ABSTRACT

TUCKER, MATTHEW BENJAMIN. Purification, Crystallization, and Structural Determination of Wild Type Zebrafish Caspase-3 and the Utilization of a Novel M13 Bacteriophage System to Determine Substrate Specificity for Zebrafish Caspase-3. (Under the direction of Dr. A. Clay Clark).

Apoptosis is the vital process regulating the equilibrium of cell proliferation and cell death in metazoan animals. Although the exact mechanism of apoptosis is unknown in every animal, this process, in all metazoan animals, is regulated by a group of conserved cysteine proteases known as caspases. Caspase-3, an executioner caspase vital to the success of apoptosis, is conserved in both humans and zebrafish. Although the two proteins only maintain a sequence identity of 61%, the crystal structure of zebrafish caspase-3 is very similar to the structure of human caspase-3; the root mean square deviation (RMSD) value of the two structures is equal to 0.462. The similarity in structure between the two homologues indicates that zebrafish may be an advantageous animal model utilized to examine potential drug candidates against caspase-3. Caspases are notorious for preferring one substrate to another based on the residues surrounding the P1 aspartate residue. Many substrates can be cleaved simply for containing an aspartate residue; however, in human caspase-3, when the sequence has an aspartate in the P4 site, that substrate turns into a highly favorable cleavage target. Currently, the sequence DEVD is the sequence determined to be the most preferential for human caspase-3. Utilization of a novel M13 bacteriophage substrate specificity model yielded the sequence DNLD as the ideal sequence cleaved by human caspase-3. Although this sequence isn't considered to be the most preferred sequence, it has been reported in previous studies to be unique substrate to only caspase 3, unrecognized by human caspases 8&9.

The M13 phage display specificity model utilized with Human caspase 3 was used to determine that zebrafish caspase-3 prefers either a valine or aspartate in the P4 site of the target sequence. The preference of valine over aspartate seemed to diminish over time, as substrate sequences became more enriched, resulting in fewer “average” substrates. Utilizing a valine in the P4 site of substrates has been a hallmark trait of human caspase-6. Therefore, it may be possible that zebrafish caspase-3 helps to preform some of the roles in zebrafish that are traditionally completed by caspase-6 in humans.

Purification, Crystallization, and Structural Determination of Wild Type Zebrafish Caspase-3 and the Utilization of a Novel M13 Bacteriophage System to Determine Substrate Specificity for Zebrafish Caspase-3

by
Matthew Benjamin Tucker

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Biochemistry

Raleigh, North Carolina

2015

APPROVED BY:

_____	_____
Dr. A Clay Clark Committee Chair	Dr. Michael Goshe
_____	
Dr. Paul Hamilton	

DEDICATION

I dedicate this thesis to my family. Education was always held in a positive, encouraging light and I can't begin to explain how that has shaped me into the person I am today.

BIOGRAPHY

Matt Tucker grew up in Randleman, North Carolina with his brother, Andrew and parents Patti and Keith. After graduating from Randleman High School in 2009, Matt enrolled in the Biochemistry program at NC State University in the fall of 2009. After Matt's sophomore year, he felt the need to explore the world of academic science and research at NC State. His Junior year involved balancing classes and conquering the world of V266 caspase-3 protein science in the Clark lab. The work progressed for two years and Matt decided he wanted to complete more work under Dr. Clark and enrolled in the Master's program in the Department of Biochemistry. Matt will be attending Duke University in the fall, pursuing a PhD in Mechanical Engineering.

ACKNOWLEDGMENTS

Family has always meant a lot to me and I think it is because I have a tremendous one. Dad, you are a rock, tough as nails, and I try to emulate your tenacity each day I wake up in the morning. Mom, you have been an inspiration for as long as I can remember; you have what seems like an infinite amount of patience and would sacrifice everything to make sure anything in my life, no matter how small, was marginally better. I'll never forget the dedication is took to attend school at night, while working, to finish you education degree. Andrew, it's ya boy, Matthew. We have the whole twin, unspoken communication thing going for us (except we literally couldn't look more different) and it is just more proof that we are unstoppable together. You are successful, like insanely successful, and at the end of the day I'll be happy to match half of what you achieve. Morgan, speaking of successful, that word can't be uttered without you getting thrown into the sentence. Seriously, you guys are a power couple; it's cool to see- let alone be related. You quickly adapted to a new academic scenario and I want to let you know that you have been a big inspiration for me. Celina, you have been absolutely great over the past four years. We have certainly matured from our college sophomore selves, but we still know how to have fun! Although the all-nighters in the library seemed brutal at the time, I think I can look back and say they were a little less grueling because you were there. I am proud of you for what you have done over the past two years and look forward to the day that you call North Carolina your permanent home. In the meantime, I am going to eat as much candy as humanly possible. Thank you to Clay Clark, Paul Hamilton, Michael Goshe, Sarah Mackenzie, and everyone in the Clark lab.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

Chapter 2

Chapter 3

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 1

Introduction

A. Importance of Apoptosis

Apoptosis, or programmed cell death, helps to ensure normal cell turnover in multicellular organisms. There are a number of diseases related to the improper execution of apoptosis. In circumstances of cancer, there is not enough apoptosis leading to cell death. However, in circumstances of neurodegenerative diseases, such as Parkinson's disease, there is too much apoptosis leading to cell death. Apoptosis is also a crucial point of interest in cardiovascular disease and autoimmune disorders (1).

B. Apoptotic Pathways

Currently, there are two main molecular pathways known that lead to apoptotic cell death. The two main pathways are the intrinsic pathway and extrinsic pathway. Figure 1 graphically depicts the two pathways. The intrinsic pathway involves non-receptor-mediated stimuli that eventually will result in the release of cytochrome c from the mitochondria. The lack of a certain signal, such as growth factors or hormones, could result in apoptosis being triggered. Also, the presence of certain signals such as radiation, toxins, or hypoxia can encourage apoptosis to occur. The released cytochrome c binds to the protein Apaf-1 and procaspase-9; the binding of all of these proteins results in the formation of a quaternary protein structure known as the "apoptosome" (2). The accumulation of procaspase-9 in a concentrated region results in caspase-9 activation. Caspase-3 is produced in the cell as an inactive dimer. Activated caspase-9 targets and activates procaspase-3, which leads to the successful execution of apoptosis in the cell (2).

The extrinsic pathway initiates apoptosis through transmembrane receptor-mediated interactions. Ligation of transmembrane death receptors including TRAIL, TNF, and Fas

leads to the recruitment of procaspase-8/10. These procaspases are recruited via the Fas-associated death domain protein, commonly known as FADD. FADD then associates with procaspase-8. After dimerization, a death-inducing signaling complex (DISC) is formed and procaspase-8 is activated. Active upstream caspase can then cleave executioner caspases, such as caspase-3 to ensure the destruction of the cell.

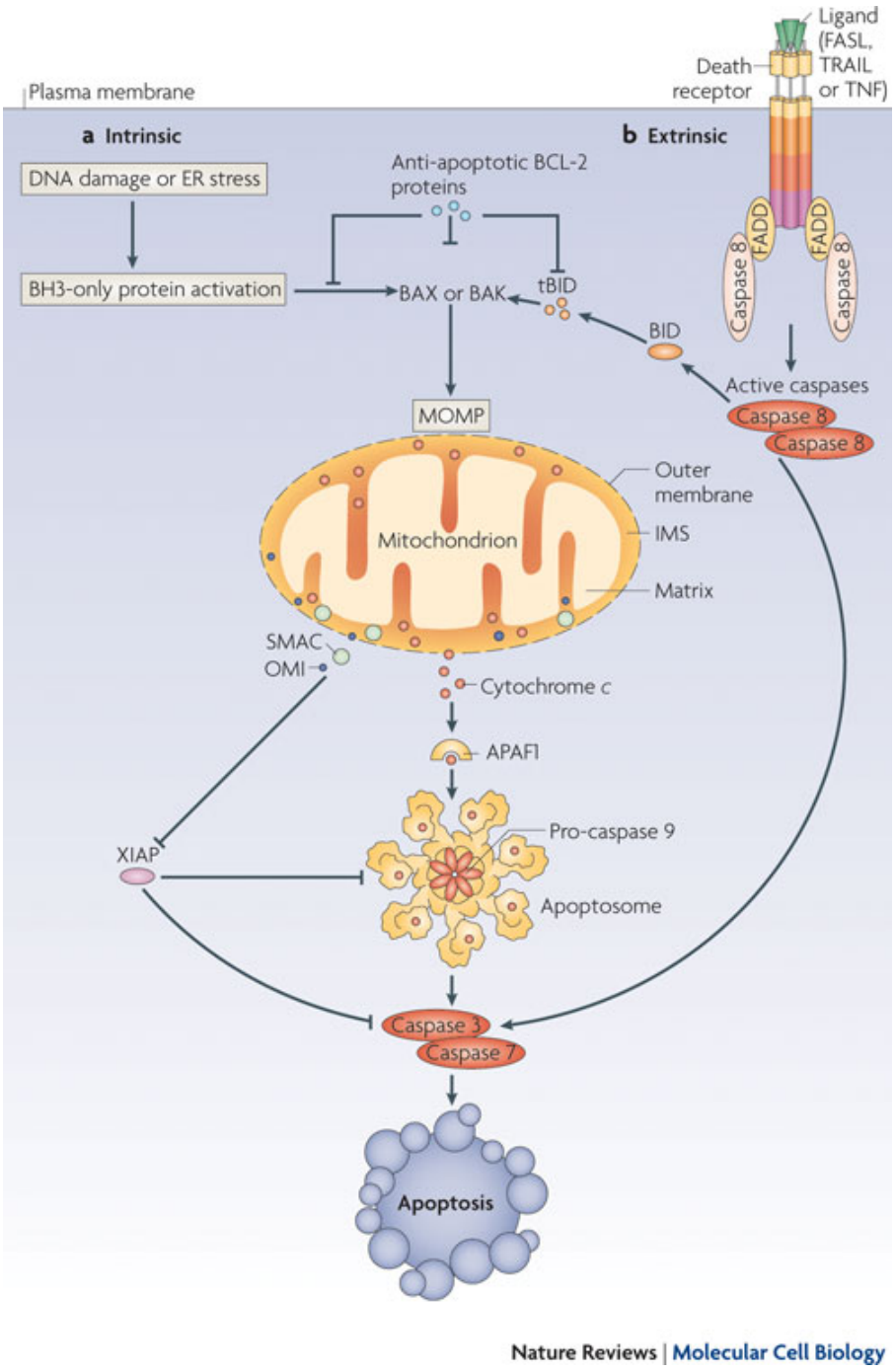

Figure 1: Intrinsic and Extrinsic pathways of apoptosis. (3)

C. Caspases are the Mediators of Apoptosis

Apoptosis is mediated by a family of proteases known as caspases. Caspases are cysteine-dependent aspartate-specific proteases; therefore, a cysteine residue is used as a nucleophile to attack protein substrates containing an aspartate residue. Hydrolysis of the peptide bond is the mechanism by which cleavage of the peptide bond occurs. All caspases share the same general primary protein structure. As seen in figure 2, caspases have an N-terminal prodomain, large subunit, an intersubunit linker, and a small subunit on the C-terminal end. Caspases are commonly divided into two general categories for apoptosis. The categories are the initiator caspases and executioner caspases. Executioner caspases include caspases 3, 6, and 7. Initiator caspases include caspases 2, 8, 9, and 10.

Initiator caspases have a longer prodomain than executioner caspases. The initiator caspases contain a prodomain with a caspase recruitment domain (CARD) or a death effector domain (DED). Initiator procaspases are found as monomers and activation occurs after dimerization of two monomeric caspase units. Executioner caspases require proteolytic cleavage in order to become an active protease, capable of cleaving substrates. Executioner caspases lack long prodomains, unlike initiator caspases.

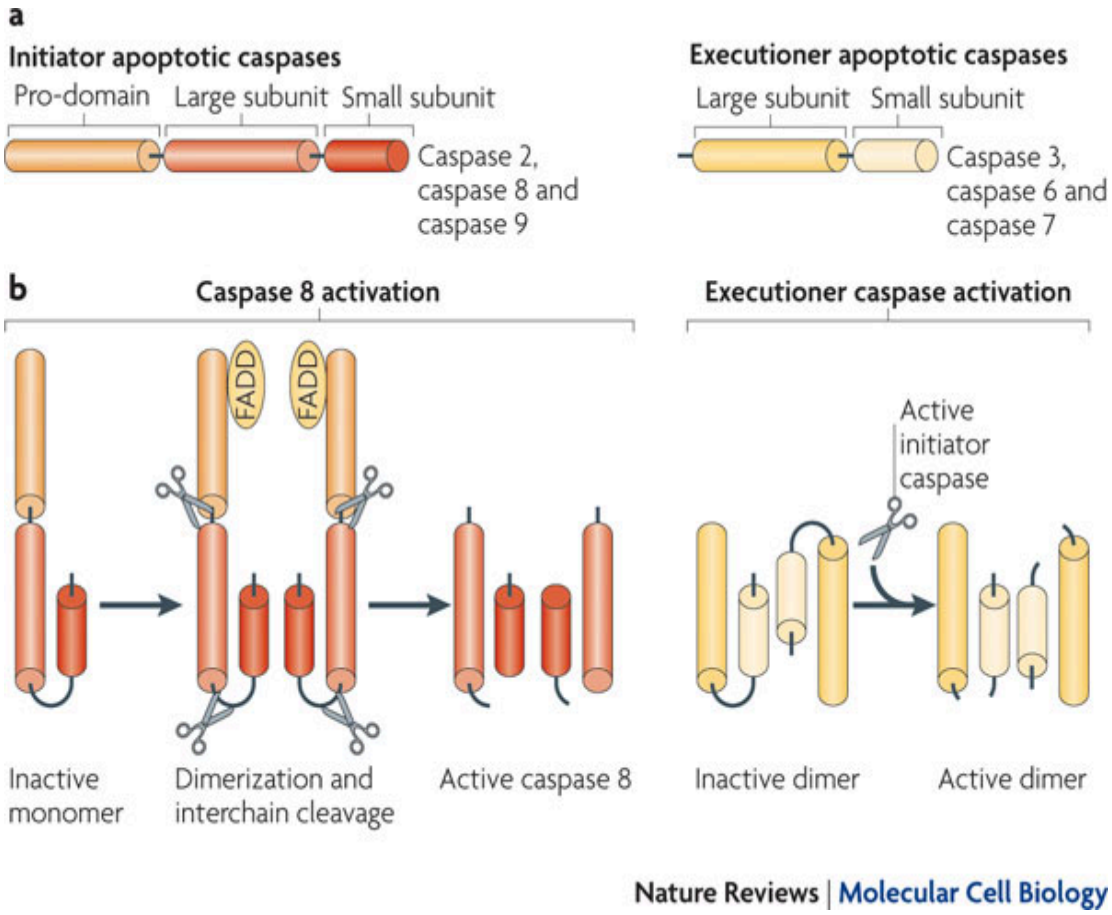

Figure 2: A) Organization and structure of the mature apoptotic caspase subfamilies B) General folded structures of initiator and executioner caspases, cleavage sites (3).

D. Human Caspase-3 Maturation and Activation

Each caspase-3 monomer contains a large subunit and a small subunit, connected by an intersubunit linker. Each caspase-3 monomer, when folded, consists of multiple beta-strands surrounded by alpha-helices. Caspase-3 is found in the cell as inactive dimer, cleavage at specific portions of the protein produces an active caspase-3 that can cleave substrates. Cleavage occurs at three specific places on each monomer in the dimer. Cleavage occurs after an aspartic acid residue at the D9, D28, and D175 positions on each monomer. As seen in figure 2, mature executioner caspases do not contain a prodomain. Cleavage at the D9 and D28 positions results in the removal of the prodomain (4,5). Cleavage at D175, in the intersubunit linker, results in the repositioning of active site loops. The D3A mutant is caspase-3 containing alanines at those cleavage positions, instead of aspartates. This results in an uncleaved, "pro-caspase" version of human caspase-3 (5).

E. Zebrafish Caspase-3

Danio rerio, otherwise known as zebrafish, are small tropical fish that have become an increasingly popular animal model to study several human diseases (6). Zebrafish embryos offer visual transparency, short reproductive cycles, and ease of drug administration (6,7). These advantages, along with those listed in Figure 3, have positioned zebrafish to become a popular animal model when researching possible treatment options for common diseases. In particular, using zebrafish to screen small molecule anti-cancer drug libraries.

Apoptosis is an evolutionarily conserved process. However, the complexity, chosen pathway, and number of caspases utilized may differ amongst various organisms. The current belief concerning apoptosis is that there are some differences in the earlier stages of

apoptosis, but there is strong activation of one or more caspases in the latter stages of apoptosis (8). Therefore, despite possible variation in caspase pathways between humans and zebrafish, analysis of zebrafish caspase-3, an executioner caspase, may shed light more light on the process of apoptosis in zebrafish and the similarities to the human caspase-3.

F. Substrate Specificity of Caspases

Caspases are cysteine proteases that cleave the peptide bond C-terminal to an aspartic acid. Caspases, along with Granzyme B, are the only endoproteases known that cleave after an aspartic acid (9). Caspase substrates are commonly identified as a sequence of amino acids, P4-P3-P2-P1-P1', where P1 is an aspartic acid, and the peptide bond between P1 and P1' is the scissile bond (9,10).

Caspases have been described as promiscuous when it comes to the substrate specificity they exhibit within the cell (9). Four hundred cellular proteins have been identified as being cleaved in a caspase specific manner (11). The only three real requirements for a caspase substrate include the aspartic acid at the P1 position and a small, uncharged amino acid at the P1' position, and for P4-P3-P2 residues are complementary for interactions in the catalytic groove of the protein (12).

Many caspases have a single sequence (P4-P3-P2-P1-P1') that is recognized as that particular caspases' consensus or preferred substrate sequence, based on activity. Ranking substrates of caspases in terms of their activity is a common practice because of a caspases' ability to cleave a suite of amino acid residues containing an aspartic acid. DEVDG is the commonly accepted sequence for caspase-3; however, the sequence DXXD, where X is any amino acid residue, is also an acceptable template (9).

Table 1: Advantages and disadvantages of various animal models in research (13)

Attribute of disease model	Model organism			
	Fly	*Zebrafish*	*Mouse*	*Rat*
Practical issues				
Husbandry infrastructure	$	$	$$$	$$$
Cost per animal per year	$	$	$$$	$$$
Characterized inbred strains	+	-	++++	+++
Outbred laboratory strains	+	+++	++	++
Anatomical similarity	-	+	++	++
Molecular or genetic similarity	+	++	+++	+++
Pathological similarity	-	++	+++	+++
Storage; for example, freezing sperm	No	Yes	Yes	Yes
Molecular biology tools				
Transgenesis*	++	++	++	++
Targeted gene modification*	+	-	++++	+
Transient *in vivo* assays*	++	++++	+	+
Allelic series from TILLING*	+++	++++	++	+
Feasibility of large-scale screens‡	++++	+++	++	+
Affordability of large-scale screens‡	++++	+++	+	-
Sequencing progress§	+++	++	+++	++
Annotation progress§	++	++	++++	++
Cell-biology tools				
Cell lines and tissue culture	++	+	++++	+
Antibody reagents	++	+	++++	++

*Reverse-genetics approach; ‡forward-genetics approach; §genome sequence; -, not relevant, or not a strength; $, $$, $$$ and +, ++, +++, relative cost ($) and strength (+) of the model in each category; ++++, outstanding strength of the model; TILLING, targeting induced local lesions in genomes.

