ISOLATION AND CHARACTERIZATION OF CATHEPSIN Z, A LYSOSOMAL CYSTEINE PROTEINASE

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(ABSTRACT)

Cathepsin Z is a cysteine proteinase found in lysosomes of human cells. It was detected in human cultured cell lines using the peptidyl diazomethane inhibitor Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. The labeling of cathepsin Z by the inhibitor was both time- and concentration-dependent, and the proteinase was found in all human cell lines examined. The characteristics of cathepsin Z were examined in U-937 cells, a human monocytic line. The labeling of cathepsin Z was blocked by pre-incubation of the cells either in non-iodinated inhibitor or in the epoxysuccinyl peptide inhibitor E-64d, a specific inhibitor of cysteine proteinases. Cathepsin Z was not immunoprecipitated by antisera specific for cathepsins B, L, or S. Cathepsin Z has been estimated to be at millimolar concentrations in lysosomes, suggesting that it is a major lysosomal proteinase. The molecular weight of cathepsin Z was calculated to be 22.4 kDa by SDS-PAGE and 45–47 kDa by native PAGE and gel exclusion chromatography, indicating that it is dimeric. Cathepsin Z is susceptible to digestion by endoglycosidase H, and oligosaccharides comprise 3.1 kDa of the reduced molecular weight. The expression of cathepsin Z was not affected by differentiation of U-937 cells with phorbol ester, unlike the expression of cathepsins B and S. Undifferentiated U-937 cells express low levels of cathepsins B and S; after differentiation, expression of cathepsins B and S is greatly increased. Cathepsin Z was purified from U-937 cells by anion exchange chromatography (Mono Q), affinity chromatography (concanavalin A), and preparatory electrophoresis. N-terminal sequence analysis of both the purified protein and fragments of the protein from a V8 digest indicates that cathepsin Z is a member of the papain superfamily of cysteine proteinases.

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Abbreviations

Aca	ε-aminocaprioc acid
APS	ammonium persulfate
bis	*N*, *N'*-methylene bisacrylamide
Boc	t-butyloxycarbonyl
BSA	bovine serum albumin
C	Celsius
CAPS	3-[cyclohexylamino]-1-propane-sulphonic acid
Cbz	benzyloxycarbonyl
cm	centimeter(s)
CM-Sephadex	carboxymethyl-Sephadex
CO_2	carbon dioxide
cpm	counts per minute
C-terminal	carboxy terminal
d	day(s)
DEAE	diethyaminoethyl
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DOC	deoxycholic acid
dpm	disintegrations per minute
DTT	dithiothreitol
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
E-64d	*N*-[*N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucyl]-3-methylbutylamine
ER	endoplasmic reticulum
EDTA	ethylenediamine tetra-acetic acid
FBS	fetal bovine serum
Fmoc	*N*-9-fluorenylmethoxycarbonyl

g	gram(s)
Glc	glucose
GlcNAc	*N*-acetylglucosamine
h	hour(s)
HCl	hydrochloric acid
HIFF	human infant foreskin fibroblasts
ID	internal diameter
kDa	kilodalton(s)
KeV	kiloelectron unit
L	liter(s)
M	molar
Man	mannose
mCi	millicurie(s)
μg	microgram(s)
min	minute(s)
μl	microliter(s)
ml	milliliter(s)
μm	micrometer(s)
mm	millimeter(s)
mM	millimolar
ng	nanogram(s)
nm	nanometer(s)
NHMec	*N*-methyl coumarin
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonyl fluoride
PVDF	polyvinylidene difluoride
R_f	relative mobility

s	second(s)
SDS	sodium dodecyl sulfate
SRP	signal recognition particle
TCA	trichloroacetic acid
TEMED	*N*, *N*, *N'*, *N'*-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
V	volts
V8	endoproteinase Glu-C
W	watts
Z	benzyloxycarbonyl

