INVESTIGATION OF THE EFFECTS OF LETHAL TOXIN NEUTRALIZING FACTOR (LTNF) ON INSULIN DEGRADING ENZYME (IDE) ACTIVITY

A thesis presented to the faculty of the Graduate School of Western Carolina University in partial fulfillment of the requirements for the degree of Master of Science in Biology

Presented by

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Abstract

Lethal Toxin Neutralizing Factor (LTNF) is a protein found in the blood stream of the Virginia opossum (*Didelphis virginiana*). LTNF confers immunity against toxins and venom from many organisms including different species of plants, animals, and bacteria. LTNF has been widely studied as a universal antivenom, but some of its other possible clinical applications have not yet been fully investigated. A small ten amino acid sequence of LTNF – called LT10 – has been shown to reduce Immunoglobulin E (IgE) antibody levels which are a type of antibody that has a role in allergies and autoimmune diseases such as Diabetes mellites, Grave's disease and Hodgkin's disease. Diabetes Mellitus is a disease that effects millions of people worldwide and prevents a person's body from properly regulating blood glucose levels. Insulin Degrading Enzyme (IDE) is a key regulator for controlling insulin levels in the body and modulation of its activity is a promising drug target for the treatment of diabetes. LT10's ability to regulate IgE levels suggests the possibility that it could be used to help treat some of these autoimmune diseases. Computational docking studies predict that LT10 can bind with Insulin Degrading Enzyme (IDE) and inhibit its activity, allowing it to be a possible treatment for diabetes. The overall goal for this research is to determine if LT10 can experimentally bind to IDE and affect the level of IDE activity. Results showed that when LT10 was incubated with IDE transfected lysate there was little to no inhibition and the effect was not consistent. When LT10 was incubated with purified enzyme there was a greater amount of inhibition at lower concentrations than at higher concentrations creating a U-shape response curve. This U-shape response curve suggests LT10 may be binding to multiple sites on IDE.

1. Introduction

1.1 Background information on Lethal Toxin Neutralizing Factor (LTNF)

Lethal Toxin Neutralizing Factor (LTNF) is a peptide obtained from North America's only marsupial, the Virginia opossum (*Didelphis virginiana*). LTNF is a substance, found in the blood stream of opossums, that makes it immune to toxins and venom from many organisms including different species of plants, animals and bacteria (Lipps, 2000). LTNF has a molecular weight of 63 kDa (Lipps, 2000). LT10 is a peptide found on the N-terminus of Lethal Toxin Neutralizing Factor that is comprised of a ten-amino acid trypsin fragment. This fragment and others are caused when LTNF is incubated with trypsin which causes fragmentation at multiple arginine and lysine residues (Lipps, 2000). The amino acid sequence for LT10 peptide is LKAMCPTPPL and it has been shown that this shortened sequence has the same neutralizing properties as the full-length LTNF protein (Lipps, 2000). LTNF also has the ability to reduce Immunoglobulin E (IgE) antibody levels which are a type of antibody that has a role in allergies and autoimmune diseases such as Diabetes mellites, Grave's disease and Hodgkin's disease. LT10's ability to regulate IgE levels suggests the possibility that it could be used to help treat some of these autoimmune diseases. It is predicted that LT10 can bind with Insulin Degrading Enzyme (IDE) and inhibit its activity (Chavan & Deobagkar, 2015).

1.2 Background on Diabetes and the Importance of Insulin

Diabetes Mellitus is a disease that affects millions of people worldwide. This disease prevents your body from properly using energy from the food you eat, which is normally done by the naturally occurring hormone insulin that helps the body use sugar for energy. Insulin helps

the sugar from the blood stream to enter the cells where it can be used for energy (Diabetes Mellitus: An Overview, 2018). When sugar leaves the blood stream, the blood sugar levels are lowered. Without insulin, a person's blood sugar levels would rise, and they would enter a state known as hyperglycemia, which can lead to severe complications such as but not limited to kidney damage, seizures, coma and cardiovascular damage (Mandal, 2019)

There are two individual types of diabetes, type 1 and type 2. Type 1 diabetes is a condition where the pancreas cells that produce insulin are damaged in some way, causing the cells to no longer be able to produce insulin to direct sugar into cells and lower a person's blood sugar levels. People with this type of diabetes must use insulin injections to control their blood sugar levels (Diabetes Mellitus: An Overview, 2018). In type 2 diabetes the pancreas is able to make the insulin but either the insulin does not work properly, or the pancreas does not produce a sufficient amount of insulin for the body. Type 2 diabetes is the more common of the two types and can sometimes be controlled with a combination of diet, exercise, and weight management but it may require the use of oral medications or insulin injections (Diabetes Mellitus: An Overview, 2018).

1.3 Background Information on Insulin Degrading Enzyme (IDE)

Although Insulin Degrading Enzyme (IDE) was first discovered to be responsible for insulin catabolism, playing a role in diabetes, later studies have shown that it also has an effect on other polypeptides such as beta-amyloid, amylin, and glucagon, implicating that IDE may also play a role in neurodegenerative diseases such as Alzheimer's Disease (AD) (Tundo, 2017). IDE is a major enzyme responsible for the degradation of many targets including insulin, glucagon, atrial natriuretic peptide, and beta-amyloid peptide (Pivovarova, 2016). IDE, also known as insulysin, is the primary regulator of glucose homeostasis and *in vivo* has shown to

contribute to the regulation of peripheral insulin as well as cerebral amyloid beta peptide (Abeta) of Alzheimer's disease (Maianti, 2014) (Fernandez-Gamba 2009). IDE may also play a role in the body's metal homeostasis – metallostasis – because the enzymatic activity of IDE is affected by metal levels. Based on structural and biochemical analyses, IDE is able to selectively recognize and degrade insulin using size and charge distribution of the catalytic chamber and the structural flexibility of substrates (Hulse, 2009).

The IDE enzyme is a 110-kDa metalloprotease – meaning that it is a protease enzyme that involves a metal, in this case zinc – that can degrade insulin rapidly into inactive fragments. IDE is a ubiquitous zinc peptidase of the inverzincin family that contains an inverted zinc binding motif (HxxEH) and IDE can be found in bacteria, fungi, plants, and animals (Tundo 2017). The IDE enzyme can exist as a monomer, dimer or tetramer with the dimer species being more catalytically active (Song 2003). The crystal structure of IDE reveals a two bowl shape halves (N- and C-terminal halves) connected by a flexible linker (Shen 2006). These halves of the enzyme can open to allow substrate entry and then close to trap the molecule into a large chamber for catalysis.

1.4 Prediction of LT10 binding to IDE

Interaction studies done using *in silico* methods have shown that IDE is a novel potential target to the structure of the LT10 peptide. Molecular docking and Molecular Dynamic (MD) simulation studies have shown that IDE and LT10 have relatively stable interactions (Chavan and Deobagkar, 2015). Results of these MD simulations suggest that LT10 could inhibit IDE activity. If LT10 has the ability to inhibit IDE activity, then this has the potential to act as a possible therapeutic treatment for diabetes and other diseases caused by IDE.