G. Phage Display

Bacteriophage are small viruses that infect and replicate within bacteria. Phage display is a method that involves cloning a particular gene for a protein or peptide into a special type of vector. The gene for a protein or peptide of interest is cloned in such a way that when expressed, it will be fused with a phage coat protein. Therefore, the protein or peptide of interest can be found on the surface of the virus. This is advantageous because of the sheer diversity of peptides and proteins the process will produce; phage display can produce up to 10^{10} diverse peptides (14).

Designing a phage display experiment relies on a selection of a bacteriophage virus and selection of host a bacterium to be infected. Various bacteriophage include M13, fd, f1, T7, T4, and lambda. M13, fd, and f1 are non-lytic and filamentous bacteriophage. T7, T4, and lambda are all lytic bacteriophage. *E. coli* is a very common host bacteria chosen to be infected by the phage; however, there are numerous strains of *E. coli* utilized (15). Polyvalent phage display involves the display of more than one copy of the protein on each phage virion. Monovalent phage display involves the display of a single copy of the protein on the phage virion (15).

Phage display has traditionally been used to study receptor and anti-body binding sites, protein-ligand interactions, and protein-protein interactions (14,15). However, phage display can be utilized to study substrate specificity for proteases as well (16,17). Instead of a full-length protein being displayed on the phage, random peptide sequences, relatively short in length, are displayed on the outside of the phage. If a protease recognizes the sequence, cleavage will occur and the affixed phage will be separated from the surface; sequences not

recognized by the protease will remain fixed to the surface (16,17). The cleaved phage are collected, used to infect a bacterial host, and the selection process is repeated for as many rounds as desired. This recycling of selected phage is known as enrichment.

There are various ways phage can be affixed to a stationary surface during substrate specificity experiments. Matthews et al. utilized an M13 phage display strategy that placed the random cleavable substrate region between the gene III protein and a variant of the human growth hormone (hGH) that binds tightly to human growth hormone binding protein (hGHbp) affixed to a polystyrene plate. Therefore, when the protease recognized a cleavable sequence, the phage was released from a polystyrene plate. Phage that did not contain a cleavable sequence remained affixed to the polystyrene plate, unable to be collected (17).

Chapter 2

Materials and Methods

A. Buffer and Materials Specifications

All Buffers were subjected to vaccum filtration before use in experiments

Activity Buffer: 20 mM Potassium Phosphate, containing 1% Sucrose, at pH 7.2.

Blocking Buffer: 1% BSA, 1X PBS, 1M NaCl, and 0.1% Tween-80.

Elution Buffer: 500 mM Imidazole, 50 mM NaCl, 50 mM Tris-HCl at pH 7.9

Lysis Buffer: 50 mM Imidazole, 50 mM NaCl, and 50 mM Tri-HCl at pH 7.9

Wash Buffer (Phage Display): 1X PBS, 1M NaCl, and 0.1% Tween-80

LB (Broth): 5 grams of yeast extract, 10 grams of granulated tryptone, and 10 grams of NaCl dissolved in 1 liter of deionized water

LB-AMP (Plates): 5 grams of yeast extract, 10 grams of granulated tryptone, 15 grams agar and 10 grams of NaCl added to 1 liter of deionized water

2XYT (Broth): 10 grams of yeast extract, 16 grams of granulated tryptone, and 5 grams of NaCl are added to 1 liter of deionized water.

2XYT (Top Agar): 7 grams of agar, 10 grams of yeast extract, 16 grams of granulated tryptone, and 5 grams of NaCl are added to 1 liter of water.

2XYT (Plates): 15 grams of agar, 10 grams of yeast extract, 16 grams of granulated tryptone, and 5 grams of NaCl are added to 1 liter of water.

B. Human Caspase-3 Protein Purification

The following description of purification of human caspase-3 can be found in Pop et al., 2003. Purification of human caspase-3 involved growing BL21 cells, transformed with a pET-21b plasmid containing a C-terminally His-tagged human caspase-3, in a 100 mL LB starter culture containing ampicillin at a concentration of 50 μg/mL. When the OD_{600} of the

100 mL culture reached 1.2, Fernbach flasks containing 1 L of LB were inoculated with LB from the 100 mL starter culture.

Liquid cultures wihthin each Fernbach was grown at 37 degrees Celsius, shaking, until the OD_{600} equaled 1.2. After the optical density was reached, 800 μL of a 1M IPTG solution was added to each flask. After induction, the cells expressed the protein for 5 h at 25 degrees Celsius. The cultures were centrifuged at 5,000 rpm for 20 min. The supernatant was disposed of and the pellets were resuspended with a total of 50 mL of lysis buffer. The solution was left stirring, at 1.6 degrees Celsius, for 4 h. The mixture was placed into a French press and the cells were lysed. The lysed mixture was centrifuged at 14,000 rpm for 30 min. The supernatant was poured over a liquid chromatography column, filled with Ni-NTA Agarose (Nickel resin).

The supernatant sat on the column for 30 min before it was allowed to flow through the column. The column, saturated with C-terminally His-tagged human caspase-3, was washed with 350 mL of lysis buffer. After the wash step, the contents of the column were eluted with 50 mL of elution buffer. The 50 mL of eluted protein solution was transferred to a dialysis bag, and placed in a 1 L solution of buffer containing 50mM NaCl and 50mM Tris at pH 7.9.

The buffer exchange lasted for 4 h. The contents of the dialysis bag were transferred to a chromatography column, containing Q-sepharose resin. Two-minute fractions were collected utilizing a 1M NaCl gradient flowing through the column. Fractions containing pure caspase-3 were consolidated into a single container.

C. Zebrafish Caspase-3 Protein Purification

Purification of zebrafish caspase-3 involved growing BL21 cells, transformed with a pGEM-T plasmid containing a C-terminally His-tagged zebrafish caspase-3, in a 100 mL LB starter culture containing ampicillin at a concentration of 50 μg/mL. When the OD_{600} of the 100 mL culture reached 1.2, Fernbach flasks containing 1 L of LB were inoculated with LB from the 100 mL starter culture.

The culture within each Fernbach was grown at 37 degrees Celsius, shaking, until the OD_{600} equaled 1.2. After the optical density was reached, 800 μL of a 1M IPTG solution was added to each flask. After induction, the cells expressed the protein for 5 h at 25 degrees Celsius. The cultures were centrifuged at 5,000 rpm for 20 min. The supernatant was disposed of and the pellets were resuspended with a total of 50 mL of lysis buffer. The solution was left stirring, at 1.6 degrees Celsius, for 4 h. The mixture was placed into a French press and the cells were lysed. The lysed mixture was centrifuged at 14,000 rpm for 30 min. The supernatant was poured over a liquid chromatography column, filled with Ni-NTA Agarose.

The supernatant sat on the column for 30 min before it was allowed to flow through the column. The column, saturated with C-terminally His-tagged zebrafish caspase-3, was washed with 350 mL of lysis buffer. After the wash step, the contents of the column were eluted with 50 mL of elution buffer. The 50 mL of eluted protein solution was transferred to a dialysis bag, and placed in a 1 L solution of buffer containing 50mM NaCl and 50mM Tris.

The buffer exchange lasted for 4 h. The contents of the dialysis bag were transferred to a chromatography column, containing Q-sepharose resin. Two-minute fractions were

collected utilizing a 1M NaCl gradient flowing through the column. Fractions containing pure caspase were consolidated into a single container.

D. Crystallization of Zebrafish Caspase-3

Zebrafish caspase-3 was dialyzed in a 1 L solution of 10 mM Tris and 1mM DTT at a pH of 8.5 for four hours. Successful crystals were grown by hanging drop vapor diffusion method at a pH of 5.1, 17.5% PEG 6000, 10 mM DTT, and 3 mM NaN_3. Protein stock and inhibitor were incubated together for 1 h in a dark environment. The protein stock utilized was at a concentration of 10 mg/mL and the inhibitor concentration was five times the protein concentration. The inhibitor utilized was Ac-DEVD-CMK. The drops on the glass slide, above the reservoir solution, were 4 microliter drops. The drops were a 1:1 ratio of reservoir solution and protein solution.

The crystal trays incubated at 18 degrees Celsius for approximately 14 days. Data sets were collected at Argonne National Laboratory on the SER-CAT synchrotron beamline.

E. Generation of Phage Sequences and Libraries

Four oligonucleotides were designed and synthesized (3 control sequences and 2 random substrate libraries). The four oligonucleotides that formed the five separate libraries are listed below. The three control sequences DEVD, DEVE, and DEVA differ only by one codon. The substrate libraries are X-5 and X-6. The construct for each library can be found in Figures 3 and 4, respectively. The sequence for the oligonucleotide encoding all five of these contructs are listed in table 2. The differing codon is underlined and in bold. The X-5 substrate library contains five random amino acid positions and a single fixed aspartate residue. The X-6 substrate library has six random amino acid positions and zero fixed

aspartate residues. Each random position is designated by the MNN codon. Oligonucleotides were ordered from Integrated DNA Technologies (IDT).

Each oligonucleotide was reconstituted in molecular grade water. One microgram of each oligonucleotide was phosphorylated and then annealed to a modified ss13 SAM33 DNA vector. The ratio of vector to oligonucleotide was 20 μg to 1 μg. Synthesis of closed, circular DNA occurred after the oligonucleotide and vector anneal. The DNA was then purified in a Microcon spin column and electroporated at 2.5 kV and 200 Ohms into *E. coli* SS320 electrocompotent cells. After electroporation, 1 mL of SOC media was added to the mixture of electroporated cells, and then transferred to a culture containing 30 mL of SOC media for 25 min at 37 degrees Celsius. A sample was taken from the culture after 25 min and the diversity of the library was determined. Diversity was determined by generating a 10-fold dilution series and placing each dilution on a plate to determine the phage titer. The remaining culture was was added to 1 L of 2XYT and placed in a shaking incubator at 37 degrees Celsius overnight. After the culture was grown overnight, the culture was centrifuged at 8,000 rpm for 20 min.

Table 2: Oligonucleotide Sequences and the libraries or sequences created

Name of Oligonucleotide/ Library	Sequence of Oligonucleotide (5' to 3')
DEVD	AGT TCT CGA AGG TCT AGA ACC **GTC** GAC CTC ATC TCC ACC TCC GTG ATG GTG ATG GTG ATG AGG CCT AGT CGA GGA GTG
DEVE	AGT TCT CGA AGG TCT AGA ACC **CTC** GAC CTC ATC TCC ACC TCC GTG ATG GTG ATG GTG ATG AGG CCT AGT CGA GGA GTG
DEVA	AGT TCT CGA AGG TCT AGA ACC **AGC** GAC CTC ATC TCC ACC TCC GTG ATG GTG ATG GTG ATG AGG CCT AGT CGA GGA GTG
Substrate (X-5)	AGT TCT CGA AGG TCT AGA **MNN ATC MNN MNN MNN MNN** TCC ACC TCC GTG ATG GTG ATG GTG ATG AGG CCT AGT CGA GGA GTG
Substrate (X-6)	AGT TCT CGA AGG TCT AGA **MNN MNN MNN MNN MNN MNN** TCC ACC TCC GTG ATG GTG ATG GTG ATG AGG CCT AGT CGA GGA GTG

X-5 Substrate Library Design

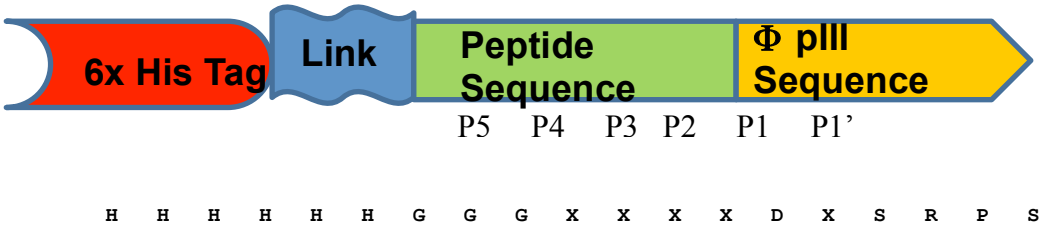

Figure 3: Amino acid representation of the X-5 peptide displayed with the pIII sequence (Image template was obtained from Dr. Paul Hamilton in a personal communication, 2014)

X-6 Substrate Library Design

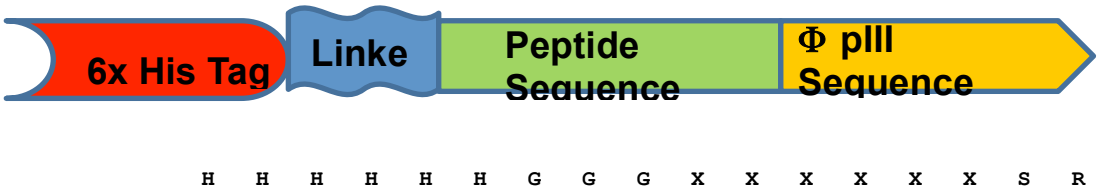

Figure 4: Amino acid representation of the X-6 peptide displayed with the pIII sequence (Image template was obtained from Dr. Paul Hamilton in a personal communication, 2014)

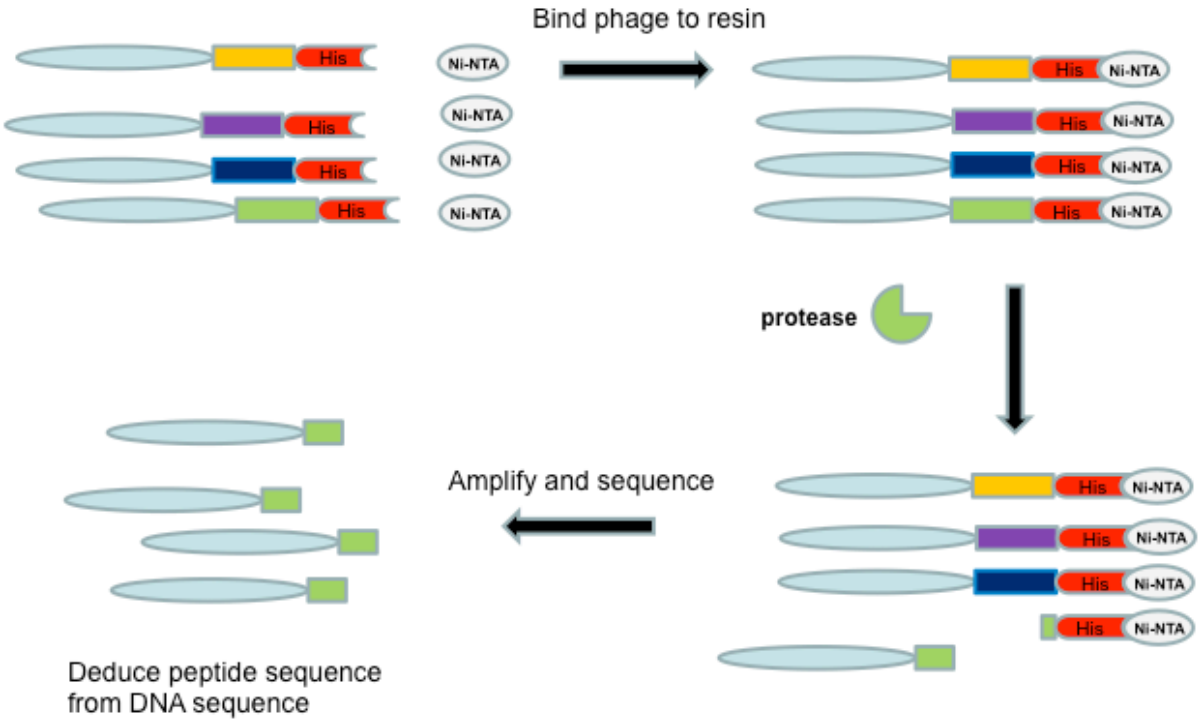

Figure 5: Figure represents a single round of selection in the M13 substrate selection protocol, from start to completion. (Image template was obtained from Dr. Paul Hamilton in a personal communication, 2014)

F. Phage Display: Caspase-3 Substrate Selection

One hundred μL of Ni-NTA sepharose bead solution was added to a microcentrifuge tube and washed with a blocking buffer solution. 1 mL of blocking buffer was added to the resin solution in the microcentrifuge tube. The tube was then centrifuged at 14,000 rpm for 1 min. The supernatant was extracted from the microcentrifuge tube and disposed of in a waste container. This buffer addition and subtraction step was repeated four times. The final blocking step involved leaving the blocking buffer on the column for 30 min at room temperature.

After the blocking buffer was applied and removed from the column four times, 150 μL of phage solution was incubated at room temperature for 45 min with the treated Ni-NTA sepharose bead solution. The addition of phage to the column marks the beginning of a round. Non-specifically bound and unbound phage were removed with 13 wash steps. 1 mL of wash buffer was added to the microcentrifuge tube. The tube was then spun down for 45 sec. The supernatant was removed and disposed of in a waste container, that occurred ten times. Next, three wash steps occurred with 1 mL of PBS. After, the was steps with PBS, the column was ready to be treated with the caspase-3 solution.

500 μL of 500 nM caspase-3 solution was added to the column and the reaction was carried out at room temperature for 4 h. After 4 h, the supernatant was removed and added to 3 mL of 2XYT media and 60 uL of *E. Coli* ER2738; the removal of supernatant marks the end of a round. Figure 5 concisely represents the steps taken before, during, and after a round of selection. Part of the supernatant is not added to the 3 mL of 2XYT media, but instead saved for serial dilution and spot titering. The 3 mL culture was grown for 4 h, shaking, and

then subjected to centrifugation at 4,000 rpm for 15 min. One hundred and fifty μL of supernatant was removed and added to 100 μL of Ni-NTA sepharose bead solution that was subject to four round of blocking buffer treatment. In total, there are five rounds of selection.

After each round of selection, serial dilutions of the final elution are plated on 2XYT plates, covered with 3 mL of 2XYT top agar containing 2% X-gal and 2% IPTG, and placed in an incubator for at least 16 h at 37 degrees Celsius. The number of plaque forming units present after each round of selection were counted and recorded. Sometimes, the plaques were cored and the DNA in the phage is analyzed.

Individual plaques from the plates were cored and grown in 3 mL of 2XYT for 4 h, shaking, at 37 degrees Celsius. After the four hour growth, the cultures were subject to centrifugation for 15 min at 12,000 rpm. Supernatant of the culture is added to a PCR reaction with both -96 and gene III primers. The PCR product was purified and DNA sequence analysis was preformed using only the gene III primer to generate a 250 bp PCR product that contained the sequence encoding the peptide.