Introduction

Proteinases, which are enzymes that degrade proteins and peptides by hydrolyzing internal peptide bonds, are classified by their mechanism of hydrolysis. There are four well-recognized mechanisms, plus a fifth class of proteinases categorized by their having a mechanism that does not fit any of the four known classes. The four mechanisms utilize serine, aspartic acid, cysteine, or a bound metal ion in their active sites. Of the cysteine proteinases, twenty families have been recognized (reviewed by Rawlings & Barrett 1994), and proteinases from six of these families have been found in eukaryotes. The largest and the best characterized family containing eukaryotic cysteine proteinases is the papain superfamily. Family members are found in a wide variety of organisms, including plants, viruses, protozoa, eubacteria, animals, and yeast. Generally, members of the papain superfamily are either lysosomal or secreted proteins; they can have either endopeptidase or exopeptidase activity, or both. All members of the papain superfamily that have proteinase activity have conserved active site cysteine and histidine residues.

Lysosomal Cysteine Proteinases

The known lysosomal cysteine proteinases, cathepsins B, H, L, and S, are members of the papain superfamily of cysteine proteinases and their mechanisms are believed to be identical to that of papain.

Synthesis of Lysosomal Cysteine Proteinases

The lysosomal cysteine proteinases are synthesized as preproenzymes and their synthesis is similar to that of secretory proteins. Synthesis begins on ribosomes in the

cytosol and the nascent peptide is targeted to the endoplasmic reticulum (ER) by a hydrophobic N-terminal signal sequence. A signal recognition particle (SRP) binds the signal sequence, and the complex of ribosome, polypeptide, and SRP are then bound to the SRP receptor on the outer membrane of the ER. After the signal sequence is inserted into the ER membrane, the SRP, and then the SRP receptor, dissociate from the ribosome and translation continues. Several ER membrane proteins form a transmembrane channel through which the polypeptide chain crosses the membrane. The polypeptide chain is co-translationally modified in the ER lumen; the signal sequence is removed by signal peptidase, N-linked carbohydrates are added to the protein at selected asparagine residues, disulfide bonds form, and folding may begin. The lysosomal cysteine proteinases have no C-terminal membrane retention sequences, hence the entire protein is transferred to the lumen of the ER.

Folding of the enzymes is completed in the lumen of the ER and, at least in the case of cathepsin L, is contingent upon the presence of the propeptide. cDNAs coding for cathepsin L molecules with altered pro-regions were constructed and expressed in COS cells. Both cathepsin L that lacked a propeptide and cathepsin L that had a propeptide from another cysteine proteinase failed to fold correctly. Both of the altered proteins failed to leave the ER and were degraded there. In addition, the altered cathepsin L underwent no mannose phosphorylation (Tao *et al.* 1994).

Glycosylation and Sorting of Lysosomal Cysteine Proteinases

The initial N-linked glycosylation that occurs co-translationally in the ER is the addition *en bloc* of one or more branched oligosaccharides [$(Glc)_3(Man)_9(GlcNAc)_2$] to the polypeptide by oligosaccharide-protein transferase. The oligosaccharides are added to asparagine residues that are part of the recognition sequence (Asn-x-Thr/Ser). There

is further modification to the glycosylation state of the proteinase in the lumen of the ER. Three glucose residues and a single mannose residue are removed from each oligosaccharide moiety.