The stability of this interaction was determined by Optimized Potential for Efficient structure Prediction (OPEP) (Chavan and Deobagkar, 2015). The OPEP score is created by taking the sum of local, nonbonded, and hydrogen bonds, $E = E_{local} + E_{nonbonded} + E_{H\text{-}bond}$ (Maupetit, 2007). Using a genetic algorithm to apply a scoring function, OPEP requires that the native structure of the interaction have the lowest energy. The lower the energy that the molecule has the better the OPEP value (Maupetit, 2007). The OPEP score for the interaction between IDE and LT10 was -5.77 and a Glide dock score of -14.697 Kcal/mol (Chavan and Deobagkar, 2015). Based on these MD simulations, there are a network of predicted hydrogen bonds between LT10 and IDE residues His 108, Asn 139, Thr 142, Lys 192, Trp 199, His 679, Arg824, Tyr 831, and catalytic Zn. Hydrophobic interactions are also predicted between LT10 and residues His 112, Phe 115, Phe 141, Gln 680, Phe 820, and Ile 832 of IDE (Chavan and Deobagkar, 2015) (Figure 1). A structure of IDE complexed with the selective peptide hydroxamate IDE inhibitor (known as asIi1) revealed interactions with His 108, Gln 111, His 112, Arg 824, and Tyr831, and Zn. Several of these interactions overlap with the predicted LT10 interactions providing further evidence as a potential IDE inhibitor (Chavan and Deobagkar, 2015). This predicted role of the LT10 peptide as an IDE inhibitor suggests the ability to use LT10 for therapeutic reasons to counter the effects of IDE. The purpose of this thesis research was to test if LT10 had the same inhibiting effects on IDE lysate compared to purified IDE and to determine an inhibition concentration 50 (IC50) value for LT10 peptide.

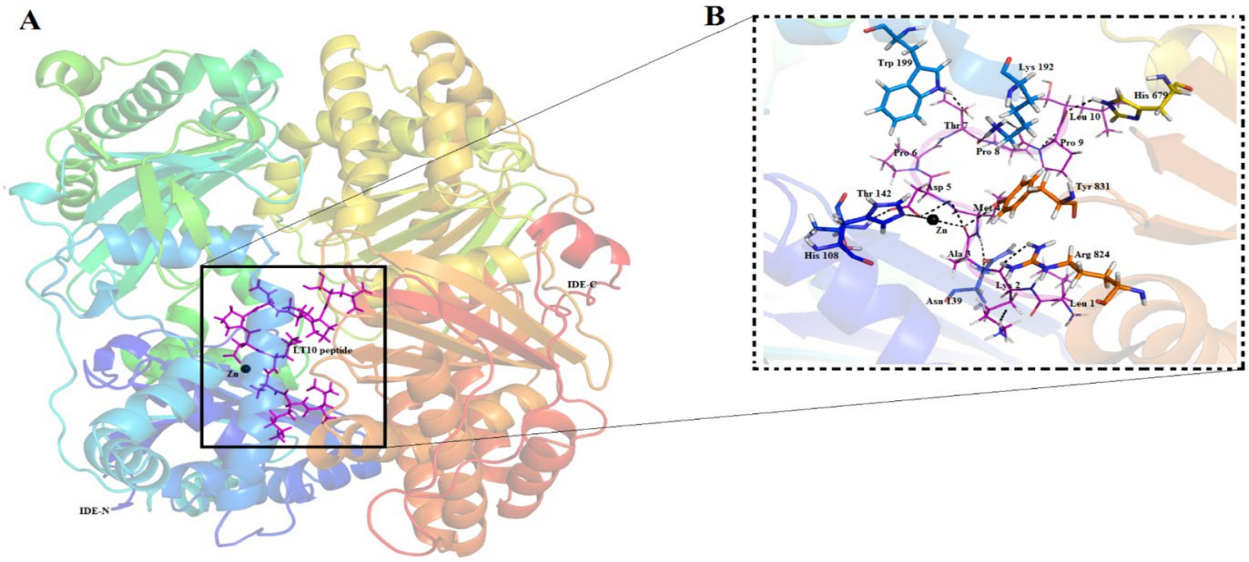


Figure 1: IDE-LT10 Docked Complex (Chavan & Deobagkar, 2015)

"**(A)** Overall structure of LT10 peptide bound to IDE. IDE represented as cartoon with labelled N- and C- termini with catalytic zinc represented as a black sphere and LT10 peptide represented as magenta sticks **(B)** Interaction details showing LT10 peptide as labelled magenta lines interacting with IDE shown as a labelled green stick." (Figure 5 - adapted from Chavan & Deobagkar, 2015).

2.Materials and Methods

2.1 Propagation and Isolation of His-tagged IDE plasmid from *E. coli* cells

Competent *E. coli* cells were transformed with His-tagged IDE (DNA plasmid: pCMV3-His-IDE, SinoBiological). The transformed *E. Coli* cells were then spread onto an LB-kanamycin agar plate and incubated overnight at 37°C to grow. The following day, in duplicate, a single colony was inoculated into a tube containing 3 ml of LB broth and incubated in a shaker at 37°C overnight to grow. The cells were then lysed, and the His-tagged IDE DNA was isolated via the Invitrogen Purelink DNA miniprep Kit. The kit consisted of a series of buffers, washes, centrifugation and elution were used to purify the DNA. The concentration of the purified DNA was measured using the Nanodrop 2000.

2.2 Determination of His-tagged IDE expression levels in HEK293 cells

Approximately 135,000 HEK293 (Human Embryonic Kidney) cells were plated, from a previously grown stock plate, onto gelatin coated coverslips in each well of a six well dish in 10% FBS-DMEM liquid media. The plated cells were incubated at 37°C overnight. The following day the cells were transfected with varying amounts of His-tagged IDE DNA: 0ng (control), 250ng, 500ng, or 1,000ng. The next day cells were then fixed to the coverslips for 15 minutes at room temperature using a 4% formaldehyde solution and stored overnight at 4°C in PBS (phosphate buffered solution).

The following day the cells were stained with a goat anti-mouse antibody (the primary staining). First, the cells were incubated at room temperature for 10 minutes in 1 ml of PBS+TritonX100 to allow the cells to be permeable to the antibody. After 10 minutes the solution was removed and 1 ml blocking solution (PBS+ 1% BSA+ Glycine+ Tween20) was

added. The cells incubated with the blocking solution at room temperature for 30 minutes to 1 hour. After the incubation period, the coverslips were then placed cell side down onto a 60µl drop of 1:500 anti-His antibody PBS mix in a humidified closed container and left to incubate at 4°C overnight.

