IDENTIFICATION OF POTENTIAL EXOSITE IN CATHEPSIN V NECESSARY FOR
ELASTIN DEGRADATION

by

LI HSUEN CHEN

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Abstract
Besides collagen, elastin is the most common connective tissue structural protein in vertebrates and similar to collagen relatively resistant to non-specific degradation. Typical elastolytic proteases are the serine-dependent pancreatic and leukocyte elastases, the Zn-dependent matrix metalloproteinase 12, and several lysosomal cysteine proteases. Among the cysteine cathepsins, cathepsins S, K and V are highly potent elastases with cathepsin V displaying the highest activity among all known mammalian elastases. Despite a shared amino acid sequence identity of over 80% between cathepsins V and L and very similar subsite specificities, only cathepsin V has a potent elastase activity whereas cathepsin L lacks it. A series of chimera mutants containing various proportions of cathepsin V and cathepsin L were constructed in an attempt to define a specific region needed for elastin degradation. It was found that retaining the peptide sequence region from amino acids 89 to 119 of cathepsin V preserves the mutant’s elastolytic activity against elastin-Rhodamine conjugates whereas the region FTVVAPGK (amino acids 112-119) contributes approximately 60% of activity retention. Several additional mutant proteins involving mutual swapping of residues VDIPK (amino acids 113-117) of cathepsin L with residues TVVAPGK (amino acids 113-119) of cathepsin V, deletion of Gly118 from cathepsin V, and insertion of Gly between Pro116 and Lys117 in cathepsin L were constructed and evaluated for their elastolytic activities. The results obtained with those mutant cathepsin proteins support the importance of the amino acid region spanning the residues from 112 to 119 in cathepsin V. Based on the 3-D structure of cathepsin V, this peptide region is located below subsite binding pocket S2 and forms a wall-like barrier which may act as an exosite for the productive binding of cross-linked elastin.
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<td>a.a.</td>
<td>Amino Acids</td>
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<td>Cat V</td>
<td>cathepsin V</td>
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<td>Cat L</td>
<td>cathepsin L</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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CHAPTER I – Overview and Introduction

1.1 Human Cathepsin Family

The human cathepsins are a family of cysteine proteases that resemble papain, a cysteine protease originally found in papaya seeds. In humans, there are eleven cathepsins whose functions encompass a whole spectrum ranging from generic housekeeping functions to specific roles in localized parts of the body (1). Human cathepsins are expressed either ubiquitously or tissue- or cell-specifically. Cathepsins such as B, C, H, X, F, L and O are found in many organs and may fulfill generic functions such as degradation or turnover of proteins, activation of proenzymes, and hormone maturation. Other cathepsins such as K, V, and S are involved in more specific functions such as bone resorption, and antigen presentation and processing.

1.2 Human Cathepsin V

1.2.1 Human Cathepsin V cDNA and Chromosomal Gene Localization

The cDNA of human cathepsin V (also known as cathepsin L2) was identified in a search for novel papain-like cathepsin sequences. A human kidney Quick Clone cDNA pool was screened for regions surrounding the conserved active site residues using degenerate primers. Out of the twenty eight hits, one unique cathepsin-like fragment was identified which displayed motifs that are characteristics of papain-like cysteine proteases. Full-length cDNA were later constructed encoding a 334 amino acid protein with a calculated molecular mass of 37329 Da (2). The open reading frame translated into a putative 17 amino acid signal peptide, a 96 amino acid propeptide and a 221 amino acid
mature peptide that contained the characteristic active site residues, C138, H277 and N301. This open reading frame was deposited in GeneBank as cathepsin V (3).

The amino acid sequence of cathepsin V is highly homologous to human cathepsin L. They share 77.5% identity for the preproenzymes and 79.5% identity for the mature proteins. Sequence identities are also shown to a lesser extend between human cathepsin V and other cathepsin members ranging from 58.6% with cathepsin K to 30.8% with cathepsin B. Furthermore, a comparison of the human preprocathepsins V and L with murine preprocathepsin L demonstrated that human cathepsin V, but not human cathepsin L, is more closely related to murine cathepsin L (Fig 1.1)(2).

The chromosomal localization of the cathepsin V gene was mapped using FISH analysis and found to be on chromosome 9q22.2, a site that is adjacent to cathepsin L (3). Both genes consist of eight exons that code for analogous regions of the cathepsins V and L structures. Moreover, the fact that cathepsin V shares almost 80% identity to cathepsin L suggests their recent evolution by gene duplication from an ancestral cathepsin-(V-L)-like gene. This close homology between two cathepsin members can also be seen in other cathepsin pairs such as cathepsins W and F, and cathepsins S and K; hence hinting on the mechanisms through which diversity within a family can be achieved (5, 6).

1.2.2 Crystal Structure of Human Cathepsin V: Overall Structure, Catalytic Machinery and Substrate Binding Pockets

1.2.2.1 Overall Structure and Catalytic Machinery

Activation of cysteine cathepsins is a crucial step in controlling the enzymes’
proteolytic activities. Recent studies have suggested that the activation of lysosomal cathepsins is indeed a combination of inter- and intramolecular process under acidic pH conditions in the lysosome (7, 8). Experimentally, the active enzymes can be obtained by pepsin treatment or autoactivation at acidic pH to remove the propeptide (9). N-terminal sequencing revealed that the first five amino acids of the mature protein are Leu-Pro-Lys-Ser-Val, suggesting that the activation leads to a 221-residue mature protein that begins with Leu 114 of the preproenzyme. The morphology of cathepsin V consists of two lobes that are roughly equal in size (Fig 1.2). The domain that consists of primarily twisted β-sheets resembles the R-domain of papain whereas the other domain, containing three α-helices, demonstrates structural homology to the papain L-domain (10). The cleft that splits the two lobes leads to the active site. The structure of cathepsin V therefore is homologous to the papain family of cysteine proteases. As it can be seen in Figure 3, a superposition of cathepsin V with papain (11), and the more closely related cathepsin L (12), K (13), and S (14) reveals their structural similarity.

Cathepsin V, like other members of the papain family of cysteine proteases, utilizes Cys 25, His 159 and Asn 175 (papain numbering) to carry out the hydrolysis of peptide bonds (Fig 1.3). This catalytic triad initializes a series of acylation and deacylation steps with the assistance of stabilization of intermediate in the oxyanion. His 159 exerts several functions in catalysis. These include forming an ion pair with Cys 25 to help maintain the deprotonated state of His 159, protonating the leaving amine and functioning as the base in the deacylation reaction (15). The oxyanion hole is primarily depending on Gln 19, a residue that is strictly conserved throughout the papain family. The side chain of Gln 19 stabilizes the carbonyl oxygen of the intermediate substrate in
both the acylation and deacylation steps along with the backbone of Cys 25 (16). Finally, Asn 175 takes the indirect role in the catalysis by positioning the His 159 correctly (17). Other studies show that Trp 177 may also play a role in catalysis. The indole ring of Trp 177 lies directly above His 159 and Asn 175 and seems to shield these residues from solvent. Additionally, evidence suggests Trp 177 may play a role in stabilizing the protonated form of His 159 (18, 19).

1.2.2.2 The Substrate Binding Pockets

While all cysteine proteases employ the same catalytic apparatus, what differentiates them is the substrate specificity. This is determined by the identity and arrangement of the amino acids that form the substrate binding pockets. The structure of cathepsin V determined using x-ray crystallographic methods in the presence of the irreversible inhibitor APC-3316 (4-Methylpiperazine-1-carboxylic acid [1-[(3-benzenesulfonyl-1-phenethylallyl)carbamoyl]-2-phenylethyl]amide) demonstrates that the inhibitor's hPhe, Phe, and N-methylpiperazine residues reside in the S1, S2 and S3 pockets, respectively, whereas the sulfone phenyl group binds in the S1' region (Fig 1.4).