Further modifications to the oligosaccharide side chains differentiate lysosomal enzymes from secretory proteins and target them to lysosomes (reviewed in Kornfeld & Mellman 1989; Hoflack & Lobel 1993). A phosphotransferase, UDP-*N*-acetyl-glucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase, recognizes a signal on lysosomal enzymes and transfers *N*-acetylglucosamine 1-phosphate to one or more mannose residues on the enzyme. For cathepsin D, a lysosomal aspartic proteinase, the recognition signal for phosphorylation is proposed to be a three-dimensional recognition domain including an essential lysine residue (residues 265−292 plus Lys 203 in cathepsin D) located on the surface of the enzyme (Baranski *et al.* 1990). When surface lysine residues on cathepsin L were covalently modified by sulfo-*N*-hydroxysuccinimide acetate, mannose phosphorylation was inhibited (Cuozzo & Sahagian 1994), providing further evidence for the involvement of the lysine residue. Baranski *et al.* (1992) demonstrated that either the recognition domain (residues 265-292) or the key lysine residue was sufficient for some phosphorylation of lysosomal enzymes, albeit at lower levels than those seen on enzymes with both determinants. Crystallographic studies have shown that the glycosylation site and the key lysine residue of cathepsin D are separated on the surface of the molecule by 28Å, but the flexible oligosaccharide chain extends across the surface of the protein from the asparagine toward the lysine, bringing the oligosaccharide to within 5Å of the ε-amino group of the lysine side chain (Baldwin *et al.* 1993). Based on this information, a model for phosphorylation of lysosomal proteinases has been proposed in which the phosphotransferase contacts the recognition domain and is able to phosphorylate

oligosaccharides at different sites on the proteinase. Because phosphotransferase is putatively a multimeric enzyme, it might phosphorylate multiple sites while in contact with a single recognition domain (Cantor *et al.* 1992; Baldwin *et al.* 1993).

The first mannose phosphorylation is thought to occur prior to the arrival of the enzyme at the Golgi apparatus in a post-ER compartment (Lazzarino & Gabel 1988); subsequent phosphorylations, if any, occur in the cis-Golgi. After the mannose phosphorylation, the *N*-acetylglucosamine residue is removed by *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase, exposing a mannose 6-phosphate monoester. The phosphoesters are formed in the medial Golgi.

When lysosomal enzymes are not phosphorylated, as occurs in patients with I-cell disease, lysosomal enzymes are secreted from most cell types (Neufeld 1991). I-cell disease is characterized by reduced or nonexistent phosphotransferase activity. Certain tissues and cell types from I-cell patients, however, have normal cellular levels of lysosomal enzymes. In a B lymphoblastoid cell line from an I-cell patient, 45% of the newly-synthesized cathepsin D is sorted to lysosomes, while the remainder is excreted, implying the existence of a sorting mechanism in some cell types that is independent of the cation-independent mannose 6-phosphate receptor (MPR). Analysis of chimeric proteins suggested that the determinant for MPR-independent sorting overlaps but is not identical to the determinant for mannose 6-phosphorylation (Glickman & Kornfeld 1993).

The phosphorylated enzymes bind to MPRs in the trans-Golgi network (TGN). The MPRs and their attached lysosomal enzymes leave the TGN in clathrin-coated vesicles and are transported to an acidic late endosomal/prelysosomal compartment, where the low pH causes the dissociation of the enzymes from the MPRs. The receptors are then recycled back to the ER or cell surface.

Maturation of the Known Lysosomal Cysteine Proteinases

The lysosomal cysteine proteinases that transit through the ER and the Golgi apparatus are enzymatically-inactive proenzymes, containing an N-terminal pro-sequence. Fox *et al.* (1992) proposed that the propeptide acts as a reversible active site inhibitor of cathepsin B. They found that a synthetic peptide that corresponded to the pro-sequence of cathepsin B inhibited enzyme activity. Interestingly, this inhibition was 160-fold weaker at pH 4.0 than at pH 6.0. To prevent degradation of the cellular synthetic apparatus, enzyme activation must not occur prior to its arrival in a low pH compartment. Stronger inhibition at non-acidic pH would help prevent activation. Mach *et al.* (1994) have also found evidence for a noncovalent association of cathepsin B and its propeptide, resulting in inhibition of the enzyme against protein substrates. When the complex was incubated at acidic pH, it dissociated, the propeptide was degraded, and enzyme activity increased.