The next day the cells were washed for 5 minutes with 1 ml of PBS, 3 times in a 6-well dish. After the washes, the coverslips were taken out of the solution and in a container were placed cell side down onto a 60µl of the secondary antibody dilution (1:1000 mouse anti-goat antibody conjugated with Alexafluor-488) and incubated at room temperature for 30 minutes to 1 hour. The cells were then stained with DAPI stain to allow visualization of the nucleus. Following the incubation period and DAPI staining, the coverslips were then mounted onto slides for analysis. The EVOS FL Auto microscope was used to image the slides using the 20X objective for each slide. For the DAPI channel the light was set on 3, the exposure set on 10ms, and the gain was set to 4.5db. The excitation and emission for Alexalfuor-488 has a maximum excitation at 499 nm and emission at 520 nm. The Alexafluor-488 dye is compatible with The GFP light cube on the EVOS FL Auto microscope. For the GFP channel, the light was set to 19, the exposure to 101ms and the gain to 7.0db. These images were used to determine the optimal amount of His-tagged IDE plasmid for subsequent transfection experiments.

2.3 Creation of HEK293 lysate for Enzymatic Assays

HEK293 cells were split into a 6-well plate, with approximately 135,000 cells per each well, and left to incubate at 37°C overnight. The following day, 4 of the 6 wells were transfected with 500ng of His-tagged IDE DNA, while 2 wells were not transfected to serve as a control. After 48 hours the cells were then lysed using a modified RIPA buffer. The RIPA buffer

contained 50 mM of Tris-HCl (pH 7.5), 150 mM of NaCl, 1% NP-40, and 1% sodium deoxycholate -/+ complete protease inhibitors (Roche). After the cells were lysed, the solution was then centrifuged in a cold room and the resulting pellet was separated from the supernatant and aliquoted into microcentrifuge tubes and frozen using liquid nitrogen to be stored at -80°C for later use. A protein Bio-Rad RC/DC Assay was used to measure total protein concentration (see section 2.4).

2.4 Determination of Protein Concentration in HEK293 Cell Lysate

The Bio-Rad RC/DC Protein Assay protocol was used to determine total protein concentration in HEK293 lysate. First 100 µl of RC reagent I was added to all of the tubes. Then samples were added to the appropriate tubes. BSA (2mg/ml) was then added to the standard tubes in volumes equivalent to 0, 2, 4, 8, 16, 24, and 32µg of protein in duplicates. Next, 100µl of RC Reagent II was added to all of the tubes. The samples were mixed and then centrifuged at max speed (13,500rpm) for 5 minutes. The supernatant was discarded. During the spinning 1ml of Reagent A was mixed with 20 µl of Assay Reagent S to ma an A/S mixture. The precipitates were resuspended in 30 µl of the A/S mixture and incubated at room temperature for 5 minutes. The samples (30µl) were then transferred to a clear 96 well plate and 200µl of Reagent B was added to each well. The plate was incubated at room temperature for 15 minutes before being read on the spectrophotometer at 650nm. The standards were prepared in duplicate according to the protocol. Samples were prepared in duplicate using 20µl and 30µl of sample. The samples included IDE lysate and Mock transfected lysate. The final concentration was calculated based on the equation determined from the linear regression of the standard curve ($y=0.0081X+0.0894$).

2.5 Determination of IDE Enzyme Activity in HEK293 Lysates

Fluorescence assays were done according to manufacturer's instructions using the SensoLyte 520 IDE Activity Assay Kit (Anaspec, Fremont, CA) with the following modifications: the IDE substrate was diluted 1000-fold in assay buffer and heated to 37 degrees Celsius. For the IDE lysate (Figure 8A) and mock lysate (Figure 8B) 20 µl of the corresponding lysate was added to each well. Then varying amounts of 10mM LT10 were added to the wells corresponding to the concentration of that well (0µM, 50µM, 100µM, 1000µM, 1500µM, 2000µM). The total volume of the well was then brought up to 50 µl using assay buffer. For the purified IDE (Figure 8C) 30µl of .1mg/mL of purified IDE, diluted 400-fold in assay buffer, was added to each well. Then varying amounts of 10mM LT10 were added to the wells corresponding to the concentration of that well (0µM, 50µM, 100µM, 1000µM, 1500µM, 2000µM). The total volume of the well was then brought up to 50 µl using assay buffer. The plate was then incubated at 37 degrees Celsius for 30 minutes. Last, 50 µl of 1000-fold diluted substrate was added to each of the wells and the assay was ran using the following settings: wavelength: Method mono/mono LM1 490/530, measurement: Mode FL Type kinetic, detection: integration 40ms PMT medium Read height 1mm. For experiments in Figure 6 the substrate used was from the SensoLyte 520 IDE Activity Kit and for the experiments in Figure 8, the substrate used was Mca-RPPGFSAFK(Dnp)-OH Fluorogenic Peptide Substrate (R&D systems). Measurements were taken every 5 minutes after 5 seconds of orbital shaking for 1hour. The following SpectraMax iD5 plate reader settings were used:

wavelength: mono/mono LM1 Ex = 490 nm/ Em = 530 nm, measurement: Mode FL Type kinetic, detection: integration 40ms PMT medium, and Read height: 1mm.

2.6 Synthesis of LT10 Peptide

The amino acid sequence for LT10 peptide is LKAMCPTPPL. The desired amount of resin was weighed and added to a syringe. The syringe was filled with dichloromethane (DCM) and shaken for 1 hour. Next the deprotection step was performed. The syringe was then filled with DMF and shaken for 1 hour. The DMF was then removed, and the syringe was filled with 15% piperidine/DMF. 2-3mL of air was left for shaking. It was shaken for 2 minutes. They syringe was then drained and refilled with 15% piperidine/DMF again leaving 2-3mL of air for shaking. This time it was shaken for 20 minutes. The resin was washed by shaking for 30 seconds with DMF 5 times. The syringe was washed with 0.2M HOBt/DMF twice and the resin was washed with 0.2M HOBt/DMF+0.001M Bromophenol Blue (BB) twice. The resin was then washed with DCM three times and then DMF three times.

Next 2 different amino acid coupling sequences were performed. First in a scintillation flask 3 equivalents of Fmoc-AA-OH and 3 equivalents of HOBt in DMF were dissolved and then activated with 3 equivalents of DIC and stirred for 5 minutes. Activated amnio acid was added to the syringe to cover the beads and shaken for 2 hours. The coupling agent was removed, and the resin was washed with DCM three times and DMF three times. The second amino acid coupling sequence started by dissolving 1.5 equivalent of Fmoc-AA-OH and 1.5 equivalent of HBTU in DMF in a scintillation flask. Then it was activated by adding 3 equivalents of DIEA and stirred for 5 minutes. The activated amino acid was added to the syringe to cover the beads and shaken for 1 hour and 20 minutes. The coupling reagent was removed, and the resin was washed with DMF three times and DCM three times.

