Biochemical Characterization of Regulators of Insulin Degrading Enzyme (IDE)

by

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This is to certify that I have examined this copy of a master's thesis by

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to my family…

ABSTRACT

Insulin-degrading enzyme (IDE) is an allosteric Zn^{+2} metalloprotease enzyme involved in the degradation of many peptides including amyloid beta (Aβ), and insulin that play key roles in Alzheimer's disease (AD) and type 2 diabetes mellitus (T2DM), respectively. Therefore, the regulation of IDE by small molecules is a rational approach for the treatment of these diseases by controlling the concentrations of substrates of enzyme. Crystal structure of IDE revealed that N-terminal of IDE has an exosite which is ~30 Å away from the catalytic region, and serves as a regulation site by orientation of the substrates of IDE to the catalytic site. Previous studies show that a 9-amino acid long peptide, bradykinin binds to the exosite and hence enhances the activity of IDE, which indicates that novel small molecules may bind to this exosite to regulate its activity. In this thesis, biochemical characterization of small molecules on the proteolytic activity of IDE was discovered using various physiological substrates of IDE. The effects of the molecules were monitored on the activity of IDE using different substrates. The resulting novel compounds, designated as D3, D4, and D6, enhances IDE mediated proteolysis of substrate V, insulin and FAβB, respectively. These selected compounds demonstrated submicromolar activation and can be utilized for the lead optimization studies to regulate substrate specific IDE activity. This study describes the first examples of a computer-aided discovery of IDE regulators, showing that *in vitro* activation of this important enzyme with drug-like small molecules is attainable.

ÖZET

İnsülin parçalayan enzim (IDE) Zn^{+2} metal proteaz ailesinden olup, 2. Tip şeker ve Alzheimer hastalıkları için önemli olan insülin ve amyloid-beta proteinlerinin kandan temizlenmesinde rol oynamaktadır. Bundan dolayı IDE' nin aktivitesini düzenlemek bu hastalıkları tedavi etmede makul bir yaklaşımdır. IDE-N bölgesinde "exosite" denen düzenleyici bir bölge bulunmaktadır. Bu bölge sübstratların katalitik bölgeye yönlendirilmesini sağlamaktadır ve katalitik bölgeden yaklaşık 30 A° uzaktadır. 9 amino asit uzunluğundaki bradikinin "exosite" bağlanarak IDE'nin aktivitesini güçlendirmiştir. Buradan yola çıkarak hesaplamalı teknikler kullanılarak bulunan 9 küçük moleküllerün biyokimyasal karakterizasyonu yapılmıştır. Aktiviteye olan etkileri flüorışıma metoduyla ve, sitotoksisite ise MTT yaşayabilme testi ile ölçülmüştür. Sonuç olarak D3, D4 ve D6 moleküllerinin sırasıyla IDE' nin sübstrat V, insülin ve FAβB kesimini artırmış oldukları deneysel olarak gözlenmiştir. Bu moleküller küçük konsantrasyonlarda aktiviteyi artırmaktadırlar ve ileriki çalışmalar için yönlendirici olabilirler. Bu çalışma ilk olarak bilgisayar yöntemleriyle bulunmuş moleküllerin deneysel olarak test edilmesini ve ilaç olabilecek öncüller olduğunu göstermektedir.

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NOMENCLATURE

IDE	Insulin Degrading Enzyme
FAβB	Fluorescein-Aβ-(1-40)-Lys-biotin
PCR	Polymerase Chain Reaction
Aβ	Amyloid-beta
FITC	Fluorescein Isothiocyanate
T2DM	Type 2 Diabetes Mellitus
AD	Alzheimer's disease
APP	β-amyloid Precursor Protein
AD	Alzheimer's disease
APOE	Apolipoprotein E
dNTP	Deoxynucleoside Triphosphates
LB	Lysogeny broth
NaH_2PO_4	Sodium phosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BSA	Bovine serum albumin
HRP	Horseradish Peroxidase
TBS	Tris Buffered Saline
ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine Tetra Acetic Acid
PVDF	Polyvinylidene Difluoride
Ni	Nickel

NTA	Nitrilotriacetic acid
PMSF	Phenylmethyl sulfonylfluoride
Taq	*Thermus aquaticus*
OD	Optic Density

Chapter 1

INTRODUCTION

According to the United States Census Bureau, world population will grow %20 in ten years, but there is %50 increase in the demographic of age 65 and over. As the life time of population increases, type 2 diabetes and Alzheimer's disease (AD) become widespread around the globe. Recent World Health Organization (WHO) indicates that 3 million people die due to diabetes per year worldwide. There is a strong relation between diabetes type 2 and AD [1, 2]. There are several approaches to the treatment for these diseases. One of the most significant methods is to regulate the catalytic activity of Insulin degrading enzyme (IDE) since IDE is involved in clearance of insulin and amyoid-β [3, 4]. Therefore, IDE is vital to development of diabetes and Alzheimer's disease.

Insulin degrading enzyme (IDE) is 110- kDa zinc metalloprotease which consists of two equal sized N- and C- terminal domains (IDE-N and IDE-C)[5-7]. IDE is an allosteric enzyme involved in the clearance of many peptides including amyloid beta (Aβ), and insulin that play key roles in Alzheimer's disease (AD) and type 2 diabetes mellitus (T2DM), respectively. Advances in molecular biology and X- ray crystallography enable us to learn the binding details of IDE with its substrates. It is possible to regulate the activity of IDE with small molecules. As a result, these molecules could be drug candidates for certain diseases.

In Chapter 2, the corresponding work in the literature is demonstrated. This chapter includes the most recent information about the structure, function, regulation of IDE and general aspects about AD and T2DM.

Chapter 3 includes materials and methods, and illustrates the details of the generation steps of obtaining IDE protein. PCR, cloning of IDE, expression and purification of IDE protein methods are explained in this chapter. In the remaining part of this chapter, *in vitro* enzymatic assays such as degradation of substrate V, FAβB, insulin- FITC and toxicity assays are presented in detail.

Chapter 4 includes the results of this work and discussion is given in the chapter 5. This thesis ends with chapter 6 which includes the future directions and conclusion of the study.

Chapter 2

LITERATURE REVIEW

2.1. Alzheimer Disease

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive formation of amyloid-beta (Aβ) and tau (τ) proteins, mitochondrial abnormalities, neuroinflammatory processes, oxidative stress, deterioration of behavioral and cognitive functions [8]. Aβ, which is formed from β-amyloid precursor protein (APP) processed by β-, and γ-secretase [9], has an early pathological role in all forms of AD. Although there has been various strategies for the treatment of AD including, anti-inflammatory approaches [10], tau pathology approaches [11], apolipoprotein E (APOE) related treatment approaches [12], metabolic dysfunction approaches [13, 14], Aβ-targeted therapeutic approaches are still in the center of AD treatment methods [15].

There are several Aβ-targeted therapeutic strategies including modulation of Aβ production, blocking of Aβ aggregation, Aβ-targeted immunotherapy, and enhancement of Aβ degradation[15]. One of the first efforts in drug development for the modulation of Aβ production is the inhibition of γ-secretase [16]. However the inhibition of γ-secretase also affects the Notch1 cleavage resulting in the blockage of thymocyte differentiation and splenic B-cell maturation and causing intestinal goblet-cell metaplasia in adult animals [17, 18]. Eisai developed a γ-secretase modulator E2012 that is still in the phase of clinical development[16]. Another Aβ modulation strategy is the design of β–secretase inhibitors;

however there is only one clinical report about a β-secretase inhibitor [19]. The second Aβ-targeted strategy, inhibiting Aβ aggregation, which can prevent the formation of toxic oligomers and plagues, is another approach getting more attractive in recent years. A very few aggregation inhibitor candidates have been determined so far, however these inhibitor candidates did not demonstrate efficacy or desirable pharmacodynamic effects [20, 21]. The third approach, Aβ-targeted immunotherapy is becoming more popular, and there are three discovered drug candidates in Phase III trials: Bapineuzumab [22, 23], Solenezumab [24] and Intravenous immunoglobin G [25, 26].Nevertheless there are many debates about the usage of immunotherapy, since some lethal side effects were observed in some phases. Hence Phase III results are needed to evaluate the efficiency of immunotherapeutics. The last approach, the enhancement of Aβ clearance is a quite reasonable strategy, since some important enzymes such as neprilysin, plasmin and IDE involved in the Aβ degradation have been identified in recent years [27]. Recently, two novel compounds were identified that stimulate the proteolysis of only short peptides of IDE synergistically with ATP using high-throughput screening [28].

2.2 Diabetes mellitus type 2

Diabetes mellitus type 2 (DM2) is a metabolic disorder which has characteristics such as high blood glucose due to insulin resistance and deficiency. DM2 has many effects such as long-term damage, heart attack, strokes and kidney failure [29].The cause of DM2 is primarily to lifestyle and genetics [30].

Many lifestyle factors are important for the development of type 2 diabetes. In one study, people maintained physical activity, had a healthy diet, did not smoke and lowered alcohol consumption, in return had lowered rate of diabetes [31]. Obesity is another important factor that contributes about %55 to type 2 diabetes [30]. In addition,

environmental toxins such as bisphenol A, constituent of some plastics, contribute to increases in rate of type 2 diabetes [32].

In addition to lifestyle, genetic factors are important causes such that having relatives with type 2 diabetes increases the risk of promoting the growth of type 2 diabetes. Besides, mutation in the islet amyloid polypeptide gene causes an earlier onset of DM2 [33, 34].

2.3 Structure, Function and Regulation of Insulin Degrading Enzyme

It was reported sixty years ago that IDE has the capability to cleave insulin [35]. The cellular localization of this process is still controversial [36]. IDE has high affinity for insulin (Km=85 nM) and cleaves it into multiple inactive fragments [37]. In addition, cathepsin D is also responsible for the degradation of insulin [38].

IDE is a 110-kDa zinc metalloprotease which is mostly found in cytoplasm of the cell and also in endosomes, peroxisomes, mitochondria, cell surface, and in secreted form [39-43]. In addition to insulin degradation, IDE also cleaves amyloidogenic peptides [3{Farris, 2003 #1206, 44], amylin, insulin growth factor-II (Fig 2.1), tumor growth factor-α, glucagon, and amyloid-β. In the figure 2.1, there is a proposed model as to how IDE binds, unfolds, and degrades its substrate. It has been shown that charge distribution of the interior surface has greater importance for substrate specificity and catalysis [7].

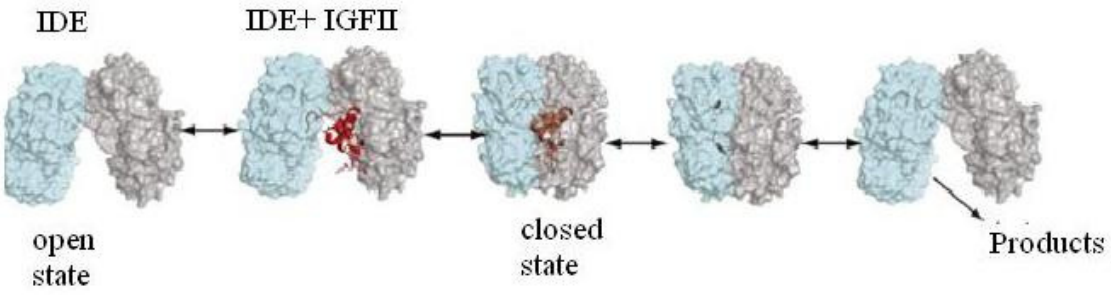


Figure 2.1: The representation of degradation of IGF-II by IDE adapted from [45].

2.3.1 Structure of IDE

IDE consists of two 56 kDa catalytic N- and C-terminal domains having four structurally homologous αβ roll domains [7]. These two N- and C-terminal domains, connected by 28 amino acid residues loop, constitute a large catalytic chamber where peptides smaller than 70 residues can fit [5]. Two halves of the IDE have higher complementarity and high surface area (Fig.2.2).

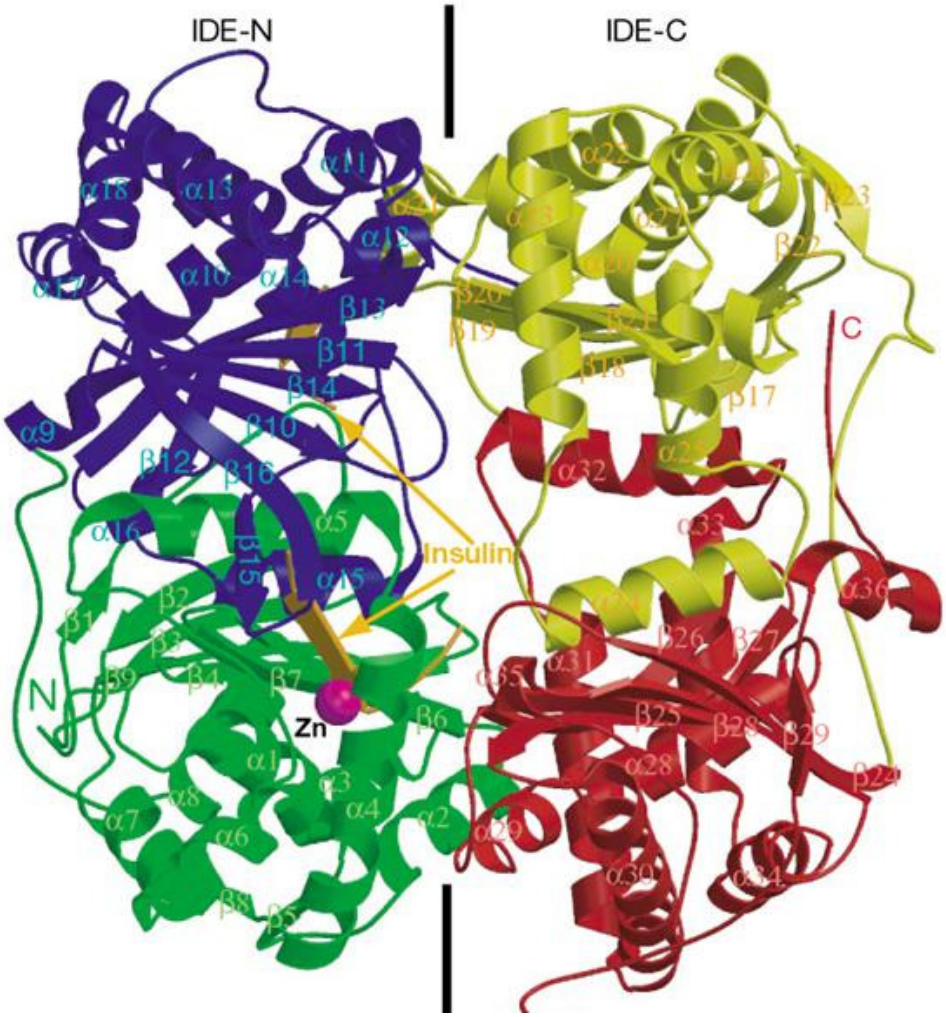


Figure 2.2: Representation of structure of IDE-N and IDE-C when binding to insulin adapted from [7]. Domains 1, 2, 3 and 4 are colored green, blue, yellow and red, respectively. Zn^{2+} and insulin B chain are colored magenta and orange, respectively.