G. Zebrafish Caspase-3 Activity Assay

Enzyme activity was measured utilizing a fluorometer and an external fluorophore attached to a peptide sequence. The activity of the protein was analyzed with caspase 3 being at a final concentration of 10 nM. 0.1% CHAPS, 10 mM DTT, and 1% Activity Buffer was utilized in each of the reactions. Substrate concentrations of Ac-DEVD-AFC utilized in the activity assays included 100, 75, 60, 50, 35, 25, 15, 10, 5, and zero micromolar. The activity assays were measured in triplicate.

Chapter 3

Results

A. Purification of Zebrafish Caspase-3

The purification of zebrafish caspase-3, from 6 Fernbach flasks, resulted in 4 mL of 100 mM caspase-3. The amino acid sequences of both human caspase-3 and zebrafish caspase-3 were compared and there was a 61% identity. Figure 7 displays the local BLAST between the two sequences. There were eleven instances where the two sequences shared four or more consecutive amino acid residues. Five of the eleven sequences contain at least one aspartic acid residue.

B. Crystallization of Zebrafish Caspase-3

The structure of wild type, cleaved zebrafish caspase-3 was determined to a resolution of 2.2 Angstroms using a molecular replacement model. The structure of zebrafish caspase-3 is shown in figure 6. In Figure 6, the homodimer of heterodimers is shown. Each monomer of the homodimer is composed of six beta strands, surrounded by five alpha helices. Heterodimers are composed of the large and small subunits.

Alignment in PyMOL of wild type zebrafish caspase-3 and wild-type human caspase-3 resulted in a RMSD value of 0.462. The alignment also uncovered a difference in the S_4 subsite between the two proteins. As seen in Figure 8, phenylalanine is present on loop four of human caspase-3. However, a threonine is present on loop four of zebrafish caspase-3 where the phenylalanine was located in human caspase-3.

C. Phage Display Control Sequences

The data found described in this section can be found in Table 3 and Table 4. When the control libraries were treated with 100 microliters of 1M imidazole, the subsequent titer resulted in pfu values ranging from $5.1X10^{10}$ pfu to $8.0\ X10^{10}$ pfu. Human caspase-3

treatments with each control sequence resulted in varying numbers. The DEVD sequence, when treated with human caspase-3, released phage correlating to a titer of 5.5 $X10^{10}$ pfu, a total equal to almost seventy percent of the imidazole elution. The DEVE and DEVA sequences did not result in the same pfu total when treated with human caspase-3. The DEVE caspase-3 sequence treatment resulted in the release of 5.0 $X10^{8}$ pfu, 1 percent of the imidazole elution. The DEVA caspase-3 sequence treatment resulted in the release of 9.8 $X10^{7}$ pfu, almost 0.2% of the imidazole elution. Zebrafish caspase-3 treatment of the DEVD control sequence resulted in the release of phage equal to a titer of 1.2 $X10^{10}$ pfu. That values equal to fifteen percent of the imidazole elution. The DEVE sequence, when treated with zebrafish caspase-3, released 2.5 $X10^{9}$ pfu. 2.5 $X10^{9}$ pfu is almost 5% of the imidazole of elution of the DEVE sequence. Zebrafish caspase-3 released phage correlating to a titer of 3.0e9 pfu, which is equal to almost 6% of the imidazole elution.

PBS elution of the various control libraries resulted in a range of fairly similar numbers. These numbers ranged from 9.5 $X10^{3}$ pfu to 3.5 $X10^{4}$ pfu. The PBS treatment of the DEVD sequence released 3.5 $X10^{4}$ pfu, which is 4.37 $X10^{-5}$ percent of the imidazole elution. The PBS treatment of the DEVE sequence released 9.5 $X10^{3}$ pfu, which is 1.82 $X10^{-7}$ percent of the imidazole elution. The PBS treatment of the DEVA sequence released phage correlating to a titer of 2.5 $X10^{4}$ pfu, which is 4.9 $X10^{-5}$ percent of the imidazole phage elution.

Figure 6: Cartoon image of wild-type zebrafish caspase-3 crystal structure inhibited with Ac-DEVD-CMK (inhibitor not shown).

```
 Score = 310 bits (795),  Expect = 1e-109, Method: Compositional matrix adjust.
 Identities = 147/241 (61%), Positives = 180/241 (75%), Gaps = 2/241 (1%)

Human  40   YSLNYPNIGHCIIINNKDFDRRTGMNPRNGTDVDAGNVMNVFRKLGYIVKVYNDQTVAQI  99
            Y ++YP +G CIIINNK+F + TGM  R+GTDVDA N+   FR L Y V+  ND T  +I
Zebra  37   YKMDYPEMGLCIIINNKNFHKSTGMTSRSGTDVDAANLRETFRNLKYEVRNKNDLTREEI  96

Human  100  MQVLTTVAHDDHSRCASLVCVLLSHGDEGVFFGTDTSVDLKSLTSLFRGDRCPSLVGKPK  159
            ++++  V+ +DHS+ +S VCVLLSHG+EG+ FGT+  VDLK +T+ FRGDRC SL GKPK
Zebra  97   VELMRDVSKEDHSKRSSFVCVLLSHGEEGIIFGTNGPVDLKKITNFFRGDRCRSLTGKPK  156

Human  160  LFFIQACRGTELDPGVETDHPDHPDIPDGRVRIPVEADFLYAYSTVPGYYSWRNTMTGSW  219
            LF IQACRGTELD G+ETD     D+     +IPVEADFLYAYST PGYYSWRN+  GSW
Zebra  157  LFIIQACRGTELDCGIETDSGVDDDMACH--KIPVEADFLYAYSTAPGYYSWRNSKDGSW  214

Human  220  FIQSLCEMMTKYGSELELLQIMTRVNHKVALDFESTSNMPGFDAKNQIPCIVSMLTKEMY  279
            FIQSLC M+ +Y  +LE + I+TRVN KVA +FES S    F AK QIPCIVSMLTKE+Y
Zebra  215  FIQSLCAMLKQYADKLEFMHILTRVNRKVATEFESFSFDATFHAKKQIPCIVSMLTKELY  274

Human  280  F  280
            F
Zebra  275  F  275
```

Figure 7: BLAST local alignment between the the primary protein sequence of human caspase-3 and zebrafish caspase-3

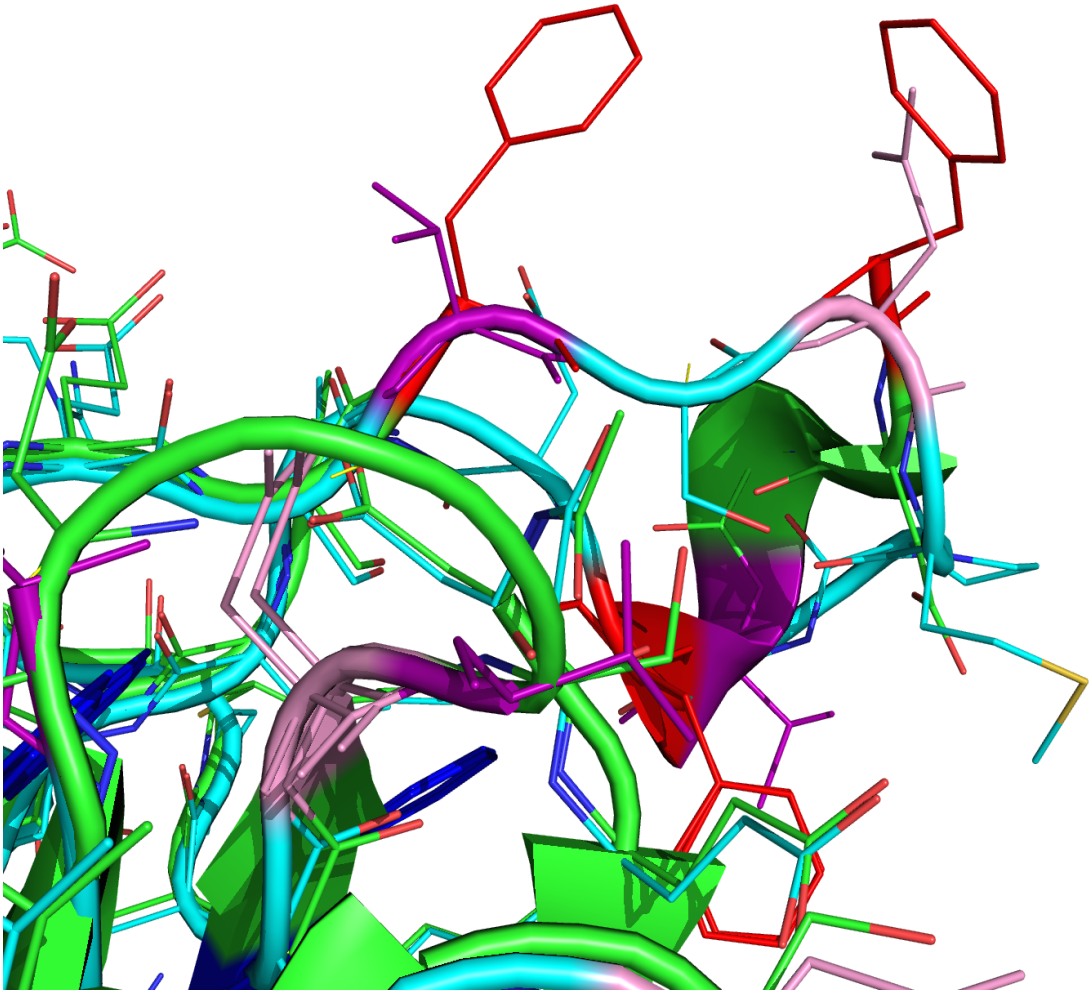

Figure 8: Image from an alignment of human caspase-3 and zebrafish caspase-3 illustrating the difference in the S4 subsite.

Table 3: Phage elution totals for control phage sequences: Positive Control, Human Caspase-3, Zebrafish Caspase-3, and Negative Control Treatments

	Imidazole (Positive Control)	Human Caspase-3	Zebrafish Caspase-3	PBS (Negative Control)
DEVD (Control)	$8.0 X10^{10}$	$5.5 X10^{10}$	$1.2 X10^{10}$	$3.5 X10^{4}$
DEVE (Control)	$5.2 X10^{10}$	$5.0 X10^{8}$	$2.5 X10^{9}$	$9.5 X10^{3}$
DEVA (Control)	$5.1 X10^{10}$	$9.8 X10^{7}$	$3.0 X10^{9}$	$2.5 X10^{4}$

Table 4: Percent phage eluted for control sequences per treatment (Human Caspase-3, Zebrafish Caspase-3, PBS) versus imidazole (positive control)

	Human Caspase-3	Zebrafish Caspase-3	PBS
DEVD (Control)	68.8%	15.0%	4.3e-5%
DEVE (Control)	9.6%	4.8%	1.83e-5%
DEVA (Control)	.2%	5.9%	4.9e-5%

D. Control Sequences Treated with Human Caspase-3 Bound to Inhibitor

As seen in Table 5, Human caspase-3, bound to Ac-DEVD-CMK, released phage resulting in a titer of 1.0 $X10^4$ pfu from the column containing the DEVD control sequence. Phage correlating to a titer of 8.82 $X10^3$ pfu were released when human caspase-3, bound to CMK, was applied to a column containing DEVE control sequence. Human caspase-3, bound to CMK, released phage correlating to a titer of 1.9 $X10^4$ pfu from a column.

Zebrafish caspase-3, bound to CMK, released phage correlating to a titer of 2.5 $X10^4$ pfu from the column containing DEVD control sequence. When CMK bound zebrafish caspase-3 was applied to the DEVE column, phage correlating to a titer of 9.5 $X10^3$ pfu were released from the column. Phage correlating to a titer of 4.1 $X10^4$ pfu were released from the DEVA column after zebrafish caspase-3, bound to CMK, was applied to the column.

E. Control Sequences Treated with Human Caspase-3 (D3A), with and without Inhibitor

Displayed in Table 6, control sequences DEVD, DEVE, and DEVA were treated with human caspase-3 (D3A). The column containing the DEVD sequence released phage correlating to a titer of 2.1 $X10^6$ pfu when exposed to the D3A human caspase-3. The column containing the DEVE sequence released phage correlating to a titer of 1.0 $X10^4$ when exposed to the D3A human caspase-3. Phage correlating to a titer of 9.8 $X10^3$ pfu were released when human caspase-3 was placed on the column containing the DEVA control sequence.

Displayed in Table 6, control sequences were exposed to human caspase-3 (D3A) plus CMK inhibitor. The DEVD control sequence, when exposed to D3A human caspase-3

plus CMK resulted in the release of phage correlating to a titer of 6.8 $X10^3$ pfu. Phage correlating to a titer of 8.8 $X10^3$ pfu were released when the column containing the DEVE control sequence was exposed to Human caspase-3 plus CMK. The column containing the DEVA control sequence released phage correlating to a titer of 1.0 $X10^4$ pfu when treated with the human caspase-3 (D3A) plus CMK inhibitor.

Table 5: Control sequences treated with wild type human caspase-3 plus CMK and zebrafish caspase-3 plus CMK

Control Sequences	Human caspase-3 + CMK	Zebrafish caspase-3 + CMK
DEVD	$1.0 X10^4$	$2.5 X10^4$
DEVE	$8.82 X10^3$	$9.5 X10^4$
DEVA	$1.9 X10^4$	$4.1 X10^4$

Table 6: Control sequences treated with Human caspase-3 (D3A) and Human caspase-3 (D3A) plus inhibitor

Control Sequence	Human Caspase-3 (D3A)	Human Caspase-3 (D3A) + CMK
DEVD	$2.1 X10^6$	$6.8 X10^3$
DEVE	$1.0 X10^4$	$8.8 X10^3$
DEVA	$9.8 X10^3$	$1.0 X10^4$

F. Substrate Libraries Control Experiments

Shown in Table 8, substrates library (X-5) and substrate library (X-6) were eluted with 250 mM imidazole. The imidazole released X-5 library phage correlating to a titer of 7.75 $X10^{10}$ pfu. The imidazole released phage yielding a titer of 3 $X10^{10}$ pfu for the X-6 library. PBS released 1.0 $X10^{5}$ pfu from the X-5 column, which is 1.2e-4 percent of the amount released by imidazole. The PBS treatment of the X-6 library resulted in the release of phage correlating to a titer of 3.6 $X10^{5}$ pfu, which is 1.2e-3 percent of the amount of phage released by imidazole.

G. X-5 Substrate Library Selection with Human Caspase-3 and PBS: Phage Titer

As seen in Table Table 7, when human caspase-3 was applied to the column containing X-5 substrate library, 3.0 $X10^{10}$ pfu were released from the column. After round 2 of phage enrichment selection, 1.2 $X10^{9}$ pfu were released after addition of human caspase-3. Round 3 human caspase-3 cleavage yielded phage correlating to a titer of $3X10^{9}$ pfu from the column. Phage correlating to a titer of 4.2 $X10^{9}$ pfu were released after the addition of human caspase-3 during round 4 of the phage enrichment selection. The final round of X-5 library enrichment selection with human caspase-3 resulted in the release of 1.0 $X10^{10}$ pfu from the column.

Also seen in Table 7, a separate experiment performed with PBS, instead of human caspase-3. The first round of X-5 selection with only PBS yielded phage release correlating to a titer of 2.1 $X10^{5}$ pfu. Round 2 of PBS selection yielded phage release correlating to a titer of 2.9 $X10^{5}$ pfu. Round 3 of phage enrichment utilizing the X-5 library and PBS treatment yielded phage release correlating to a titer of 6 $X10^{5}$ pfu. PBS released phage

correlating to a titer of $6X10^6$ pfu after round 4 of the X-5 library enrichment. Round 5 of the X-5 library enrichment released phage correlating to a titer of $8X10^6$ pfu when treated with PBS.

H. X-6 Substrate Library Selection with Human Caspase-3 and PBS: Phage Titer

Shown in Table 7, human caspase-3 was applied to a column containing the X-6 substrate library; after the first round of human caspase-3 exposure, phage correlating to a titer of 5.0 $X10^{10}$ pfu were released from the column. After round 2 of the X-6 enrichment experiment, phage correlating to a titer of 1.1 $X10^9$ pfu were released after human caspase-3 treatment. Human caspase-3 released phage correlating to a titer of 3.1 $X10^9$ pfu from the column containing X-6 library after round 3 of selection. Round 4 selections yielded a release of phage correlating to a titer of 2.5 $X10^9$ pfu after human caspase-3 was applied to the column containing the enriched X-6 phage library. Round 5, the final round of phage selection, yielded a release of phage correlating to a titer of 1.1 $X10^{10}$ pfu after human caspase-3 was applied to the column containing the X-6 phage library.

Also shown in Table 7, a separate enrichment experiment, utilizing PBS instead of human caspase-3, was carried out with the X-6 library. After round 1, PBS released phage correlating to a titer of 3.1 $X10^5$ pfu from the column containing the X-6 library. After round 2, PBS released phage correlating to a titer of 3.0 $X10^6$ pfu from the column containing the enriched X-6 library. PBS released phage correlating to a titer of 4.0 $X10^6$ pfu from the column containing X-6 library. After round 4, phage correlating to a titer of 8.0 $X10^6$ pfu were released after the column containing the enriched X-6 library was exposed to PBS. The final round, round 5, resulted in the release of phage correlating to a titer of 9.3 $X10^6$ pfu

after the column containing enriched X-6 library phage were exposed to PBS.

I. X-5 Substrate Library Selection with Human Caspase-3: Sequences

Table 9 displays all collected sequences from the five rounds of selection with the X-5 library. Rounds 1 & 2 of the X-5 substrate library selection process yielded sequences that all contained at least one aspartate residue between the glycine linker and fixed SRP in pIII viral coat protein. There were no conserved sequences found between the two rounds. However, round 2 contained two sequences containing more than two aspartate residues in the sequence. All three of the sequences contained two amino acids between the aspartate residues. Therefore, when the substrate sequences were aligned, the aspartates were in the P4 position and the P1 position. Table 10 contains all sequences aligned from P4-P1'.

Round 3 of the X-5 substrate library included sequences that all contain an aspartate. 14 sequences contained two aspartate residues. Thirteen of the fourteen sequences contained two amino acid residues between the aspartate residues. One of the fourteen sequences contained two consecutive aspartate residues. None of the sequences in round 3 appeared in round 1 or round 2.

All sequences from round 4 of the enriched X-5 substrate library selection with Human caspase 3 contained an aspartate residue. Fourteen sequences contained two aspartate residues. All fourteen of the sequences have two residues in between the aspartate residues. The sequences, DNLD and DLVD are conserved from round 3. None of the sequences in round 4 appeared in round 1 or round 2.

All sequences from round 5 of the enriched X-5 substrate library selection with human caspase-3 contained an aspartate residue. Twenty sequences contained two aspartate

residues. Nineteen of the twenty sequences have two amino acid residues between the aspartate residues. One of the twenty sequences has three amino acid residues between the aspartate residues. None of the sequences in round 5 appeared in round 1 or round 2. The sequences, LILD and DTSD are conserved from round 3. The sequence, DKMD is conserved from round 4. The sequences, DNLD and DLVD are conserved from round 3, round 4, and round 5.