The S1 pocket, formed by Asn 64, Gly 23, Gly 65 and the carbonyl oxygen of Cys 63, is rather shallow, which suggests a small contribution to the substrate specificity. The S2 pocket on the other hand is well defined. It is situated in the R-domain with a floor constructed from Ala 133, Gly 160 and the side chain of Met 68, while the walls of this site are formed by Lys 155, Asn 156, Leu 157, Phe 67, the main chain of Asp 158, and the carbonyl oxygen of Gly 66. Finally, lying on the far side of the pocket is Ala 205 whose methyl group "closes" the wall of this site. A unique feature of the S2 pocket of
cathepsin V is utilization of Phe 67 to determine the width of this S2 pocket whereas the length of this pocket is defined by the position of Ala 205. When compared to other cathepsins, the S2 pocket of cathepsin V is rather narrower and longer than those of cathepsins S and K. Due to its depth, the S2 binding pocket can accommodate larger residues that may otherwise only weakly associated to other cathepsins; therefore the S2 pocket plays a major role in the substrate specificity of papain-like cathepsins. Furthermore, since the variability of S2 among cathepsins is rather obvious, it therefore serves as a potential target in the design of specific inhibitors (10). The S3 binding pocket of cathepsin V has its bottom defined by Gly 65, 66 and part of the Asn 60 side chain. Its sides are created by the side chain of Phe 67, Arg 70, and Gln 61, and the main chain carbonyls of Asn 64 and Gly 59. When comparing to other cathepsins, this pocket demonstrates a different shape mainly because of the three side chains that form the sides of this site, and also because of the difference in the positioning of the main chain of Gly 59. Additionally, the conservation of S3 pocket across the papain family is rather little, leading to a spectrum of properties associated with this pocket. As a result, the S3 pocket might serve to be an interesting point for inhibitor design. Finally, the S1' binding pocket, which is formed by the side chain of Trp 177, the side chain of His 159, both the side chain and the carbonyl carbon of Asp 158 and Ala 136, is conserved across much of the papain family. This is likely due to the fact that residues forming this pocket are also involved in the catalytic machinery, which is highly conserved. This suggests the specificity of S1' binding pocket will be similar throughout the papain family (10).
1.2.3. Tissue Distribution and Functions of Human Cathepsin V

Brömme et al. have screened several immune tissue-related organs and found that the transcript for human cathepsin V is uniquely expressed in thymus and testis (2). In addition, others had claimed the expression of human cathepsin V in the corneal epithelium (3, 20). The specific and high expression level of cathepsin V in the thymus suggests a role in the regulation of autoimmunity. In fact, as Tolosa et al. pointed out, cathepsin V is involved in the degradation of invariant chain (Ii) that stabilizes the MHC Class-II complex in human thymus (21). In pathological circumstances, cathepsin V might be a valuable diagnostic marker for colon tumors (3), and it had also been described as participating in elastolytic activity of activated macrophages as well as playing a role in atherosclerosis along with lysosomal cathepsins L, K and S (22, 23).

1.3. Elastin Degradation by Human Cathepsin V

1.3.1. Elastin

Elastin is the extracellular matrix protein that imparts elastic recoil to tissues such as skin, lung, and large blood vessels. The elastic properties of elastin have been explained in terms of entropic contribution. It has also been demonstrated that the basic mechanism is compatible with the classical theory of rubber elasticity (24, 25). Elastin is composed mainly of neutral amino acids such as glycine, alanine, valine and proline, and is made by cross-linking numerous soluble tropoelastin molecules. The lysine residues found in elastin are responsible for the cross-link formation where some lysine ε-amino groups are enzymatically oxidized to form α-amino adipic acid δ-semialdehyde (26).
Subsequently, through either aldol condensation or Schiff reactions, lysino-norleucine, desmosine, and isodesmosine cross-links are chemically synthesized (27–30). Due to its cross-linked nature and extreme hydrophobicity, it is believed to be the most stable protein in mammalian bodies (31–33). While its elasticity has been known for decades, its detailed structure is not known primarily due to its insolubility under physiological conditions. To date, it had been reported that the building block of elastin, tropoelastin, confers an α-helical conformation (34). However further research is needed to predict the cross-linking sites in tropoelastin, and thereby, the possible confirmation of insoluble elastin.

1.3.2. Human Cathepsin V in Atherosclerosis

Atherosclerosis is a disease affecting arterial blood vessels. It is a chronic inflammatory response in the walls of arteries. It occurs in large part due to the accumulation of macrophages. It is then promoted by low density lipoproteins without adequate removal of fats and cholesterol from the macrophages by functional high density lipoproteins (HDL). Atherosclerosis is commonly referred to as "hardening" of the arteries.

Atherosclerosis is generally characterized by the enlargement of arterial intima (the innermost layer of artery) and subsequent deposition of lipids which leads to the formation of plaques. The infiltration of macrophages and smooth muscle cells (SMC) contributes to the formation of atherosclerotic lesions. Notably extra-cellular degrading enzymes such as matrix metalloproteinases, and cysteine proteases are secreted by both of these cell types (35–41). These extra-cellular degrading enzymes destroy the two
main underlying components of arterial walls, the elastins and the collagens. Upon degradation, the blood vessels are weakened, facilitating the formation and the later rupture of the atherosclerotic plaque. The cysteine proteases responsible for the abovementioned degradation are cathepsins V, S and K (42, 43). These enzymes account for ~60% of the total elastin degradation. Of this, approximately two thirds of the degradation occurs extracellularly by cathepsin K and S, whereas the remaining one third is associated to cathepsin V. Among these three cathepsins, cathepsin V demonstrates the most potent elastolytic activity (22).

1.3.3. The Comparison of Human Cathepsin V and Cathepsin L

While the expression of cathepsin V is restricted to thymus, testis, and corneal epithelium, human cathepsin L is a ubiquitously expressed enzyme. Even though these enzymes display differing expression patterns, cathepsin V shares almost 80% amino acid sequence identity with cathepsin L. Moreover, their chromosomal localization also suggests a close linkage as cathepsin V has been mapped to chromosome 9 at 9q22.2, a site adjacent to the cathepsin L locus (2). This is a suggestion of possible divergence of cathepsin V and L from a cathepsin V, L-like ancestor. Furthermore, substrate profiling also seems to point in the same direction. Studies employing a library of synthetic substrates to probe the specificity of each substrate binding pocket of several cysteine proteases from the papain family reported that the S1, S2, and S3 subsites of cathepsin L and V are highly similar and exhibit similar substrate preferences (44).

Nonetheless, cathepsin V is described as the most potent elastase known, where cathepsin L demonstrates only a minimal elastolytic activity (22). Based on their identical
mode of catalysis and similarity in substrate preferences, we hypothesize the presence of a specific exosite in cathepsin V which facilitates the binding of elastin and thus rendering its elastolytic activity.
Figure 1.1 A multiple amino acid sequence alignment between human cathepsin V, human cathepsin L, and mouse cathepsin L. Dark shaded background represents identical amino acids whereas similar amino acids are light shaded, and unrelated residues have a white background. Arrowheads mark the putative cleavage sites between the signal sequence and the propregion, A17-V18, and between the pro- and the mature regions of the protease (D113-L114). Typical for cathepsins, the second amino acid residue adjacent to the processing site of cathepsin V is a proline. (P115) (1)
Figure 1.2 Ribbon model of human cathepsin V (PDB code 1FH0). The coil represents α-helix whereas the arrow stands for β-strands. The black stick molecule in the middle represents the inhibitor APC-3316. The smudge yellow portion at the bottom represents the bridge area (aa 113 to 119), and the brown region on the left is from aa 89 – 104.
Figure 1.3 Superposition of human cathepsin V (Purple) with human cathepsin K (yellow), and S (red), and the mature part of the human procathepsin L (blue) (Figure Adapted from Ref 10).
Figure 1.4 View of the cathepsin V binding site with the structure of bound APC-3316 represented as a stick model. The three hydrogen bonds between main-chain atoms of the inhibitor and the enzyme are shown as dotted white lines. It can be seen that the hPhe, Phe, and N-methylpiperazine moieties of the inhibitor are structurally homologous to the P1, P2, and P3 residues of a natural substrate and are bound in the S1, S2 and S3 pockets of this protease, respectively. The sulfone phenyl group binds in the S1' region.
References


CHAPTER II – Manuscript

2.1 Introduction

The human cathepsin family is a group of papain-like cysteine proteases whose function may range from generic housekeeping functions such as protein degradation and turnover to highly specialized functions such as bone resorption and antigen presentation (1). Among the eleven human cathepsins, cathepsin V had been found expressed in thymus, testis, cornea and epidermis (2–4). Its involvement in converting the MHC/class II-associated invariant chain ß2m into CLIP suggests cathepsin V is the protease regulating the generation of antigen-presentable αβ–CLIP in human thymus (3).

Elastin is the extracellular matrix protein that imparts elastic recoil to tissues such as skin, lung, and large blood vessels (5). Similar to collagen, elastin confers its resistance to proteolytic degradation largely owing to its structure. It consists of numerous cross-linked molecules of soluble tropoelastin whose structure had recently been solved as an α-helical conformation (6). The result is an insoluble elastin whose contribution to tissue’s elasticity and tensile strength is evident. Among the known mammalian elastases such as serine-dependent pancreatic and leukocyte elastases, the Zn-dependent matrix metalloproteinase 12, and several lysosomal cysteine proteases, human cathepsin V had been reported as one of the most potent elastase (4). Moreover, along with cathepsins K, and S, cathepsin V was also expressed by macrophages found in atherosclerotic plaques where they contribute to the degradation elastin that may be directly related to the formation of and the subsequent rupture of plaque (4). While recent studies have indicated that elastolytic cathepsins K and S may carry out the degradation of elastin through a series of adsorption and desorption to elastin in a non-catalytic

A version of this chapter will be submitted for publication.
manner as a mean to commence the proteolysis of elastin (7), no further information concerning the mechanism employed by these elastases could be elucidated due to elastin’s insolubility in aqueous solution.