2.2 Structural basis for substrate selectivity of IDE

There are two unique features of IDE substrates. One of the most significant is the presence of disulfide bonds in certain IDE substrates (Fig.2.3). These disulfide bonds may

affect the binding of the substrate to catalytic site. Second unique feature is the cleavage of insulin into multiple inactive fragments (Fig.2.3). The cleavage sites are nonrandom and products are stochastic [7].

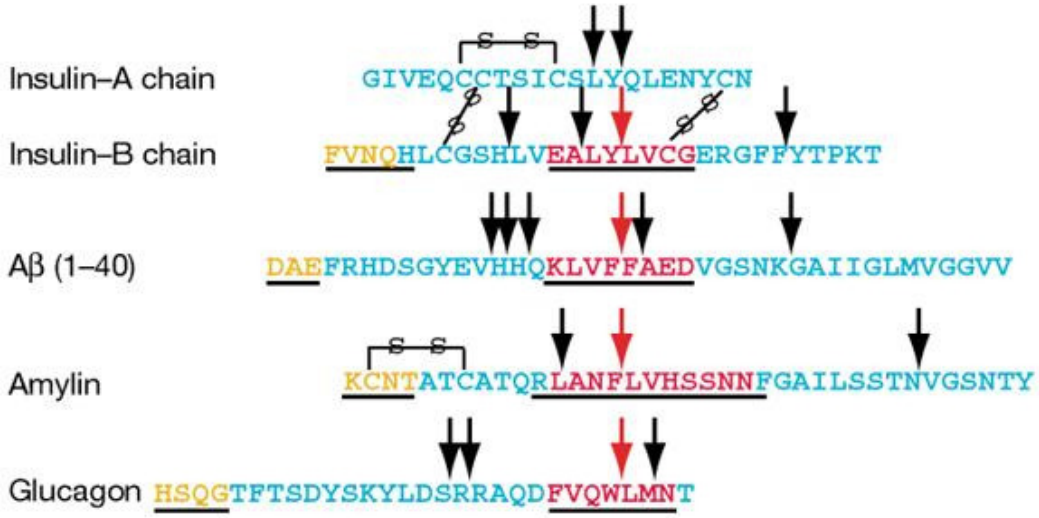


Figure 2.3: Substrate selectivity of IDE adapted from[7].Dash lines shows the disulfide bonds between cysteine residues and arrows represent the cleavage sites. The red colored region shows the cataltytic cleft binding region.

Cleavage mechanism of amylin, insulin, and glucagon provides information about IDE activity on its substrates. These substrates are smaller than 70 residues and change their conformation from α-helix to β-strand [7]. With the help of this conformational change, substrates bind to the catalytic site and exosite with noncovalent interaction. When the enzyme catches the substrate, the N-terminal of the peptide attaches to the exosite which has regulatory function and is a conserved region [7, 46]. The exosite directs the C-terminal

end of substrates to the catalytic site. In addition, small peptides like bradykinin bind to the exosite [47] as a result it activates IDE [48].

Furthermore, IDE-C has no activity as itself; IDE-N shows only %70 activity of intact IDE. Therefore, IDE needs all subunits for restoring whole enzyme activity [49]. Besides, C-terminal end of IDE facilitates substrate recognition. In addition, the selectivity of substrates is strongly influenced by size, shape, and charge distribution within the large internal chamber of IDE [47].

2.3.3 The Regulation of IDE activity

There are several regulatory mechanisms that control the catalytic activity of IDE:

- Open and closed conformation of IDE
- Gene expression
- Oligomeric state
- Ions and molecules
- Cellular,tissue and organ distribution
- Oxidative stress

First, based on substrate-bound and substrate-free crystal structures of IDE, the enzyme can be in open or closed state [5, 47].The structural solution of IDE shows that IDE normally remains in close state. It is thought that closed state is the rate limiting step for the proteolytic activity of IDE, since substrates have no access to bind to the exosite or to the catalytic site. When IDE is in open state, substrates such as Aβ are entrapped by the enzyme. The substrate undergoes conformational change, which is then followed by the closed state. In this closed state, IDE cleaves Aβ and releases the products of proteolysis by going towards the open state. These steps are called “Catalytic Cycle of IDE” (Fig.2.4). In

addition, the cleavage of substrates into multiple fragments is not clear which IDE performs in only one cycle.

There are several factors that regulate the catalytic cycle. Firstly, high surface complementarity of IDE-N and IDE-C may facilitate the closed state of IDE [46]. Additionally, charge characteristics of interior surfaces favor IDE in its closed conformation. As a result of this property, mutations that disrupt the interactions between IDE-N and IDE-C could increase the proteolytic activity of the IDE.

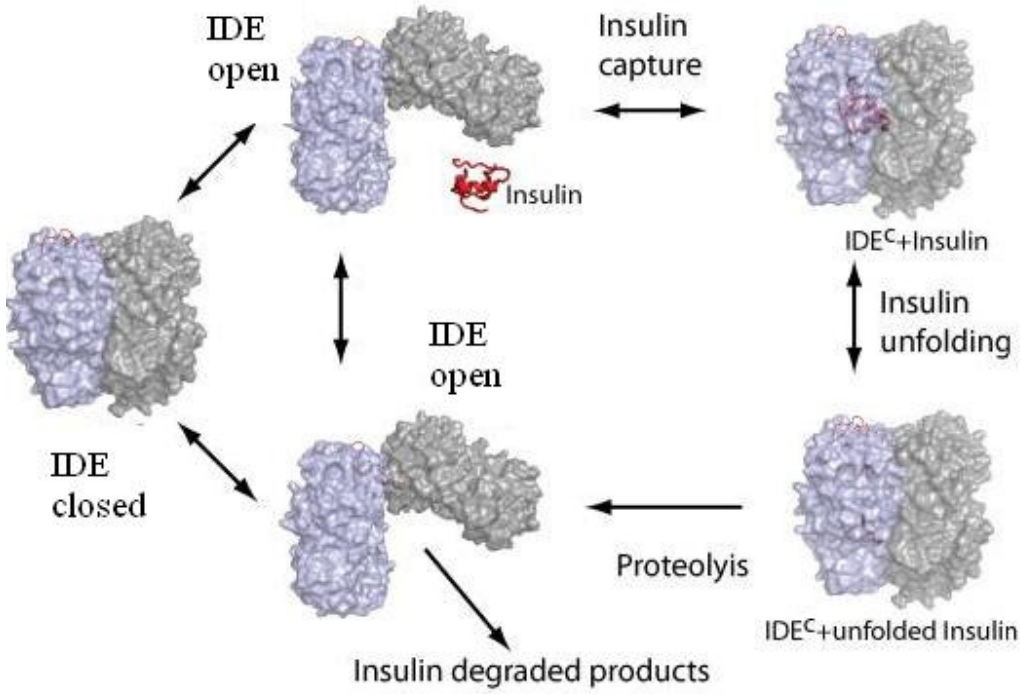


Figure 2.4: Representation of catalysis of insulin with IDE adapted from[71].

Secondly, IDE is highly expressed in kidney and liver tissues at the genetic level which is related to the fact that IDE degrades insulin [50]. In addition, enzymatic activity of IDE in different rat tissues has the following sequence:

Liver > pancreas > kidney > testis > adrenal gland > spleen > ovary > lung > heart > muscle > brain > fat [51]. This is consistent with the degradation of insulin with IDE since highest enzymatic activity is noted in liver and kidney. As a result of these facts, genetic regulation may play a key role in the expression of IDE in different tissues. However, IDE gene expression factors are not found yet, instead small molecules such as synthetic retinoic acid analogs may regulate IDE expression [52].

Moreover, IDE is an allosteric enzyme which exists in equilibrium of dimers and tetramers and dimeric form has higher activity than tetrameric form [48]. Dynorphin B, a small peptide substrate, has been identified to increase the cleavage of Aβ while inhibiting the degradation of insulin at the same time [48].

IDE activity is also regulated by other factors besides certain peptides. Muscles in which Ca^{+2} is depleted have lower insulin degradation and IDE activity [53]. In addition, IDE activity is also decreased by the free long chain fatty acids and acyl-CoA [54]. This means that fatty acid concentration has direct effect on catalytic activity of IDE. Besides, IDE activity is affected by ATP [55, 56]. With the addition of ATP, insulin degradation is decreased. ATP behaves like an allosteric regulator such that it promotes IDE to stay in dimeric form rather than tetrameric form. As a result, ATP favors the transition from closed to open state [46].

Furthermore, IDE activity is also regulated within different cellular compartments and tissue distribution. IDE is mostly located in the cytoplasm of the cell about %95[3, 39]. Although, IDE is highly concentrated in the cytoplasm, still the location where insulin degradation takes place is not known [57]. It is thought that the degradation process may take place in the cytosol, or at the cell surface, or through the action of secreted IDE. For instance, it is shown that primary microglia cells secrete IDE [70].

Other modulators of IDE activity are oxidative stress and nitrosative stress [58, 59]. IDE is sensitive to these stresses because of its two features. Firstly, IDE has a metal ion on

or near the active site which makes the enzyme lose its catalytic activity due to oxidative damage [60]. Secondly, IDE has many cysteine residues which easily react with the free radicals.

In conclusion, IDE activity is regulated by many factors such as conformational state, presence of ATP, oligomeric state, ion and molecules, cellular and tissue distribution, and oxidative stresses. As a result of these known factors, and the fact that small peptides like bradykinin regulate the IDE activity, a computer aided approach was used for screening small molecules as a separate study in our group. Two small chemical compounds are discovered and confirmed with experimental methods that regulated the activity of the IDE towards degradation of substrate V, insulin and amyloid-beta.

Chapter 3

Materials and Methods

This chapter includes methods used in this study for both purification and characterization of IDE protein. First, PCR and cloning methods are presented. Then expression and purification of protein, SDS-PAGE, western blotting and affinity chromatography methods are described. And finally, proteolysis assay is presented.

Materials. cDNA of human insulin degrading enzyme (IDE) was obtained from Prof. Richard A. Roth from Stanford University. Substrate V was purchased from R&D systems, Inc. (Minneapolis, MN), FITC from Sigma, fluorescein-Aβ-(1-40)-Lys-biotin (FAβB) from Anaspec Corp (San Jose, CA), 1, 10- phenanthroline from Sigma, and Neutravidin- coated agarose beads from Thermo Scientific.

3.1. Cloning of human Insulin Degrading Enzyme (IDE)

3.1.1. Polymerase Chain Reaction (PCR)

cDNA of hIDE was PCR amplified with primer sets that contains unique restriction enzyme sites XhoI, NotI and hexa-histidine tag (6xHis). The primers that have been used for amplification of hIDE cDNA are forward primer: 5'-CTTGCGGCCGCAATGCGGTACCGGTACCGGCTAGCGTG-'3 and reverse primer: 5'-GTGCTCGAGGAGTTTTGCAGCCATGAA-'3.

PCR reaction is performed in a total volume of 50 µl containing 100 ng of plasmid, 40 pmol of each primer, 0, 2 mM dNTPs, and 2 unit of Taq DNA polymerase. Amplification reaction was performed with the conditions 95 ℃ for 30s, 55 ℃ for 30s and 72 ℃ for 3min for 33 cycles. The reaction mixture was kept at 95 ℃ for 4 min before the first cycle, and after the 33rd cycle additional extension period is applied at 72℃ for 10 min. Finally the amplified products were visualized under the agarose gel electrophoresis by running on 1% agarose gel at 100V for 15 min.