After the first amnio acid was added to the sequence, 50% acetic anhydride in pyridine (10mL per 1g of resin) was added and shaken for 20 minutes. Then the acetylation reagent was removed, and the resin was washed with DMF five times. At the end of the amino acid chain, deprotection was repeated using deprotection process already mentioned. The resin was then washed with DMF three times and DCM eight times. The TFA cleaving solution was added to the syringe leaving 2-3mL of air for shaking and was shaken for 5 hours. It was then washed with TFA five times, and the filtrate and washings were collected and concentrated with a stream of nitrogen/argon. The product was extracted by cold ether (4C) and the suspension was kept 1 hour in the freezer, then centrifuged for 7 to 10 minutes. The extraction was repeated 3 to 5 times for scavenger residue. The cleaved peptide was treated for 2 hours in 6% acetic acid to complete protecting group cleavage. The product was centrifuged again, and the ether was decanted. The products were dissolved into 1M acetic acid and lyophilized.

3. Results

3.1 Expression of IDE enzyme in HEK293 cells

The overall goal of the research was to measure the enzymatic activity of IDE from cell lysate in the absence and presence of LT10 peptide. HEK293 cells were transfected with varying amounts of His-tagged IDE plasmid (pCMV3-His-IDE: SinoBiological) using Continuum transfection reagent (Figure 2). Cells transfected with 500ng of plasmid had the per cell highest expression of IDE based on the immunofluorescence images (Figure 2). Based on these results, all subsequent experiments used cell lysate made from cells transfected with 500ng of IDE plasmid for use in enzymatic assays (see materials and methods).

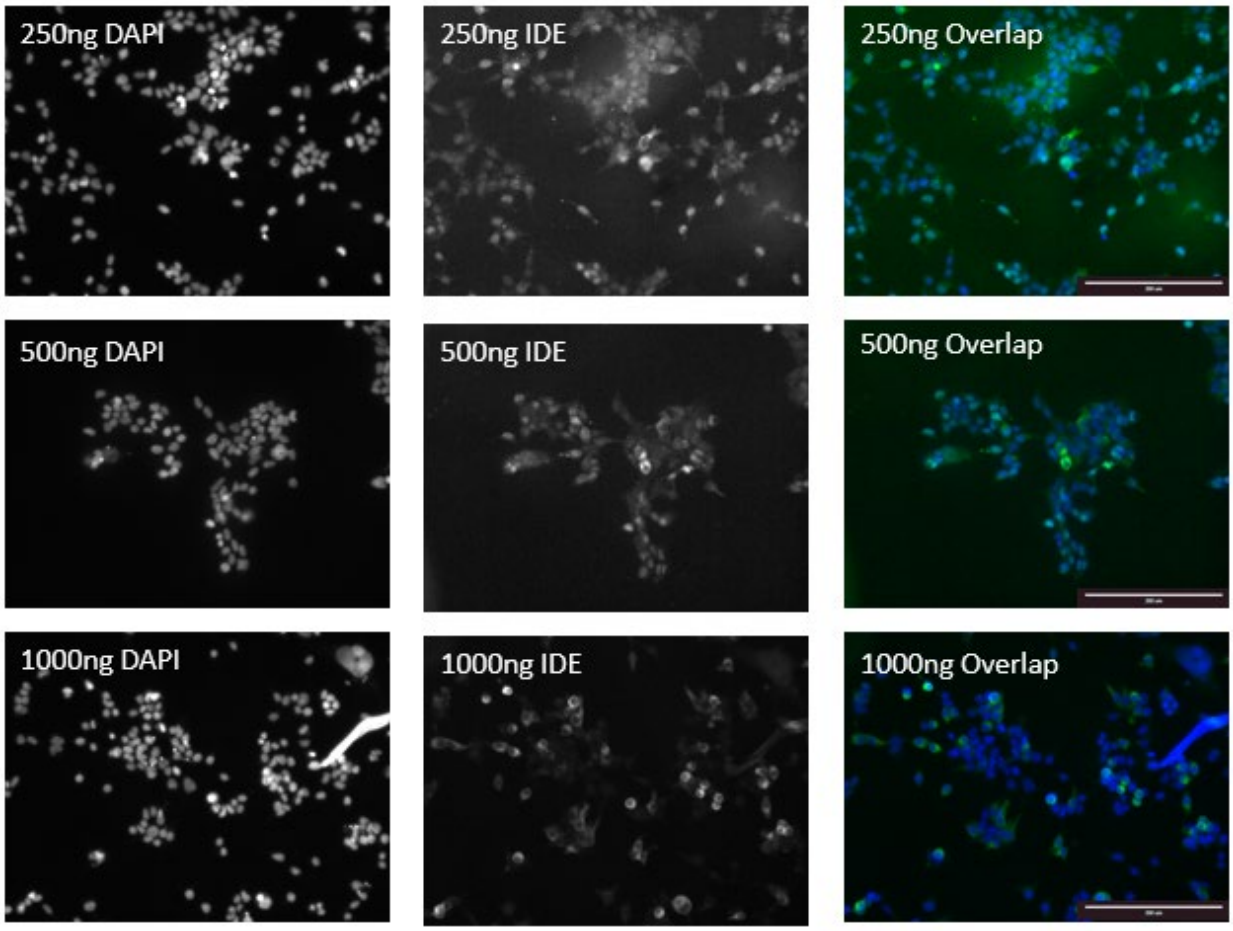
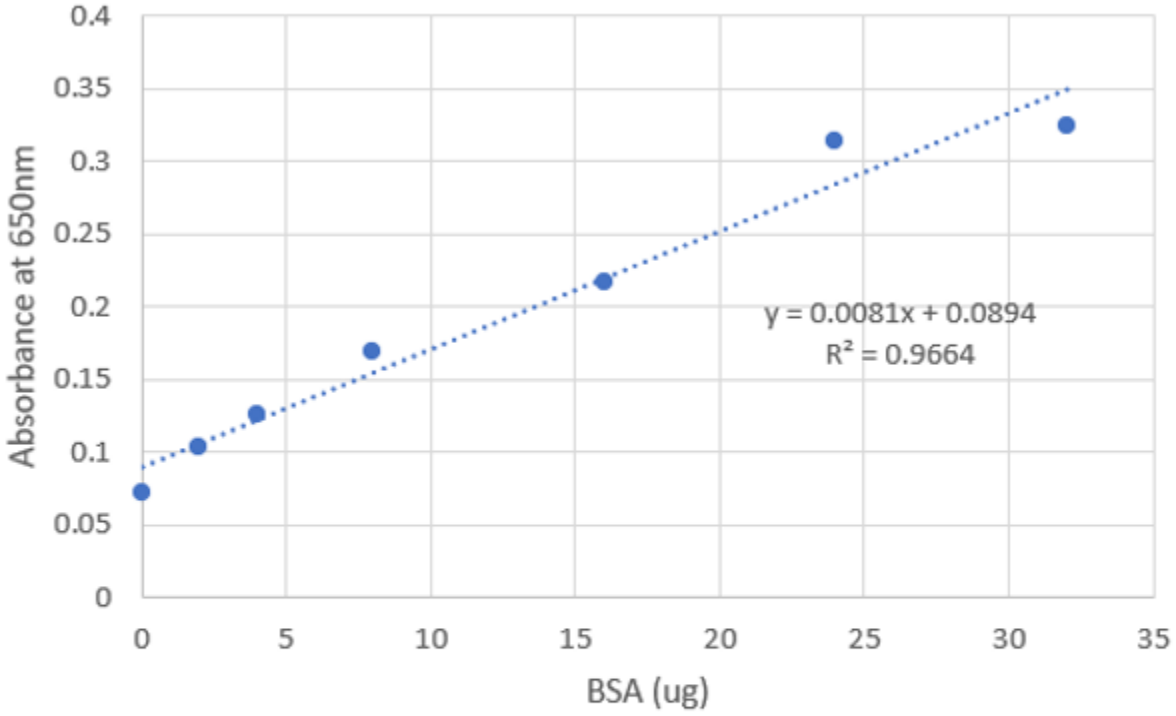