While human cathepsin V demonstrated potent elastolytic activity against elastin conjugates such as elastin- Congo Red, and elastin-rhodamine, its highly similar counterpart, human cathepsin L, however only exhibits minimal elastolytic activity against these substrates. An amino acid sequence alignment has shown that human cathepsins V and L share 78% identity in their amino acids sequence, and subsite specificity characterization studies using peptide substrate libraries has also revealed highly similar substrate preferences (8). This information suggests that distinct amino acid sequence differences are responsible for the different elastin-degrading capabilities of both cathepsins. In this study, we have determined a region whose existence directly relates to the cathepsin V’s potent elastolytic activity, and within which a short domain lying directly under the S2 subsite contributes to the majority of its elastolytic activity.

2.2 Experimental Procedures

Recombinant Mutant Cathepsins: Cloning, Expression, Activation and Purification. Wildtype cathepsin V recombinant cDNA was previously prepared (8). The recombinant mutant cathepsins were constructed by PCR amplification using Pfu-polymerase (Fig. 2.1) (Fermentas, ON) and primers outlined in Table 1. The PCR constructs were ligated into the XhoI and NotI sites of the P.pastoris expression vector pPIC9 (Invitrogen, CA) rendering direct fusion of the proenzyme sequence with the yeast
secretory α-factor pheromone signal sequence. The vector was then linearized with SacI and subsequently electroporated into P. pastoris GS115 using a standard protocol (9).

The resulted His+ Mut+ clones that carried the recombinant mutant enzymes were selected and tested for productivity. Among which, the clone that produced the highest level of activity against the synthetic substrate, Z-FR-MCA, (Alexis, Switzerland) was selected for expression through fermentation. Briefly, the clones bearing recombinant mutants were first inoculated in 5 mL of MD (Dextrose 2% v/v) and grown for 24hrs at 30°C, followed by resuspending the cell pellet into 50 mL of BMGY ((1% w/v) yeast extract, 2% (w/v) peptone, 100mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base, 4x 10-5% (w/v) biotin, 1% (v/v) glycerol) followed by a 12hrs incubation at 30°C. The cells were again resuspended into 500 mL of BMGY and grown until O.D.600 reached 6.0 before they were resuspended into 1.5 L of BMMY ((1% w/v) yeast extract, 2% (w/v) peptone, 100mM potassium phosphate (pH 6.0), 1.34% (w/v) yeast nitrogen base, 4x 10-5% (w/v) biotin, 0.5% (v/v) methanol)). At this point, the cells were subjected to induction with 0.5% (v/v) methanol every 24hrs while aliquots of the supernatant were withdrawn to test for enzyme activity. The cells were harvested on the 4th day of induction (96hrs post first induction). Cells were separated by centrifugation and the clear supernatant was concentrated 50-fold using an Amicon Ultrafiltration membrane (Millipore, MA) to about 30 mL.

The concentrated yeast supernatant containing the recombinant proenzyme was adjusted to pH 4.0 using glacial acetic acids (Fisher Scientific, NH) and mixed with pepsin to a final concentration of 0.6 mg/mL (Sigma-Aldrich, MS). The activation mixture was incubated at 37°C, and monitored for activity using Z-FR-MCA as a
fluorogenic substrate in 100mM sodium acetate buffer, pH 5.5, supplemented with 2.5mM EDTA and 2.5mM dithiothreitol. The activation was stopped by bringing the pH to 5.5 and ammonium sulfate was added to a final concentration of 2M. After centrifugation at 4,000g, the cleared supernatant was loaded on an n-butyl Sepharose column (GE Healthcare, U.K.) and washed with 100 mM sodium acetate, pH 5.0, containing 2M ammonium sulfate, 0.5mM EDTA and 0.5mM dithiothreitol until A280 reached baseline. Recombinant mutant proteins were eluted from the column using a linear gradient starting with 100 mM sodium acetate, pH 5.0, containing 2M ammonium sulphate and 0.5mM EDTA and 0.5mM dithiothreitol and ending with 100 mM sodium acetate, pH 5.5, containing 0.5mM EDTA and 0.5mM dithiothreitol. The recombinant mutant enzymes were the major 280 nm absorbing peaks, as monitored by activity against Z-FR-MCA. The eluted protease activity containing fractions were combined and further concentrated 10-fold using Amicon Ultra concentrator (Millipore, MA) to about 1.5mL and diafiltered with 100mM sodium acetate buffer, pH 5.5, supplemented with 0.5mM EDTA and 0.5mM dithiothreitol. The final solution was divided into 100uL aliquots for storage at -80°C.

Recombinant Cathepsin V, Cathepsin L, and Mutant Cathepsins Assay with Carbobenzyox-(Z)-Phe-Arg-Methylcoumarylamide Substrate and Enzyme Kinetics. Initial rates of methylcoumarylamide substrate hydrolysis were monitored in 1cm cuvettes at 25°C in a Perkin-Elmer fluorimeter at excitation and emission wavelengths of 380 and 450 nm, respectively. The assay buffer contained 100mM sodium acetate, 2.5mM EDTA and 2.5mM dithiothreitol at pH5.5. The active site concentration of
purified cathepsins was determined by E-64 titration. A pretest was done first to isolate the appropriate range of inhibitor concentrations. Briefly, portions of purified aliquot of cathepsins (5 μL) were diluted appropriately and initial rates against Z-FR-MCA were measured. The range of E-64 was pre-determined by mixing 20 μL of E-64 (various concentrations: 100 μM, 10 μM and 1 μM), 20 μL of diluted cathepsins and 40 μL of assay buffer, and incubated for an hour at 25°C before testing for remnant activity against Z-FR-MCA. The selected range then consisted of 10 to 12 points of E-64 concentrations. The titration was commenced with the abovementioned concentrations of E-64 mixed with diluted cathepsins and assay buffer in the same fashion as in the pre-test. The remnant initial activity would then be plotted on a spreadsheet to determine the x-intercept which represents the concentration of E-64 needed to achieve 100% inhibition thus suggesting the concentration of cathepsin active site. The kinetics constant $k_{cat}$ and $K_m$ of recombinant wildtype and mutant cathepsins were determined by the following: appropriate dilutions were made on cathepsins to achieve appropriate initial activities. The diluted cathepsins were mixed with different concentrations of Z-FR-MCA and assay buffer to construct a Michaelis-Menten representation of cathepsins which was analyzed using nonlinear regression program Prism to obtain the kinetics constants (Refer to Appendix for detail on processing a Michaelis-Menten graph).

Elastin Degradation Assay Using Elastin-Congo Red and Elastin-Rhodamine Conjugates and HPLC Degradation Profile. The cathepsins were diluted appropriately to incubate with either elastin-congo red (Sigma-Aldrich, MS) or elastin-rhodamine (EMD, Germany) (final concentration 10mg/mL) and assay buffer in MaxQ 5000 floor
shaker at 37°C at 200 rpm (Geneq Inc, QC). Samples of reaction mixture were withdrawn at time points 0', 30', 60', 90' and 120' and mixed with E-64 to stop the reaction. The activity was also recorded at the same time points. The samples were centrifuged at top speed on a bench-top accuSpin Microcentrifuge machine (Fisher Scientific, NH) to precipitate undigested particles of elastin-conjugates. Supernatants were loaded unto appropriate plates for O.D.490 measurement using $V_{\text{max}}$ Kinetic Microplate Reader (Molecular Devices, CA) or fluorescence measurement with excitation and absorption of 570 nm and 590 nm using Gemini plate reader (Molecular Device, CA). All measurements were carried out in triplicates. The HPLC degradation profile of bovine neck elastin was carried out as follow. Diluted cathepsins were mixed with bovine neck elastin (EPC, MS) (final concentration of 10mg/mL) and assay buffer, and incubated in MaxQ500 floor shaker at 37°C at 200 rpm for 18hrs. The reaction was stopped with E-64, and the mixture was centrifuged at top speed to precipitate undigested elastin particles. The supernatant was injected into System Gold Detector HPLC (Beckman Coulter, CA) for analysis. The degraded fragments were loaded onto a reversed phase C-18 analytical column (Phenomenex, CA) and eluted with a linear gradient starting with 0.1% trifluoric acid and ending with 90% acetonitrile supplemented with 0.1% trifluoric acid. The resulted spectra were analyzed with the 32 Karat program (Beckman Coulter, CA).

2.3 Results

2.3.1 Initial Approach to the Identification of Elastin-Binding Site in Cathepsin V

In order to identify the structural region responsible for elastin degradation, initial efforts focused on dividing the cathepsin V sequence into four roughly equal regions.
Since it is known that cathepsin L demonstrates only a minor elastolytic activity, corresponding regions of cathepsin L based on sequence alignment were substituted into cathepsin V to observe for loss of elastolytic activity. Three mutant vectors, namely M1, M2 and M3, were constructed with standard cloning protocols using primers spanning the boundary of the divided regions mentioned above. M1 has a.a. 1 to 54 from cathepsin L, and the rest from cathepsin V. M2 contains roughly half of cathepsin L (a.a. 1 to 121) and half of cathepsin (a.a. 121 to 220). Finally, M3 contains the fragment from cathepsin L until a.a. 171 while the rest being cathepsin V. All three mutant constructs were confirmed with automated sequence analysis to contain the correct sequences.