3.1.2 Cloning of Human Insulin Degrading Enzyme to Pet 21b

In order to clone amplified IDE gene to bacterial expression vector pET21-b (Fig 3.1), the PCR product and plasmid were digested with XhoI and NotI restriction enzymes. The digestion was performed in a total volume of 100 µl, composed of 1µg plasmid, 1x buffer O. 2 U of XhoI, and 1 U of NotI for 16h at 37 ℃. The samples were gel-purified with the gel purification kit (Qiagen) by a protocol suggested by the manufacturer. The ligation was performed in a total volume of 10 µl containing 50 ng of pET21-b, 100 ng of PCR product, 1x DNA ligase buffer (MBI Fermentas) and 1 unit DNA ligase (MBI Fermentas) for 1h at room temperature. The ligation was performed with 1:3 molar ratio of vector: insert. Then, ligation product was transformed to *E.coli DH5α* by the heat shock and seeded on the LB agar plate with 100ug/ml ampiciline. Then, several colonies were picked up and inoculated in 2 ml of liquid LB medium supplemented with 100ug/ml ampiciline at 37 ℃ for 16h to perform isolation of plasmid DNA by miniprep. The presence of the insert was checked with restriction digestion with EcoRI. The digestion was performed in a total volume of 20 µl containing 300ng of plasmid DNA, 1x EcoRI buffer (MBI Fermentas) and 0.2 unit EcoRI (MBI Fermentas) for 1h at 37 ℃. Then, digestion was analyzed with the agarose gel electrophoresis. The digested samples were run on 1% agarose gel at 120V for 10 min.

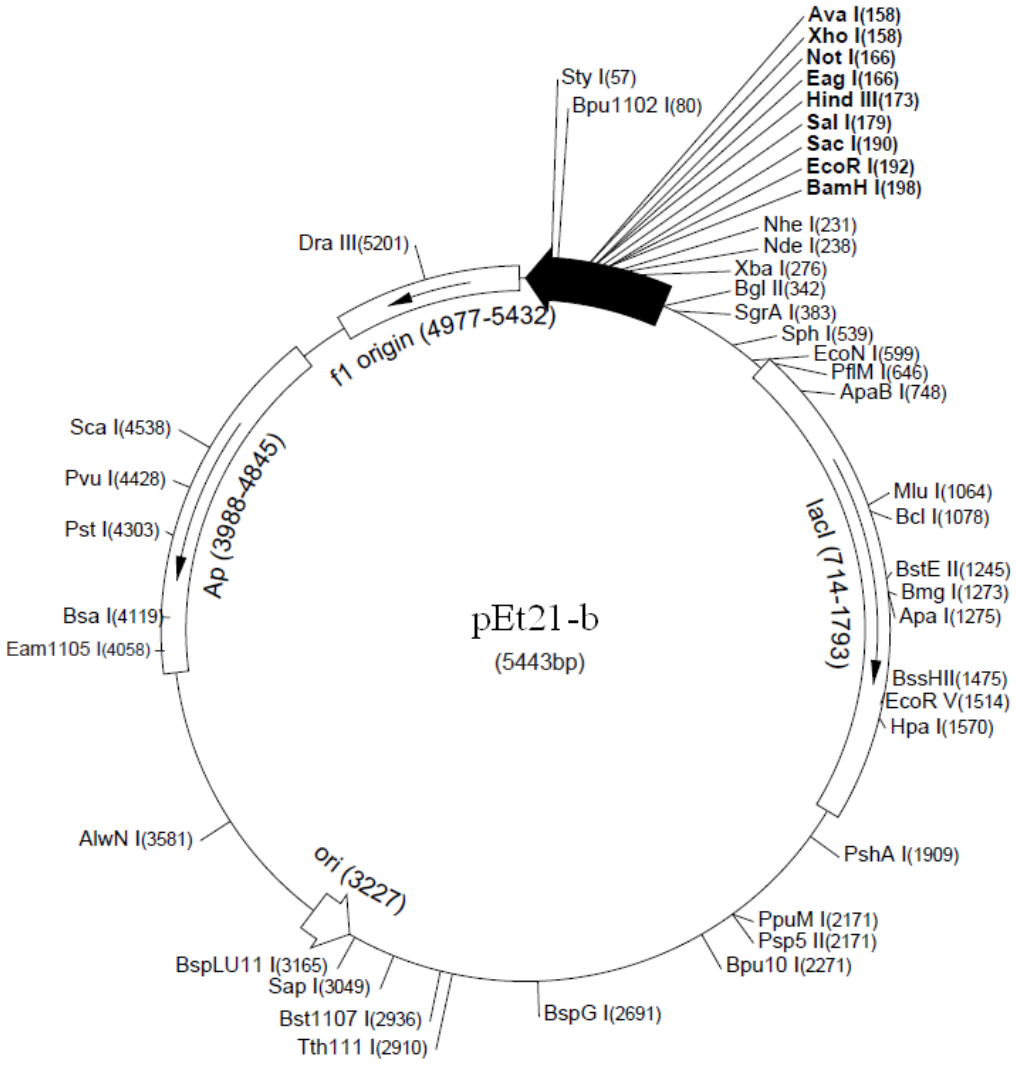


Figure 3.1: pET21-b vector map.

3.2 Expression and Purification of Human Insulin Degrading Enzyme

In order to express IDE proteins; first, the IDE expression plasmid was transferred to *E.coli* BL21 cells by heat shock and seeded on the LB agar plate with 100ug/ml ampicillin. The next day, the single colony possessing pET21b-hIDE plasmid was picked up and inoculated in LB medium supplemented with 100ug/ml ampicillin at 37 ℃ for 16h. Next, the culture was diluted 1:100 and inoculated in LB/ ampicillin medium. After the OD A_{600} reached 0, 6-0, 8, the protein expression was induced with 0,4mM IPTG for 3 hours at 37 ℃. The cells were gathered by centrifugation at 9000g for 5 min. The pellet was treated with lysis buffer containing 50 mM NaH_2PO_4, 300mM NaCl, 10 mM imidazole, pH 8.0, 100mM PMSF, 50 mM protease inhibitor cocktail and 0.1mg/ml lysozyme at 4 ℃. Then, bacterial cell wall was degraded by sonication for 8 × 15 sec. The cell lysate was centrifuged at 10000g for 30 min at 4 ℃. Since IDE is a cytosolic protein, the supernatant was kept for further purification.

3.3 Affinity Chromatography

First, the column containing Ni^{+2} resins was generated with lysis buffer containing 50 mM NaH_2PO_4, 300mM NaCl, 10 mM imidazole, pH 8.0. Then, the supernatant of the lysate was passed through the column about 300 µl per minute. After all supernatant passed, the column was washed with washing buffer composed of 50 mM NaH_2PO_4, 300mM NaCl, 30 mM imidazole, pH 8.0. The hIDE-6XHis was eluted (Fig. 3.2) with elution buffer containing 50 mM NaH_2PO_4, 250mM NaCl, 300 mM imidazole, pH 8.0. The eluted protein was dialyzed against 100mM NaCl, 50mM $MgCl_2$, 10 mM HEPES and %50 Glycerol (v/v) for overnight at 4 ℃ by using dialysis tubing cellulose membrane (MWCO 12 000) and stored at -20 ℃.

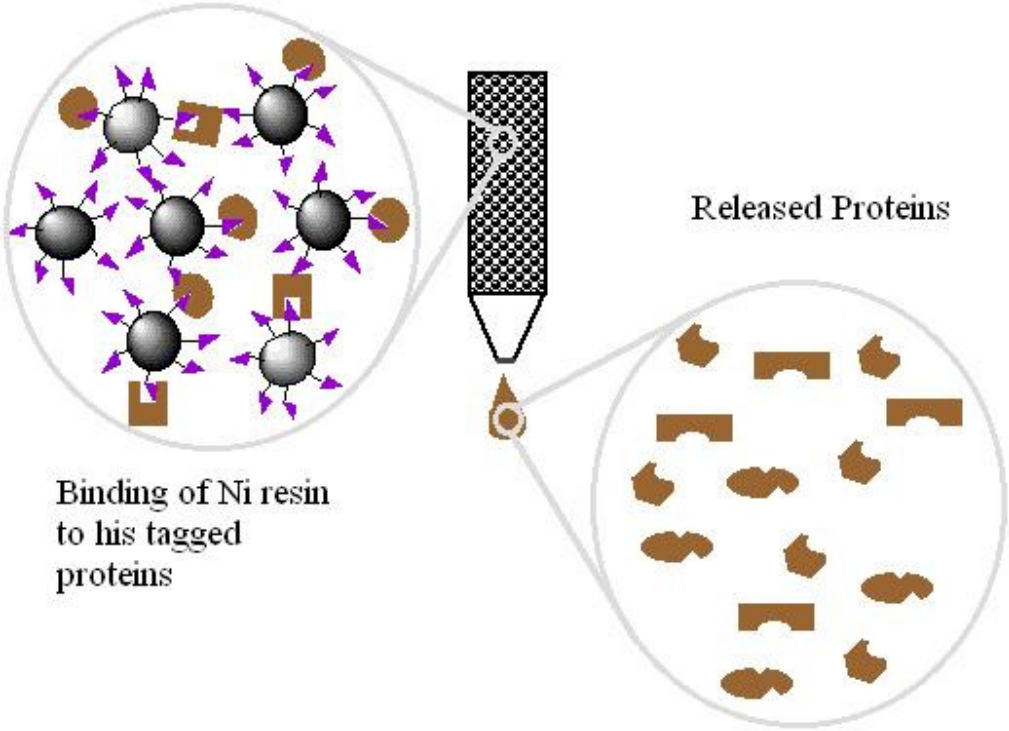


Figure 3.2: Representation of affinity chromatography.

3.4 SDS-PAGE and Western Blotting

Electrophoretic separation and analysis of the proteins was performed by SDS-PAGE followed by western blotting. Briefly, protein samples were electrophoresed on a 10% SDS-PAGE. Gels subjected to 150 V for 1.5 h for the separation of proteins. Then, gel was transferred to polyvinylidene difluoride membrane (Biotrace PVDF, Pall Corporation, FL, and USA) with transfer cell at 100V for 1h. After pre-blocking with BSA, the membrane was incubated with anti-His (1:1000 diluted in 0.01 % Tween20/ TBS) and mouse-HRP conjugated secondary antibody (1:10000 diluted in 0.01% Tween20/ TBS) for 1 h,

respectively. Proteins were visualized by ECL plus system according to manufacturers instruction.

3.5 Enzymatic assays of compounds by IDE

Proteolytic activity of IDE and *in vitro* effects of the potential compounds on IDE activity were monitored by using fluorogenic substrate V (7-methoxycoumarin-4-yl-acetyl-NPPGFSAFK-2, 4-dinitrophenyl), insulin-FITC and FAβB.

3.5.1 Degradation of substrate V: 105 µl of 4 µM substrate V, 0.02 mg/ml IDE were dissolved in 30 mM potassium phosphate, pH 7.3, mixed with various concentrations (0-35 mM) of newly found compound. The assay was conducted at 37 °C for 2.5 h. The hydrolysis of substrate V was measured using a Tecan Safire2 microplate reader with excitation and emission wavelengths set at 300 and 395 nm, respectively.

3.5.2 Degradation of Insulin-FITC by IDE: The proteolysis of insulin by IDE was monitored by a fluorescent polarization assay using FITC-labeled insulin. FITC was attached to the insulin as described above. 80 ng of human recombinant IDE was mixed with 10 nM FITC-insulin in total 100 µl potassium phosphate buffer, pH 7.3. Reaction was done at 37 °C for 2.5 h. The fluorescence increase is monitored by using multi-label plate reader.

3.6 FAβB degradation assay: FAβB (50 nM) peptide was dissolved in a buffer composed of 50 mM HEPES, 100 mM NaCl, % 0,05 bovine serum albumin, pH 7,4. The

reaction was initiated in total 100 µl with 20 µM drugs by adding 5 nM recombinant hIDE for 2,5h at 37 °C. The reaction was stopped by adding 2mM of protease inhibitor 1, 10-phenanthroline. Uncleaved FAβB was precipitated with Neutravidin- coated agarose beads by gentle rocking for 30 min. and centrifugation for 10 min at 14000 x g. The supernatant was transferred to the black 96 well plates and fluorescence increase (488 excitation, 525 emission) was measured with multi-label plate reader.

3.7 FITC binding to insulin: Briefly, 4.5 mg fluorescein isothiocyanate (FITC) was dissolved in 200 µl acetone. Then, 2 mg of insulin was dissolve in 4 ml of 0.1 M Na-carbonate, pH 9.1 and 0, 2 mM EDTA which prevents the aggregation of insulin. Then FITC in acetone was added dropwise to the insulin with constant stirring. The reaction was done at room temperature for 20 h. The unbound FITC is removed by using dialysis cassette with a cellulose membrane (MWCO 3500) against 1X PBS solution. Finally, FITC is stored at – 20 °C.

3.8 MTT assay for evaluating cell viability

The cytotoxicity assay was performed using human cervical cancer HeLa cells. Cells were cultured at 37 °C under a humidified atmosphere of 5% CO_2 in medium supplemented with 10% fetal serum and dispersed in replicate 96- well plates with 2.5×10^4 cells/well. Compounds were then added. After 24 hours exposure to the chemical compounds, cells viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenpyltetrazolium bromide (MTT) assay [61]. MTT (5 mg) was dissolved in 1X PBS, 200 µl medium and 50 µl MTT in PBS was added to the wells. The cells were cultured at 37 °C for 3, 5 h. Finally,

the pellet was dissolved in ethanol and DMSO mixture, 1:1 ratio. The optical density (OD) of the wells was determined using an ELISA plate reader at a test wavelength of 600 nm and a reference wavelength of 630 nm. Each test was performed in triplicate measurements.

Chapter 4

RESULTS

4.1. Cloning of hIDE to pET21-b

First, hIDE cDNA was cloned to the pET21-b which contains and presences of insert was verified by digesting with Hind III.As demonstrated in figure 4.1, samples in lane 1, 2, 4 and 6 contains two fragments of 5500 and 2500 base pair. These results indicate that hIDE was successfully cloned into pET21-b (3300 bp+ 5442 bp).