Figure 2: HEK293 cells transfected with IDE plasmid

HEK293 cells transfected with His-tagged IDE DNA and incubated for 48 hours before formaldehyde fixation, staining (anti-His antibody and DAPI), and imaging with EVOS FL microscope (see materials and methods). Depicted are grey scale images acquired with the DAPI and GFP (IDE) light cubes on EVOS FL microscope and the third images are the resulting overlay of the two channels (pseudo-colored). The scale bar equals 200 microns.

3.2 Measuring Protein Concentration of Cell Lysate

Equal amounts of cell proteins were used in the fluorescence-based IDE activity assays for normalization purposes (see section 3.4). Therefore, total protein levels were measured from the HEK293 lysates using a BioRad RC/DC kit according to manufacturer's instructions. A standard curve was created using BSA standards (Figure 3). A linear regression was performed on the BSA standard curve, and the equation obtained (Y=0.0081X+0.0894) was used to calculate the total protein concentration in lysates. Two different volumes of lysate were measured (20µl and 30µl) to determine the average protein concentration. The total protein concentration in both the mock transfected lysate (.705 µg/µl) and the IDE lysate (.675 µg/µl) were very similar (Figure3). These concentration values were used to calculate the amount of protein used in the IDE enzymatic assays (see section 3.4 and 3.5).



Amount of lysate	Mock lysate average absorbance	IDE lysate average absorbance
20µL	0.1956 -/+ 0.0216	0.20025 -/+ 0.05035
30µL	0.27365 -/+ 0.02045	.2532 -/+ 0.0206

Figure 3: Measuring the protein concentration of mock transfected lysate and IDE transfected lysate.

A protein assay was performed according to the Bio-Rad RC DC Protein Assay protocol. The graph depicts the BSA standards and linear regression performed. Samples of 20 µl and 30 µl of both Mock transfected lysate and IDE transfected lysate were also prepared according to the protocol. This assay allowed for the calculation of total protein per µl of each type of lysate.

3.3 Optimizing the amount of lysate to use in IDE experiments.

The Sensolyte 520 assay kit was used to measure IDE enzyme activity in mock and IDE transfected lysates. The Assay works on the principle of Forster Resonance Energy Transfer (FRET). This assay works by having a dye on one end of the kit's substrate peptide and a quencher on the opposite end. The quencher keeps the dye from fluorescing in the intact peptide. Upon peptide cleavage, the dye is able to fluoresce, and the fluorescent signal is proportional to enzyme activity (Figure 4).

Mock and IDE transfected lysates were measured using the SensoLyte 520 IDE Activity Assay Kit per manufacturer's instructions. Zero to twenty microliters were used in the assay to determine the optimal signal-to-noise ratio. Addition of increasing amounts of IDE transfected lysate led to increase signal from 200,000 to 300,000 relative light units (RLU). In contrast, the signal stayed the same, except for a spike with ten microliters, upon addition of increasing amounts of mock transfected lysate (Figure 5). However, it is important to note there was a substantially larger range of values for mock compared to IDE lysate (Figure 5, error bars). These results confirmed that IDE activity could be measured in lysate and the 20µl volume was chosen for subsequent experiments.

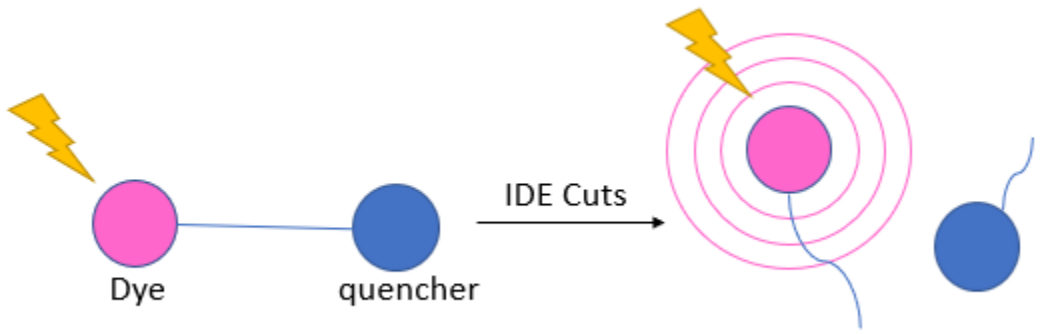


Figure 4: Principle of Sensolyte 520 IDE activity assay

One end of the peptide has a dye (represented in magenta) while the other end contains a quencher (represented in blue). The quencher keeps the dye from fluorescing when both are attached to the peptide. When IDE cuts the peptide, the dye is able to give off a fluorescent signal that can be measured.