In addition to preventing enzyme activity, the pro-sequence may be responsible for membrane association of the proenzyme. Procathepsins L and D bind microsomal membranes at acidic pH, but mature cathepsins L and D show no association with microsomal membranes over a broad pH range (McIntyre & Erickson 1991). The membrane association is independent of MPRs and a 43 kDa integral membrane protein, called the lysosomal proenzyme receptor (LPR), has been identified as the mediator responsible for the binding of procathepsin L to microsomal membranes (McIntyre & Erickson 1993).

Binding of procathepsin L to microsomal membranes at acidic pH is inhibited by two synthetic peptides; the peptides, of 9 and 24 residues, are based on the N-terminal sequence of procathepsin L (McIntyre *et al.* 1994). A six-residue sequence that resembles the vacuolar sorting sequence from yeast proproteinases has been proposed to

be a signal sequence for binding of lysosomal proenzymes to microsomal membranes at acidic pH. The motif is S-x-x-(R/K/H)-x-L, but in mammalian cathepsins L, B, and D, the third, fourth, and fifth residues are all positively charged at pH 5. The LPR may be a second stage in the targeting of lysosomal enzymes to lysosomes. Since the binding occurs only at acidic pH, only enzymes that have previously been targeting to acidic compartments will bind to the LPR. In addition, the binding site on the proenzyme must be on a part of the enzyme that is not needed for activity, e.g., a propeptide sequence, so that the binding mediator on the proenzyme may be removed or altered when the enzyme reaches its destination and is activated and solubilized (McIntyre *et al.* 1994). In addition to their targeting role, it has been suggested that LPRs may play a role in activation of lysosomal proenzymes, aiding in the proteolytic removal of the prosequence at acidic pH (McIntyre & Erickson 1993).

During transport of the proteinases to lysosomes, the prosequence is removed, but the location and mechanism of this activation is still under investigation. Recombinant rat procathepsins B and S expressed individually in yeast (*Saccharomyces cerevisiae*) can be activated autocatalytically by a unimolecular mechanism (Mach *et al.* 1993; Brömme *et al.* 1993a), but incubation of procathepsin B with mature cathepsins D, L, or B also results in processed cathepsin B (Rowan *et al.* 1992). In *in vitro* experiments on the maturation of purified cathepsin B, Kawabata *et al.* (1993) found that cathepsin D was responsible for the processing of procathepsin B to the mature form. In both autocatalytic activation and activation by other cathepsins, additional trimming of up to fourteen residues by exopeptidases was required to achieve the same N-terminus as that found *in vivo*. The location of activation has been proposed to be lysosomes (Nishimura *et al.* 1988) and/or an endosomal/prelysosomal compartment (Roederer *et al.* 1987).

After their arrival in lysosomes, the proteinases undergo further processing. Cathepsin B (human) is processed from a 33 kDa single-chain form into a disulfide-bonded two-chain form of 24 and 5 kDa (Mach *et al.* 1992). Cathepsin H (human) is processed from a 28 kDa single-chain form into a disulfide-bonded two-chain form of 22 and 6 kDa; additionally, there is an octapeptide disulfide-bonded to the heavy chain (Ritonja *et al.* 1988). Cathepsin L (human) is processed from a 30 kDa single-chain form into a disulfide-bonded two-chain form of 24 and 5 kDa (Mason *et al.* 1985). Mature human cathepsin S, however, is believed to be a single polypeptide of 24 kDa (Kirschke *et al.* 1989; Wiederanders *et al.* 1992). Recent evidence, however, indicates that cathepsin S may also have a two-chain form (Mountz 1994).