Table 7: Phage released from columns containing X-5 and X-6 libraries. Phage released with either Human caspase-3 or PBS.

Substrate Library	Round 1	Round 2	Round 3	Round 4	Round 5
X-5	3.0e10	1.2e9	3.0e9	4.2e9	1.0e10
X-6	5.0e10	1.1e9	3.1e9	2.5e9	1.1e10
X-5(PBS)	2.1e5	2.9e5	6.0e5	6.0e6	8.0e6
X-6(PBS)	3.1e5	3.0e6	4.0e6	8.0e6	9.3e6

Table 8: Phage released from columns containing X-5 and X-6 libraries. Phage released with Positive Control (imidazole) or Negative Control (PBS).

Substrate Library	Imidazole (Positive)	PBS (Negative)
Substrate (X-5)	7.75e10	1.0e5
Substrate (X-6)	3e10	3.6e5

Table 9: Amino Acid Sequences collected from all five rounds of the X-5 phage selection using Human caspase-3.

Sequence #	Round 1	Round 2	Round 3	Round 4	Round 5
1	VAQPDS	FDLLDT	PDGVDS	NDLVDS	SDLVDS
2	IYPSDS	FGMLDS	GDLSDS	LDISDS	LNILDS
3	AWVQDS	VISVDS	LVGSDG	GDEPDA	SGLSDS
4	NIYNDS	GHLVDS	ILILDL	IISSDS	GDNLDT
5	SAQPDS	PSLLDS	ADQSDS	IDLVDS	IDNLDS
6	TPSLDS	TDAMDS	ISMADI	LIYWDS	TDTSDS
7	WNIGDL	PNAVDS	LDGSDS	GSELDG	ADENDS
8	VKLPDS	AQAMDS	GSPSDS	GDSTDG	LDNLDS
9		IVAPDS	WTIPDS	QDTSDG	GDTPDS
10		HNILDA	IDRPDT	GDNLDS	GINLDT
11			QDIVDG	SDNLDS	SDNLDG
12			GIQQDG	LDATDS	IYVTDS
13			TVRMDS	LLAIDS	LDNLDS
14			GDTSDG	PTAMDG	SDNLDG
15			TDNLDS	TGRIDS	NTNLDS
16			GNESDG	IDHLDT	GPWVDS
17			PNLSDS	VNILDS	GDNLDG
18			IGGQDS	GDLQDS	SWTFDS
19			LLILDG	SDSPDS	GDILDL
20			IDVGDS	TDNLDS	LDNLDS
21			TSQRDS	TDLVDA	TDSFDS
22			ADNLDS	FLGNDS	AISLDS
23			PDLVDS	SLLTDS	ADGPDS
24			IPINDT	PTFSDS	PDLYDS
25			TGLDDS	TSTSDS	WDKMDT
26			GDLTDS	GGAPDS	PILVDS
27			PDNLDG	VDKMDT	PTSRDG
28			TIPGDS	PARPDS	TGGIDS
29			TGGQDS	TDAPDS	TLILDS
30			LIILDT	TDQNDS	TDNLDS
31			PPTSDS	TPIRDS	GDARDS
32			VGTNDS	VLINDG	PYMKDI
33			KINTDT	IPNHDS	LTNIDN
34			GHNLDS	MIPLDT	VPILDT
35			CIPNDS	VDNLDT	LGNLDG

Table 10: Amino Acid Sequences collected from all five rounds of the X-5 phage selection using Human caspase 3.

Sequence #	Round 1	Round 2	Round 3	Round 4	Round 5
1	AQPDS	DLLDT	DGVDS	DLVDS	DLVDS
2	YPSDS	GMLDS	DLSDS	DISDS	NILDS
3	WVQDS	ISVDS	VGSDG	DEPDA	GLSDS
4	IYNDS	HLVDS	LILDL	ISSDS	DNLDT
5	AQPDS	SLLDS	DQSDS	DLVDS	DNLDS
6	PSLDS	DAMDS	SMADI	IYWDS	DTSDS
7	NIGDL	NAVDS	DGSDS	SELDG	DENDS
8	KLPDS	QAMDS	SPSDS	DSTDG	DNLDS
9		VAPDS	TIPDS	DTSDG	DTPDS
10		NILDA	DRPDT	DNLDS	INLDT
11			DIVDG	DNLDS	DNLDG
12			IQQDG	DATDS	YVTDS
13			VRMDS	LAIDS	DNLDS
14			DTSDG	TAMDG	DNLDG
15			DNLDS	GRIDS	TNLDS
16			NESDG	DHLDT	PWVDS
17			NLSDS	NILDS	DNLDG
18			GQDIS	DLQDS	WTFDS
19			LILDL	DSPDS	DILDL
20			DVGDI	DNLDS	DNLDS
21			SQRDS	DLVDA	DSFDS
22			DNLDS	LGNDS	ISLDS
23			DLVDS	LLTDS	DGPDS
24			PINDT	TFSDS	DLYDS
25			GLDDS	STSDS	DKMDT
26			DLTDS	GAPDS	ILVDS
27			DNLDG	DKMDT	TSRDG
28			IPGDS	ARPDS	GGIDS
29			GGQDS	DAPDS	LILDS
30			IILDT	DQNDS	DNLDS
31			PTSDS	PIRDS	DARDS
32			GTNDS	LINDG	YMKDI
33			INTDT	PNHDS	TNIDN
34			HNLDS	IPLDT	PILDT
35			IPNDS	DNLDT	GNLDG

J. X-5 Substrate Library Selection with PBS: Sequences

Sequences from enriched X-5 substrate phage library when treated with PBS, taken from various rounds of the selection, contained an aspartate in a majority of the samples. The sequences can be observed in Table 11. In round 1, five of the seven sequences contained an aspartate residue. The two sequences that did not contain an aspartate residue were mutated phage, missing part of the glycine linker, most of the random residues (including the fixed aspartate), and part of the histidine tag.

Round 2 yielded sequences that contained aspartate residues in six of the seven sequences analyzed. The single sequence had a partially deleted glycine linker (one residue missing) and two random amino acid positions were deleted. There were no conserved sequences from round 1 found in round 2.

Round 3 sequences contained aspartate residues in four of the seven sequences analyzed. One of the three sequences that did not contain aspartate also lacked an entire glycine linker. Two of the three sequences missing an aspartate had all but two amino acids deleted from the region between the linker and the fixed SRP. The fixed aspartate was deleted in both cases. There were no sequences conserved from round 1 or round 2.

Round 4 sequences contained aspartate residues in four of the eight sequences analyzed. Two of the four sequences missing an aspartate residue were also missing some of the glycine linker, most of the random residues, and histidine tag. The other two sequences were also missing most the random residues, including the fixed aspartate, but had an intact glycine linker and histidine tag. There were no sequences conserved from round 1, round 2, or round 3.

Round 5 sequences contained aspartate residues in five of the 8 eight sequences analyzed. Two of the three sequences had numerous deleted portions. The entire glycine linker was deleted, most of the random residues, and all but two residues in the histidine tag. The other sequence lacking an aspartate was also missing most of the random sequence, but maintained a glycine linker and histidine tag. The sequence, WTYD was found in round 5 and also round 3.

K. X-6 Substrate Library Selection with Human Caspase-3: Sequences

As seen in Table 12, all sequences from round 1 of the X-6 substrate library selection with Human caspase 3 contained an aspartate in the random region. Only one sequence out of ten contained two aspartates. That single sequence contained two amino acid residues between the aspartate residues. Only one of the sequences analyzed utilized the glycine linker as part of the recognition sequence before cleavage.

All sequences from round 2 of the X-6 substrate library selection with Human caspase 3 contained an aspartate residue in the random region. None of the sequences analyze contained more than one aspartate residue. There are no sequences from round 2 that match a sequence analyzed from round 1.

All thirty-eight sequences from round 3 of the X-6 substrate library selection with Human caspase 3 contained an aspartate residue in the random region. None of the sequences analyzed from round 3 appeared in round 1 or round 2. Ten of the thirty-eight sequences utilized the fixed residues surrounding the random substrate region for sequence recognition purposes. Seen in Table 13, thirteen of the thirty-eight sequences contained two aspartate residues in the random region. All thirteen sequences that contain two aspartate residues have

exactly two residues between the aspartate residues. One sequence contains three aspartate residues.

All thirty-seven sequences from round 4 of the X-6 substrate library selection with human caspase-3 contained an aspartate residue in the random region. Sixteen sequences contain two aspartate residues in the random region. As seen in Table 13, all sixteen of those sequences contain two amino acid residues between the two-aspartate residues. There is one sequence that contains three aspartate residues. DLVD and DNLD are two sequences that appear in round 4 and round 3. There are no sequences in round 4 that appear in round 1 or round 2.

All sequences analyzed from round 5 of the X-6 substrate library selection with Human caspase 3 contained an aspartate in the random region. Twenty-two sequences contain two aspartate residues. Twenty-one of those twenty-two sequences have aspartates separated by two amino acid residues. The sequences DLVD and DNLD are found in round 3, round 4, and round 5. The sequences DVSD, DESD, and DGTD are found in round 3 and round 5. There is one sequence, GGVD that is found in round 1 and round 5. There are no sequences found in both round 2 and round 5.

Table 11: Sequences from released from five rounds PBS treatment to X-5 and X-6 libraries. Sequences are aligned from tag to fixed SRP region.

#	Round 1 (X-6)PBS	Round 2	Round 3	Round 4	Round 5
1	HHHHHHG GGWYTFAA SRP	HHHGGGIPY DFASRP	HHHHHHSSP RHSRP	GGGHHKYV F	HHHHHH GGGIIAY PNSRP
2	HHHGGIYW SRP	HHHGGTIYS RP	HHHHHHGG GTTSSWSRP	HHHHHHGG GPHTPASRP	HHHHHH GPISRP
3	HHHHHHG GGTWPIFAS RP	HHHHHHGG GNMTCSWSR P	HHHHHHGG MITGWSRP	HHHHHHGG VNSRP	HHHHHH GGGPLK SRP
4	HHHHHHG GGIPFSRP	GGAPNSHHH H	GGGWMFGIP SRPHHHHHH	GGITRKSNS RPHHHH	GGGFYIS AWSRPH HHHHH
5	HHHHHHG GGPWVGH RSRP	HHHHHHGG GVGGYTSRP	HHHGPKHIP RFSRP	HHHGGIPPF HNSRP	HHHGGG PAQWYI SRP
#	Round 1 (X-5) PBS	Round 2	Round 3	Round 4	Round 5
1	HHHGGAGI SRP	HHHHHHGG YKIRSRP	HHHHHHPIW TTTSRP	HHGGYSRP	HHTWCS RP
2	HHHHHGG YGGGSRP	HHHHHHGG GFGHIDPSRP	HHHHHHGG GICSRP	HHHGRPAS RP	HHWAPR SRP
3	HHHHHHG GGGGGPD MSRP	HHHHHHGG GASCYDRSR P	HHHHHHGG GNWSRP	HHHHHHGG GWPISSRP	HHHHHG GGITSRP
4	HHHHHHG GGHVTYDA SRP	HHHHHHGG GGGPKDTSR P	HHHHHHGG GLGRWDQS RP	HHHHHHGG GYSSRP	HHHHHH GGGWW YTDFSRP
5	HHHHHHG GGPLKHDE SRP	HHHHHHGG GGIGSDASRP	HHHHHHGG GVCTYDASR P	HHHHHHGG GYRLIDQSR PGLGTDISR P	HHHHHH GGGPLIT DRSRP
6	HHHHHHG GGYRFGDC SRP	HHHHHHGG GGLPFDFSRP	HHHHHHGG GGGYRDMS RP	HHHHHHGG GPNIKDKSR P	HHHHHH GGGHHT FDISRP
7	HHHHHHG GGKLIPDTS RP	HHHHHHGG GGGGTDPSR P	HHHHHHGG GPWTYDSSR P	HHHHHHGG GWQASDI	HHHHHH GGGREY PDISRP

Table 12: Amino Acid Sequences collected from X-6 phage selection using Human caspase 3.

Sequence Number	Round 1	Round 2	Round 3	Round 4	Round 5
1	GLTTDT	GHNLDS	QTSIDS	DMKDTG	GISLDS
2	IARDSS	TSSPDH	GKSYSD*	FNTQLD*	IDTLDS
3	LLTDSI	TINMDS	AADGAD*	PARPDS	PDGTDS
4	TNGDST	QMLDLT	NIGLDS	AGDGS*	ADALDS
5	TIQGDS	FLGDNI	RDLVDT	LDVLDA	LVSEDS
6	RAHSDS	SLMDST	INTDSD*	LSDAVD*	IDLEDS
7	PGPDTT	NTPDGG	LSDGAP*	GDTSDA	YADAED*
8	DARDST	TDSFGI*	EVGDSV	IDTTDV	VDGLDS
9	GGLDSI		LDVLDS	FNIEDA	MMGEDG
10	VDILTT*		INSVDA	WILTDS	NDLVDA
11			GTQSD*	LDLADS	NMSMSD*
12			NDETDS	MHSYAD*	TEERPD*
13			VDVSDT	YITSDS	NLSFED*
14			TTLSDS	LDLLDS	IDESDS
15			STLLDS	IMLSDS	VDNLDS
16			PNVSDS	DAVDTP	LATPLD*
17			GIDESD*	DASTSN*	GGDNLD*
18			LPYVDS	LDNVDT	SLVDSA
19			SWDDWD*	TDLTDS	DVSDAT
20			QDGTDA	DTNDAY	GTFSDS
21			GRPDSA	PYWDST	ADNLDT
22			NDLIDS	ITNDGS	DNLDGS
23			WPNIVD*	DVLDSR	GGSLDS
24			GIDNLD*	LILDGI	DENDSS
25			APIDTI	INTDSG	TSTDSA
26			PDISDS	LISSDS	PDLTDT
27			SSPNGD*	RHVLDT	DSSVFD*
28			GNDIPN *	ADQPDG	LYDSTS*
29			DNIDAT	PSLDSA*	HNLDSI
30			SSNIDT	DQADIS	GGVDVS
31			DNLDSS	GDNLDS	GQAMDS
32			PYETID*	TFSDSG	DVLDSG
33			GGNYDA	GEPDAS	GGDNLDS
34			PRIDTS	VDSVDA	IDTLDG
35			VDTGIA	GDDVDS	VDNLDS
36			DNLDTA	DTSFHI*	IIDNLD*
37			GNHLID*	HPDSTA*	VEHLDA
38			VILLDS		

Table 13: Amino Acid Sequences collected from X-6 phage selection using Human caspase 3. The sequence represented from P4 through the P1' position

Sequence Number	Round 1	Round 2	Round 3	Round 4	Round 5
1	LTTDT	HNLDS	TSIDS	DMKDT	ISLDS
2	IARDS	SSPDH	SYSDS	TQLDS	DTLDS
3	LLTDS	INMDS	DGADS	ARPDS	DGTDS
4	TNGDS	QMLDL	IGLDS	GAGDG	DALDS
5	IQGDS	FLGDN	DLVDT	DVLDA	VSEDS
6	AHSDS	SLMDS	TDSDS	DAVDS	DLEDS
7	PGPDT	NTPDG	GLSDG	DTSDA	DAEDS
8	DARDS	GGTDS	EVGDS	DTTDV	DGLDS
9	GGLDS		DVLDS	NIEDA	MGEDG
10	GGVDI		NSVDA	ILTDS	DLVDA
11			TQSDS	DLADS	SMSDS
12			DETDS	SYADS	ERPDS
13			DVSDT	ITSDS	SFEDS
14			TLSDS	DLLDS	DESDS
15			TLLDS	MLSDS	DNLDS
16			NVSDS	DAVDT	TPLDS
17			DESDS	GGGDA	DNLDS
18			PYVDS	DNVDT	SLVDS
19			DDWDS	DLTDS	DVSDA
20			DGTDA	DTNDA	TFSDS
21			GRPDS	PYWDS	DNLDT
22			DLIDS	ITNDG	DNLDG
23			NIVDS	DVLDS	GSLDS
24			DNLDS	LILDG	DENDS
25			APIDT	INTDS	TSTDS
26			DISDS	ISSDS	DLTDT
27			PNGDS	HVLDT	SVFDS
28			GGNDI	DQPDG	GLYDS
29			DNIDA	PSLDS	HNLDS
30			SNIDT	DQADI	GGVDV
31			DNLDS	DNLDS	QAMDS
32			ETIDS	TFSDS	DVLDS
33			GNYDA	GEPDA	DNLDS
34			PRIDT	DSVDA	DTLDG
35			GGVDT	DDVDS	DNLDS
36			DNLDT	GGGDT	DNLDS
37			HLIDS	GHPDS	EHLDA
38			ILLDS		

L. X-6 Substrate Library Selection with PBS: Sequences

As seen in Table 11, out of the twenty-five X-6 substrate library sequences analyzed, only one of the sequences contained an aspartate residue. The single sequence containing an aspartate residue was from the second round.

Five rounds of sequences contain numerous flaws deviating from the design. For instance, there are residues from the glycine bridge deleted, or the entire glycine bridge is deleted. Some sequences have fewer than six residues in the random or substrate region. Various sequences from the PBS selection have part or the entire fixed SRP region deleted from the sequence. Numerous sequences from the PBS selection are missing part or all of the histidine tag.

A number of sequences had only one of these partial or whole deletion events; however, some sequences had multiple deletion events. For instance, sequence 5 from round 4 has a single glycine deleted from the glycine and three histidine residues deleted from the histidine tag.

M. X-5 Substrate Library Selection with Zebrafish Caspase-3 and PBS: Phage Titer

As seen in Table 14, in an experiment similar to the human caspase-3 enrichment the X-5 substrate library was enriched over a series of five rounds utilizing zebrafish caspase-3. After the first round, phage correlating to a titer of $1.2X10^{10}$ pfu were released by zebrafish caspase-3 from the column containing X-5 substrate library. After round 2, phage correlating to a titer of $1.9\ X10^{9}$ pfu were released by zebrafish caspase 3. After zebrafish caspase-3 was applied to a column containing enriched X-5 substrate library phage, phage correlating to a titer of $1.0\ X10^{9}$ pfu were released from the column. After round 4, phage correlating to a

titer of 7.3 $X10^9$ pfu were released from a column containing enriched phage by zebrafish caspase-3. After the final round, round 5, phage correlating to a titer of 9.8 $X10^9$ pfu were released from the column containing enriched phage.

In a separate experiment, X-5 substrate library was enriched over a series of five rounds, utilizing PBS. After round 1, phage correlating to a titer of 4.1 $X10^5$ pfu were released with PBS from a column containing X-5 substrate library. PBS released phage correlating to a titer of 2.95 $X10^5$ pfu from a column containing enriched X-5 substrate library, after round 2. After round 3, PBS released phage correlating to a titer of 8.4 $X10^5$ pfu from a column containing enriched X-5 substrate library. After round 4, PBS released phage correlating to a titer of 1.1 $X10^6$ pfu from a column containing enriched X-5 substrate library. After round 5, the final round of selection, PBS released phage correlating to a titer of 9.3 $X10^6$ pfu from a column containing enriched X-5 substrate library.