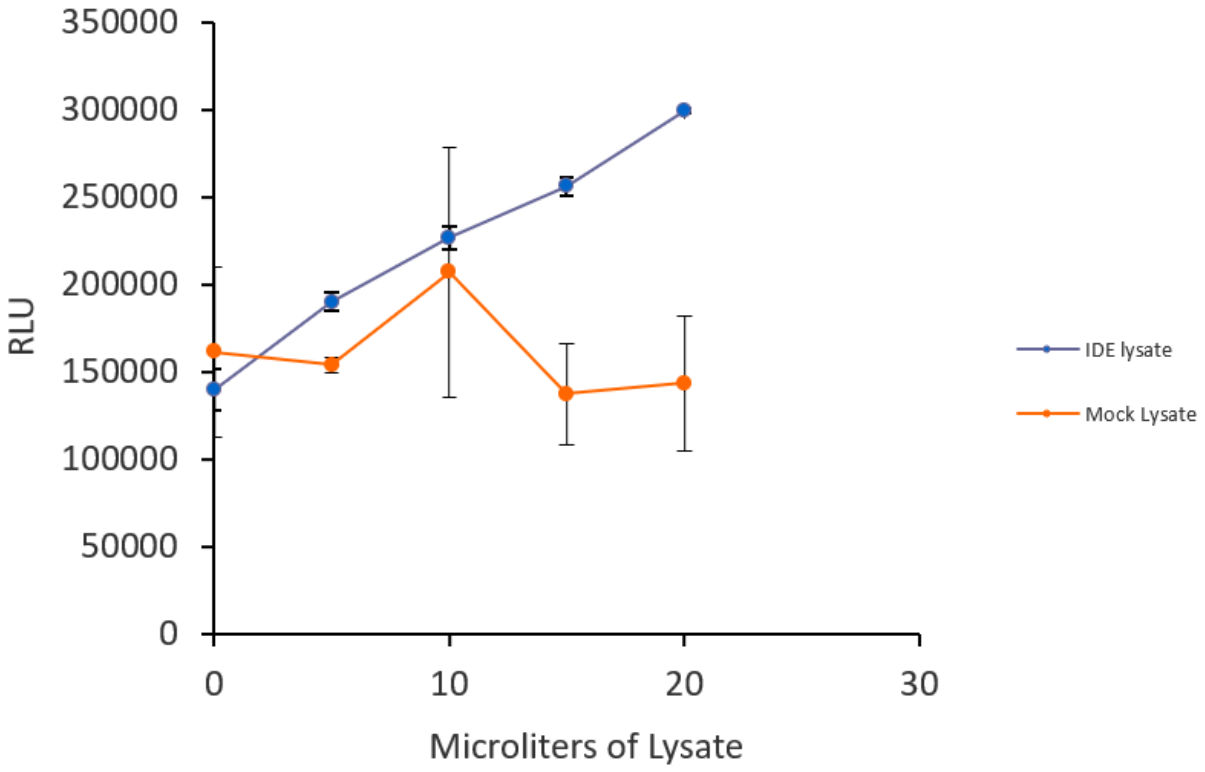


Figure 5: Optimization of Mock transfected and IDE transfected lysate to use in experiments.

Varying amounts of both Mock transfected and IDE transfected lysate were incubated with substrate and ran on the SpectraMax iD5 plate reader with the following settings: wavelength: Method mono/mono LM1 490/530, measurement: Mode FL Type kinetic, detection: integration 40ms PMT medium Read height 1mm. Error bars are standard deviation of the replicates.

3.4 Inhibition of IDE lysate activity with EDTA

EDTA is a known inhibitor of IDE activity. It is a metal chelate that can bind and sequester metals from enzymes. IDE transfected lysates were measured in the absence or presence of increasing concentrations of EDTA (Figure 6). Enzyme activity decreased to approximately 50% in the presence of 500μM and 20% in the presence of 1500μM EDTA. There was greater variance at the 100μM concentration compared to the other concentrations based on the error bars depicted (Figure 6). This larger variance is most likely due to one measurement that appeared to be an outlier (Figure 6, replicate 5). It is possible that this "outlier" was produced because of a bubble in that well during measurement but the exclusion of the data point could not be justified and thus was included in the analysis. It is not uncommon for bubbles to form during assay measurement and lead to distortions of the fluorescent signal due to light scattering. Regardless, these results demonstrated that the enzymatic activity was metal dependent as expected.

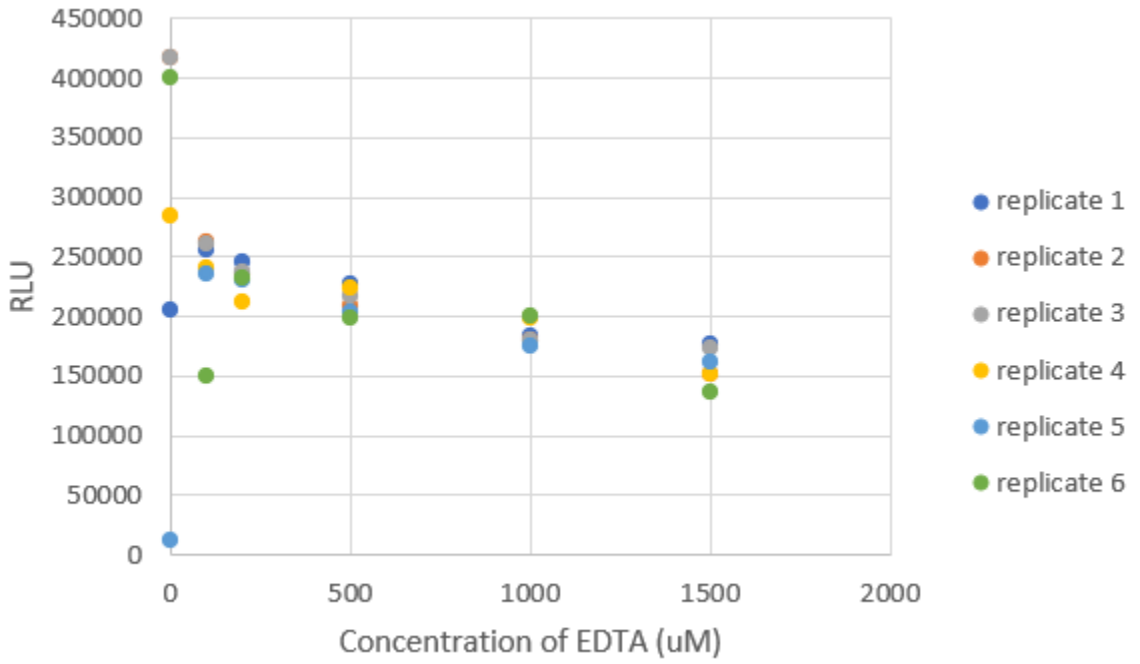


Figure 6: Inhibition of IDE activity by EDTA

IDE lysate's enzymatic activity was measured in the presence of the known inhibitor EDTA. IDE lysate was incubated with increasing amounts of EDTA for 30 minutes before the substrate was added and the activity was measured for 30 minutes. The graph depicts the results of the assay after 30 minutes with the substrate incubated at 37 degrees Celsius. Each point on the graph represents 1 replicate and replicates are pooled from two separate experiments.

3.5 Measurement of IDE enzymatic activity in absence and presence of LT10 peptide

LT10 peptide was synthesized using a solid phase technique (see materials and methods) and the sequence of the peptide was confirmed by mass spectroscopy (Figure 7). The enzymatic activity measured in IDE lysate increased in the presence of LT10 peptide (Figure 8A). The activity increased approximately 1.5-fold in the presence of 2000μM LT10 peptide. The activity was measured at both 30 minutes and 60 minutes after addition of substrate and similar results were obtained. Interestingly, the activity measured in the mock transfected lysate appeared to follow a U-shaped response with inhibition at low concentrations (100-1000μM) and increased signal at higher concentrations (2000μM) (Figure 8B). Importantly, the overall signal for the mock was 2-3 times lower compared to the IDE lysate similar to the optimization experiment (Figure 5). Samples that contained no lysate and only buffer and substrate had readings of 100,000 – 150,000 RLUs. These results suggest there is endogenous IDE in HEK293 lysate and/or other endogenous enzymes, such as cathepsins, that are weakly cleaving the substrate.