The catalytic activity of the chimera proteins was tested using the synthetic fluorogenic substrate, Z-Phe-Arg-MCA. All three constructs possessed functional active site as depicted by the specificity constant, $k_{cat}/K_m$ obtained (Fig. 2.2). The activities were in the range of the parent cathepsins V and L.

We hypothesized that an exosite present in cathepsin V, but absent from cathepsin L, is responsible for binding of elastin thus rendering the elastolytic activity of cathepsin V. In other words, the chimeric mutant that contains the potential exosite sequence will demonstrate elastolytic activity. Evaluation of the mutants’ elastolytic activity was carried out using elastin-rhodamine as a substrate. Figure 2.3 shows the residual elastolytic activity of both wildtype and mutant cathepsins based on the activity of cathepsin V. It can be seen that M1, in comparison to M2 and M3, demonstrates the most potent elastin degrading activity. M2 and M3, on the other hand, perform in roughly the same level with wildtype cathepsin L. When comparing the two wildtype cathepsins V and L, cathepsin V clearly exhibits roughly 5-times more activity against elastin-
rhodamine. Furthermore, while it can be seen that M1 displays an increased elastolytic activity when compared to wildtype cathepsin V, the activity actually lies close to the experimental error of cathepsin V.

Moreover, while the amount of fluorescent rhodamine certainly points to the mutant’s elastolytic ability, it does not provide any information regarding the locations of cleavage sites in elastin. In other words, the mechanism of elastin degradation could not be compared using fluorogenic elastin conjugates. Hence, to ensure the mutant degrades elastin in the same fashion as wildtype, elastin degradation against unlabeled bovine neck elastin followed by HPLC analysis was carried out. Figure 2.4 shows the HPLC profile of cathepsin V and M1 obtained by running degraded fragments of elastin through a reversed phase C-18 column. The peaks correspond to different sizes of degraded fragments, and the intensity represents the quantity of fragments. A comparison of HPLC profiles between cathepsin V and M1 suggests they both employ similar, if not identical, mechanisms while degrading elastin as the peaks in both profiles almost overlap each other. The same observation was also obtained from HPLC profiles of M2, M3 and wildtype cathepsin L. These data indicate that the amino acid sequence 54 – 121 is responsible for the elastolytic activity in cathepsin V.

2.3.2 Dissection in Region from Amino Acid 54 to 121 in Cathepsin V

Further dissection of the 54-121 amino acid region was carried out to zoom into amino acid residues responsible for the formation of the putative exosite. For this purpose, two more primers were designed: M4 divides the region further into two segments, and M5 was created based on both structure analysis and sequence alignment. Upon
examining the crystal structure of cathepsin V (11), it can be seen that amino acid 112 to 119 of cathepsin V lies directly under its S2 subsite and forms a bridge-like structure. This region defines the bottom of one of the entrances that lead to the active site cleft and is therefore potentially interesting.

The expressed mutant cathepsins M4 and M5 both exhibit functional active sites; M4 even displays an exceptionally high value of specificity constant against Z-FR-MCA (Fig. 2.2). The elastolytic activity of both mutants was assessed with elastin-rhodamine conjugate. M4, while containing portion from cathepsin L until amino acid 89, retains full elastolytic activity when compared to wildtype cathepsin V. On the other hand, M5, when compared to M2, has the cathepsin L sequence till 112 and thus contains the cathepsin V-specific bridge area, is only able to recover 60% of the elastin degrading activity (Fig 2.4). The result suggests the involvement of the bridge region (a.a. 112 to 118) in retaining the majority of the wildtype’s elastolytic activity and the participation of residues from the M4 region (a.a. 89 to 113) for the remaining elastolytic activity.

Although the bridge region is certainly significant in retaining a vast amount of the parental cathepsin V’s elastolytic activity, its inability to recover the full activity suggests a partial or incomplete mechanism might be employed due to loss of region from a.a. 89 to 113. After subjecting bovine neck elastin fragments degraded overnight by M5 to HPLC analysis, it can be seen that while both profiles are highly similar to each other, few peaks are either missing or shown in lower intensity in the profile of M5 (Fig. 2.6). This further confirms that a compromised binding mechanism might be in effect resulting in such incomplete digestion pattern. On the other hand, M4 which demonstrates full elastolytic activity was expected to employ a complete binding
mechanism resembling that used by the wildtype cathepsin V (Fig. 2.5). The HPLC profile of M4 suggested that even though fewer discrepancies were seen when compared to M5, there were still few peaks that are missing (Fig. 2.7). Combining both the elastin assay and HPLC profiles, it can be inferred that while both regions are significant in pertaining full elastolytic activity, other residues must have aided in the binding of elastin thus generating a slightly different degradation pattern.

2.3.3 A Parallel Approach to Probe the Bridge Region

As it has been shown that retaining the bridge region recovers the majority of cathepsin V’s elastolytic activity, the next round of mutations were mainly made to further characterize this region. In one instance, the bridge region was exchanged between cathepsin L and cathepsin V to further assess its role as a potential elastin binding domain. In another, sequence alignment of cathepsins S, K, and V demonstrated that there is a proline residue followed by either a negatively charged amino acid a deletion connecting to a glycine residue which lies ahead of a lysine residue (Fig. 2.8). This trend is however not observed in cathepsin L as the proline in the bridge region is directly followed by a lysine residue. Due to proline’s unique structure and glycine is characteristic small size, the lysine that follows the glycine seems to orient its side chain outward whereas such orientation of the lysine side chain is not observed in cathepsin L; rather, it appears that the lysine side chain is pointing inward (Fig. 2.9). It thus suggests the involvement of glycine 118 in cathepsin V in orienting the subsequent lysine away and possibly creating either an antenna for elastin binding while opening up the width of the entrance into the active site cleft for elastin. To test this hypothesis, glycine 118 was
deleted from cathepsin V while an extra glycine residue was inserted in between proline 116 and lysine 117 in cathepsin L.

2.3.3.1 Bridge Exchange Between Cathepsins V And L

The results obtained from M4 and M5 suggests that the bridge is directly involved in retaining approximately 60% of the wildtype elastolytic activity in cathepsin V. It was thus designed to mutually swap the residues VDIPK (amino acids 113 – 117) in cathepsin L with residues TVVAPGK (amino acids 113 – 119) from cathepsin V to assess its effect in either creating or diminishing the elastolytic activity, respectively. The mutants were cloned and expressed using standard protocol. The kinetics analysis on both mutants against synthetic substrate Z-FR-MCA was carried out, and the resulted $k_{cat}/K_m$ values suggest the proper formation of active site and retention of subsites preferences found in their corresponding wildtype cathepsins. Mutant cathepsin V bearing the residues VDIPK from cathepsin L (M8) was assayed against elastin-rhodamine. The swapping of the bridge area seemed to contribute to a 60% loss of elastolytic activity (Fig. 2.10), and to alter the degrading pattern as is evident from the HPLC profile (Fig. 2.11). Several characteristic elastin fragments seen in the profile of cathepsin V are not found in that of M8 which suggests incomplete degradation. On the other hand, however, mutant cathepsin L carrying residues TVVAPGK of cathepsin V (M9) did not seem to reflect the mirror-image effect one would expect. The introduced bridge region in M9 was unable to generate any significant increase in elastolytic activity as the amount of fluorescence generated by rhodamine upon cleavage was comparable to the result obtained from degradation by wildtype cathepsin L (Fig. 2.10). Upon examining the M9’s HPLC profile
on the degraded elastin fragments however, it can be seen that while most of the characteristic peaks seen in cathepsin L’s degradation profile were not observed, a characteristic peak resembling one from cathepsin V’s profile was found (Fig. 2.12). So even though no observable elastolytic activity was concluded through this mutation, pattern of elastin degradation was certainly altered which supports the region’s involvement in elastin degradation.

2.3.3.2 Glycine 118’s Involvement In Elastolytic Activity

In parallel to the bridge exchange, the effect of glycine 118 was evaluated, and two mutants were created. M6 contains a single glycine 118 deletion from the wildtype cathepsin V, and M7, serving as the counterpart in this assessment, carries a glycine insertion in between proline 116 and lysine 117 in cathepsin L. Both mutants’ activities against Z-FR-MCA were assessed and kinetics study was carried out; both results suggested the retention of parental wildtype cathepsins’ specificity constant (Fig. 2.2). Further elastin degradation assay showed the same trend found in M8 and M9. Glycine-deleted M6 demonstrated a 25% decrease in elastolytic activity (Fig. 2.13), and the HPLC profile generated indicates a slight change in the size of fragments obtained (Fig. 2.14). These results pointed toward the involvement of glycine 118 in elastin degradation; however, the expected effect of glycine insertion was not manifested in the case of M7. The elastin degradation assay by M7 using elastin-rhodamine demonstrated that inserting a glycine is not sufficient to restore the activity (Fig. 2.15). This is compatible with results obtained from M9; since swapping of the bridge region did not restore any elastolytic activity, glycine insertion should not as well. Furthermore, much like the
effect brought by the bridge region of cathepsin V, the degradation profile of M7 against bovine neck elastin also pointed toward the involvement of that region as the glycine inserted certainly altered the local conformation of that area (Fig. 2.16).