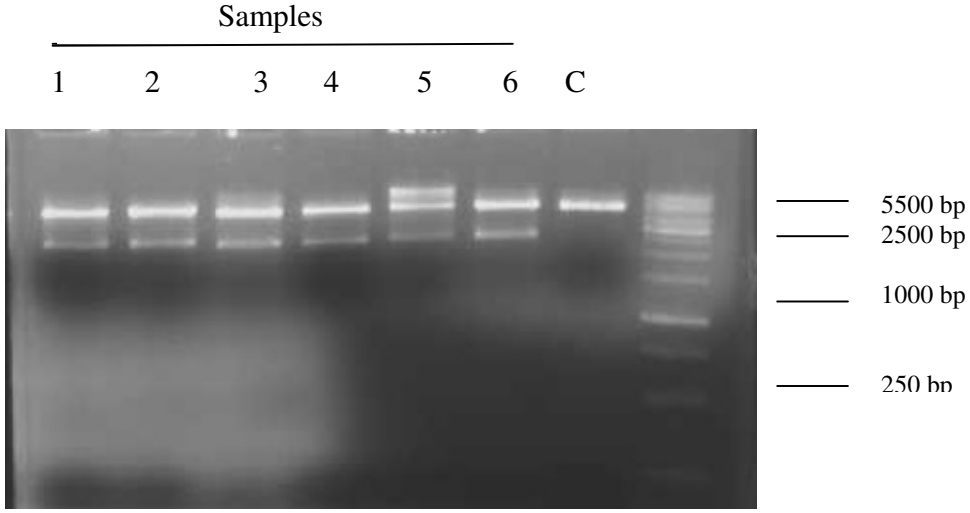


Figure 4.1. Hind III cleavage of the cloned hIDE-pET21b. There 6 samples and one control lane.

4.2. Expression and purification of hIDE

Initially, optimization experiments were carried out under different experiment conditions to maximize the over expression of hIDE. pET21b-hIDE plasmid were transferred to *E.coli* BL21 cells. Then, several colonies were picked, and transferred into

the liquid LB containing appropriate amount of ampicillin. When the OD_{600} values for cells reached to 0.6-0.8, IPTG was added to final concentration of 400 µM to initiate expression. Next, cells were placed into different conditions consisting of 37 ºC 3h, 6h, room temperature 24h and 4 ºC 48h. As a result, the maximum expression of IDE was observed at 37 ° C for 3 hours (Figure 4.2, lane2). In order to check the stability of protein western analysis was performed on sample using the anti-HIS IgG. As shown in figure 4.3, the presence of a single band with molecular weight of 110 kDA indicated stability of hIDE in *E.coli* (Figure 4.3).

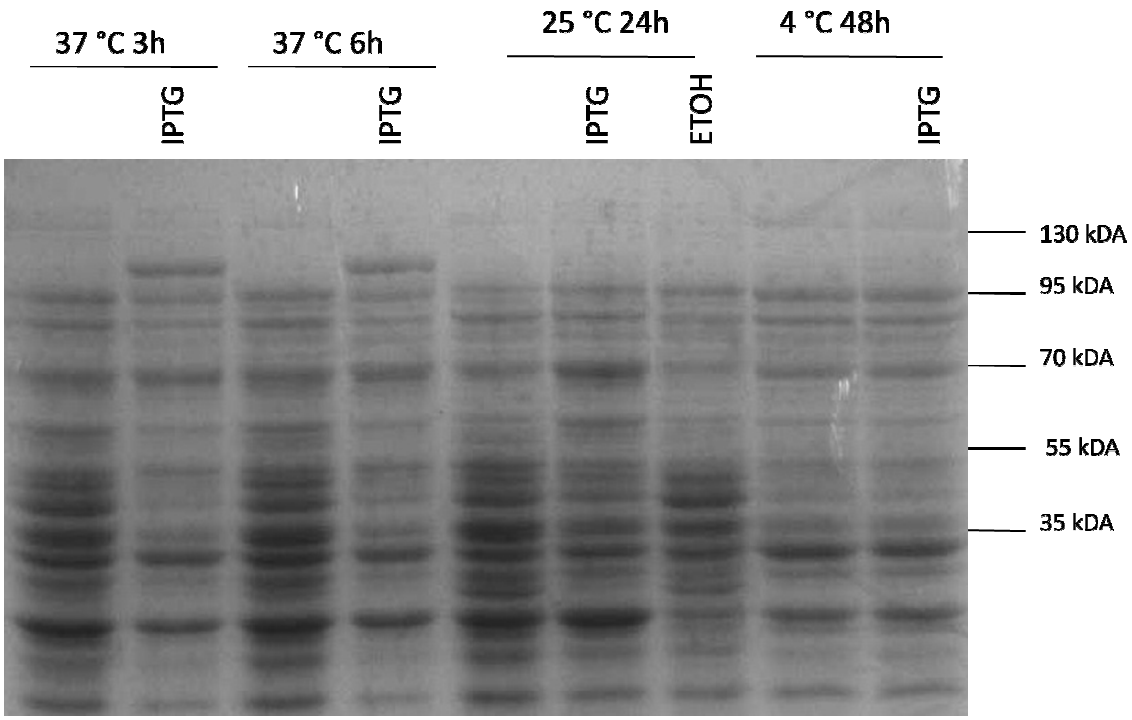


Figure 4.2. Expression of hIDE at different temperatures and times.

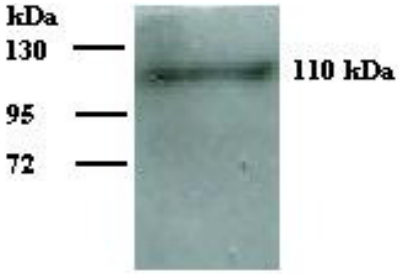
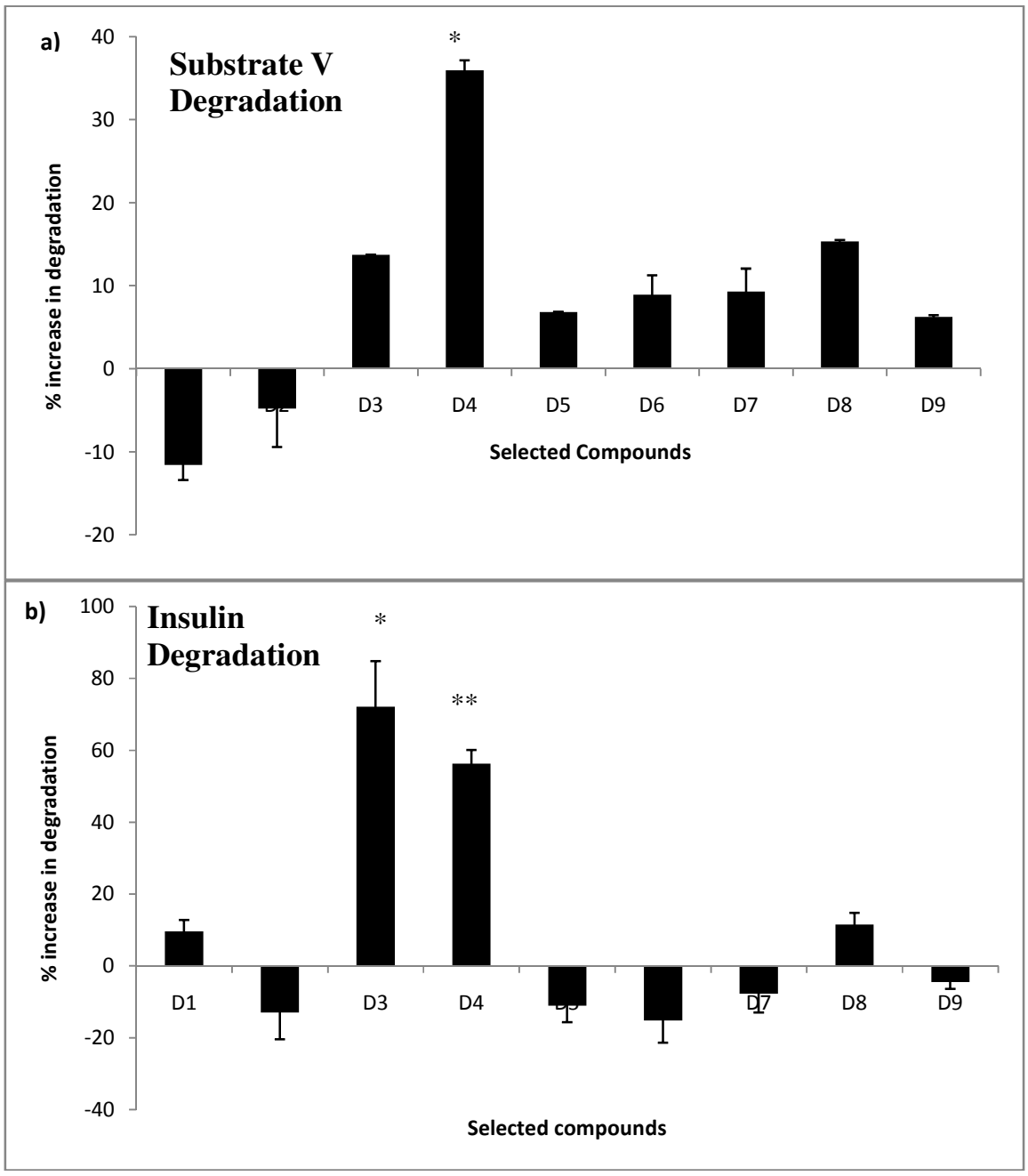


Figure4.3. Western blot analysis of IDE.

4.3. In vitro enzymatic assays

4.3.1. Enzymatic assay of hIDE using substrate V, FAβB and insulin-FITC

IDE activity was measured in presence of different substrates including fluorogenic-substrate V (7-methoxycoumarin-4-yl-acetyl-NPPGFSAFK-2, 4-dinitrophenyl) [62] , insulin-FITC, and FAβB using recombinant hIDE, and a previously developed homogeneous fluorescence-based assay [47, 63, 64]. Initially, enzymatic assay were carried out in the presences of 9 small molecules with concentration of 20 μM in order to identify activation or inhibition effect of those compounds on IDE activity. We identified three compounds, D3, D4 and D6 that enhanced the activity of IDE towards degradation of insulin, substrate V and FAβB, respectively (Figure 4.4). Both D3 increases IDE-mediated insulin degradation by 72% (Figure 4.4.b).On the other hand D4 molecule is not only enhances IDE-mediated substrate V (%36) and but also enhances insulin degradation up to 60% (Figure 4.4.a, b). Also, D6 molecule increases the FAβB degradation by 20% (Figure 4.4.c). Next, EC_{50} values of compounds were calculated using proteolysis assays. Quantitative experimental observation of specific activity versus compound concentration were fit to a sigmoidal plot (Hill, 4 parameter equation), and EC_{50} of D4 were found as 7.5 μM and 13.6 μM for substrate V and insulin degradation, respectively (Figure 4.5.a,b). EC_{50} value for D3 on insulin degradation was approximately 20.04 μM (Figure 4.5.c), whereas EC_{50} value for D6 on FAβB degradation was about 1.91 μM (Figure 4.5.d).


a)
Substrate V Degradation
% increase in degradation
40
30
20
10
0
-10
-20
*
D3
D4
D5
D6
D7
D8
D9
Selected Compounds
b)
Insulin Degradation
% increase in degradation
100
80
60
40
20
0
-20
-40
*
**
D1
D3
D4
D8
D9
Selected compounds

