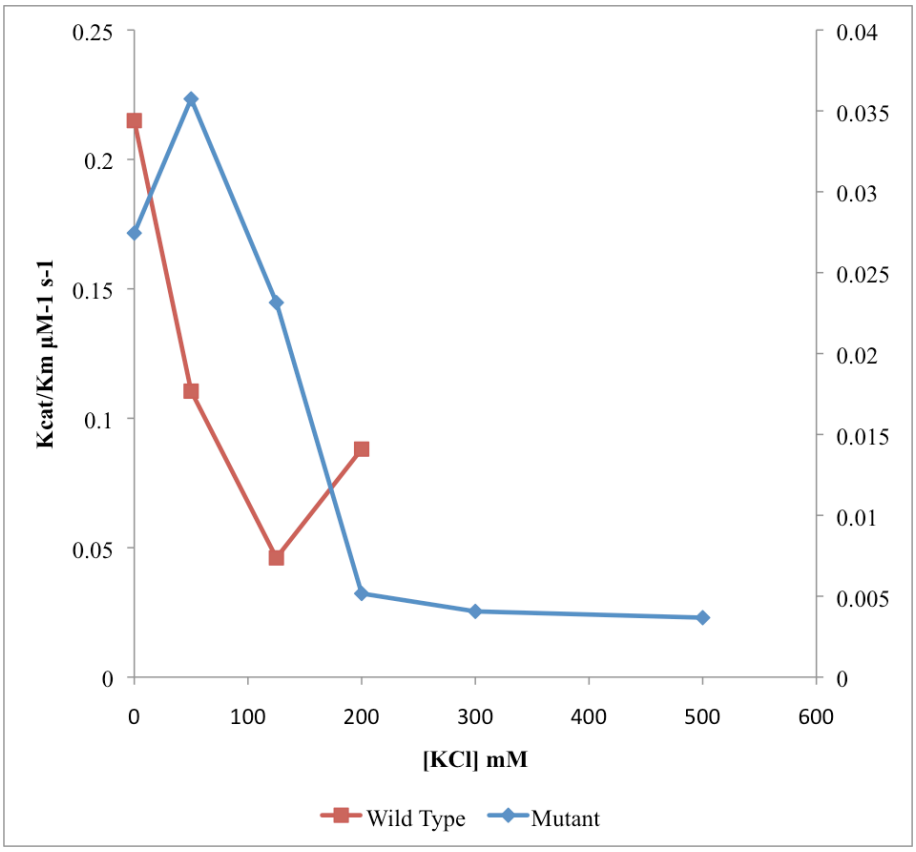

Figure 3. Kcat/Km over different salt concentrations. Includes both wild type and mutant.

Salt Concentration Study

Salt studies were done using fluorescence assays, **Figure 3** shows an over lay of both the wild type and mutant in different salt concentrations. The wild type TOP showed high activity with no KCl and sharp decrease with the increase of KCl concentration. Previous studies indicate that activity increases with slight increases in salt, followed by a decrease in activity [13]. The trend seen with previous studies is seen with the mutant TOP; with a slight increase then decrease of activity with increasing KCl concentrations.

Discussion

Molecular modeling indicates that the 165 and 111 amino acid pair is more

conserved through species than 168 and 107. This suggests that residues at 165 and 111 are probably more important in the closing of the enzyme during activity. The molecular and blast search suggests that these surface residues aid in the conformational change.

The changes of surface residues affect the kinetics of enzyme, indicated by the kinetic assays. The K_{cat} in the mutant enzyme decreased and the K_m did not show a significant change (**Table 1),** which suggests that there is no effect on the binding of substrate. This result indicates that the change in surface residues have no direct effect on the binding of MCA substrate. The decrease in K_{cat}, however, indicates that conformational change is disrupted by the mutation. Previous studies show that MCA requires the conformational change for cleavage [5]; this can possibly cause the cleavage rate to decrease. This then indicates that the mutated enzyme is not efficiently closing. When the enzyme does not fully close it causes residues that are involved in cleavage to be farther apart. When the residues are further it takes longer for the MCA to get cleaved, thus the effect is a lower K_{cat}. This lowering in the K_{cat} causes the overall efficiency to decrease with the MCA substrate (**Table 1**).