2.4 Discussion

The amino acid sequence of cathepsin V bears approximately 80% identity to that of cathepsin L, and has been found in testis, cornea, and thymus (9, 12, 2). Cathepsin V has been described as a major protease in corneal epithelium (2). Moreover, it was reported that cathepsin V is involved in the conversion of MHC/class II-associated invariant chain Ii into CLIP and therefore suggesting its role in the generation of antigen-presentable αβ –CLIP complexes in human thymus (3). The crystal structure of cathepsin V has also been solved (11), and its active sites and sub-pockets were thoroughly studied. While there are a growing number of reports on cathepsin’s function and structure, less is known about its elastolytic activity. Cathepsin V is described as the most potent mammalian elastase to-date, its elastolytic activity against elastin-conjugate in comparison to other enzymes was found to be the highest (4). Human cathepsin L, on the other hand, only demonstrated a minor elastolytic activity. Furthermore, a substrate specificity profiling studies on various human cathepsins reported that the substrate preferences of both human cathepsins V and L are highly similar (8). These observations raise the question what structural difference between cathepsins V and L is responsible for the high elastolytic activity of cathepsin V.

Nonetheless, one potential concern that lies in this study is how to contribute the elastolytic activity observed to the structural difference but not to the difference in the
enzyme's protease activity in general. This question can be answered with the kinetic behaviors of the mutants. First, both cathepsins V and L employ an identical catalytic triad to carry out the proteolysis. While cathepsins V and L have different turnover rates, the enzyme kinetics of all mutants are compatible with their parental cathepsins. So, a mutant enzyme behaves more like either one of the parental cathepsins when the proportion of that parental cathepsin becomes greater. However, the elastolytic activity observed is independent of such trend in enzyme kinetics. When comparing the $k_{cat}/K_m$ value of M5 with wildtype cathepsin L against synthetic Z-Phe-Arg-MCA, M5 and cathepsin L demonstrate comparable specificity constants (Fig 2.2). However, their elastolytic activities against elastin-rhodamine display a 2-folds difference (Fig 2.5). This suggests that while the mutant may behave like wildtype cathepsin L, the added region from cathepsin V has "created" the elastolytic activity. This independence of elastolytic activity to the enzyme's protease activity can also be seen when comparing M5 and M3 where they clearly behave similarly in the Z-Phe-Arg-MCA hydrolysis, but differ greatly in their elastolytic activity (Fig 2.2, 2.3 and 2.5). This observation supports our original hypothesis that an elastin binding domain must exist to render cathepsin V its elastin degrading activity. Furthermore, it has been reported that human cathepsin L demonstrates potent activity against soluble ETNA-elastin but displays minimal adsorption to insoluble elastin (7). This finding is compatible with our hypothesis in that human cathepsin L must lack the elastin binding domain thereby is unable to adsorb to and degrade polymerized elastin efficiently. Moreover, human cathepsin L's lack of an elastin binding domain and its potent elastolytic activity against soluble elastin also helps
distinguishing the insoluble elastin degrading ability of cathepsin V as a structure related phenomenon but less as a general protease activity issue.

The investigation on elastolytic activity has not been an easy task mainly due to elastin’s insolubility in aqueous phase, and its random pattern of linking and cross-linking of tropoelastins. As a result, one cannot predict the overall structure of the polymerized elastin; and for this reason, elastolytic activity of enzymes are assayed either using fluorogenic or chromogenic elastin conjugates or by labeling degraded elastin fragments with fluorogenic chemicals such as fluorescamine. In this study, we set out to pinpoint the potential elastin binding domain in human cathepsin V by cloning chimeric mutants and mutants involving point-mutation and region swapping. Our sequential analysis has pinpointed the potential elastin binding domain to amino acids 89 to 119 in cathepsin V with the TVVAPGK (amino acids 113 – 119) region contributing most to the elastolytic activity found in cathepsin V (Fig. 2.16). The TVVAPGK domain which resembles a bridge-like structure is located beneath the S2 subsite pocket. Being located right at the entrance leading to the active site cleft, it is tempting to speculate that the bridge directs the bound elastin directly into the cleft where the active site residues could carry out the cleavage reaction. We assessed the importance of this domain by mutually swapping this domain between human cathepsins V and L, designated M8 and M9. It was clearly seen that without the bridge domain, M8 lost 50~60% of its elastolytic activity whereas switching the bridge domain to cathepsin L did not rescue any noticeable elastolytic activity (Fig. 2.10). The inability to generate elastolytic activity in cathepsin L led us to consider the involvement of other domain; however, the decrease in elastolytic activity found in M8 indicated that the TVVAGPK domain played a role in elastin
degradation. Furthermore, this domain is also interesting because the extending arms of lysine residue 119 pointing away from the active site cleft (Fig. 2.9) may service two possible purposes. First, the lysine side chain may act as an antenna that inserts into and hold the elastin in place; secondly, its orientation may also clear up the "passage way" leading to the active site. The opposite is observed in human cathepsin L where the side chain of lysine\textsuperscript{117} is oriented inwardly facing the active site cleft. This configuration may have contributed to the inability of cathepsin L to bind to elastin molecule. From the overlay of the bridge domains and sequence alignment of human cathepsins V and L, it was found that a glycine residue immediately following a proline residue is present in cathepsin V but not L. Due to the unique structure of proline and the size of glycine, such arrangement may have resulted in the orientation of the succeeding lysine. So, a glycine deletion and insertion was carried out in cathepsins V and L, respectively, and designated as M6 and M7 mutants to evaluate the impact of such arrangement. It was found that the deleted glycine correlates with a 25% decrease in cathepsin V's elastolytic activity (Fig. 2.13) suggesting its potential role in elastin degradation. On the other hand, while inserting a glycine residue into cathepsin L did not generate an increase in elastolytic activity, the HPLC profile of elastin degradation by M7 revealed a elastin degradation pattern that differs from that of wildtype cathepsin L and resembles that of wildtype cathepsin V (Fig. 2.15). Taken together, even though no information concerning the mechanism employed by these mutants during elastin degradation could be inferred, the participation of the TVVAPGK domain and of the glycine residue in the reaction was evident. Other residues that may play a role in elastin degradation are found within amino acid 92 to 104 (Fig. 2.8 and 2.16). Residues that differ between cathepsin V and L within
that region show more neutral amino acids in cathepsin V than in cathepsin L. Since elastins are consisted mainly of neutral amino acids, a neutral surface of the region would serve as a potential binding patch whereas the charged residues in the same region of cathepsin L would not favor such interaction. It is therefore of great interest to further explore the involvement of this region in elastin degradation.

It had been reported that cathepsins K, S and L may proceed in elastin degradation through the following steps: first the enzyme adsorbs to elastin molecule in a nonproductive manner, and the adsorbed enzyme then interacts with other elastin sites to form a catalytically productive complex. Upon cleavage and release of the products, the enzyme may either remain adsorbed on the surface of elastin or dissociates from it (7). It was noted that such adsorption step was not observed in cathepsin L probably because the adsorption/desorption proceeds in rapid cycle. One would however expect cathepsin L to demonstrate high elastolytic activity if such speculation is true. Because cathepsin L had been shown to demonstrate high degrading activity against soluble elastin, if there indeed is an interaction between cathepsin L and the elastin molecule, then the subsequent catalytic step should commence spontaneously and rapidly as well thus producing a potent elastolytic activity. The result reported from our laboratory however was not compatible with such option as only minimal elastin degradation was observed for human cathepsin L (4). Therefore one may consider that the inability to detect any adsorption/desorption be contributed to its lack of a strong elastin-binding domain. However, if that is the case, how do we explain the inability to rescue cathepsin L’s elastolytic activity after swapping the potential elastin-binding domain? It can be argued that since elastin is mainly consisted of neutral amino acids, thus possibly possessing a
surface with neutral electrostatic potential. Human cathepsin V has been reported to possess only a few localized negatively charged patches on its surface while its surface including the substrate binding region is mostly neutral thereby potentially facilitating the hydrophobic interaction with elastin. However, the electrostatic potential of cathepsin L is negative in extended regions of the surface including the active site cleft (9). Since elastin is relatively large comparing to cathepsin L, the negative surface potential may not allow strong adsorption of the enzyme to the surface of elastin in spite of the introduced elastin-binding domain, hence the observed lack of increased elastolytic activity. On the other hand, cathepsin V, after swapping of the bridge-region (TVVAPGK) with cathepsin L, demonstrated compromised elastolytic activity because the introduced region may weaken elastin binding. Upon more considerations, the fact that two potential elastin binding domains in cathepsin V are not adjacent to each other but still contribute to elastolytic activity can be explained as well. Since elastin, being polymers of tropoelastins are vastly cross-linked to each other, may possess multiple extending “pods” of tropoelastins and is so much larger in size in comparison to cathepsin V, it will make perfect mechanistic sense for cathepsin V to possess multiple elastin-binding domains on different facets to involve in a three-dimensional interaction with elastin as cathepsin V “dives” into the gaps between tropoelastins. So, combining the adsorption/desorption model proposed in another study (7), an overall putative model of elastin binding by cathepsin V may involve the following steps. First, weakly charged cathepsin V adsorbs on the surface of elastin in a non-catalytically productive manner facilitated by the insertion into and subsequent fastening of ε-amino side chain of lys119 to elastin, as well as elastin interaction found on the neutral patches in the region of
amino acids 91 to 104, followed by the spontaneous docking of tropoelastin into the neutral active site cleft where the catalytic step takes place (Fig. 2.17).