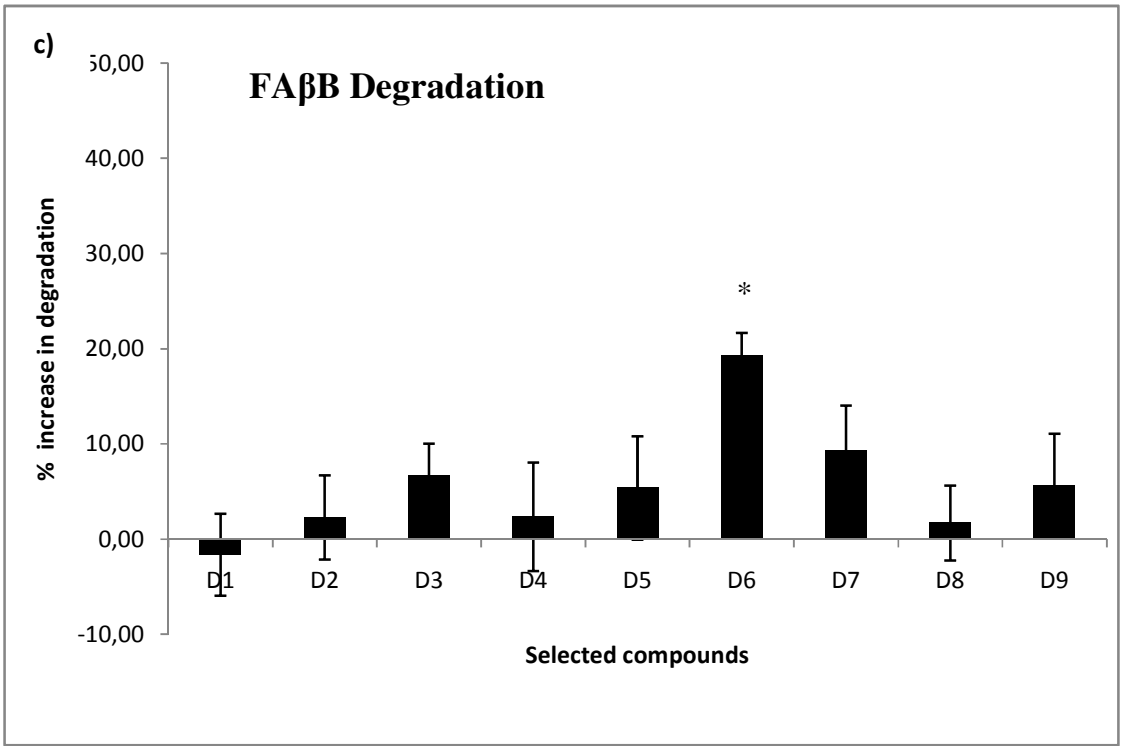
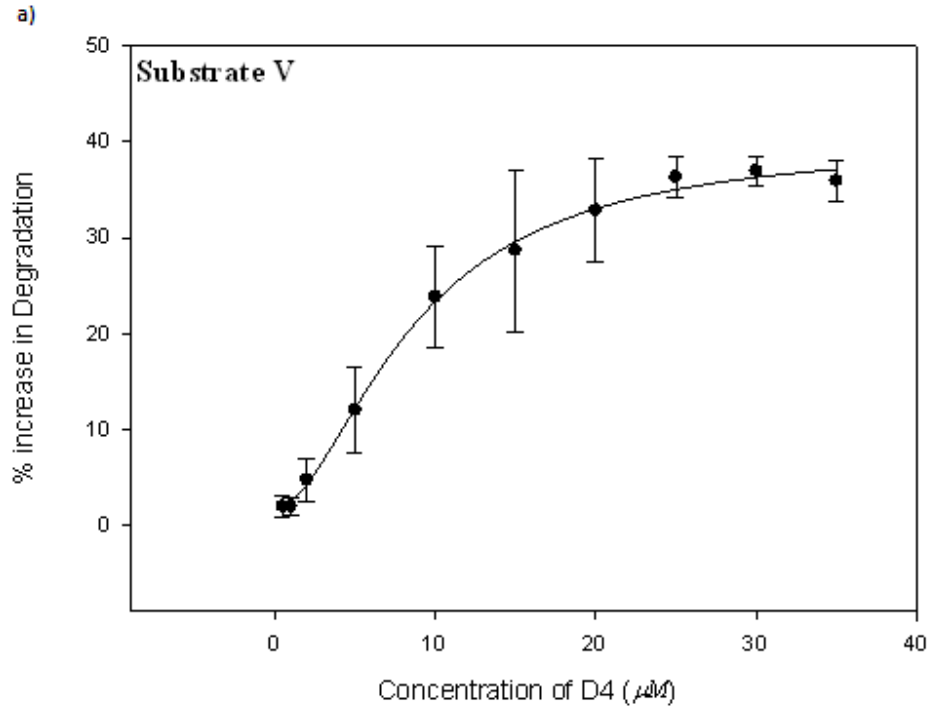
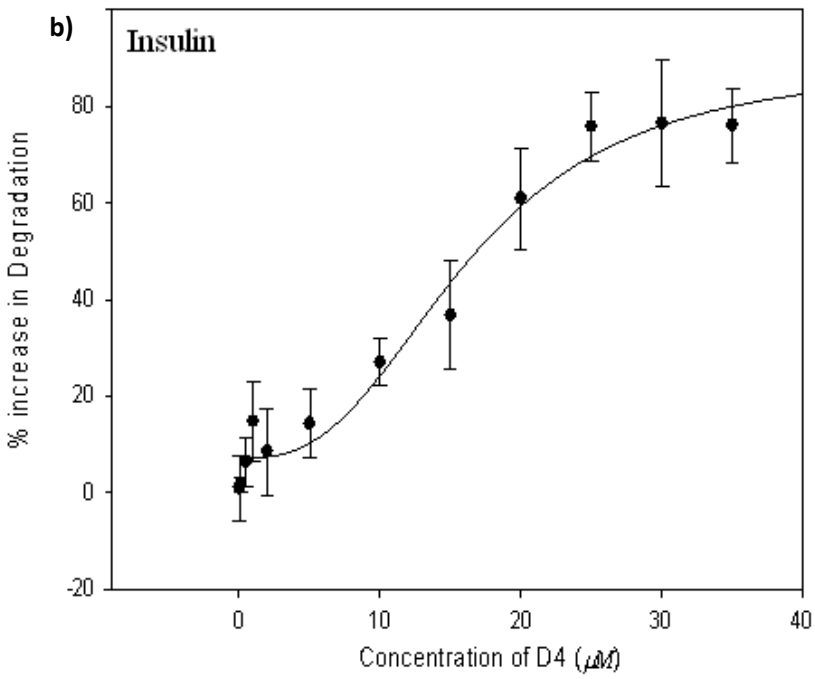
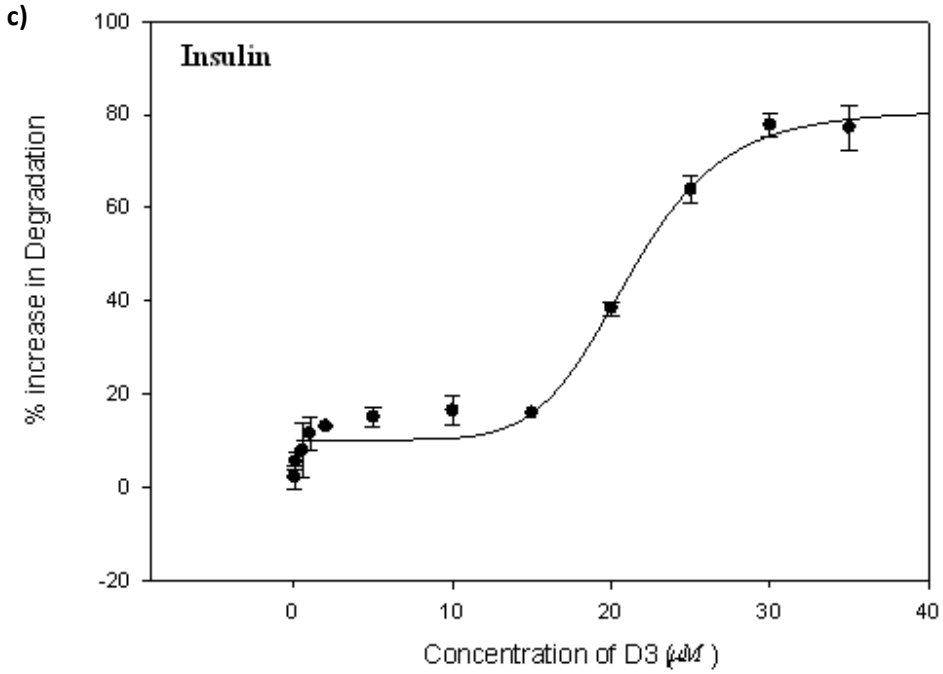


Figure 4.4. The effect of selected compounds on the proteolytic activity of IDE. **a)** Compound D4 shows ~%36 increase in the magnitude of IDE activity for substrate V degradation(*p<0.0006). **b)** Compound D3 and D4 shows ~ 72% and 60% increase respectively in the magnitude of IDE activity for insulin degradation(*p<0.02, **p<0.003). **c)** Compound D6 increases the IDE-mediated FAβB degradation ~20% (*p<0.0002). Data are mean ±SEM for 2 independent experiments.


a)
Substrate V
% increase in Degradation
Concentration of D4 (μM)
0
10
20
30
40
50


b)
Insulin
% increase in Degradation
80
60
40
20
0
-20
0
10
20
30
40
Concentration of D4 (μM)


c)
Insulin
% increase in Degradation
100
80
60
40
20
0
-20
0
10
20
30
40
Concentration of D3 (μM)

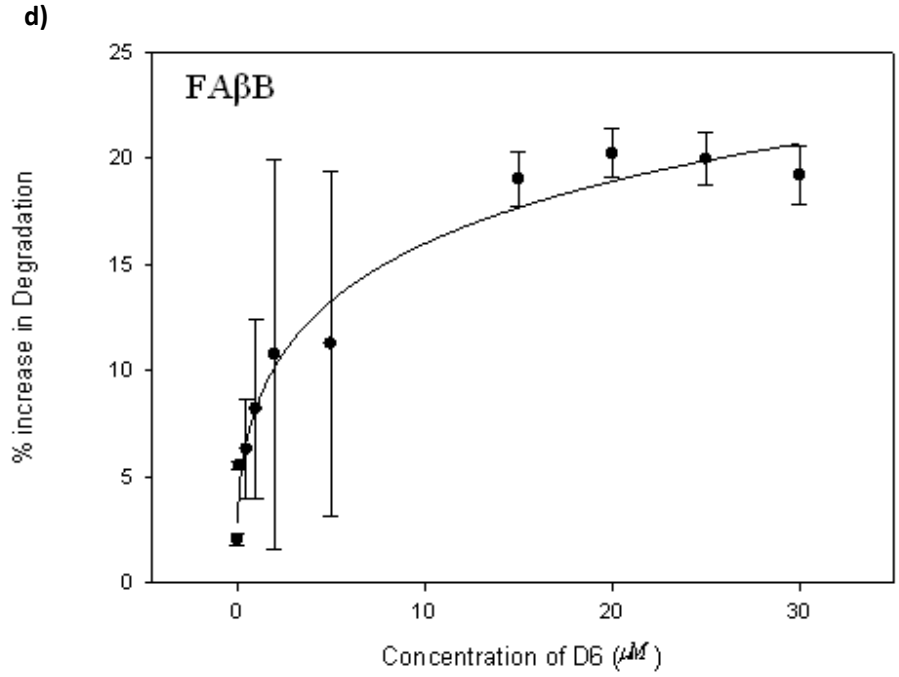


Figure 4.5. Measurement of the increase of IDE activity for **a)** substrate V and **b)** insulin degradation in the presence of D4 at concentrations ranging from 0.02 to 40 μM provided an activation constant of 8.6 μM**. c)** Increase in IDE activity for insulin degradation in the presence of D3. **d)** Increase in IDE activity for FAβB degradation in th presence of D6. Data are mean ±SEM for 3 independent experiments (p<0,0001).

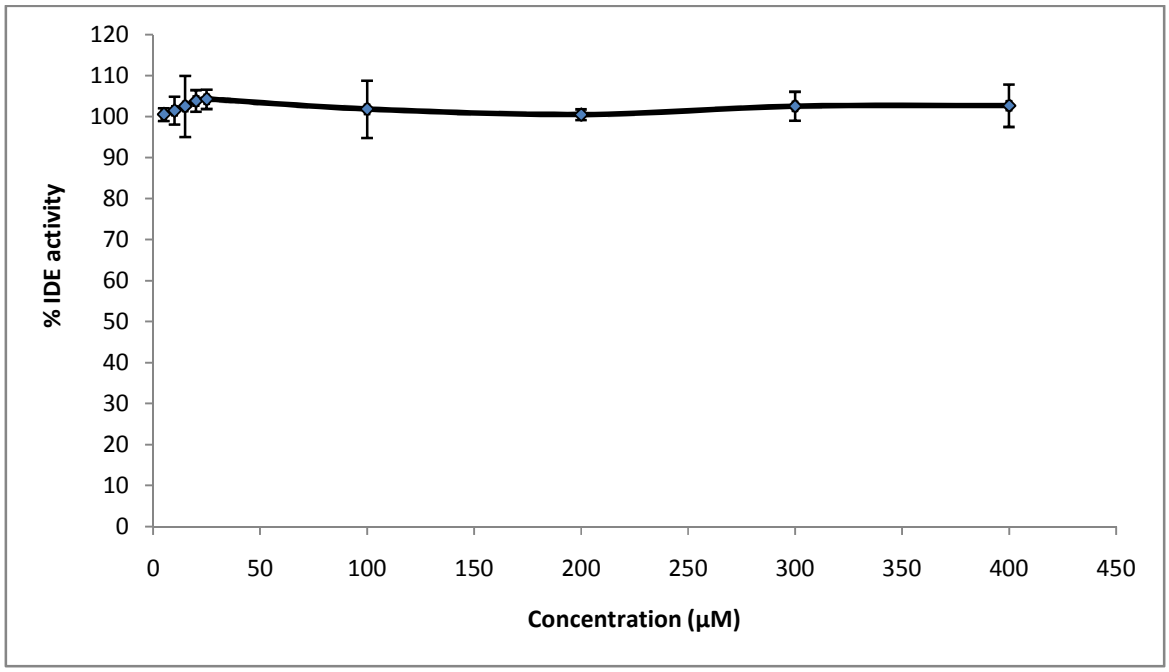
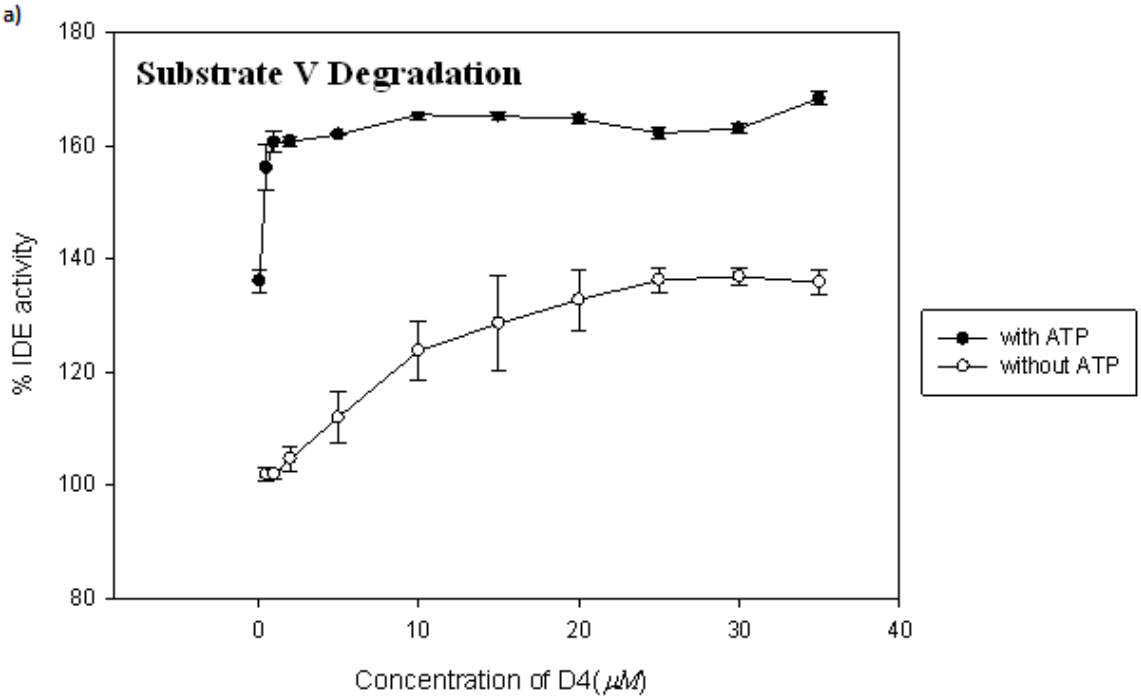


Figure 4.6. Effect of ATP on substrate V degradation. The activation of IDE was tested in the presence of different concentrations of ATP. Data are mean ±SEM for 2 independent experiments.