For the purified IDE we see that at lower concentrations there is inhibition of activity, but the higher concentrations do not show as much inhibition creating a “U” shaped curve similar to the mock lysate (Figure 8B). This could indicate that higher concentrations do not necessarily mean better inhibition and in fact appear to activate the IDE enzyme.

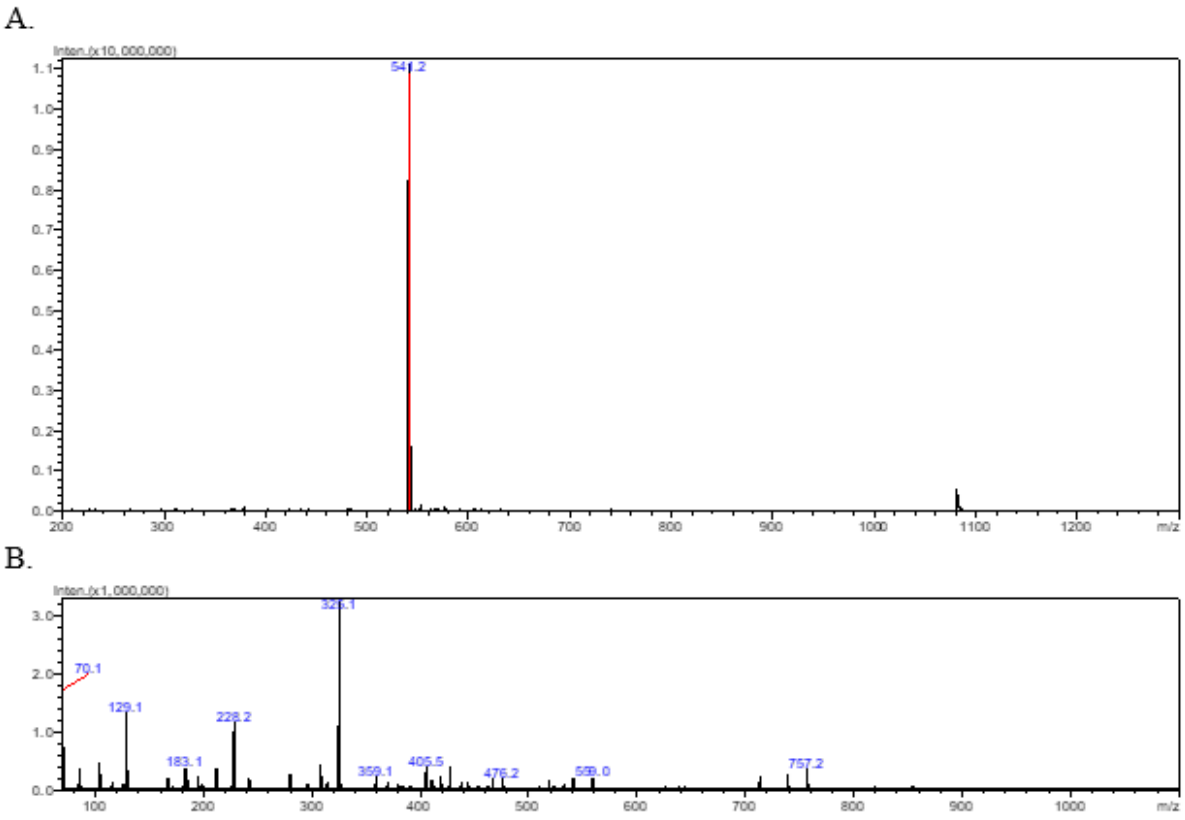


Figure 7: Confirmation of LT10 peptide sequence by liquid chromatography mass spectroscopy (LCMS).

LT10 peptide was dissolved to 1mg/ml in water and 1µl was injected onto LCMS instrument. A. Mass Spectrum of the LT10 peptide with m/z 541.2 as (M+2)2+ peak. B. Product ion scan: Precursor 541.3. The calculated molar mass of LT10 is 1080.6 g/mole and the calculated (M+2)2+ peak is predicted to be 541.3. Observed (M+2)2+ peak from the MS chromatogram is 541.2 confirming correct synthesis of peptide.

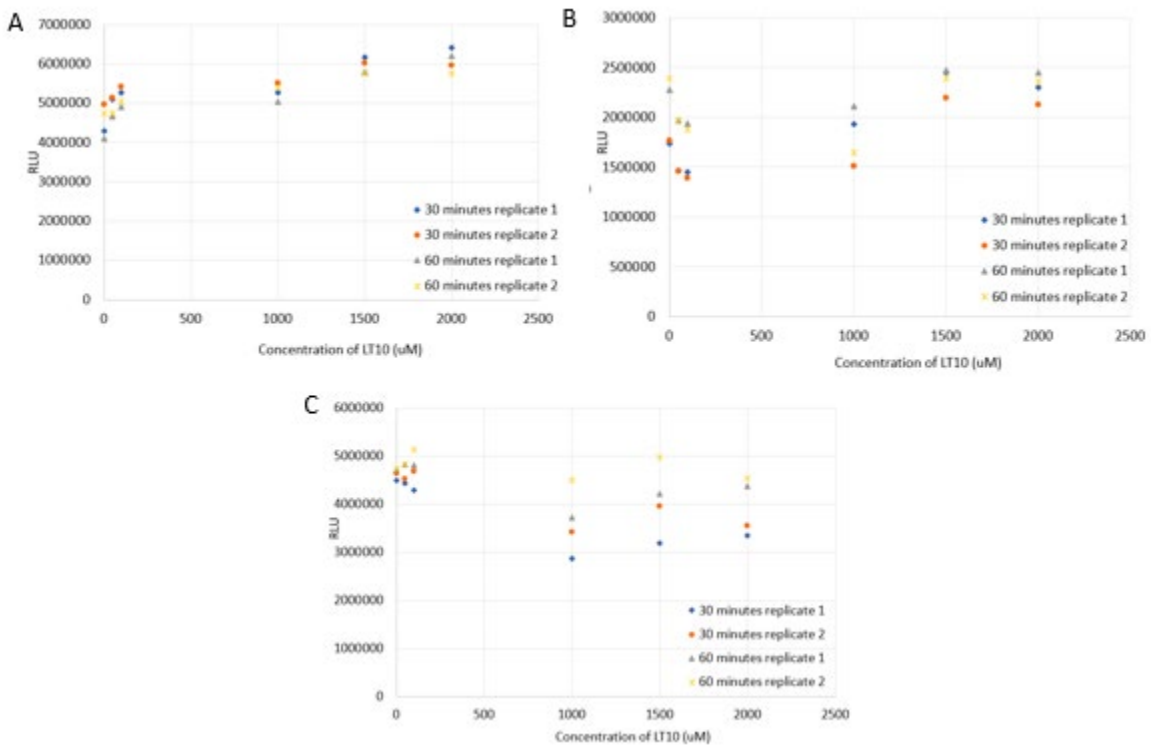


Figure 8: LT10 inhibition of IDE Enzymatic Activity.