N. X-6 Substrate Library Selection with Zebrafish Caspase-3 and PBS: Phage Titer

As seen in Table 14, X-6 substrate library was enriched over a series of five rounds utilizing zebrafish caspase-3. After the first round, phage correlating to a titer of 9.9 $X10^9$ pfu were released by zebrafish caspase-3 from the column containing X-6 substrate library. After round 2, phage correlating to a titer of 1.5 $X10^9$ pfu were released by zebrafish caspase-3. After zebrafish caspase-3 was applied to a column containing enriched X-6 substrate library phage, phage correlating to a titer of 2.6 $X10^9$ pfu were released from the column. After round 4, phage correlating to a titer of 4.5 $X10^9$ pfu were released from a column containing enriched phage by zebrafish caspase 3. After the final round, round 5, phage correlating to a titer of $1.4X10^{10}$ pfu were released from the column containing enriched phage by zebrafish caspase-3.

In a separate experiment, as seen in Table 14, X-6 substrate library was enriched over a series of five rounds, utilizing PBS. After round 1, phage correlating to a titer of 3.1 $X10^4$ pfu were released with PBS from a column containing X-6 substrate library. PBS released phage correlating to a titer of 5.3 $X10^6$ pfu from a column containing enriched X-6 substrate library, after round 2. After round 3, PBS released phage correlating to a titer of 4.5 $X10^6$ pfu from a column containing enriched X-6 substrate library. After round 4, PBS released 7.2 $X10^6$ pfu from a column containing enriched X-6 substrate library. After round 5, the final round of selection, PBS released phage correlating to a titer of 9.3 $X10^6$ pfu from a column containing enriched X-6 substrate library.

O. X-5 Substrate Library Selection with Zebrafish Caspase 3: Sequences

As seen in Table 15, sequences from round 3, round 4, and round 5 of the X-5 library selection, using zebrafish caspase-3, all contained an aspartate in the region between the glycine linker and fixed SRP. Table 16 lists sequences starting with the P4 position and ending with the P1' position. Six sequences analyzed in the third round contain two aspartate residues. Five of these six sequences contain two amino acid residues between each aspartate. Fifteen sequences contained a valine and an aspartate separated by two amino acid residues. The valine is N-terminal to the aspartate residue.

In round 4, thirteen sequences contained two aspartate residues. Eleven of the thirteen sequences have two amino acid residues separating the aspartate residues. Eleven sequences analyzed in round 4 contain a valine and aspartate residue separated by two amino acid residues. The valine is N-terminal to the aspartate residue. The sequences, VLPD, DTFD, DNLD and VLRD appear in round 4 as well as round 3.

In round 5, there are thirteen sequences that contain two aspartates. Twelve of the thirteen sequences have two amino acid residues between the aspartate residues. Six sequences contain two amino acid residues between a valine residue and an aspartate residue. The valine residue is N-terminal to the aspartate residue. The sequence, TTLD, is found in round 3 and round 5. The sequences, DNTD, YFTD, and VEAD are found in round 4 and in round 5. As seen in Table 16, the sequences, DNLD and VLRD are found in round 3, round 4, and round 5.

P. X-6 Substrate Library Selection with Zebrafish Caspase-3: Sequences

As seen in Table 17, sequences from round 3, round 4, and round 5 of the X-6 library selection, using zebrafish caspase-3, all contained an aspartate in the region between the glycine linker and the fixed SRP. Five sequences analyzed in the third round contain two aspartate residues. Four of these five sequences contain two amino acid residues between each aspartate. Five sequences contained a valine and an aspartate separated by two amino acid residues. The valine is N-terminal to the aspartate residue.

In round 4, ten sequences analyzed contained two aspartate residues. Seven of the ten sequences contain two amino acid residues between the aspartate residues. Six sequences contain an aspartate residue and valine residue separated by two amino acid residues. The valine is N-terminal to the aspartate residue. The sequences, DNLD and TYTD are found in both round 3 and round 4.

In round 5, the final round of selection, eight sequences contain two aspartate residues. Six of the eight sequences contain two amino acid residues separating two aspartate residues. Eleven sequences contain a valine residue and an aspartate residue separated by two

amino acid residues. The valine residue is N-terminal to the aspartate residue. The sequences, VELD and VLPD are found in round 3 sequences and round 5 sequences. As seen in Table 18, the sequences, DNLD and TYTD are found in round 3, round 4, and round 5.

Table 14: Phage released from columns containing X-5 and X-6 libraries after Zebrafish caspase-3 or PBS treatment.

Substrate Library	Round 1	Round 2	Round 3	Round 4	Round 5
X-5	1.2e10	1.9e9	1.0e9	7.3e9	9.8e9
X-6	9.9e9	1.5e9	2.6e9	4.5e9	1.4e10
X-5(PBS)	4.1e5	2.9e5	8.4e5	1.1e6	8.3e6
X-6(PBS)	3.1e4	5.3e6	4.5e6	7.2e6	9.3e6

Table 15: Amino Acid Sequences collected from X-5 phage selection using Zebrafish caspase 3.

Sequence #	Round 3	Round 4	Round 5
1	LTTLDS	GVAADT	GTGSDS
2	RVTEDS	GTSFDS	YVLRDT
3	SDMSDS	TVLPDS	MDNTDF
4	LVSNDN	YVLRDS	IVVSDS
5	GITVDS	LDNPDS	GLSNDN
6	GDSTDI	YVLRDT	LTTLDS
7	NVVYDT	PDRTDL	GAITDS
8	LVTDDS	PSSPDN	GDNADS
9	LSSYDG	MDNTDS	GHIVDS
10	ITVSDS	ITDLDS	GDGLDA
11	RPHQDS	KDQLDS	GVSTDH
12	TVLPDT	MVAADG	LVGTDS
13	AVAVDN	GYFTDS	GDTTDS
14	GDVPDS	YVLRDT	GGDLDA
15	YVLPDS	GPAMDS	GYFTDS
16	AGEPDT	GTSHDS	GGSTDH
17	LVHPDS	GVRPDS	YVLRDT
18	PGNIDT	IVEADT	GDTTDS
19	AVISDS	GHDLDS	AGIPDS
20	IVTTDS	IDNLDA	GDEPDT
21	TVLADT	GGAIDT	GDNLDS
22	AVRMDS	GDNADS	KRMVDT
23	GGITDS	YVLRDG	GDALDT
24	GVASDQ	GVLRDT	ADNLDS
25	GLITDS	GIHVDT	GDHLDT
26	PYSIDA	AGISDT	MMILDL
27	TDNLDS	GDYEDS	KLAIDS
28	GVAHDS	GHDLDS	TVEADS
29	AVAVDT	TDTFDS	ADNLDS
30	TDTFDI	KDQLDT	GDELDG
31	TSLVDG	YVLRDT	AMILDL
32	YVLRDG	TVLPDS	GDILDT
33	GILTDS	TDMSDT	VAGIDS
34	QFEIDI	SDNLDS	ALTLDS
35	VNIVDG	LTTVDA	VQNLDT

Table 16: Amino Acid Sequences collected from X-5 phage selection using Zebrafish caspase 3.

Sequence #	Round 3	Round 4	Round 5
1	TTLDS	VAADT	TGSDS
2	VTEDS	TSFDS	VLRDT
3	DMSDS	VLPDS	DNTDF
4	VSNDN	VLRDS	VVSDS
5	ITVDS	DNPDS	LSNDN
6	DSTDI	VLRDT	TTLDS
7	VVYDT	DRTDL	AITDS
8	VTDDS	SSPDN	DNADS
9	SSYDG	DNTDS	HIVDS
10	TVSDS	TDLDS	DGLDA
11	PHQDS	DQLDS	VSTDH
12	VLPDT	VAADG	VGTDS
13	VAVDN	YFTDS	DTTDS
14	DVPDS	VLRDT	GDLDA
15	VLPDS	PAMDS	YFTDS
16	GEPDT	TSHDS	GSTDH
17	VHPDS	VRPDS	VLRDT
18	GNIDT	VEADT	DTTDS
19	VISDS	HDLDS	GIPDS
20	VTTDS	DNLDA	DEPDT
21	VLADT	GAIDT	DNLDS
22	VRMDS	DNADS	RMVDT
23	GITDS	VLRDG	DALDT
24	VASDQ	VLRDT	DNLDS
25	LITDS	IHVDT	DHLDT
26	YSIDA	GISDT	MILDL
27	DNLDS	DYEDS	LAIDS
28	VAHDS	HDLDS	VEADS
29	VAVDT	DTFDS	DNLDS
30	DTFDI	DQLDT	DELDG
31	SLVDG	VLRDT	MILDL
32	VLRDG	VLPDS	DILDT
33	ILTDS	DMSDT	AGIDS
34	FEIDI	DNLDS	LTLDS
35	NIVDG	TTVDA	QNLDT

Table 17: Amino Acid Sequences collected from X-6 phage selection using Zebrafish caspase 3.

Sequence #	Rd 3	Rd 4	Rd 5
1	GGMRDV	GGVRPD*	SVNLDT
2	TLVQDL	GAIDTT	TTGIDM
3	GCGIWD*	DNADST	KSTDGG
4	IRILDL	IHVDSG	VLNAND*
5	GDYADV	GHDLDS	DSNDGR
6	IVELDL	VGNDTG	TVELDT
7	DSQDNQ	DEYDST	DERSDT
8	TVLDLI	GDHLDS	DQLDST
9	NVSAFD*	DGLDIT	PVLPDG
10	IFDKDA	GYFTDS	NLKIDG
11	HVLPDR	GLSTDH	PTPDRI
12	PARSDL	GDTTDS	LATYTD*
13	AVDGSD*	GIHPDG	GGVLRD*
14	GGYSID*	GDPDTS	VREDSG
15	GGLDST	KLADSS	ALTYDT
16	GITDAT	MILDLR	GGVLRD*
17	VRMDSS	DHLDPS	VLADSS
18	AGGTDS	GDNLDI	NQIDST
19	SDNLDT	TSHDSG	RHTSDV
20	GGVTTD*	VLDLSI	STDNLD*
21	GVLDTS	GPAMDS	GNIDSS
22	SILLDG	VRPDST	AVGNDT
23	GYEEDY	LATYTD*	SVTEDS
24	GNSDYA	MVEADS	VLEDSP
25	LNTQID*	VTSDER	HPDLDV
26	GTTDST	PIQEDS	FDNTDG
27	GGADAG	VILDLP	VVYDTS
28	LATYTD*	DNLDGT	PSSPDN
29	GNTLDY	GQMDLQ	LDSTDI
30	GNVDAT	WVINPD*	VNILDS
31	LILVDS	TSNLDN	AVQLPD*
32	TGVELD*	VVTNLD*	NDGVIP*
33	NVILLD*	ILIPDA	IDNLDG

Table 18: Amino Acid Sequences collected from X-6 phage selection using Zebrafish caspase 3.

Sequence #	Rd 3	Rd 4	Rd 5
1	GMRDV	VRPDS	VNLDT
2	LVQDL	GAIDT	TGIDM
3	GIWDS	DNADS	KSTDG
4	RILDL	IHVDS	NANDS
5	DYADV	HDLDS	DSNDG
6	VELDL	VGNDT	VELDT
7	DSQDN	DEYDS	ERSDT
8	TVLDL	DHLDS	DQLDS
9	SAFDS	DGLDI	VLPDG
10	FDKDA	YFTDS	LKIDG
11	VLPDR	LSTDH	PTPDR
12	ARSDL	DTTDS	TYTDS
13	DGSDS	IHPDG	VLRDS
14	YSIDS	GDPDT	VREDS
15	GGLDS	KLADS	LTYDT
16	GITDA	MILDL	VLRDS
17	VRMDS	DHLDP	VLADS
18	GGTDS	DNLDI	NQIDS
19	DNLDT	TSHDS	HTSDV
20	VTTDS	GVLDL	DNLDS
21	GVLDT	PAMDS	GNIDS
22	ILLDG	VRPDS	VGNDT
23	YEEDY	TYTDS	VTEDS
24	GNSDY	VEADS	VLEDS
25	TQIDS	VTSDE	PDLDV
26	GTTDS	IQEDS	DNTDG
27	GGADA	VILDL	VVYDT
28	TYTDS	DNLDG	SSPDN
29	NTLDY	GQMDL	DSTDI
30	GNVDA	INPDS	NILDS
31	ILVDS	SNLDN	QLPDS
32	VELDS	TNLDS	GGNDG
33	ILLDS	LIPDA	DNLDG

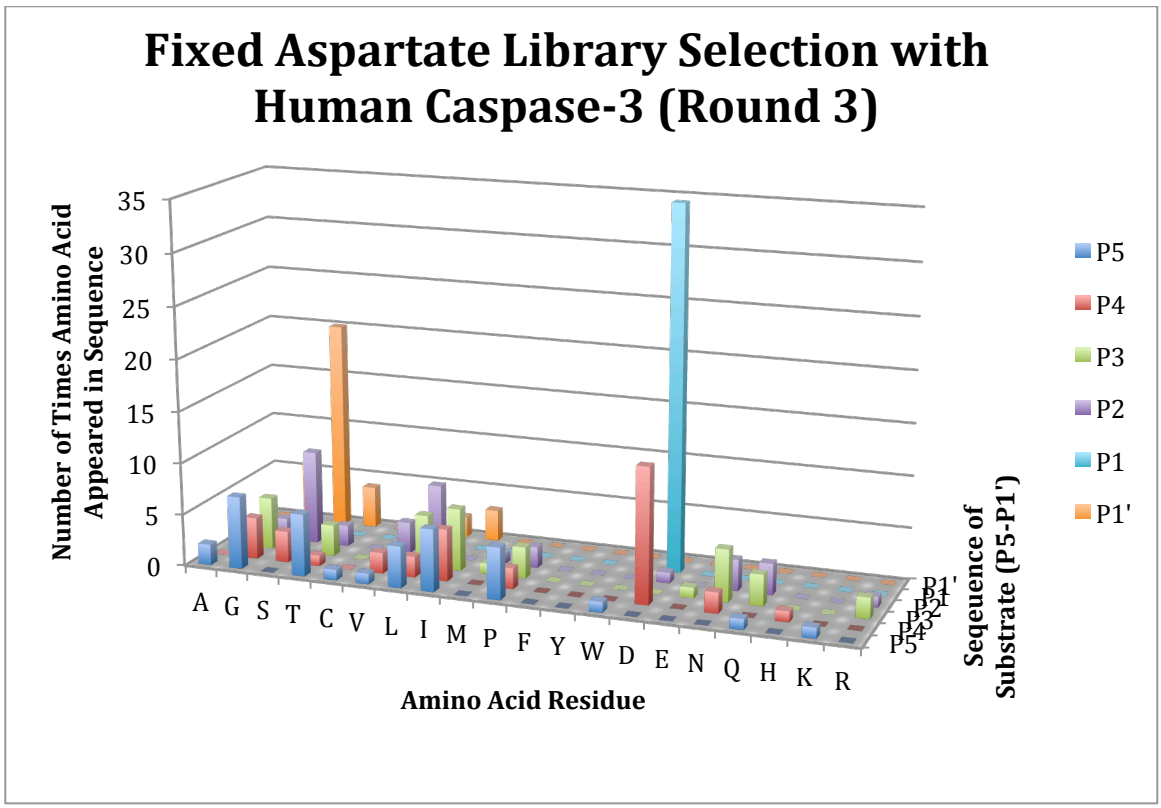

Figure 9: Sequence preference of human caspase-3 for the fixed aspartate library (X-5) after round 3.

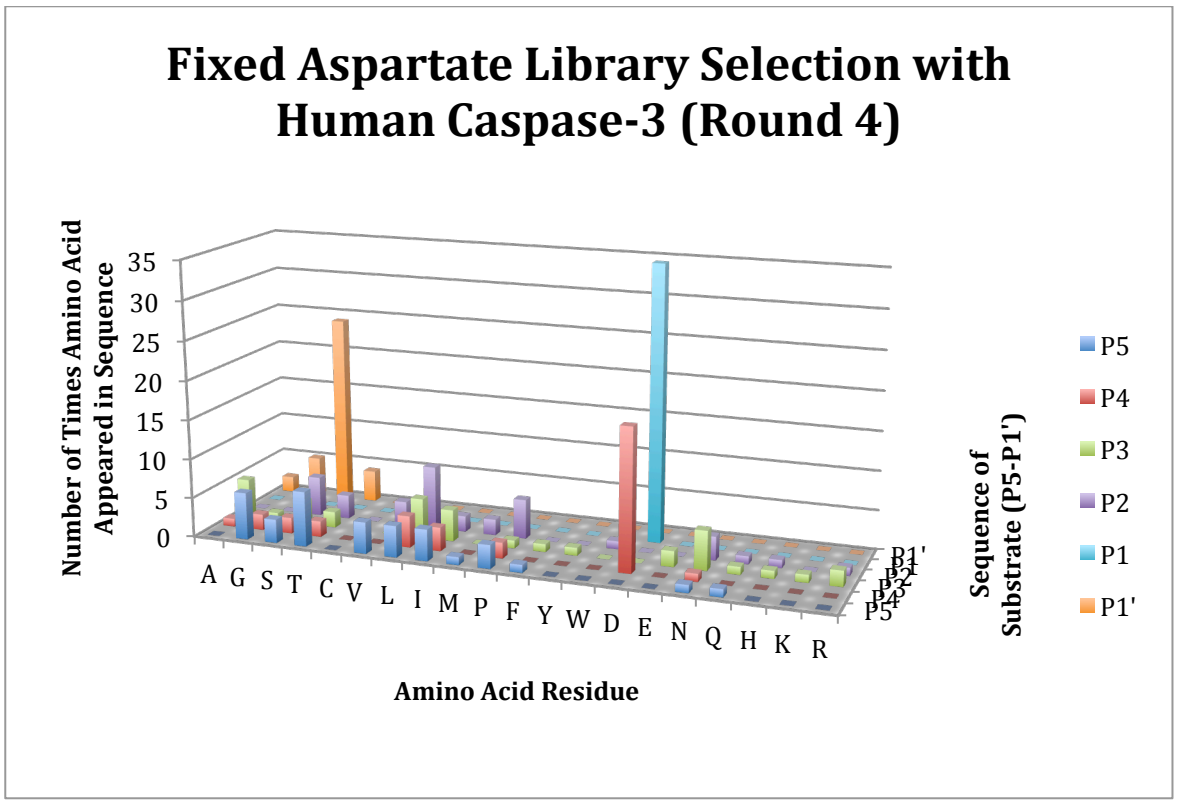

Figure 10: Sequence preference of human caspase-3 for the fixed aspartate library (X-5) after round 4.