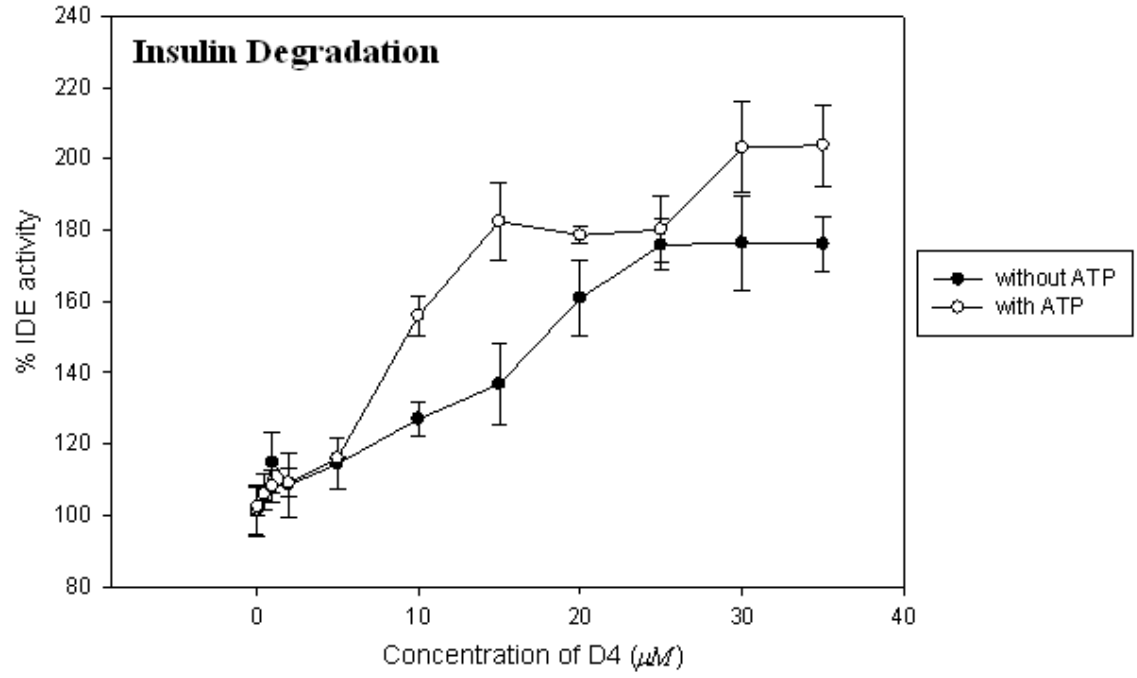
It has been showed that the activity of hIDE is enhanced even more in presence of ATP along with activators [28].To such effect with novel activators identified in this study, assay was performed in presences of varying amount of ATP. The activity of IDE increases by 36% in the presence of 0.1mM of ATP and 0.1 µM of D4, and the activity increases suddenly by 56% with 0.5 µM of D4 (Figure 4.7a). However, activity increase (60%) remained stationary for the concentration higher than 1 µM of D4 (Figure 4.7). Consequently, IDE activity can be considered as independent on D4 concentration after 1 µM of D4 in the presence of 0.1M ATP. The effect of ATP on substrate V degradation was tested and though ATP causes an increase in IDE activity itself, there is no change in IDE

activity in the presence of different concentrations (0-400 μM) of ATP (Figure 4.6). Cabrol *et al.[28]* found a synergetic effect of ATP with discovered compounds, thereby we repeated fluorogenic assay with different concentrations of the novel compounds in the presence of ATP. Also, IDE activity for insulin degradation is not affected by ATP (Figure 4.7.b). The other novel compound, D3 enhanced substrate V degradation by 15%, whereas this compound was more potent on degradation of insulin with an increase in the proteolysis of 72%. Finally, it was observed that ATP had no effect on FAβB degradation in the presence of D6.

a)


Substrate V Degradation
% IDE activity
180
160
140
120
100
80
0
10
20
30
40
Concentration of D4(μM)
with ATP
without ATP

b)


Insulin Degradation
% IDE activity
240
220
200
180
160
140
120
100
80
0
10
20
30
40
Concentration of D4 (μM)
without ATP
with ATP

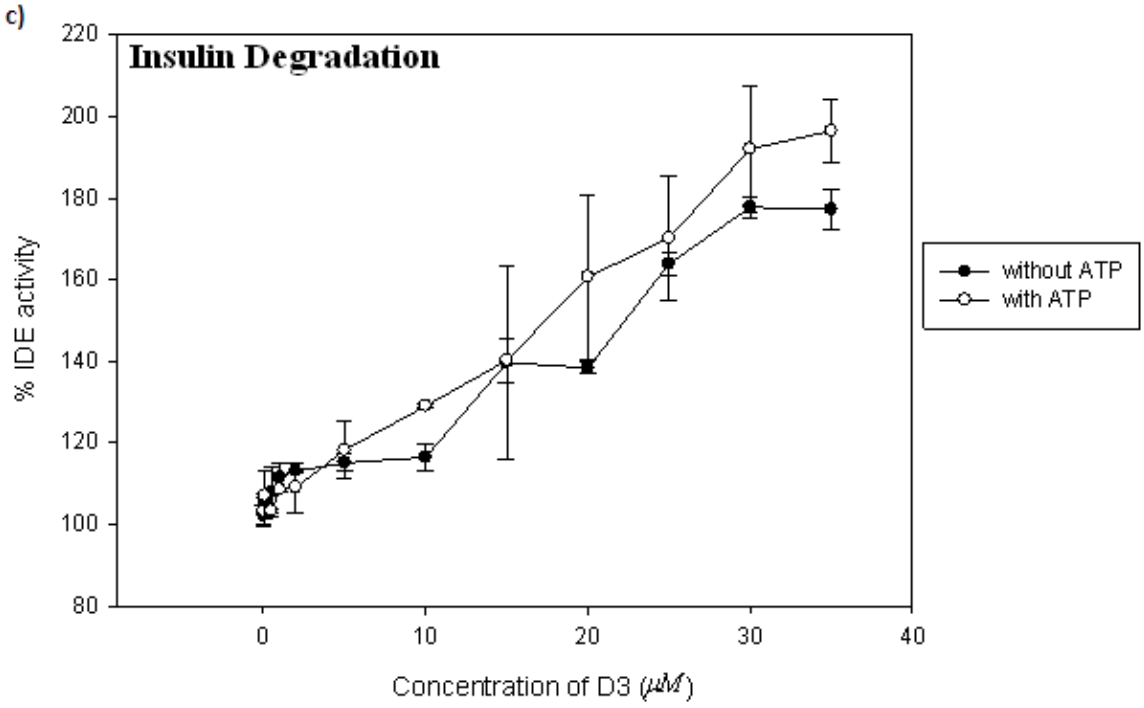


Figure 4.7. The effect of compound D4 on IDE mediated hydrolysis of **a)** substrate V and **b)** insulin when tested in the presence and absence of ATP (0.1 mM). **c)** The effect of D3 on IDE mediated hydrolyis of insulin in the presence of ATP (0.1 mM). Data are mean ±SEM for 2 independent experiments.

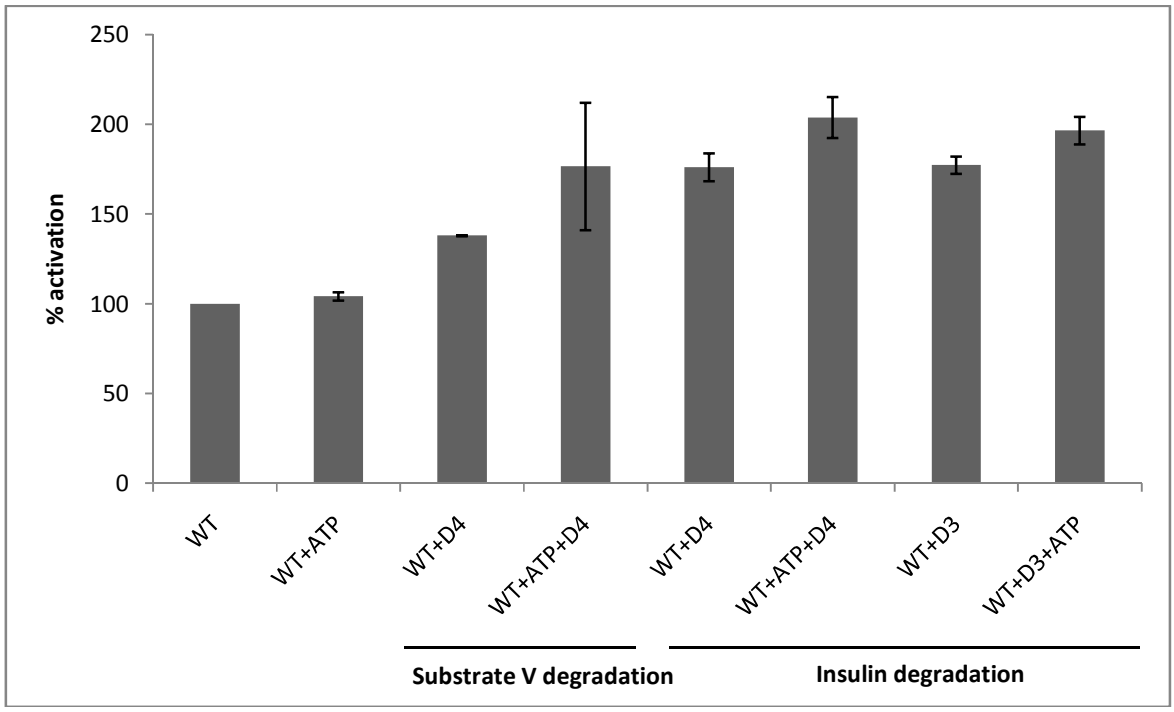
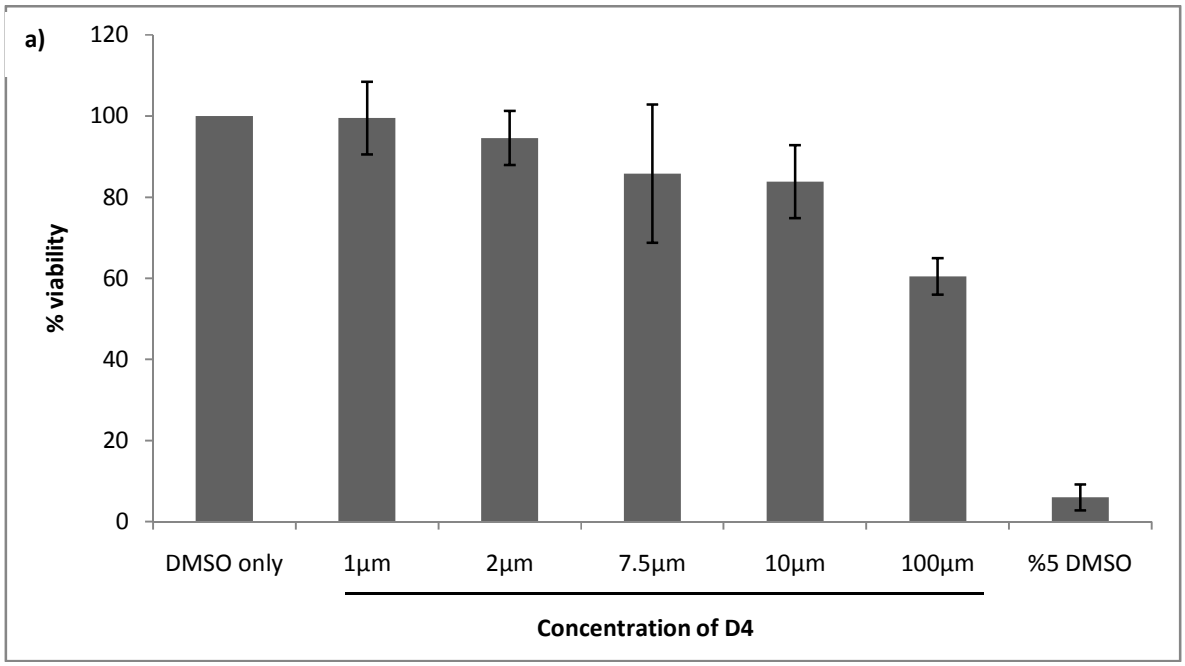


Figure 4.8. Overall results of experiments showing the synergetic effect of ATP. The maximum activity obtained for all experiments are provided. All activities are normalized to wild type activity.