The identification of elastin-binding domains in cathepsin V might be exploited for inhibitor design. Cathepsin V was found to degrade elastin in pathological conditions such as atherosclerosis (4); however, its involvement in the generation of antigen-presentable αβ–CLIP complexes in immune system suggests its important role in humans (3). Therefore, the design of a specific inhibitor that will diminish its elastolytic activity perhaps through interruption of elastin binding while still allowing it to carry out its regular functions might be beneficial. Identifying the elastin binding domains is simply the first step. As the mechanistic information of elastin degradation slowly unfolds, the approaches to develop elastolytic activity-specific inhibitor will eventually be realized.
Table 2.1

PCR Primers for mutant cathepsins

A compilation of all primers used in constructing mutant cathepsins. PCR reactions were carried out using a standard protocol. The melting temperature was 95°C, annealing temperatures vary according to the individual primers and the extension temperature was 72°C. An initial 5 minutes heat-start and final 8 minute extension step was also applied.

<table>
<thead>
<tr>
<th>Mutant Cathepsins</th>
<th>Primer Sequences</th>
<th>Mutation</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant 1</td>
<td>5’-CAC TGA GCG AGC AGA ATC TGG-3’</td>
<td>Cat L: aa 1 to 54</td>
<td>57.9</td>
</tr>
<tr>
<td>Forward</td>
<td>Reverse</td>
<td>Cat V: aa 47 to 221</td>
<td>57.9</td>
</tr>
<tr>
<td>Mutant 2</td>
<td>5’-GGA GAA GCC CCT GAT GAA AGC AG -3’</td>
<td>Cat L: aa 1 to 127</td>
<td>60.0</td>
</tr>
<tr>
<td>Forward</td>
<td>Reverse</td>
<td>Cat V: aa 119 to 220</td>
<td>60.0</td>
</tr>
<tr>
<td>Mutant 3</td>
<td>5’-GAT CAT GGT GTT CTG GTG GTT GG-3’</td>
<td>Cat L: aa 1 to 171</td>
<td>58.1</td>
</tr>
<tr>
<td>Forward</td>
<td>Reverse</td>
<td>Cat V: aa 163 to 220</td>
<td>58.1</td>
</tr>
<tr>
<td>Mutant 4</td>
<td>5’-GGC CTG GAC TCT GAG GAA TCC TAT CC-3’</td>
<td>Cat L: aa 1 to 90</td>
<td>60.1</td>
</tr>
<tr>
<td>Forward</td>
<td>Reverse</td>
<td>Cat V: aa 82 to 221</td>
<td>60.1</td>
</tr>
<tr>
<td>Mutant 5</td>
<td>5’-TCT GTT GCT AAT GAC ACC GGC TTT-3’</td>
<td>Cat L: aa 1 to 112</td>
<td>59.0</td>
</tr>
<tr>
<td>Forward</td>
<td>Reverse</td>
<td>Cat V: aa 105 to 221</td>
<td>59.0</td>
</tr>
<tr>
<td>Mutant 6</td>
<td>5’-ACA GTG GTC GCA CCT AAAG GAG -3’</td>
<td>Cat V gly118</td>
<td>58.9</td>
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<tr>
<td>Forward</td>
<td>Reverse</td>
<td>Knock-out</td>
<td>58.9</td>
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<tr>
<td>Mutant 7</td>
<td>5’-GTG GAC ATC CCT GGA AAG CA -3’</td>
<td>Cat L glycine</td>
<td>57.4</td>
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<td>Forward</td>
<td>Reverse</td>
<td>Insertion between Pro116 and Lys117</td>
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<tr>
<td>Flanking Primers</td>
<td>5’-GAC TGG TTC CAA TTG ACA AGC-3’</td>
<td>N/A</td>
<td>54.3</td>
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<tr>
<td>Forward</td>
<td>Reverse</td>
<td>5’-GCA AAT GGC ATT CTG ACA TCC-3’</td>
<td>54.8</td>
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</table>
Table 2.2

The enzyme kinetics of both wildtype and mutant cathepsins.
The $k_{cat}$ and $K_m$ value of each enzyme was calculated using GraphPad Prism program through non-linear regression algorithm. The substrate used was the synthetic fluorogenic substrate Z-FR-MCA using condition outline under “experimental procedure”.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin V</td>
<td>25.18 ± 0.83</td>
<td>11.45 ± 0.08</td>
<td>2.20 ± 0.07 x 10$^6$</td>
</tr>
<tr>
<td>M6</td>
<td>18.19 ± 0.24</td>
<td>7.04 ± 0.29</td>
<td>2.58 ± 0.03 x 10$^6$</td>
</tr>
<tr>
<td>M8</td>
<td>26.82 ± 0.86</td>
<td>8.29 ± 0.99</td>
<td>3.24 ± 0.10 x 10$^6$</td>
</tr>
<tr>
<td>M1</td>
<td>11.24 ± 0.48</td>
<td>11.20 ± 0.83</td>
<td>1.00 ± 0.04 x 10$^6$</td>
</tr>
<tr>
<td>M2</td>
<td>17.72 ± 1.43</td>
<td>3.00 ± 1.56</td>
<td>5.91 ± 0.48 x 10$^6$</td>
</tr>
<tr>
<td>M3</td>
<td>23.5 ± 0.1</td>
<td>3.12 ± 1.54</td>
<td>7.54 ± 0.02 x 10$^6$</td>
</tr>
<tr>
<td>M4</td>
<td>24.92 ± 0.35</td>
<td>1.15 ± 0.07</td>
<td>2.17 ± 0.31 x 10$^7$</td>
</tr>
<tr>
<td>M5</td>
<td>16.18 ± 0.42</td>
<td>2.54 ± 0.20</td>
<td>6.36 ± 0.17 x 10$^6$</td>
</tr>
<tr>
<td>M9</td>
<td>7.72 ± 0.03</td>
<td>2.00 ± 0.12</td>
<td>3.86 ± 0.01 x 10$^6$</td>
</tr>
<tr>
<td>M7</td>
<td>24.76 ± 0.86</td>
<td>1.89 ± 0.22</td>
<td>1.31 ± 0.45 x 10$^7$</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>11.57 ± 2.37</td>
<td>1.33 ± 0.33</td>
<td>8.72 ± 1.78 x 10$^6$</td>
</tr>
</tbody>
</table>
Figure 2.1 A schematic diagram showing the various proportions of cathepsins V and L in mutants. Both cathepsins L (blue) and V (red) have their catalytic units marked and named in red. The only difference to their S2 subsite is also marked and identified in black. (Leucine in cathepsin L and proline in cathepsin V). The box indicates regions that were shown to be responsible for any elastolytic activity. A cross-mark (x) in Mutant 6 indicates glycine^{118} deletion and a plus sign (+) in Mutant 7 represents a glycine addition.
Figure 2.2 The \( k_{cat}/K_m \) comparison of selected mutant and wildtype cathepsins against Z-FR-MCA. Appropriate dilution was made on cathepsins to achieve decent initial activity. The diluted cathepsins were mixed with different concentrations of Z-FR-MCA and assay buffer to construct a Michaelis-Menten representation of cathepsins which was analyzed using nonlinear regression program Prism to obtain the kinetics constants. All values are derived from measurements of triplicates.
Figure 2.3 The elastolytic activity of mutant and wildtype cathepsins using elastin-rhodamine conjugates at 120 minutes. 10mg/mL of elastin-rhodamine was incubated at 37 °C with 1 μM of enzyme with constant stirring of 200 rpm in activity buffer containing 100mM sodium acetate, 2.5mM EDTA and 2.5mM dithiothreitol at pH5.5. Aliquots of sample were withdrawn from the reaction mix at t = 0, 30, 60, 90, and 120 minutes, then E64 was added and the mutants tested for absorbance at 590 nm. All measurements were taken in triplicates. The $p > 0.31$ between Cat V and M1; $p > 0.05$ between Cat L and M2, and $p > 0.08$ between Cat L and M3.
Figure 2.4 The HPLC profiles of wildtype cathepsin V and M1. Cathepsins (final concentration 1 μM) were mixed with bovine neck elastin (EPC, MS) (final concentration of 10mg/mL) and assay buffer, and incubated in MaxQ500 floor shaker at 37 °C at 200 rpm for 18hrs. The reaction was stopped with E-64, and the mixture was centrifuged at top speed to precipitate undigested elastin particles. The supernatant was injected into System Gold Detector (Beckman Coulter, CA) for HPLC profile. The degraded fragments were loaded onto a reversed phase C-18 analytical column (Phenomenex, CA) and eluted with a linear gradient starting with 0.1% trifluoric Acid and ending with 90% acetonitrile supplemented with 0.1% trifluoric acid. The chromatograms were analyzed with 32 Karat program. It can be seen that both profiles almost superimpose each other suggesting M1 retains all the residues needed to exercise the full elastin-binding mechanism.
Figure 2.5 The elastolytic activity of mutants 2, 4, 5 and wildtype cathepsins using elastin-rhodamine conjugates at 120 minutes. 10mg/mL of elastin-rhodamine was incubated at 37 °C with 1 μM of enzyme under constant shaking of 200 rpm in activity buffer containing 100mM sodium acetate, 2.5mM EDTA and 2.5mM dithiothreitol at pH5.5. Aliquots of sample were withdrawn from the reaction mix at t = 0, 30, 60, 90, and 120 minutes, added with E64 and tested for absorbance at 590 nm. Enzyme activities against Z-FFR-MCA were also recorded at the above time points to monitor their residual activities. All measurements were taken in triplicates with standard deviation shown as error bar. While M4 retains full elastolytic activity, M5 only possesses 60% of the original elastolytic activity. However, when compared to M2, M5 which has only 8 amino acids more from cathepsin V demonstrates a dramatic increase in elastolytic activity. \( P > 0.96 \) between Cat V and M4; \( p < 0.02 \) between Cat V and M5; and \( p > 0.05 \) between Cat L and M2.
Figure 2.6 The HPLC profiles of wildtype cathepsins V, L, and M5. Several peaks that can be seen from the profile of human cathepsin V are missing from the profile of M5. Also, the highest peaks that overlap each other in both profiles seem to be different in intensity. This suggests that while the bridge region is important in generating 60% of elastolytic activity, it does not provide full binding mechanism resembling one employed by wildtype human cathepsin V.
Figure 2.7 The HPLC profiles of wildtype cathepsins V, L and M4. Few peaks are missing from the profile of M4, yet M4 still demonstrates to retain full elastolytic activity. It suggests that while other residues might be involved in achieving a complete binding mechanism, they may not be as crucial in generating elastolytic activity. Also, it can be seen that the highest peaks are almost equal in intensity even though other peaks are lower for M4.
Figure 2.8 Multiple amino acids sequence alignment of human cathepsins K, S, V and L. All sequences were extracted through CBI database. The sequences were aligned using the Clustal multiple sequence alignment algorithm. Identical residues are marked with an asterisk, related residues are marked with two dots and those of less homology with one dot. All amino acids sequences contain the pre-pro-region, pro-region and the mature enzyme. The letters highlighted in red, are the first residue of the mature enzyme and the region highlighted in blue are the Pro-X-Gly-X segments. Notice that all but cathepsin L possess the glycine residue in the blue region. The residues shown in bold and red colour are the ones differing between cathepsins L and V that may contribute to the elastin degrading ability of cathepsin V.
Figure 2.9 The superposition of human cathepsins V and L. These two structures were obtained from the RCSB Protein Data Bank with the designated number 1FH0 (human cathepsin V) and 1ICF (human cathepsin L). The overlay was done using PyMol program. The bridge region is represented as a stick model for both cathepsin V (yellow) and cathepsin L (red). It can be seen that the region mostly superimposes each other as it moves from left to right until proline (in white box). The lysine side chain immediately follows the proline residue in cathepsin L orients inward (red arrow) while the lysine side chain in cathepsin V following the glycine (gray) residue is imposing outwardly (yellow arrow).
Figure 2.10 The elastolytic activity of mutants 8, 9 and wildtype cathepsins using elastin-rhodamine conjugates. 10mg/mL of elastin-rhodamine was incubated at 37 °C with 1 μM of enzyme under constant shaking of 200 rpm in activity buffer containing 100mM sodium acetate, 2.5mM EDTA and 2.5mM dithiothreitol at pH5.5. Aliquots of sample were withdrawn from the reaction mix at t = 0, 30, 60, 90, and 120 minutes, added with E64 and tested for absorbance at 590 nm. All measurements were taken in triplicates with standard deviation shown as error bar. M8 retains approximately 60% of activity where M9 demonstrates comparable activity to cathepsin L. P < 0.04 between Cat V and M9, and p < 0.04 between Cat L and M9.
Figure 2.11 The HPLC profiles of wildtype cathepsins V, L and M8. The profile of M8 displays high similarity to wildtype cathepsin V with only a few peaks that are lower in intensity relative to other peaks. The highest peak is also significantly lower for M8.