A. IDE lysate's enzymatic activity measured in the presence of increasing amounts of LT10 peptide. **B**. Mock transfected lysate's enzymatic activity in the presence of increasing amounts of LT10 peptide. **C.** Purified IDE's enzymatic activity in the presence of varying amounts of LT10 peptide. This experiment was done in duplicate and activity was measured at the 60-minute timepoints. There are two time points represented on this graph, 30 minutes and 60 minutes. The two replicates for 30 minutes are represented by a blue diamond and an orange circle. The two replicates for 60 minutes are represented by a grey triangle and a yellow X.

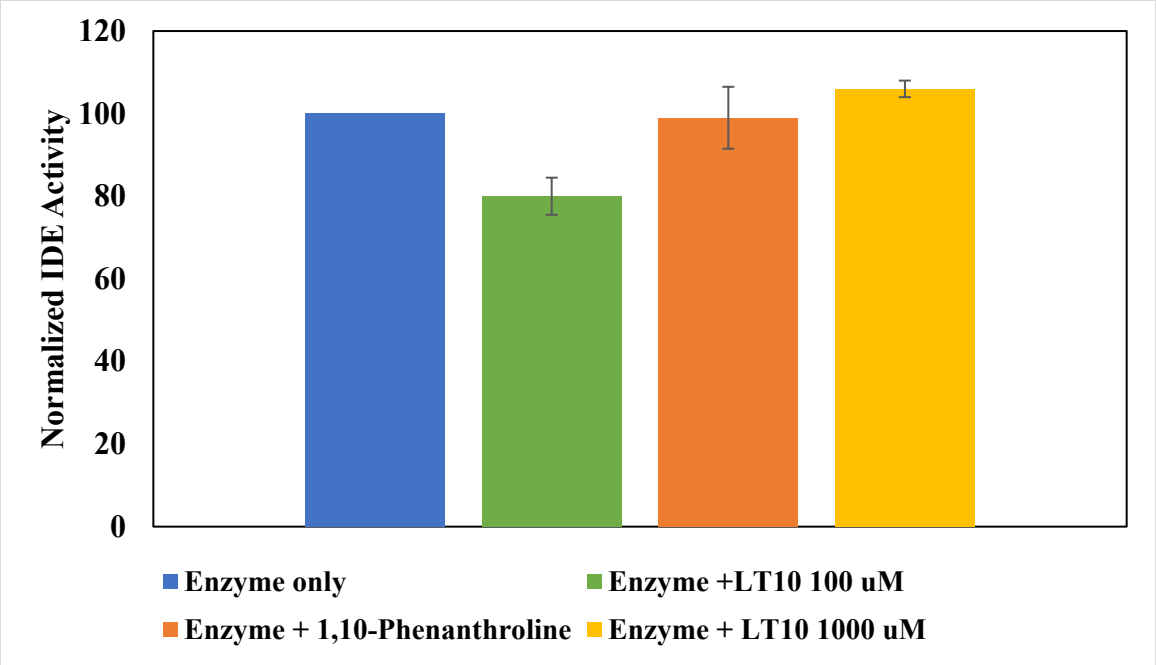


Figure 9: LT10 Peptide can Inhibit IDE Enzymatic Activity (Preliminary Data – from Rice 2018)

IDE enzymatic activity measured in the absence (blue bar), or presence of 100 μM LT10 peptide (green bar), 1000 μM LT10 peptide (yellow bar), or 1, 10 – Phenanthroline (orange bar). Enzyme only activity was normalized to 100% for comparison to enzyme with inhibitors. Fluorescence was measured on a Biotek Plate Reader with Ex/Em = 490/520 nm in duplicate. All samples were measured in duplicates and then averaged. (Error range: LT10 100 μM ± 4.5%, 1, 10 – Phenanthroline ± 7.5%, LT10 1,000 μM ± 2%)

Discussion

IDE transfected HEK293 lysate exhibited greater activity (2-3 fold) compared to the mock transfected lysate. The activity measured in the IDE transfected lysate could be inhibited in a dose-dependent manner by the chelator EDTA (Figure 6). This demonstrated that the enzymatic activity was metal dependent. Previous experiments performed by S. Rice observed inhibition of purified IDE enzyme in the presence of 100 μM LT10 (Rice 2018). Surprisingly, none of the concentrations of LT10 tested here with the lysate inhibited activity and increased activity was observed (Figure 8A).

There was a difference in the absolute RLU values between the optimization and LT10 experiments (Figure 5 & Figure 8). However, these differences are probably due to the use of a different substrate. The substrate used for the experiment in Figure 8 was Mca-RPPGFSAFK(Dnp)-OH Fluorogenic Peptide Substrate from R&D systems instead of the Anaspec peptide (Figure 5 & 6). The change in peptide was necessitated due to availability and financial considerations. The R&D peptide sequence can be recognized and cleaved by ECE-1, ACE, Cathepsin A, Cathepsin X/Z/P, Neprilysin, and Insulysin. Because of this, it is possible that activity in the Mock transfected lysate could be due to endogenous enzymes such as Cathepsins or Neprilysin that recognize and cleave the substrate and/or endogenous IDE. The potential for endogenous enzyme activity could be an argument to use purified IDE in future experiments (Figure 8B).

For the experiment done with LT10, there was little to no inhibition of activity in the IDE lysate (Figure 8A). There was not consistent inhibition in lysate and that could be due to the peptide binding to other proteins in the sample and causing interference or the expression level of the exogenous IDE. The inhibition of purified IDE enzyme at low (100 – 1000 μM, Figure

8C) and apparent activation at higher concentrations (>1000μM, Figure 8C) was in agreement with S. Rice's results in Figure 9 (Rice, 2018). This observed activation produced a U-shaped response curve. This biphasic (non-monotonic) response seen for the purified enzyme and the mock lysate upon incubation with different amounts of LT10 could be the phenomenon hormesis (Conolly, 2004). These results suggest that LT10 could bind to additional sites besides the predicted binding at the active site. This could be caused by the enzyme undergoing allosteric activation where the peptide can bind to a distal site distinct from the substrate binding site and cause "heterotrophic" activation of the enzyme. This phenomenon has been observed for the binding of Bradykinin peptide to IDE (Song 2003). The U-shaped response of IDE upon incubation with LT10 could be similar to Bradykinin peptide's activation of IDE. If this is the case then future experiments could be performed to determine if IDE oligomerization is altered in the presence of LT10. Importantly, it would be useful to test other proteases in our assay to confirm that the effect seen is not an artefact of the assay and to repeat the experiment with purified IDE enzyme to confirm the results. LT10 could be a potential activator of IDE, at certain concentraetions, and thus could be a potential candidate for modulating beta-amyloid processing to treat Alzheimer's. These preliminary experimental results have uncovered a potential activating role for LT10, in addition to inhibitor function, that was not predicted from *in silico* studies.

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