Conversely, the mca-Bk substrate yields different results. Instead of a decrease there was significant increase in K_{cat} and virtually no change in K_m (**Table 2**). This indicates that the substrate-enzyme interaction is affected differently between MCA and mca-Bk. The increase of K_{cat} indicates an increase in the cleavage rate. This is probably because mca-Bk operates under a different mechanism than MCA [5]. Under the assumption that the enzyme does not close efficiently, this might explain why the increase in K_{cat} is seen. If the enzyme does not close fully then the enzyme will probably not need as much time to open to

release the products, in turn increasing the rate of cleavage for mca-Bk.

A different mutant was assayed with mca-Bk. This mutant contains four mutations where the charges were just swapped. The hypothesis was that the activity would be similar to the wild type since the charges could still interact to aid in the conformational change. Interestingly there was large increase in K_{cat} and a slight change in K_m, but the K_{cat}/K_m was relatively the same as the wild type (**Table 2**). This indicates that enzyme does not fully close even when the charges are still able for interaction. However, compare to the other mutant it behaved more like the wild type.

The inhibition studies further support the idea that this mutation keeps the enzyme from fully closing. The K_i was determined by activity assays with inhibitor present. **Table 1** shows the low K_i with the MCA substrate compare to the high K_i with the mutant enzyme. A low K_i indicates that the enzyme-inhibitor complex is high. Therefore with an increase in K_i it suggests that the enzyme-inhibitor complex is not forming as well. In other words the data suggest that the inhibitor is a good competitor for the wild type enzyme and not a good competitor for the mutant enzyme. This is probably due to the open-close hinge mechanics of the enzyme. Since the mutation causes the enzyme not to close sufficiently it might not hold the inhibitor as well. For an enzyme-inhibitor complex to form the enzyme must be able to bind and stay bound otherwise the inhibitor will fall out.

Interestingly, there was not a significant change in the K_i between the wild type and the mutant for the mca-Bk substrate **(Table 2**). This indicates that the enzyme has a higher affinity for the mca-Bk substrate than the MCA substrate. With a higher affinity it suggests that the inhibitor is a bad competitor, thus a higher K_i value. The high K_i value for the mutant can be

explained with the open-close hinge mechanics. Again since the enzyme is not able to close sufficiently it might not be able to hold on to the inhibitor as well.

Conversely, the quadruple mutant shows a decrease in K_i with the mca-Bk substrate that indicates that this mutant can perform the open-close hinge mechanic better than the double mutant. However, the quadruple mutant shows that it has a better affinity towards the inhibitor than the wild type. This indicates that the switch of residues causes the affinity for mca-Bk to go down; perhaps the mutant altered an interaction within. In other words this data reveals that the structural change causes the enzyme to work less effectively even though the change is not significant.

To further investigate the activity of the mutant enzyme a salt study was done. The salt studies (**Figure 3**) reveal interesting data that is inconclusive. This study indicates that the wild type enzyme has a sharp decrease of activity with the increase of salt. This could suggest that the ions are interacting with the enzyme causing the enzyme not to feel its ionic attraction. When it fails to feel the ionic attraction the residues do not interact as effectively. Interestingly, a previous study shown that the optimal salt concentration is about 0.125 N [13], which this result did not show. This could be the different substrate specificity. When different substrates are used the enzyme might interact differently. The enzyme starts to increase activity around 200 mM KCl. This suggests that favorable interactions between the ions and enzyme are occurring. This could be because the ions are reducing unfavorable interactions between enzymes that causes them to be inactive [13].

Conversely, the results for the mutant replicate more of what previous study has shown. The difference between the wild type and the mutant at the lower

concentration of salt could be explained by the lost of salt bridge formation in the mutant. When the residues were changed, the lip interaction and presumably the salt interaction were also changed. Thus introducing salt in solution increases the interaction of the lip, making it more active. When too much salt is added it suggests that an ionic barrier is formed between the lips [13] that cause a decrease in activity. Also when the enzyme was assayed in higher salt concentrations it began to plateau, which suggests the salt interaction is affecting the formation of the substrate-enzyme complex. It may somehow shield the enzyme from interacting with residues causing activity to go down.

In summary, these results indicate that the lip-surface residues are important in conformational change and enzyme activity. It also provides further support that mca-Bk operates under different mechanics than other substrates such as MCA. These results reveal the importance of certain residues, which leads to better understanding of the TOP enzyme.

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