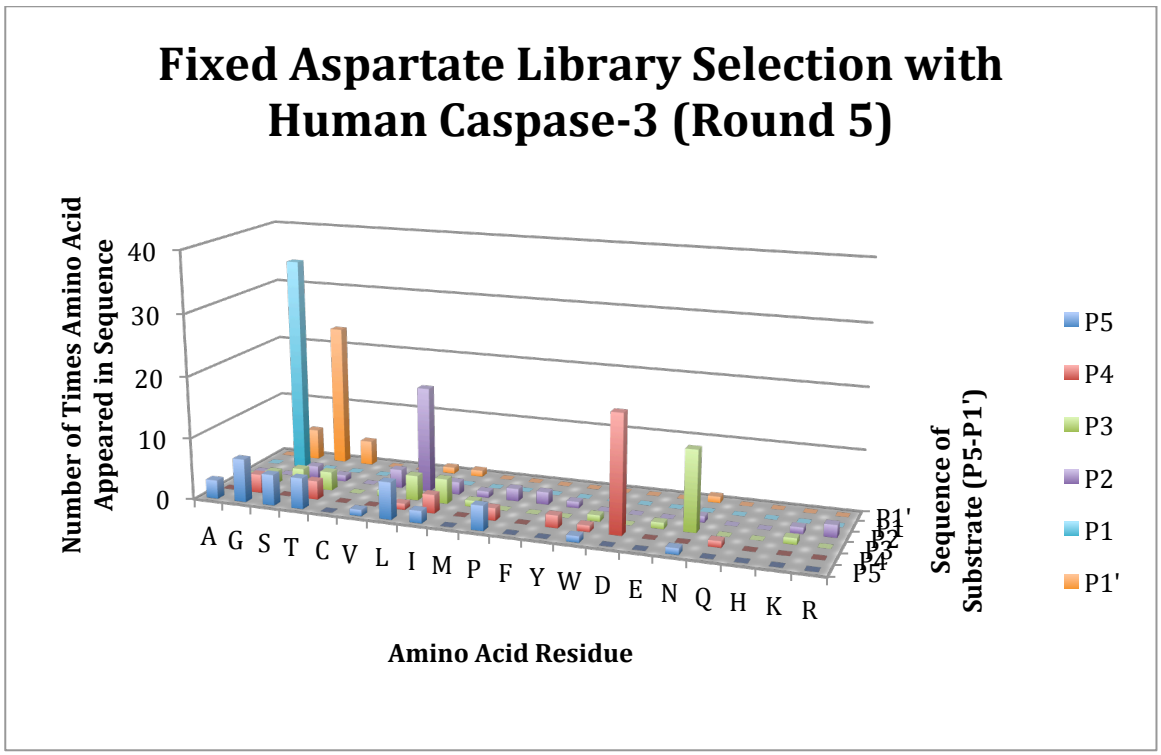

Figure 11: Sequence preference of human caspase-3 for the fixed aspartate library (X-5) after round 5.

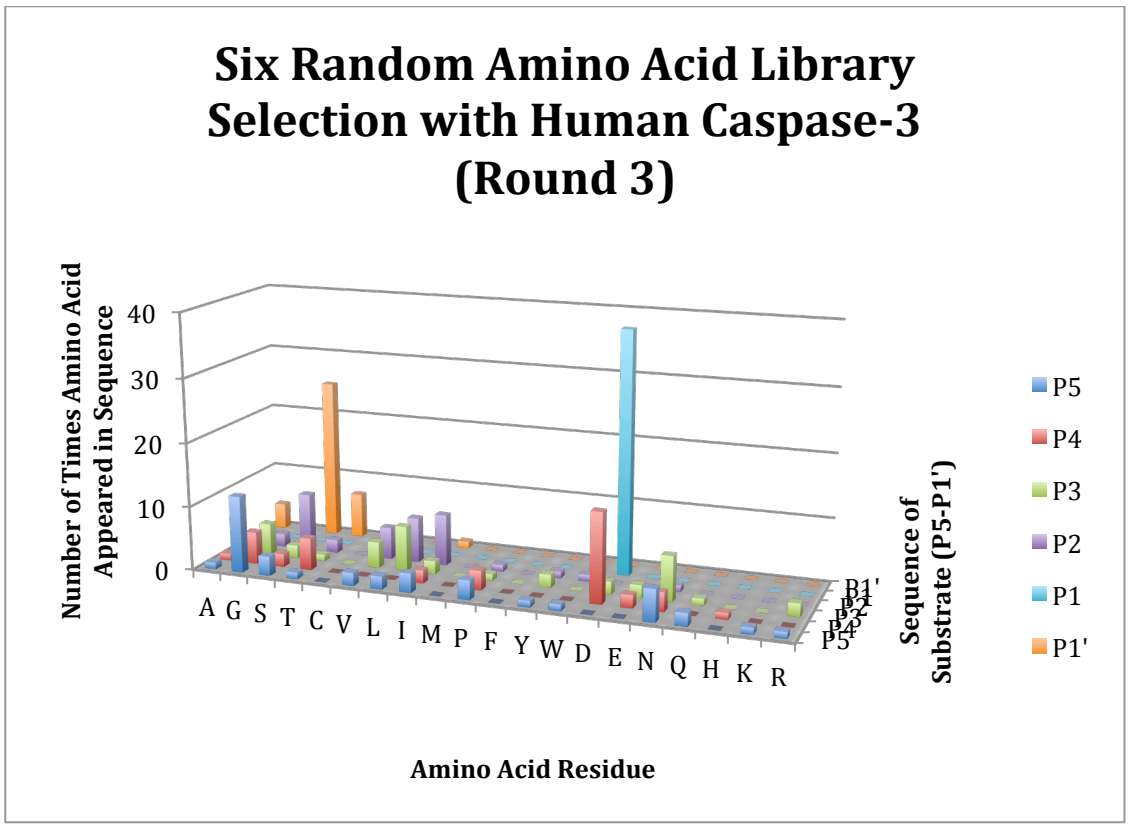

Figure 12: Sequence preference of human caspase-3 for the six random amino acid library (X-6) after round 3.

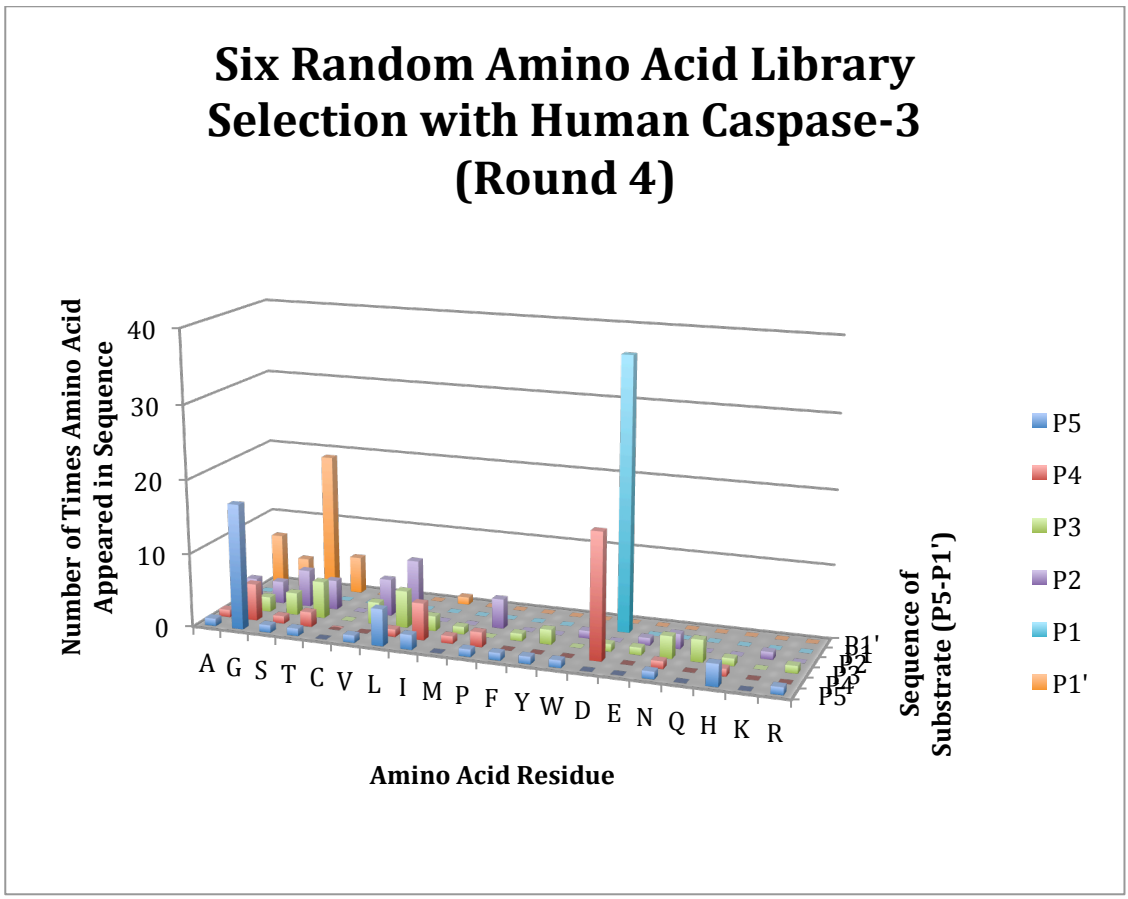

Figure 13: Sequence preference of human caspase-3 for the six random amino acid library (X-6) after round 4.

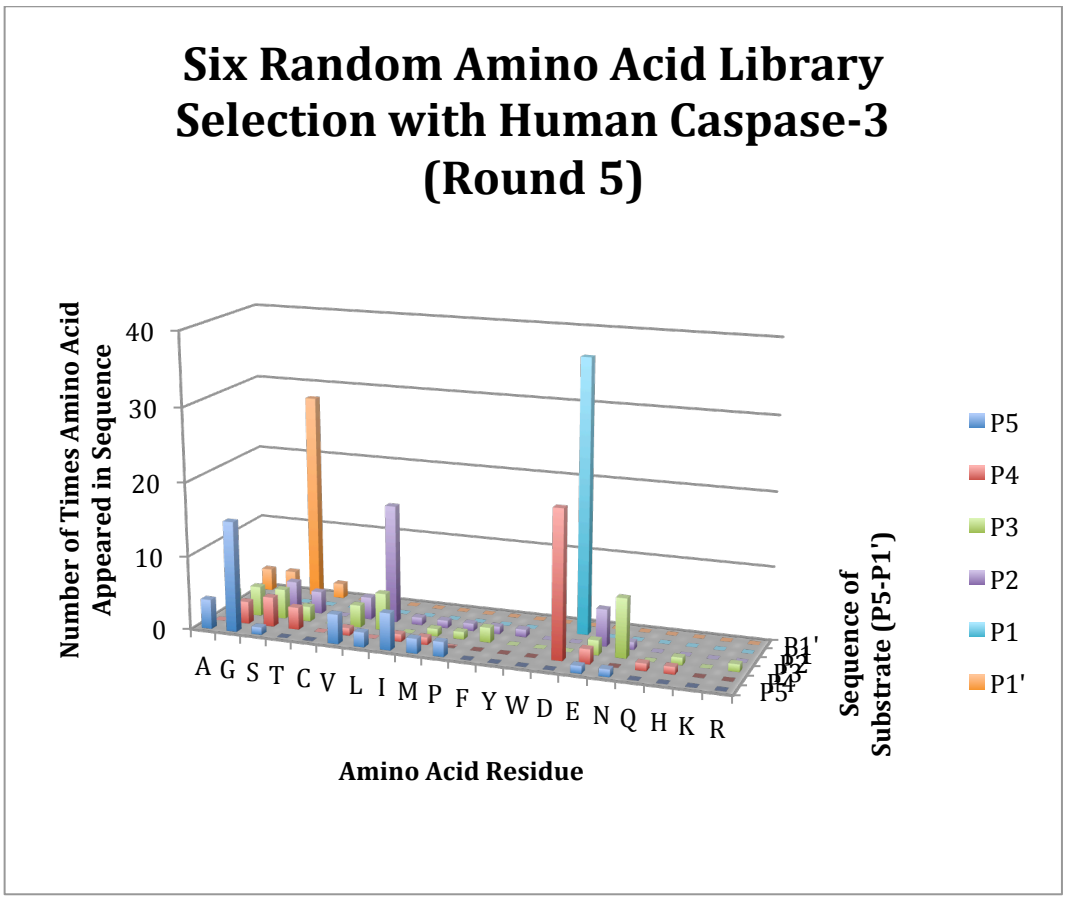

Figure 14: Sequence preference of human caspase-3 for the six random amino acid library (X-6) after round 5.

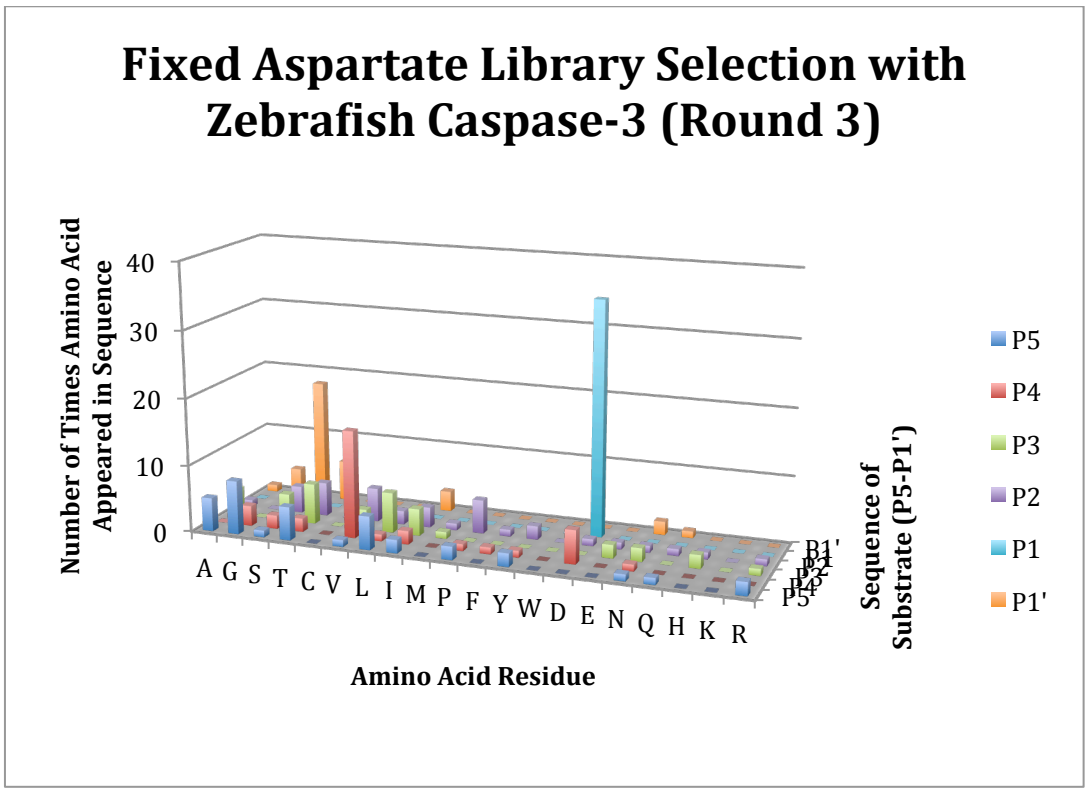

Figure 15: Sequence preference of zebrafish caspase-3 for the fixed aspartate library (X-5) after round 3.

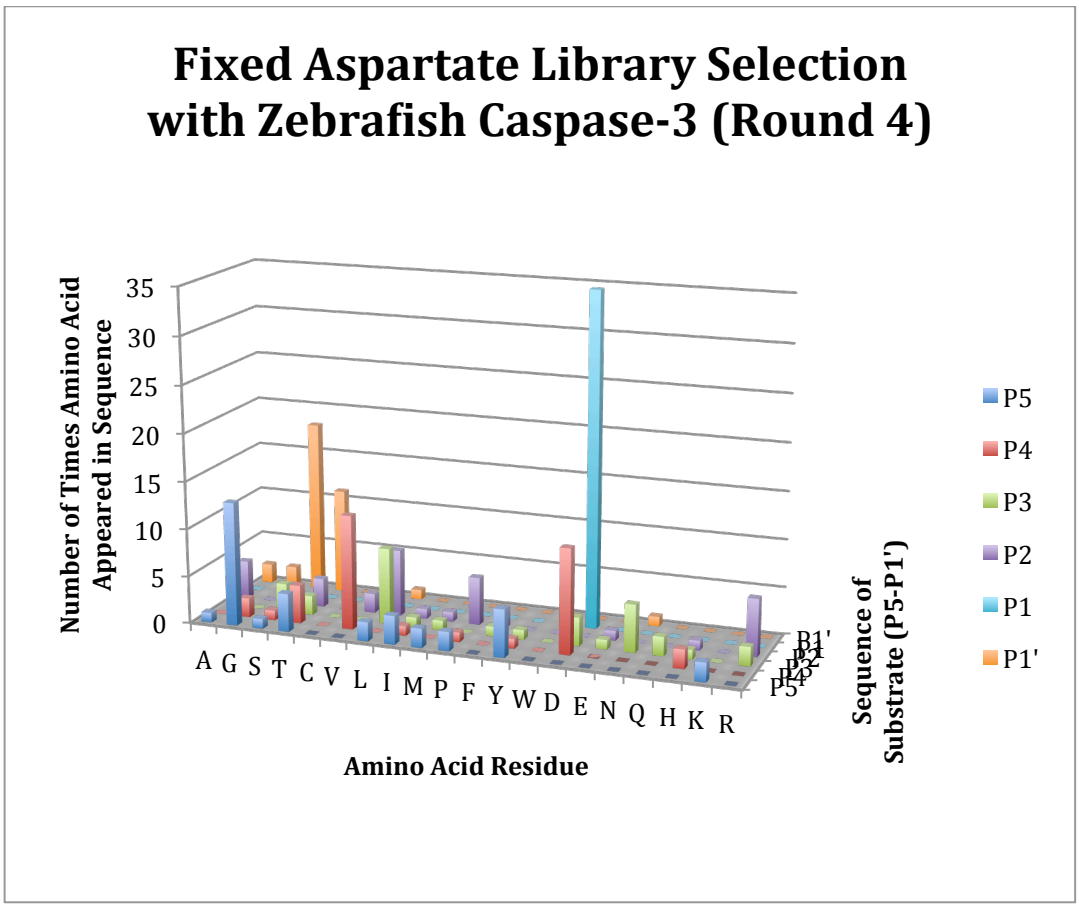

Figure 16: Sequence preference of zebrafish caspase-3 for the fixed aspartate library (X-5) after round 4.