4.4. Cell viability Tests

The activity of D4 was characterized in a cellular assay to establish that it does not have any cytotoxic effects. MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide or thiazolyl blue) assay with HELA cells was used to determine whether the small molecule affects cell viability.[61] In this method, a purple formazan dye forms as a result of cleavage of the yellow tetrazolium MTT within metabolically active cells. The resulting precipitate of the intracellular formazan can be dissolved in a detergent solution and quantified spectrophotometrically at 595 nm. The cytotoxicity of D4 was measured by

incubating HELA cells in the presence of D4 at concentrations ranging from 1 to 100 µm for 24 h. Cell viability was measured using the MTT method after a 12 h culture. The extent of cell death was expressed relative to a control containing DMSO. It was found that D4 did not affect viability significantly up to a concentration of 2 µm, and at higher concentrations as high 100 µm, cells remained 60% viable (Figure 4.9.a). It was also observed that approximately 85% of cells remained viable at a concentration of 7.5µM, which was closer to the EC_{50} value of the compound. Another novel compound, D3 did not demonstrate any toxicity at high concentrations (Figure 4.9.b).However; it was observed that D6 showed toxicity at higher concentrations (Figure 4.9.c). On the other hand, D6 was found to be non toxic at its EC_{50} value (1.91 µM). Besides D4, D3 did not demonstrate toxicity effects as it was shown that HeLa cells were still viable even at higher concentrations of D3.



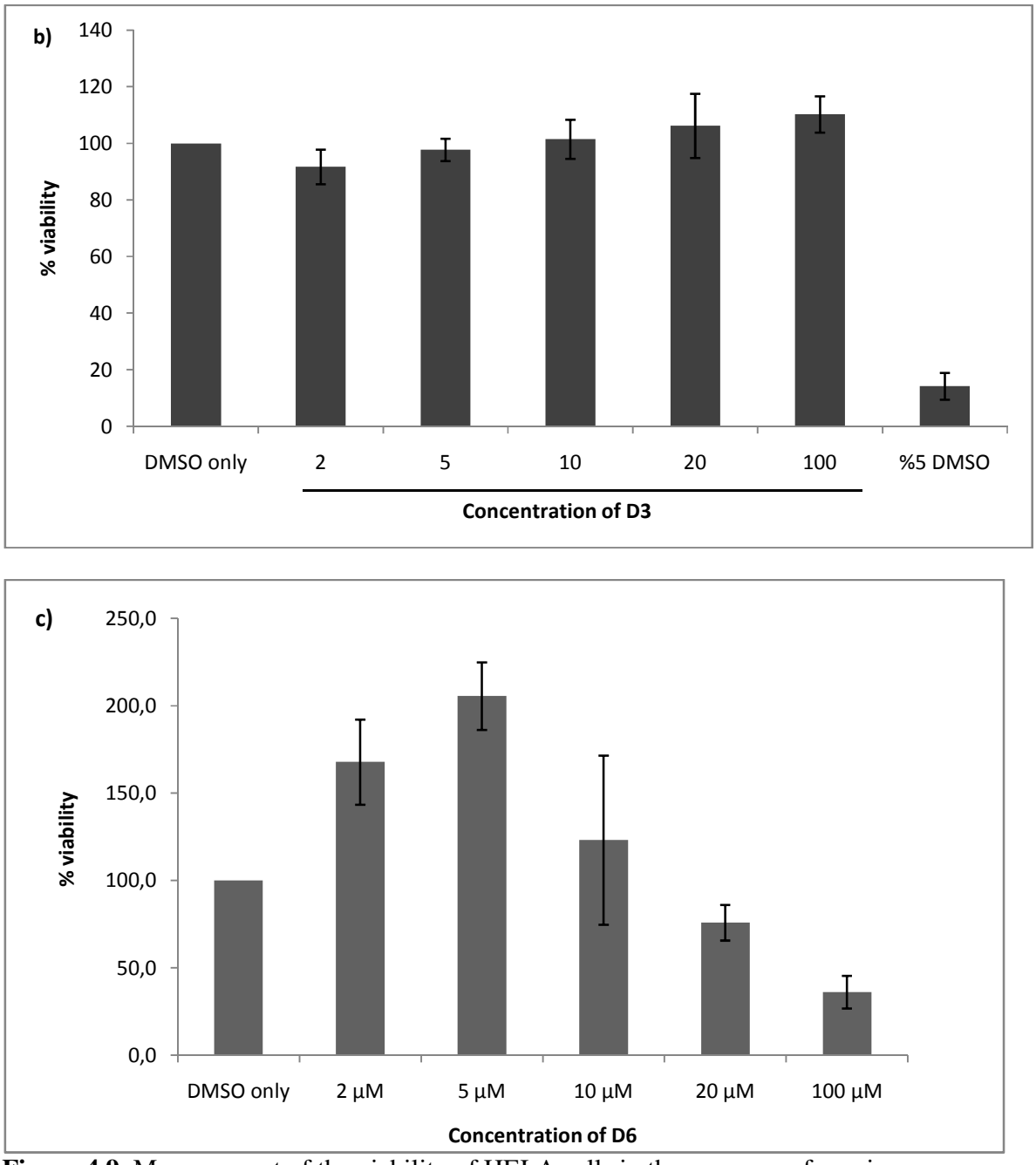


Figure 4.9. Measurement of the viability of HELA cells in the presence of ranging

concentrations of **a)** D4 and **b)** D3 **c)** D6 from 1 to 100 μM. Data are mean ±SEM for 6 independent experiments.

Chapter 5

DISCUSSION

In the present study the effects of 9 molecules obtained through structure–based drug design on the activity of hIDE were characterized. Characterization included *in vitro* enzymatic assays consisting of degradation of substrate V, insulin-FITC, FAβB which were followed by toxicity assay for selected compounds. Consequently, three drug-like compounds, D3, D4, and D6 (Figure 5.1) were discovered that enhanced the activity of IDE towards degradation of substrate V, insulinand amyloid-beta at a 50% affectivity constant in the submicromolar level.

Although there has been various strategies for the treatment of AD including, anti-inflammatory approaches [10], tau pathology approaches [65], apolipoprotein E (APOE) related treatment approaches [66], metabolic dysfunction approaches [13, 14], Aβ-targeted therapeutic approaches are still in the center of AD treatment methods. [67] There are several Aβ-targeted therapeutic strategies including modulation of Aβ production, blocking of Aβ aggregation, Aβ-targeted immunotherapy, and enhancement of Aβ degradation. [67] In this paper the last approach, enhancement of Aβ degradation strategy is followed by designing regulators to enhance the activity of insulin degrading enzyme.

It is already known that ATP enhances the IDE activity of some substrates and it sometimes show synergistic effect with the drug-like compounds. [68] In this study it was observed that activity of IDE for substrate V degradation did not change in the presence of ATP (Figure 4.6). The concentration dependent D4 experiment was repeated in the absence and presence of ATP; and the activity of IDE showed different trends in these cases.

Interestingly, IDE-mediated substrate V activity increased in the presence of 0.1M ATP and D4 (Figure 4.7.a), nevertheless this activity did not change as function of D4 concentration. However, insulin degradation was not significantly affected by ATP when the test was repeated in the presence of D3 and D4 (Figure 4.7.a, b). Also, it was observed that the difference between the conditions that is in the absence of ATP and in the presence of 0.1 mM of ATP for insulin degradation was not significant as substrate V degradation. On the other hand, *in vitro* enzymatic assay was also conducted to check the effects of selected compounds on FAβB degradation. It was observed that only D6 enhanced the proteolytic activity of IDE for FAβB degradation. However, maximum increase activity of IDE was recorded as 20 % in the presence of D6. This result brings out the necessity for further optimization in D6 structure towards higher affinity for Aβ degradation in the future studies.

On the light of these observations it can be speculated that the binding site of ATP does not overlap with the site where D4 binds. One possible mechanism is that ATP affects the transition between open to close conformation of IDE, and this change on the structure may also affect the binding of D4 to the exosite. Another possibility is that ATP may cause an increase in the hydrodynamic radius of IDE which may result in a decrease for the affinity of D4 [69]. The two compounds discovered by Cabrol et al. [68] were found to be effective in the presence of ATP, as they speculated that all these molecules bonded to one or more sites located within the internal chamber of IDE. This mechanism reduces the possibilities of different protein and ligand conformations by decreasing the accessible volume to substrates. [47] On the other hand, ATP does not show any effect on catalytic activity of IDE for in the presence of compounds D3 and D6.

After the identification of activator role of D3 on substrate V, selected 9 compounds were tested to determine any effect that these might have on longer peptide substrates such as, insulin and FAβB. The novel compounds D3 and D4, both increased IDE mediated insulin degradation by approximately 72% and 60%, respectively. Interestingly, D3 had almost no effect on substrate V degradation. This unique feature makes D3 specific to insulin degradation. Also, D6 was found to be specific towards FAβB degradation, since it had no effects towards degradation of remaining substrates (Figure 4.4).

Another important result of this study is that the novel compound D3 does not show toxicity (Figure 4.9.b) whereas D4 demonstrates low toxicity at lower concentration; however D4 and D6 are toxic at higher concentration, ≥ 100 μM (Figure 4.9.a, c). New derivatives of the novel compounds which may be nontoxic can be identified by similarity search or pharmacophore modeling as a future work.

The significance of this study from a biomedical perspective, developed IDE regulators demonstrated novel and potentially important effects on short (substrate V) and long peptides (insulin, Aβ). Thus IDE regulators with appropriate pharmacokinetic properties may be useful for Alzheimer's disease by virtue of enhancing the clearance of amyloid-β from plasma. Finally, the regulators that we discovered constitute important new tool for the experimental manipulation of IDE, which might be critical for routine experimental and clinical applications that involve short and long peptide substrates of IDE.

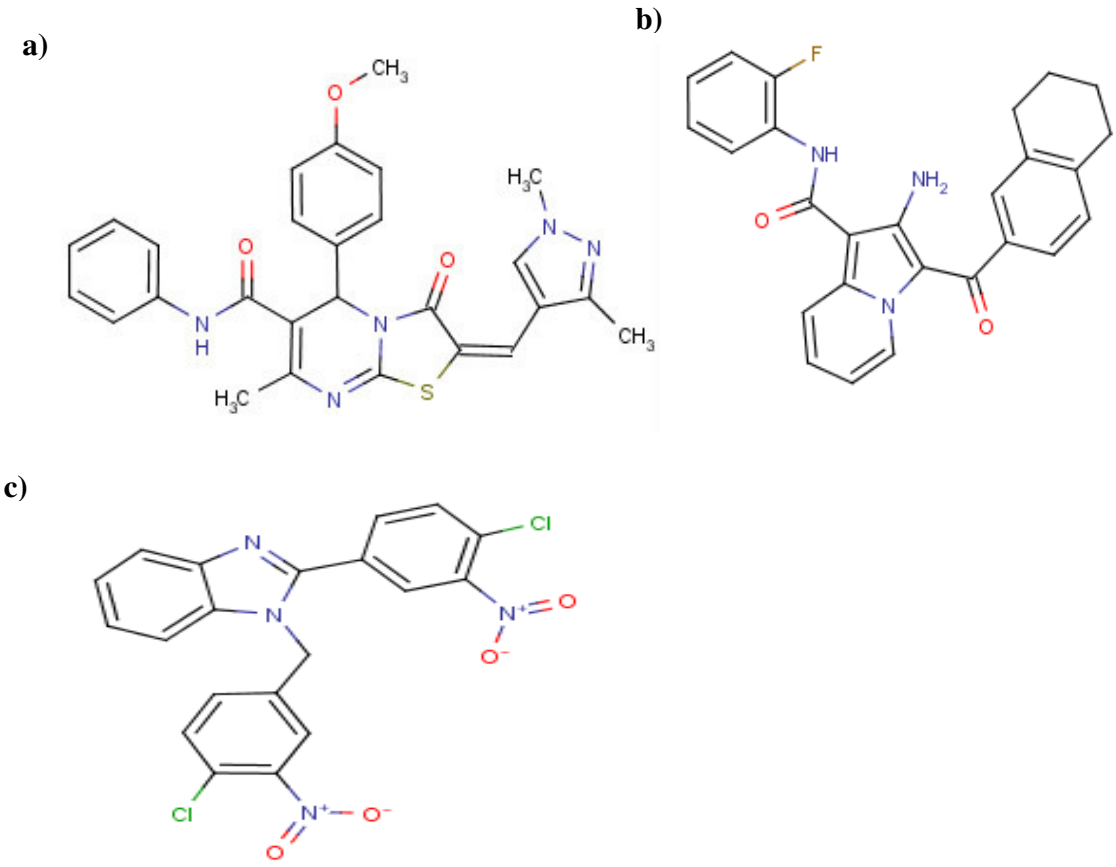


Figure 5.1. The structures of discovered potential drugs.**a)** D3 **b)**D4 **c)**D6

Chapter 6

CONCLUSION

At the outset of this study, computer aided discovery of novel compounds that regulate the activity of IDE were identified by performing in vitro enzymatic and cell viability assays. The proposed compounds probably bind to exosite region that plays an important role in activity regulation of IDE by orienting the peptides to the catalytic site. Considering the potency and low toxicity of the lead compounds, it is worth to optimize these compounds to obtain an efficient drug-like IDE activator. Future efforts will be undertaken to improve the activity of IDE by following the goal of lead optimization of the novel compounds.

Furthermore, these selected 9 compounds may be used for cleavage of other substrates of IDE such as glucagon, insulin-like growth factor (IGF)-II, amylin, transforming growth factor-α (TGF-α) and reduced amylin. There may be significant enhancement of degradation by IDE or other new compounds may be efficient. These studies will also determine the most efficient strategy for up-or down- regulation of IDE. As a result, rational way to cure the Alzheimer's disease or type 2 Diabetes mellitus will be understood by found small molecules which are stable and nontoxic.