Figure 2.12 The HPLC profiles of wildtype cathepsins V, L and M9. M9 which contains the bridge region of cathepsin V demonstrates an increase in intensity of the highest peak even though no other degraded fragments are observed.
Figure 2.13 The elastolytic activity of mutants 6, 7 and wildtype cathepsins using elastin-rhodamine conjugates at 120 minutes. 10mg/mL of elastin-rhodamine was incubated at 37 °C with 1 μM of enzyme under constant shaking of 200 rpm in activity buffer containing 100mM sodium acetate, 2.5mM EDTA and 2.5mM dithiothreitol at pH5.5. Aliquots of sample were withdrawn from the reaction mix at t = 0, 30, 60, 90, and 120 minutes, added with E64 and tested for absorbance at 590 nm. All measurements were taken in triplicates with standard deviation shown as error bar. It can be seen that M6 retains approximately 75% of the wildtype elastolytic activity, whereas insertion of glycine in cathepsin L (M7) did not rescue elastin-degrading activity. $P < 0.03$ between Cat V and M6, and $p > 0.93$ between M7 and Cat L.
Figure 2.14 The HPLC profiles of wildtype cathepsins V, L and M6. Carrying a glycine deletion, the HPLC profile of M6 demonstrates a degradation pattern that is highly similar to the wildtype but with few missing peaks and a less intense highest peak. This confirms the deletion of glycine compromises the elastolytic activity possibly because the mutant was unable to bind to certain residues of elastin.
Figure 2.15 The HPLC profiles of wildtype cathepsins V, L and M7. After inserting a glycine between proline 116 and lysine 117 in cathepsin L, the mutant although did not generate any detectible results from elastin-rhodamine assay, it is evident that the binding pattern is more like cathepsin V than cathepsin L. It suggests the importance of glycine in the possibly changing the orientation of the succeeding lysine side chain thus allowing partial binding and degradation of elastin.
Figure 2.16 The distribution of potential elastin binding domains in human cathepsin V. Shown here is a PyMol rendered surface representation of human cathepsin V. The domains potentially correlate with the elastolytic activity are colored in steel blue, royal blue and yellow whereas the rest was left as red. The steel blue domain spans amino acid 113 to 119, TVVAPGK, in cathepsin V is located directly at the entrance to the active site cleft. The royal blue patch represents glycine^{118} which may involve in elastin binding by orienting the subsequent lysine^{119}. The yellow domain spanning amino acid 89 to 104 is where the neutral patch is located. Notice how the two domains are not adjacent to each other which is mechanistically reasonable as elastin in its natural form is a polymerized tropoelastin with complicated cross-linking, therefore by placing the elastin binding domains on different facets of the enzyme it allows the enzyme to interact the macromolecule in a three-dimension fashion.
Figure 2.17 A schematic representation of the proposed elastolytic mechanism of cathepsin V. Binding is initiated through binding of the side chain of lysine$^{119}$ (dark blue) of cathepsin V (light blue) to one of the extending pod of elastin molecule (gray). Adsorption happens when the elastin-binding domains (green: bridge-region; red: neutral patch) of cathepsin V contact with other extending pod of elastin molecule. Catalysis takes place as a pod of elastin falls into the active site cleft. The final dissociation of enzyme from the elastin molecule occurs immediately after the catalysis leaving a cleaved product.
References


CHAPTER III – Conclusion and Recommendations for Further Work

The human cathepsins are a family of papain-like cysteine proteases found to perform an array of functions. While for some, the functions and distributions are identified; some members of the family are rather new. (1). Human cathepsin V is one of the later additions to the cathepsin family as its identification and characterization was only carried out in the past ten years (2). The amino acid sequence of cathepsin V bears a 80% identity to that of cathepsin L, and its expression has been found in testis, cornea, epidermis, and thymus (2, 3, 4) suggesting a potential role in immune response. The crystal structure of cathepsin V has been studied (5), and its active sites and sub-pockets thoroughly characterized. While there are a growing number of reports on cathepsin’s function and structure, less is known about its elastolytic activity. Cathepsin V is described as the most potent mammalian elastase to-date (6). Human cathepsin L, on the other hand, only demonstrated minimal elastolytic activity. Moreover, a substrate specificity profiling study on various human cathepsins revealed significant similarity between the substrate preferences of both human cathepsins V and L (7). Based on these observations, it prompts us to hypothesize that a structural difference between cathepsins V and L must exist that causes the potent elastolytic activity of cathepsin V.