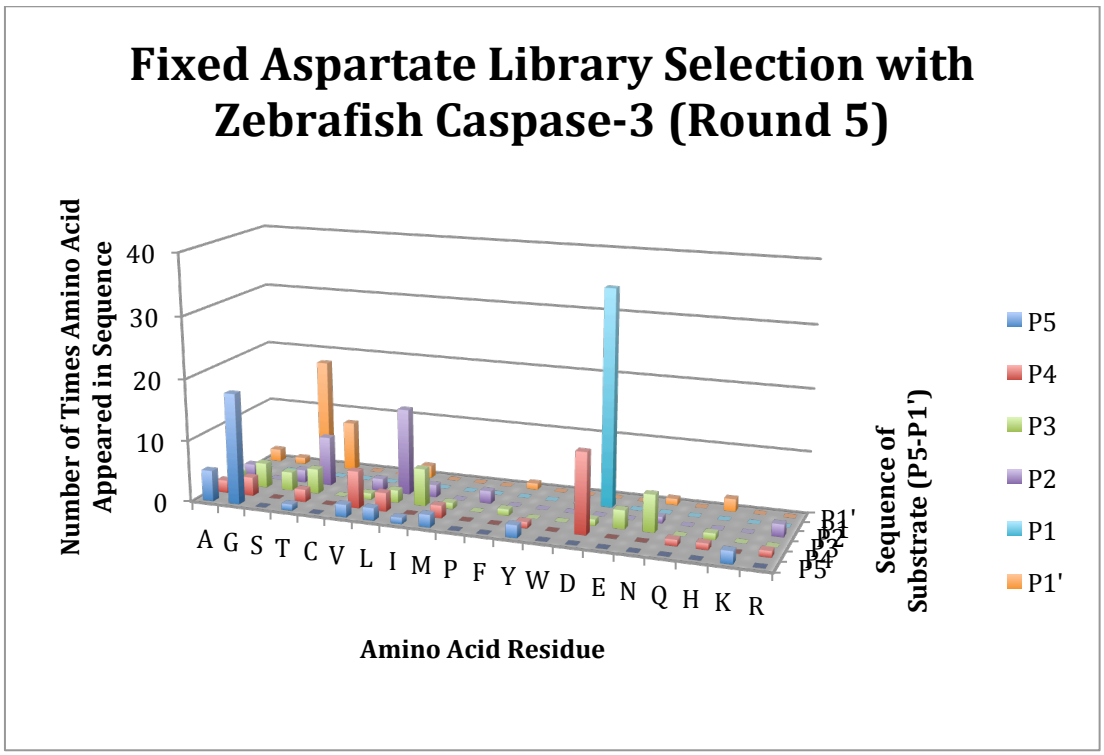

Figure 17: Sequence preference of zebrafish caspase-3 for the fixed aspartate library (X-5) after round 5.

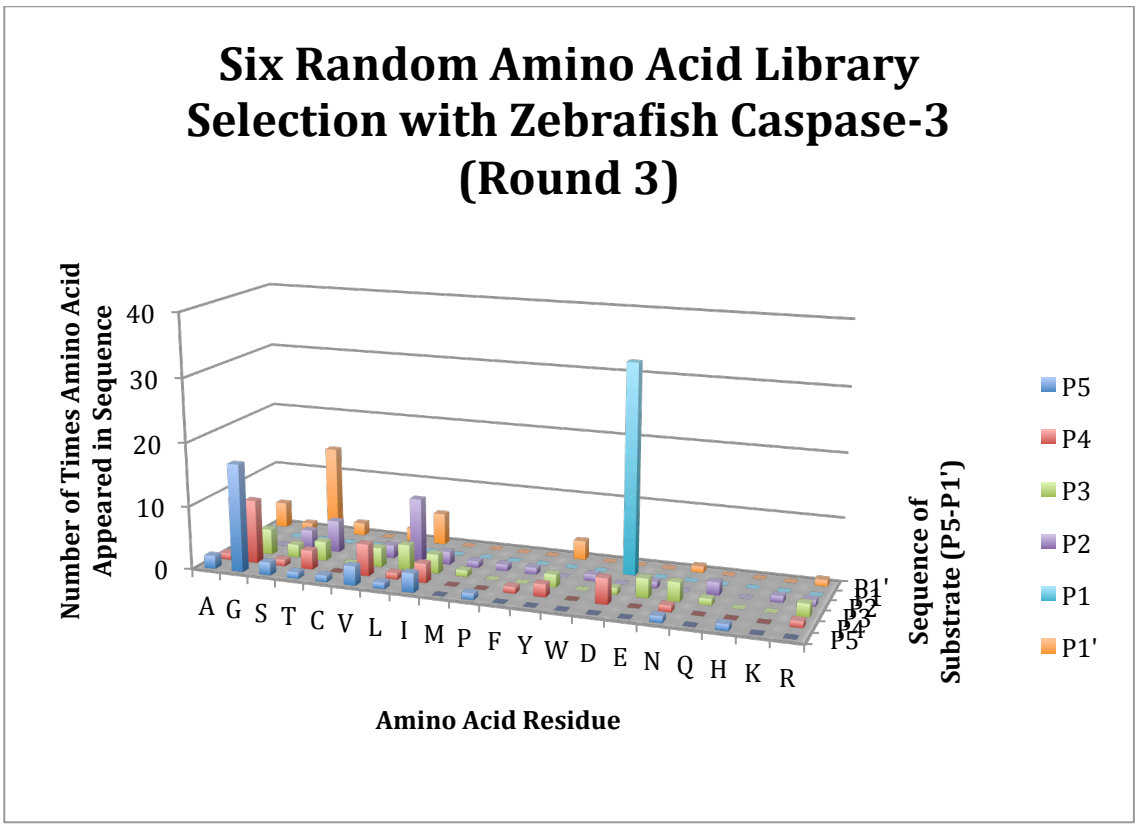

Figure 18: Sequence preference of zebrafish caspase-3 for the six random amino acid library (X-6) after round 3.

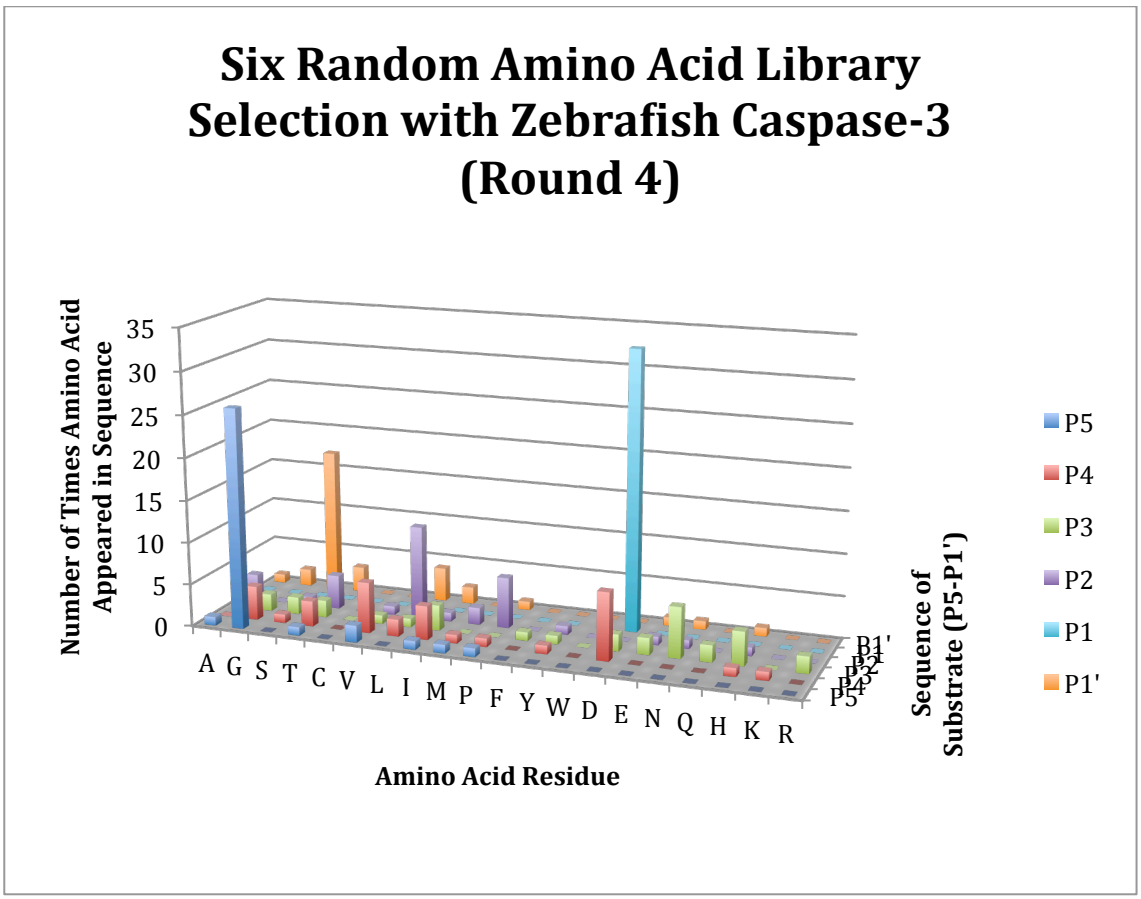

Figure 19: Sequence preference of zebrafish caspase-3 for the six random amino acid library (X-6) after round 4.

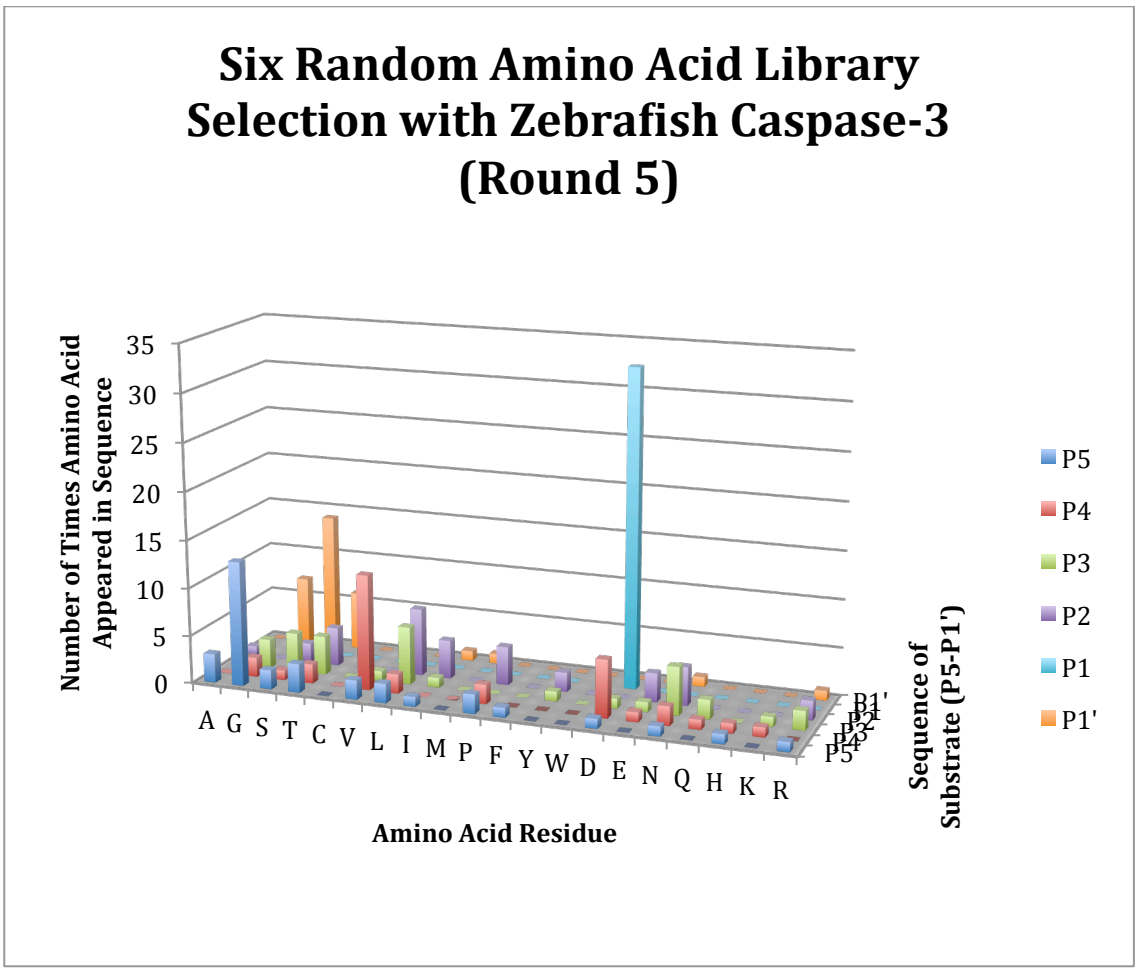

Figure 20: Sequence preference of zebrafish caspase-3 for the six random amino acid library (X-6) after round 5.

Chapter 4

Discussion

Purification of Zebrafish Caspase-3

The process of purifying wild type zebrafish caspase-3 was quite similar to the process of purifying wild type human caspase-3. In particular, the duration of induction, after the addition of IPTG, was 5 h in both cases. Caspase induction times vary from caspase to caspase. Therefore, purification and induction similarity may indicate the proteins share similar properties or similar functions.

Crystallization of Zebrafish Caspase-3

The crystal structure of zebrafish caspase-3, inhibited by Ac-DEVD-CMK, shared structural similarities with the crystal structure of human caspase-3 crystalized with Ac-DEVD-CHO. CMK is an irreversible inhibitor and aldehydes are reversible inhibitors of caspase-3. The structure of the crystallized protein does not change drastically depending on the inhibitor utilized. Both structures shared the feature of each monomer containing six beta strands, surrounded by five alpha helices. Proteins conserved regions involved in catalysis including a large and small subunit, a catalytic cysteine and a histidine (likely used to deprotonate the cysteine). Differences in residue identity and residue flexibility exist primarily in the S_4 subsite in the active site of the protein. This could indicate that the S_4 subsite in zebrafish casapase-3 is different to accommodate a different residue in the P4 site, besides aspartate; instead of a phenylalanine, there is a threonine present in the zebrafish caspase-3 S_4 subsite. Refer to Figure 8 to observe the difference between the subsites in the proteins.

Control Sequence Experiments

When each control sequence was treated with 250 mM imidazole, this theoretically eluted all of the phage bound to the column. The imidazole molecules displace the phage, by outcompeting the tagged protein for binding sites. The total phage eluted from each column, after imidazole treatment, phage correlating to a value between 5.1 $X10^{10}$ pfu and 8.0 $X10^{10}$ pfu. Therefore, it is likely that the maximum number of phage that can realistically bind to the column falls somewhere in that range.

Conversely, the simple PBS solution, void of caspase-3 or imidazole, released phage correlating to a number that falls between 9.5 $X10^{3}$ pfu and 3.5 $X10^{4}$ pfu. This PBS treatment acted as a negative control, to determine the basal level of phage coming off of the column. Therefore, for any given treatment to a control seqeuence, phage correlating to titer of at least 1.0 $X10^{4}$ pfu will be collected from the experiment, regardless of the treatment utilized.

The three control sequences: DEVD, DEVE, and DEVA are utilized to determine the validity of the caspase-3 treatment. Because the sequences can be correlated to known enzyme activity levels, there should be differences in elution from the columns containing various sequences. Caspase-3 should be able to recognize and cleave the first sequence, DEVD, but should not be able to cleave the DEVE or DEVA sequences. The data from the experiments indicates a difference in cleavage, based on sequence. However, abnormalities in the data for zebrafish are discussed below.

Human caspase-3 treatments of columns containing the DEVE sequence yielded pahge elution values that differed from the human caspase-3 phage elution values of DEVD by approximately by two orders of magnitude. These data suggest possibly three things for

human caspase-3. The first possibility is that human caspase-3 has the potential to cleave after a glutamate residue, but it isn't a highly preffered substrate. The second possibility is that the control sequence is being cleaved with a recognition sequence of GGGD, utilizing the glycine linker upstream of the aspartate residue. The third possibility is that there is a combination of the first two scenarios. One could test this hypothesis by synthesizing tetrapeptide sequences and executing activity assays.

Control Sequencess Treated with Caspase-3 Bound to Inhibitor

A concern for this method of substrate specificity detection arises when one considers that there is a possibility for the histidine tag on the purified recombinant protein to displace the histidine tag on the phage. Although this is unlikely, due to the avidity advantage polyvalent phage have over a protein's single histidine tag, an experiment was performed to determine if this was a valid concern.

Ac-DEVD-CMK, a covalent inhibitor, was incubated with each protein and applied to individual columns, each containing a control library. The phage released from each control column were fairly similar, DEVD and DEVA columns released phage correlating to a titer of $1.0X10^4$ pfu for each protein. The DEVE column released approximately $9.0\ X10^3$ pfu for each protein. The phage elution values for each of the control sequencess are very similar to the PBS treatment phage elution values, and orders of magnitude lower than the elution by uninhibited human caspase-3 and zebrafish caspase-3.

The phage release values of the inhibited caspase treatment matching pfu values of the PBS treatment indicate that proteolytic cleavage, not histidine tag displacement, is responsible for the release of control phage from the column. Because the active site of each

caspase is covalently bound to an inhibitor, the protein is unable to release phage by cleavage of the displayed amino acid sequence. Therefore, the release values of inhibited caspase are equal to the basal levels of elution established by PBS treatment experiments. If the values of phage release for the DEVD control sequence when treated with inhibited caspase were equal to value between 1.0 $X10^{10}$ and 5.0 $X10^{10}$ pfu, it would mean that a non-cleavage event was responsible for releasing the phage from the column.

Control Sequencess treated with Human Caspase-3 (D3A), with and without Inhibitor

There are three cleavage sites in human caspase-3. Two cleavage sites involve removal of a prodomain, and the third cleavage site is in the intersubunit linker of the catalytic domain. Caspases are the chief proteins responsible for cleaving caspase-3 for activation. Therefore, when these residues are mutated to alanines, cleavage does not occur and the protein is suspended in an uncleaved, but weakly active version of pro-caspases-3.

The DEVD control sequence results from this experiment showed approximately a two orders of magnitude drop in the number of phage released from a column containing the control sequence when exposed to D3A protein with and without the covalent inhibitor, CMK, respectively. Uninhibited D3A showed a greater number of phage released compared to the basal release levels established for the DEVD control sequence. This indicates that the D3A protein is capable of cleaving and releasing DEVD control phage from a nickel column. The reduction in the number of phage released, after addition of inhibited caspase, provides more evidence that the release is linked to cleavage events.

The DEVE and DEVA control sequences have approximately the same amount of released phage, with or without the inhibitor bound to the D3A protein. First, this provides

more evidence suggesting release of phage from each column occurs because of a proteolytic cleavage event. Secondly, this shows that human caspase-3 (D3A) might only recognize DEVD and not GGGD, or that the activity of the caspase 3 is reduced so much that it is not possible to resolve the difference in phage released due to cleavage of a fair substrate versus the background phage eluted ata a consistent rate.

Substrate Libraries Control Experiments

The X-5 and X-6 libraries were subject to a treatment with a 250 mM imidazole solution, or in a separate experiment, a treatment with 1X PBS. The imidazole treatment was utilized to determine the column capacity of phage for each library. The number of phage released from the X-5 and X-6 columns was approximately equal to the positive control phage elution values established for the three control sequences. Both libraries and all three control sequences had positive control values in the mid to upper 10^{10} pfu range. Therefore, the maximum number of phage that can be released from a column, given these experimental conditions, should not be greater than 8 $X10^{10}$ pfu.