Finally, this study is promising as it may be possible to regulate catalytic activity of IDE using small molecules, and that these molecules could be potent drug candidates. Besides, in further studies cleavage of Aβ is performed by using bradykinin or kallidin with these selected small compounds since bradykinin has a regulatory role such that binds to exosite and enhances the activity of IDE. Screening of compounds may be performed under

conditions that contain a complex mixture of substrates [28]. As a result, by mimicking this complex of substrates, new compounds may be discovered to enhance the catalytic cleavage of Aβ by IDE.

Supplementary 1

PCR Reactions

For 50 µl reaction mixture:

Reagents	Quantity
10X Taq Buffer (Fermentas)	5 µl
10X MgCl2 (25 mM)	5 µl
4 mM dNTP mix	1 µl
Forward Primer (10 pmol/ml)	2 µl
Reverse Primer (10 pmol/ml)	2 µl
DNA template	1 µl
Taq DNA Polymerase (1 to 2 units)	~0.1 µl
Sterile & deionize water	complete to 50 µl

Table1: PCR reagents and quantities.

PCR Program:

Temperature	Time	Cycle
95 ºC	4 min	1
95 ºC	30 sec	30
55 ºC	30 sec	
72 ºC	1.5 min	
72 ºC	10 min	1
4 ºC	~	1

Table 2: Time needed for PCR program.

Supplementary 2

Restriction Digestion and Alkaline Phosphatase Treatment

Following buffers are used for respective enzyme digestion:

Restriction Enzymes	Fermentas buffer
Not I	Buffer O
EcoR I	EcoR I Buffer
Hind III	Buffer R
Xho I	Buffer R
BamHI	BamHI Buffer

Table3: The names of restriction enzymes and buffers.

Restrictions are done at 37 ℃ water-bath, for 1 hour or more depending on needs. Alkaline phosphatase treatments are carried out after digestions when necessary. After digestion, 1 µl Shrimp Alkaline Phosphatase (SAP, Fermentas) is added to the mixture and incubated in 37 ℃ for 30 min. SAP is inactivated by incubating at 65 ℃ for 10 min.

Supplementary 3

Ligation with T4 Ligase and Heat-Shock Transformation

Fermentas T4 ligase enzymes and buffer are used for ligation. Reactants are as follows:

Reactants	Quantity
Linearized vector DNA	~ 10 ng
Insert DNA	~ 30 ng to 60 ng
10X ligation buffer	1 µl
T4 DNA ligase	1 µl
Sterile & deionized water	complete to 10 µl

Table4: Reactants and quantities of ligation.

Mixture is incubated at room temperature for 90 minutes.

After ligation is completed, DNA is transferred to *E.coli* by heat-shock as follows:

1. Thaw competent cells on ice. Keep DNA on ice until cells are thawed.
2. Add DNA to cells under sterile conditions and mix by inversion. Keep cells on ice for 10 min.
3. Transfer cells to water-bath at 42 ºC for 90 sec. Then, transfer cell on ice and incubate for 5 min. Add 1 ml LB.
4. Incubate at 37 ºC, 200 RPM shaker for 1 hour. Spread cells on selective agar plates and keep in 37 ºC incubator overnight.

Supplementary 4

Mini-prep Protocol:

1. Grow bacterial colonies overnight in LB with antibody. (I do 5 ml cultures)
2. Take out 1.5 ml bacteria and spin down for 60 sec. Remove supernatant.
3. Add 150 µl Solution 1. Vortex to resuspend pellet.

4. Add 150 µl Solution 2. Vortex and wait 1-2 min.

5. Add 150 µl Solution 3.Vortex.

6. Centrifuge for 10 min.

7. Remove the supernatant and put in new tube.

8. Add 3X volume of 95% Ethanol (I like to use cold Ethanol).

9. Centrifuge for 15 min.

11. Discard supernatant.

12. Add 1000 µl 70% Ethanol. Spin 5 min, (RT or 4 C) and remove supernatant

13. Dry pellet, we do this in the hood (in a speedvac dry >10 min).

14. Resuspend in 50 µl TE or water.

Solution 1

50 mM glucose	0.9 g glucose (2.25 ml of 40% glucose)
25 mM Tris-Cl (pH 8.0)	2.5mL 1M Tris-Cl (pH 8.0)
10 mM EDTA (pH 8.0)	2.0 ml 0.5M EDTA (pH 8.0)
	Water up to 100 ml

Solution 2

0.2 N NaOH	2 ml 10 N NaOH
1% SDS	5 ml 20% SDS
	Water up to 100 ml

Solution 3

3 M potassium acetate	29.44g potassium acetate or 60 ml 5M KoAc
11.5% acetic acid	11.5 ml glacial acetic acid
	Water up to 100 ml

Supplementary 5

Expression and purification human insulin degrading enzyme (hIDE):

1. hIDE-pET21b plasmid is transferred to *E.coli* BL21 strain by heat shock transformation.
2. One colony on the agar plate is picked and grown in 10 ml LB with ampicillin overnight.
3. Add 10 ml of culture to 1 liter of LB with ampicillin (1:100 diluted)
4. Incubate at 37 ºC in the shaker for 2 hours, until OD reaches 0, 6-0, 8.
5. Test OD using LB-ampicillin as a blank (λ= 600 nm)
6. Add IPTG for a final concentration of 400 mM and continue to incubate at 37 ºC for 3 hours.
7. Transfer culture to centrifuge tubes (250 ml) and centrifuge at 9000 rpm for 5 min at 4 ºC in the swinging bucket rotor
8. Resuspend the pellet in 5 ml of phosphate buffered saline (PBS). Cells can be frozen at this point for several months at -20 ºC.

5.1. Preparation of cell lysate:

1. To melt pellet on the ice waiting for 30 min.

2. Resuspend pellet in 10 ml of lysis buffer, 100 µl phenylmethanesulfonylfluoride (PMSF), 50 µl proteinase inhibitor coctail and 500 µl of lysozyme (20 mg/ml) at 4 ºC.
3. Take it to the 50 ml falcon tube.
4. Sonicate for 8 times for 15 sec bursts (%60-70) with probe sonicator on ice.
5. Transfer culture to centrifuge tubes (50 ml) and centrifuge at 10000 rpm for 30 min at 4 ºC in the swinging bucket rotor.
6. Save supernatant.

5.2. Preparation of column chromatography:

1. Make slurry of Ni^{+2} resins in the lysis buffer.
2. Using a pipette to load the resin suspension onto the column.
3. Allow the material to settle till the required level.
4. Wash the column thoroughly with 2 to 3 column volumes of lysis buffer before loading supernatant onto the column.

5.3. Column run:

1. The supernatant is loaded at a slow rate onto the column.
2. Wash buffer is applied onto the column for 5 column.
3. The bound proteins are eluted with increasing concentrations of salt called elution buffer.
4. Elutes are collected as fractions.
5. The fractions can be analyzed by running on SDS-PAGE for purity.

Lysis Buffer:

50 mM NaH_2PO_4

300 mM NaCl

10 mM imidazole

Adjust pH to 8.0 using NaOH

Wash Buffer:

50 mM NaH_2PO_4

300 mM NaCl

20 mM imidazole

Adjust pH to 8.0 using NaOH

Wash Buffer:

50 mM NaH_2PO_4

300 mM NaCl

250 mM imidazole

Adjust pH to 8.0 using NaOH

5.4. Reuse of Ni –NTA Resin:

1. Wash the column with 2 volumes of Regeneration Buffer (6M GuHCl, 0.2 M Acetic acid).
2. Wash the column with 5 volumes of H_2O.
3. Wash the column with 3 volumes of 2% SDS.
4. Wash the column with 1 volume of 25% EtOH.
5. Wash the column with 1 volume of 50% EtOH.
6. Wash the column with 1 volume of 75% EtOH.

7. Wash the column with 5 volumes of 100 % EtOH.
8. Wash the column with 1 volume of 75% EtOH.
9. Wash the column with 1 volume of 50% EtOH.
10. Wash the column with 1 volume of 25% EtOH.
11. Wash the column with 1 volume of H_2O.
12. Wash the column with 5 volumes of 100 mM EDTA, pH 8.0.
13. Wash the column with 5 volumes of H_2O.
14. Recharge the column with 2 volumes of 100 mM $NiSO_4$.
15. Wash the column with 2 volumes of H_2O.
16. Wash the column with 2 volumes of regeneration buffer.
17. Equilibrate with 2 volumes of a lysis buffer.

Supplementary 6

Preparation of SDS-PAGE reagents:

- **Ammonium persulfate (APS):** Dissolve 10 mg of APS in 1ml water. Prepare fresh daily.
- **N,N'-tetramethylene-ethylenediamine (TEMED) :** Use from TEMED bottle and keep it at 4ºC.
- **30 % Acrylamide/0.8 % Bisacrylamide:** Mix 30 gram acrylamide and 0, 8 gram bisacrylamide in total volume of 100 ml distilled water. Store it at 4ºC in the dark.
- **4X Tris-CI/SDS, pH 6.8:** Dissolve 6.05 gram tris base and 4 ml of 10% SDS in 40 ml distilled water. Adjust pH to 6.8 with HCl. Add distilled water to 100 ml total volume and store at 4ºC.

- **1X SDS Running buffer:** Dissolve 15.1 g Tris base, 72 g glycine and 5 g SDS in about 800 ml distilled water. After solutes are dissolved, bring the total volume to 5 L.
- **2X SDS Sample Buffer:** Mix 30 ml 10 % SDS, 10 ml glycerol, 5 ml 2-mercaptoethanol, 12.5 ml of 4X Tris-Cl/SDS, pH 6.8 and 5-10 mg bromophenol blue. Bring the volume 100 ml with distilled water. Divide into 1 ml aliquots and keep it at -20 ºC.

Coomassie Staining

Reagents: 0.1 % Coomassie Blue R
12 % Acetic acid
50% Methanol

Procedure:

The dye is dissolved in methanol and acetic acid is added. The solution is completed to final volume of 1L with distilled water.

Destaining Solution:

30% methanol
7% acetic acid

Destaining solution is prepared complete final volume of 500 ml and gel is washed wit this solution until background is clear.

SDS-PAGE Protocol

Pouring Resolving Gel:

1. Clean glass plates with soap and water, then with ethanol. Assemble the glass plates and spacers.
2. Transfer the degassed resolving gel to a beaker and add APS and TEMED. Mix and quickly add the resolving gel solution to the center of plates to a height about 4 cm from the top.
3. Quickly add isopropanol to the top of this until the level reaches the top of the plates. Isopropanol will prevent oxygen from getting into the gel which could oxidize it and inhibit polymerization.
4. The resolving gel should polymerize in 15 minutes.

Pouring Stacking Gel:

1. Pour off isopropanol. Pour water several times into the gel plate space to rinse off all the isopropanol.
2. To polymerize the stacking gel, add APS/TEMED, mix, then pour on top of the polymerized resolving gel.
3. Insert the comb straight on down, then pour a little more stacking gel on the sides of the comb to fully seal it. Remove any bubbles from underneath the comb, if possible, by moving the comb gently from side to side so the bubbles get into the space in between and float up.
4. The stacking gel should polymerize in 10 to 15 minutes.

Load the Gel:

1. Attach the large gel plates containing the polymerized gel to the apparatus via the clips provided.
2. Pour Tris-glycine electrophoresis buffer into the upper and lower chambers.
3. Flush the wells with a syringe just before loading to get rid of any unpolymerized polyacrylamide that may seep in.
4. Load samples and protein marker.
5. Run large thin gels at a constant current of 90 V. After the dye front enters the resolving gel, you can turn the current up to 130 V. Where you stop the gel will depend on how big the smallest protein is that you want to visualize. If you wait until the dye front just flows out of the gel, it should take about 2 1/2 - 3 hours for a large, thin gel to run.

Supplementary 7

Western Blot Protocol

Transferring the gel:

1. While the gel is running, prepare the transfer buffer, and cut out a piece of nitrocellulose and four pieces of Whatmann filter paper for the transfer.
2. Cut out the Whatmann filter papers so they each are slightly larger than the gel in each dimension--about 1/4 to 1/2 cm larger. Cut out the nitrocellose so that it is slightly smaller than the Whatmann filter papers but still covers the entire surface of the gel.
3. Float the nitrocellulose filter on the surface of a tray of methanol and allow it to wet from beneath by capillary action.

4. Remove the plates from the apparatus. Remove the spacers and pry off one of the plates by inserting a spatula and twisting the plate up.
5. Transfer the gel by running at 100 V for 1 hour. Make sure to have the cooling unit on at 4 C so that cold water can circulate through the tank while the transfer is taking place. Otherwise, if the buffer gets hot, bubbles of air may come out of solution and become trapped in the sandwich.

Probing the blot with an antibody:

1. Remove the blot from the transfer unit and block by placing in BSA for 1 hour with shaking.
2. Incubate in primary antibody on our rocker for one hour. Typically, the primary antibody is diluted 1:1000.
3. Wash the blot three times with PBST, 5-10 minutes each time.
4. Incubate the blot in secondary antibody for one hour on a rocker. Most of our primary antibodies are from mice, so the HRP antimouse antibody from the ECL kit can be used. The blot can be incubated in 10 mL of wash buffer that is 2% BSA and to which secondary antibody has been added to a 1:5000 dilution.
5. Wash the blot four times with wash buffer, 5-10 minutes each time. Between washes, rinse with distilled water.
6. Toward the end of the washes, prepare for exposing the blot to film.

Solutions

Resolving Gel:

1650 µl Monomer

1250 µl 1.5M Tris pH 8.9

2 ml distilled water

50 µl APS

3 µl TEMED

Stacking Gel:

330 µl Monomer

250 µl 0.5 M Tris

1.4 ml distilled water

20 µl APS

2 µl TEMED

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