Nonetheless, one potential concern that lies in this study is how to contribute the elastolytic activity observed to the structural difference but not to the difference in the enzyme’s protease activity in general? This question can be answered taking advantage of the kinetic behaviors of the mutants. Both cathepsins V and L employ identical catalytic sites to carry out proteolysis even though their substrate preference may differ slightly. However, the kinetics of all mutants is compatible with their parental cathepsins. The
mutant behaves more like one parental cathepsin or the other along with increasing proportion of either parental cathepsin. While there is a trend in the mutants’ kinetic behavior, the elastolytic activity is independent of such trend. Furthermore, it has been reported that human cathepsin L demonstrates potent activity against soluble ETNA-elastin but displays minimal adsorption to insoluble elastin (8). This finding is compatible with our hypothesis that human cathepsin L must lack the elastin binding domain thus unable to adsorb to and degrade polymerized elastin efficiently. Therefore, human cathepsin L’s lack of an elastin binding domain and its potent elastolytic activity against soluble elastin altogether also helps distinguishing the insoluble elastin degrading ability of cathepsin V as a structure related phenomenon. However, two obstacles lying in the investigation of elastolytic activities: 1) elastin is insoluble in aqueous phase, and 2) its pattern of cross-linking of tropoelastins is random. One hence cannot predict the overall structure of the polymerized elastin and the assay of elastolytic activity are almost always carried out either using fluorogenic or chromogenic elastin conjugates or by labeling degraded elastin fragments with fluorogenic chemicals such as fluorescamine.

In this study, we set out to pinpoint the potential elastin binding domain in human cathepsin V by cloning chimeric mutants and mutants involving point-mutation and domain-swapping. Our sequential analysis has pinpointed the potential elastin binding domain to amino acids 89 to 119 in cathepsin V where TVVAPGK (amino acids 113–119) seems to correlate with majority of elastolytic activity found in cathepsin V. The TVVAPGK domain (bridge-like) is an interesting one because of its close proximity to the active site cleft and the orientation of the lysine side chain. It is located right at the entrance leading to the active site cleft, and therefore may direct the bound elastin into
the active site cleft for degradation. The importance of this domain was assessed by mutual swapping between human cathepsins V and L, designated M8 and M9. And it was clear that the loss of the domain from cathepsin V constitutes an approximately 50–60% loss of elastolytic activity. However, implementing this same domain in cathepsin L did not rescue any noticeable elastolytic activity. This absence of elastolytic activity in cathepsin L led us to consider the involvement of another domain.

One other point of interest in this bridge-like domain is the orientation of the side chain of lysine$^{119}$ as it points away from the active site cleft. We believed that this specific orientation may service two possible purposes: first, the ε-amino group may act as an “antenna” that inserts into and hold the elastin in place; and secondly, this orientation may clear up the “passage way” leading to the active site thus allowing easier access of elastin for degradation. The opposite is observed in human cathepsin L where the ε-amino group of lysine$^{117}$ is orienting inwardly facing the active site cleft and may have contributed to the inability of cathepsin L to adsorb to elastin molecule.

Following the sequence alignment in the bridge-like domain of both human cathepsins V and L, it was found that a glycine residue immediately following a proline residue is present in cathepsin V but not in L. Due to the unique structure of proline and the size of glycine, such arrangement may have resulted in the orientation of the succeeding lysine. So a glycine deletion and insertion was carried out in cathepsin V and L to evaluate the impact of such arrangement. It was found that the deleted glycine correlates with a 25% decrease in cathepsin V’s elastolytic activity suggesting its potential role in elastin degradation. On the other hand, while inserting a glycine residue into cathepsin L did not generate an increase in detectible elastolytic activity, the HPLC
profile of elastin degradation by this mutant suggested a degrading pattern similar to wildtype cathepsin V but not L. Taken together, even though no information concerning the mechanism employed by these mutants during elastin degradation could be inferred, the participation of the TVVAPGK domain and of the glycine residue in elastin degradation was evident. Furthermore, as was mentioned earlier, our results seemed to suggest the involvement of other residues in elastin degradation, and these residues are found within amino acid 92 to 104. The difference between cathepsins V and L within that region show more neutral amino acids in cathepsin V than in cathepsin L. Since elastins are consisted mainly of neutral amino acids, a neutral surface of the region would serve as a potential binding patch whereas the mainly charged residues in the same region of cathespin L would not favor such interaction. It is therefore of great interest to further exploring the involvement of this region in elastin degradation.

It had been hypothesized that cathepsins K, S, and L may proceed in elastin degradation through the following steps: first the enzyme adsorbs to elastin molecule in a nonproductive manner, and the adsorbed enzyme then interacts with other elastin sites to form a catalytically productive complex. Upon cleavage and release of the products, the enzyme may either remain adsorbed on the surface of elastin or dissociates from it (8). It was pointed that such adsorption step was not observed in cathepsin L probably because the adsorption/desorption proceeds in rapid cycle. One would however expect cathepsin L to demonstrate high elastolytic activity if such speculation is true. Because cathepsin L had been shown to demonstrate a high degrading activity against soluble elastin, if there indeed is an interaction between cathepsin L and elastin molecule, then the subsequent catalytic step should commence spontaneously and rapidly thus displaying a potent
elastolytic activity. The result reported from our laboratory however was not compatible with such option as only minimal elastin degradation was observed for human cathepsin L (6). Therefore one may consider that the inability to detect any adsorption/desorption be contributed to its lack of a strong elastin-binding domain. However, if that is the case, how do we explain the inability to rescue cathepsin L’s elastolytic activity after swapping the potential elastin-binding domain? It can be argued that since elastin is mainly consisted of neutral amino acids, it possibly possesses a surface with neutral electrostatic potential. Human cathepsin V has been reported to possess only a few localized negatively charged patches on its surface while majority is weakly positive and the active site cleft is entirely neutral. However, the electrostatic potential of cathepsin L is negative in extended region of the surface including the active site cleft (2). Since elastin is relatively large comparing to cathepsin L, the negative surface potential may not allow strong adsorption of the enzyme to the surface of elastin in spite of the introduced elastin-binding domain, hence the observed lack of increased elastolytic activity. On the other hand, cathepsin V, after swapping of the bridge-region (TVVAPGK) with cathepsin L, demonstrated compromised elastolytic activity because the introduced region may weaken elastin binding.

Taken together the results and the potential participation of other residues in elastin degradation, one may question the possibility of multiple elastin-binding sites. Upon more considerations, however, the fact that two potential elastin binding domains in cathepsin V are not adjacent to each other but still contribute to elastolytic activity are not impossible. Since elastin, being polymers of tropoelastins vastly cross-linked to each other, may possess multiple extending “pods” of tropoelastins and is so much larger in
size in comparison to cathepsin V, it will make perfect mechanistic sense for cathepsin V to possess multiple elastin-binding domains on different facets to involve in a threedimensional interaction with elastin as cathepsin V “dives” into the gaps between tropoelastins. So, combining the adsorption/desorption model proposed by Novinec et al. (8), an overall putative model of elastin binding by cathepsin V may involve the following steps. First, weakly charged cathepsin V adsorbs on the surface of elastin in a non-catalytically productive manner facilitated by the insertion into and subsequent fastening of $\varepsilon$-amino side chain of lys119 to elastin, as well as elastin interaction found on the neutral patches in the region of amino acids 91 to 104, followed by a subsequent spontaneous docking of tropoelastin into the neutral active site cleft where the catalytic step takes place. As a result of the proposed model, several further works should be carried out in order to confirm or to alternate the model. First, the region between amino acids 91 to 104 should be examined to evaluate the involvement of elastin interaction through patches of neutral domains. Secondly, as Lys$^{119}$ is suggested to initiate the adsorptive step through its side chain, it will be interesting to assess its effect on elastin binding by alanine substitution.

The identification of elastin-binding domains in cathepsin V serves significant purposes in inhibitor design. Cathepsin V was found to carry out elastin degrading activity in pathological condition such as atherosclerosis (6); however, its involvement in degradation of MHC-II complex in immune system suggests its important role in humans (9). Therefore, a specific inhibitor that will diminish its elastolytic activity perhaps through interruption of elastin binding while still allowing it to carry out its regular functions might be highly beneficial. Through the identification of the elastin binding
domain in cathepsin V, studies on the mechanisms of elastin degradation can be understood. Altogether, these findings shall shed light on the design of specific inhibitor.
References


The enzyme kinetics of both the wildtype and mutant cathepsins described in this thesis was determined as following. The data were collected as outlined in Experimental Procedure, followed by the utilization of the Prism program (GraphPad Inc.). In the Prism program, data were first input to construct a Michaelis-Menten graph as the one shown above for Mutant 6 where \( V_{\text{max}} \) and \( K_m \) will be calculated. After the Michaelis-Menten graph was constructed, appropriate information such as the concentration of active enzyme in the reaction would be input in order to calculate the \( k_{\text{cat}} \) from \( V_{\text{max}} \). The value obtained for \( k_{\text{cat}} \) will again need to be further converted to the correct unit as the y-axis of a Michaelis-Menten graph is based on the absorbance measured over 1 minute which was used by the spectrophotometer. Therefore, appropriate unit conversion would be
commenced to convert the absorbance measured over 1 minute, to concentration of substrate degraded per second, i.e., μM/second.