Conversely, the PBS treatment was utilized to determine the number of pfu that are released simply due to the addition and extraction of a buffered solution to the column. The PBS treatment was used to determine the basal release level for each column. The number of phage released after addition of PBS to the X-5 and X-6 columns was slightly greater than the values for the control libraries, but still less than one order of magnitude. Therefore, if the number of phage released from a X-5 or X-6 column is less than or equal to 10^5 then there is probably no cleavage resulting in the release of the phage.

X-5 Substrate Library Selection with Human Caspase-3 and PBS: Phage Titer

Over the course of five rounds, X-5 phage was loaded onto a column and released with the treatment of human caspase-3. The number of phage released after round 1 almost equaled the positive control value for the substrate library, but was only approximately 40% of the contents of the column. There is a tenfold decrease in the number of phage released in round 2, from the column containing the enriched phage from round 1. The number of phage released from the column containing the enriched product slowly trends upward as the rounds progress, finishing with a round 5 release equal to 33% of the release after round 1.

PBS treatments, in a separate column, were completed in series with the human caspase-3 treatments to act as a negative control. Over the course of five rounds, a trend of more phage being released as the rounds increased developed. However, the greatest release of phage after PBS treatment, which occurred in round 5, was only $9.3X10^6$ pfu.

These data illustrate that the same experiment, preformed side-by-side, changing only the presence of protein versus buffer, result in a four order of magnitude difference between the release of phage at round 5 for each treatment. However, there is a strange anomaly occurring after round 1 treatment and release. After round 1 there was a decline in the number of phage released, followed by a steady increase in phage released after round 2. It is difficult to speculate why this phenomenon occurred, but one might argue it has something to do with the number of *E. coli* cells available to be infected. $1.0X10^8$ *E. coli* cells are available for infection during propagation. Therefore, the number of phage able to infect bacterial cells may outnumber the available bacterial cells able to be infected. The number of phage released may increase over the rounds after round 2 because of more cleavable phage being

selected and propagated in the bacteria. If a sequence is more likely to be cleaved, it will show up as a function of enrichment.

X-6 Substrate Library Selection with Human Caspase-3 and PBS: Phage Titer

In an experiment, the same one performed with the X-5 library, the X-6 substrate library phage release data was very similar to the X-5 substrate phage release data. The X-6 library released more phage after round 1 than the X-5 library, but the same trend of the second round reduction in the number of phage released and then steady increase held constant.

The negative control data, utilizing PBS as the treatment versus a solution containing caspase-3, resulted in phage release values similar to values seen in the X-5 PBS treatments. The pfu values for both libraries were almost identical for each treatment. The theory for the reduction in pfu from round 1 to round 2 is the same for the X-5 library as it is for the X-6 library.

X-5 Substrate Library Selection with Human Caspase-3: Sequences

The aspartate at the P4 position becomes more common as the rounds of enrichment continue. This was a predicted trend, considering human caspase-3 prefers substrates with the DXXD sequence. Also, until round 5, there is not much definition for the P2 and P3 residues. These data also match the DXXD template that defines caspase 3-substrate specificity. The P1' position is dominated by serine and glycine residues. This follows the theory that caspase substrates have a small residue in the P1' position.

DNLD was a sequence that was conserved in the three rounds of selection. More importantly, DNLD was very prominent in the fifth round. This indicated that DNLD was the

preferred substrate for human caspase-3 utilizing the X-5 substrate library. DEVD is the established, "preferred" substrate, but DNLD has been investigated in the past for its strong interactions with human caspase-3 (30).

DNLD has been shown to be well recognized by caspase-3, but not well recognized by caspase 7; caspase 8 and caspase 9 do not recognize the DNLD sequence well at all. The theory for the prolific recognition and cleavage of DNLD by caspase-3 has to do with the asparagine and leucine residue in the DNLD sequence. The interaction of asparagine with serine in the S_3 subsite and leucine in the hydrophobic S_2 subsite help to explain why the binding of DNLD to human caspase-3 is so strong (30).

X-6 Substrate Library Selection with Human Caspase-3: Sequences

After five rounds of selection, the X-6 substrate library selection process resulted in a P1 and P4 aspartate residue. The P3 site did not offer a preferred residue and the P2 site has multiple residues that are likely, but seems that a leucine was the most preferred after five rounds.

The fact that there was not a more defined, definite consensus sequence discovered by this library, like the DNLD sequence revealed by the X-5 library, is not very surprising. The X-5 library offered a fixed aspartate residue at a position that likely enabled an accelerated selection process for each residue position in the tetrapeptide. The fixed positions in the peptide, surrounding the variable regions, were often utilized as part of the recognition sequence in the X-6 substrate library.

All sequences from the X-6 library selection contained at least one aspartate residue. This is a positive sign for the method for two reasons. First, a sequence that is released by a

caspase should have an aspartate residue within the sequence; otherwise it is difficult to reason how the caspase could have released the phage from the column. Secondly, very few proteases are aspartate specific. Therefore, when the results all return with an aspartate residue, it further confirms the action of human caspase-3.

X-5 & X-6 Substrate Library Selection with PBS: Sequences

X-5 substrate library selection utilizing PBS resulted in some full, intact sequences that contained a variety of amino acids. However, a large portion of the sequences selected had deleted portions of the peptide construct. These deleted portions included the glycine linker, fixed aspartate portions, random amino acid positions, and the histidine tag. There were no sequences present that seemed like ideal caspase substrates, showing the difference of results between protein and PBS treatments.

X-6 substrate library selection utilizing PBS resulted in numerous sequences with deleted portions. These deleted portions included the glycine linker, the random residue positions, and the histidine tag. Sequences that contained the whole, intact peptide often include a mixture of various types of amino acid residues and lack the presence of an aspartate residue.

Although there are no conserved sequences present from the PBS treatments of either library, most sequences do share similarities. Sequences from both libraries tend to be mutated peptides, lacking all of the portions present in the sequences released by a caspase. These types of sequences are probably present when substrate libraries are treated with caspase, but are greatly outnumbered by the sequences released by caspase-3.

X-5 Substrate Library Selection with Zebrafish Caspase-3 and PBS: Phage Titer

The number of phage released from the column containg the X-5 substrate library, with zebrafish caspase-3, mirror the trend observed when human caspase-3 was placed on a column containing X-5 substrate library. There is a first round release of phage correlating to a titer of roughly 10^{10} pfu, but then a ten fold decrease in round 2. The phage release is approximately constant from round two to round three. Starting with round four, there is a steady increase in the number of phage released, all the way through round five. The number of phage may increase over the rounds following round 2 because of more cleavable phage being selected and propagated in the bacteria. If a sequence is more likely to be cleaved, it will show up as a function of enrichment.

In a separate experiment, completed in series with zebrafish caspase-3 treatments, columns containing X-5 substrate library were treated with PBS. The final round, round 5, resulted in a release of phage equaling $8.3X10^{6}$ pfu. The rise in the number of phage released from the column over a series of rounds indicates that as the target substrate phage are being enriched, so are phage that simply are released from the column.

X-6 Substrate Library Selection with Zebrafish Caspase-3 and PBS: Phage Titer

The number of phage released for the X-6 substrate library, with zebrafish caspase-3, mirror the trend observed when human caspase-3 was placed on a column containing X-6 substrate library. There is a first round release of phage equaling a titer of $1X10^{10}$ pfu, but then a tenfold decrease in the number of phage released after the second round. The number of phage released steadily increases by each round starting with round three.

The X-6 substrate library was treated with PBS, instead of zebrafish caspase-3, and

the values of phage released from the column increased over the course of the five rounds. After five rounds, the number of phage released from the column by PBS treatment was approxmatley three orders of magnitude lower than the treatment of the X-6 library with zebrafish caspase-3. This difference in phage indicates the zebrafish caspase-3 treatment lead to a proteolytic release of the X-6 phage from the column.

X-5 Substrate Library Selection with Zebrafish Caspase-3: Sequences

X-5 library selection, over the course of five rounds indicated that the P4 position in the recognition sequence is either a valine or aspartate residue, with aspartate being in the P1 position. After round three, there is a greater population of valine in the P4 position than aspartate. However, by the fifth round aspartate is in the P4 position more frequently than a valine residue. As the rounds increased, the P3 and P2 position went from being undefined to having a leucine present in the P2 position.

These data may indicate that when there are numerous, diverse substrates available for zebrafish caspase-3 to cleave, the protein will tend to cleave substrates with a valine in the P4 position. However, as substrates of greater specificity to the zebrafish caspase-3 increase, the protein will begin to cleave substrates than contain an aspartate residue in the P4 position, or will cleave these substrates more quickly than ones not containing an aspartate in the P4 position.

X-6 Substrate Library Selection with Zebrafish Caspase-3: Sequences

X-6 library selection, over the course of five rounds, did not produce the same results as the X-5 library selection. Analysis of the sequences from each round of X-6 treatment indicated a selection of either an aspartate or a valine in the P4 position; however, the

frequency of the residues occurring at the P4 position was fairly low compared to the frequency that those amino acids appeared in the X-5 experiments. Unlike the X-5 sequence results, the X-6 sequence results did not produce a consensus residue in the P2 position. The frequency of recurring amino acid residues, from the X-6 substrate library, in the P3 and P2 positions, were lower than the than the frequency of recurring amino acids in those same positions in the X-5 substrate library.

Unlike the X-5 library results, there was not a switch from valine in the P4 position to an aspartate in the P4 position. However, the explanation behind this discrepancy could be due to the fact that there were still too many average substrates due to the lack of a fixed aspartate, guiding the selection process along. If more rounds of enrichment are executed, it may increase the frequency of an aspartate being located in the P4 position of the sequence. However, more rounds of enrichment may also lead to the deletion of genes in the phage display, causing complications with the experiment.

An aspartate was present in the random region of each sequence collected. As stated above, if zebrafish caspase-3 released a phage not containing an aspartate residue, it would be troubling considering caspases are aspartate directed proteases.

Chapter 5

Conclusion

Human caspase-3 and zebrafish caspase-3 were similar in terms of structure and purification procedure. Not only is the structure very similar, but also the two proteins tend to target substrates containing two aspartate residues separated by two random amino acid residues. Therefore, the active sites likely bind substrates in a similar way. The only caveat being that zebrafish caspase-3 seems to utilize a valine residue in the P4 position. However, there is research to suggest that there are substrates cleaved by human caspase-3 that contain a valine residue in the P4 position. This similarity of structure and substrate preference leads one to assume that utilizing zebrafish as an animal model to discover possible drugs that will bind to and activate the caspase 3 zymogen, pro-caspase-3, will have a fruitful future.

The creation and validation of an M13 phage display substrate specificity system, that utilizes immobilized nickel ion affinity chromatography binding to a poly-Histidine tag displayed on the viral coat of the bacteriophage, was a vital aspect of the research. Although the method did not select for the most readily cleaved substrate found in academic literature, DEVD, it did discover a sequence that is recognized well by caspase-3 but not caspases 7,8, and 9; unlike DEVD, DNLD was shown to be cleaved by caspase-7 poorly, and is almost unrecognizable to caspases 8&9 (29).

The application of the M13 phage display substrate model with zebrafish caspase-3 yielded promising results. However, the results were not as conclusive with zebrafish caspase-3 as they were with human caspase-3. In the earlier rounds a valine at the P4 site was preferred, but an aspartate existed at the P4 site in numerous sequences as well. The fifth round of selection yielded a greater propensity of aspartate residues in the P4 site versus a valine residue; valine residues were the second most popular choice. The P3 site did not have

a clear consensus although amino acids isoleucine and asparagine were the first and second most preferred options, respectively. The P2 site seemed to gravitate towards the leucine residue in the P2 site, but threonine and alanine were also present multiple times at that position.

The lack of a clear, conclusive tetrapeptide result for the zebrafish caspase-3 consensus sequence may mean numerous things. First, it could point to the fact that the method is very sensitive to different proteins. Perhaps the elements of the experiment including buffer composition, rounds of enrichment, time of exposure to protein, and concentration of the protein could have been altered to give different results. After all, this method was developed and fine-tuned for human caspase-3, not zebrafish caspase-3. A second possibility is that zebrafish caspase-3 has many different roles when it come to cleaving substrates than a human caspase-3 does. If this is true, it may recognize a suite of sequences, not having a very strong preference for one over another, making the process of enrichment less effective at finding a single substrate sequence. Despite falling short of attaining a true tetrapeptide consensus sequence, the discovery of D/V-X-L/T-D is an indication that the method could be used to help flesh out protease substrate tendencies.

As mentioned previously, the ability for zebrafish caspase-3 to vigorously recognize sequences containing a valine in the P4 site suggests that there may be an additional set of responsibilities for zebrafish caspase-3 in terms of cleavage events. It has been posited in previous literature that zebrafish caspase-3 may complete roles in zebrafish that would be accomplished by caspase-6 in humans (28).

The difference in substrate libraries, X-5 and X-6, illustrates a vital aspect about

phage display substrate selection. The presence of a fixed amino acid helps to anchor and guide the selection of phage that are advantageous substrates. I feel that this is especially advantageous when looking at a protein with an ability to recognize and cleave substrates as ubiquitously as caspase 3. Although the pfu release values did not show a steady climb in value, as one would expect would be the result of enrichment, there was evidence of enrichment with the sequencing data. The same sequences and more similarities in sequences began to appear as the rounds progressed, for both libraries, for both proteins. Accommodating for slight changes in conditions and exposure, I feel this substrate specificity method utilizing M13 bacteriophage is an advantageous tool to utilize the substrate preferences of various proteases, at a relatively cheap cost.

REFERENCES

1. Chamond, R.R, Anon, J.C, Aguilar C.M., and Pasadas, F.G. "Apoptosis and Disease." European Journal of Allergy and Clinical Immunology 14 (1999):367-74.

2. Chinnaiyan, A.M. "The apoptosome: heart an soul of the cell death machine." Neoplasia 1.5 (1999): 5-15.

3. Tait, Stephen, and Douglas Green. "Mitochondria and cell death: outer membrane permeabilization and beyond." Nature Reviews Molecular Cell Biology 11 (2010):621-32.

4. Bose K, Pop C, Feeney B, Clark A.C. "An Uncleavable Procaspase-3 Mutant Has a Lower Catalytic Efficiency but an Active Site Similar to That of Mature Caspase-3." Biochemistry 42.42 (2003): 12298-12310.

5. Pop C, Feeney B, Tripathy A, Clark AC. "Mutations in the procaspases-3 dimer interface affect the activity of the zymogen." Biochemistry 42.42 (2003): 12311-20.

6. Parng, C, Seng. WL, C Semino and MP McGrath. "Zebrafish: a preclinical model for drug screening." Assay and Drug Devlopment Technologies 1.1 (2002): 41-8.

7. Valencia C.A., Bailey C, Liu R. "Novel zebrafish caspase-3 substrates." Biochemical and Biophysical Research Communications 361 (2007): 311-16.

8. Crawford, ED, Seaman, JE, Barber, AE, David, DC, Babbitt, PC, Burlingame, AL, and JA Wells. "Conservation of caspase substrates across metazoans suggests hierarchical importance of signaling pathways over specific targets and cleavage site motifs in apoptosis." Cell Death and Differentiation 19.12 (2012): 2040-8.

9. Timmer JC, Salvesen GS. "Caspase Substrates." Cell Death and Differentiation 14 (2007): 66-72.

10. Keri, Gyorgy, and Istvan Toth. *Molecular Pathomechanisms and New Trends in Drug Research*. Florida: CRC Press, 2002. Print.

11. Fischer, U, Janicke, RU, and K Schulze-Osthoff. "Many cuts to ruin: a comprehensive update of caspase substrates." Cell Death and Differentiation 10.1 (2003): 76-100.

12. Pop C, Salvesen G. "Human Caspases: Activation, Specificity, and Regulation." The Journal of Biological Chemistry 284.33 (2009): 21777-81.

13. Lieschke Graham & Currie Peter. "Animal models of human disease: zebrafish swim into view." Nature Reviews Genetics 8 (2007): 353-67.

14. Azzazy, H, and Edward Highsmith. “Phage display technology: clinical applications and recent innovations.” Clinical Biochemistry 35.6 (2002): 425-45.

15. Clarkson, Tim, and Henry B. Lowman. Phage Display: A Practical Approach. New York: Oxford University Press, 2004. Print.

16. Matthews D, Goodman L, Gorman C, Wells J. “A survey of furin substrate specificity using substrate phage display.” Protein Science 3 (1994): 1197-1205.

17. Matthews DJ, Wells JA. “Substrate phage: selection of protease substrates by monovalent phage display.” Science 260. 5111 (1993):1113-7.

18. Boatright, K.M., and Salvesen, G.S. “Mechanisms of caspase activation.” Curr. Opin. Cell Biol. 15 (2003): 725-731.

19. Demon D, Van Damme P, Berghe T, Vandekerckhove J, Declercq W, Gevaert K, Vandenabeele P. “Caspase substrates: easily caught in deep waters?” Trends of Biotechnology 27.12 (2009).

20. Earnshaw W, Martins L, Kaufmann S. “Mammalian Caspases: Structure, Activation, Substrates, and Functions During Apoptosis.” Annual Rev. Biochem. 68 (1999):383-424.

21. Fang B, Fu G, Agniswamy J, Harrison RW, Weber IT. “Caspase-3 binds diverse P4 residues in peptides as revealed by crystallography and structural modeling.” Apoptosis 14.5 (2009): 741-52.

22. Garcia-Calvo M, Peterson E, Leiting B, Ruel R, Nicholson D, Thronberry N. “Inhibition of Human Caspases by Peptide-based and Macromolecular Inhibitors.” The Journal Biological Chemistry 273.49 (1998): 32608-13.

23. Salvesen G, Dixit V. “Caspases: Intracellular Signaling by Proteolysis.” Cell 91 (1997): 443-46.

24. Sedlacek R, Chen E. “Screening for prtease substrate by polyvalent phage display.” Combinatorial Chemistry & High Throughput Screening 8.2 (2005): 197-203.

25. Grutter, M. “Caspases: key players in programmed cell death.” Current Opinion in Structural Biology 10 (2000): 649-55.

26. Hsu H, Tsai K, Sun Y, Chang H, Huang Y, Yu H, Lin C, Mao S, Yang A. “ Factor Xa Active Site Substrate Specificity with Substrate Phage Display and Computational Molecular Modeling.” The Journal of Bioloigical Chemistry 283.18 (2008): 12343-53.

27. MacKenzie S.H, Clark A.C. “Targeting cell death in tumors by activating caspases.” Current Cancer Drug Targets 8 (2008): 98-109.

28. Talanian R, Quinlan C, Trautz S, Hackett M, Mankovich J, Banach D, Ghayur T, Brady K, Wong W. “Substrate Specificities of Caspase Family of Proteases.” The Journal of Biological Chemistry 272 (1997): 9677-82.

29. Yoshimori A, Sakai J, Sunaga S, Kobayashi T, Takahashi S, Okita N, Takasawa R, Tanuma S. “Structural and functional definition of the specificity of a novel caspase-3 inhibitor, Ac-DNLD-CHO.” BMC Pharmacology 7.8 (2007).