Purification and Characteristics of Lysosomal Cysteine Proteinases

Cathepsin B has been purified from human liver (Barrett & Kirschke 1981). The tissue is homogenized and autolysed overnight under acidic conditions. The enzyme is then purified by ion exchange and covalent chromatography on DEAE-cellulose and Sepharose-aminophenylmercuric acetate, respectively. Human cathepsin B has been sequenced (Ritonja *et al.* 1985) and cloned (Chan *et al.* 1986). The pre- and pro-peptide sequences are 17 and 62 amino acids long, respectively, and the mature enzyme is 254 residues. There is a glycosylation site near the N-terminus of the mature enzyme, but the protein does not bind to concanavalin A. It is likely that there is further trimming of the oligosaccharide side chain in lysosomes. Analysis indicates only a small amount of carbohydrate is present in the mature enzyme (Barrett & Kirschke 1981). Processing from a single- to a two-chain form is not required, as the two forms show similar catalytic activity for the sheep enzyme (Mason 1986a). Cathepsin B has been crystallized, and its structure defined (Musil *et al.* 1991).

Cathepsin H has been purified from human hepatocytes (Schwartz & Barrett 1980). Liver is homogenized and subjected to autolysis as for cathepsin B. The protein is precipitated with acetone, and then purified by anion exchange (DEAE-cellulose), hydroxylapatite, and affinity chromatography (concanavalin A), followed by gel filtration. More recently, Popovic *et al.* (1993) purified cathepsin H from human kidney extract in a single step, using affinity chromatography. They used low molecular weight cysteine proteinase inhibitors from potatoes (PCPIs) coupled to Sepharose and eluted cathepsin H with a citrate buffer. Human cathepsin H has been sequenced (Ritonja *et al.* 1988) and cloned (Fuch *et al.* 1988); it has a 92 amino acid pro-sequence and two potential glycosylation sites. The mature enzyme is 220 amino acids long, and has N-linked glycosylation, as demonstrated by its binding to concanavalin A.

Cathepsin L has been purified from human hepatocytes (Mason *et al.* 1985). The tissue is first homogenized and subjected to autolysis as above. The protein is precipitated using ammonium sulfate and then purified using anion exchange and hydrophobic interaction chromatography on CM-cellulose and phenyl-Sepharose. The final step in the purification is gel filtration. Human cathepsin L has been sequenced (Ritonja *et al.* 1988) and cloned (Gal & Gottesman 1988; Joseph *et al.* 1988; Chauhan *et al.* 1993). Cathepsin L has pre- and pro-sequences of 17 and 96 amino acids, respectively, and the mature protein consists of 217 amino acids with one potential glycosylation site. Cathepsin L is glycosylated, but binds poorly to concanavalin A (Mason *et al.* 1985).

Cathepsin S has been purified from human spleen (Kirschke *et al.* 1989; Wiederanders *et al.* 1992). The tissue is homogenized and the protein precipitated with ammonium sulfate. The sample is then purified using cation exchange chromatography on CM-Sephadex, followed by gel filtration. The protein is then applied to a

chromatofocusing column; the final step in the purification is hydrophobic interaction chromatography on phenyl-Sepharose. The complete protein sequence from purified human cathepsin S has not been determined, however the sequence of purified bovine cathepsin S contained no glycosylation or glycosylation sites (Ritonja *et al.* 1991). Human cathepsin S has been cloned and expressed. Shi *et al.* (1992) found that the cDNA predicts a pro-enzyme of 331 amino acids and the expression yielded a 28 kDa mature enzyme. Wiederanders *et al.* (1992) found that the cDNA predicted pre- and pro-enzyme sequences of 15 and 99 amino acids, respectively, and a mature cathepsin S of 217 amino acids. In their case, however, expression yielded a mature cathepsin B of 24 kDa. This corresponds to the molecular weight found in human spleen (Kirschke *et al.* 1989). Both groups found only one potential glycosylation site, located in the pro-region of the enzyme.

When the sequences of the four known lysosomal cysteine proteinases are compared, there are regions of strong conservation, particularly in the sequences surrounding the active site cysteine and histidine (Cys 25 and His 159 in papain; Figure 1.1). Another residue important for catalysis is Asn 175 (papain numbering), and there is also conservation around that residue. There is much less similarity in the central portion of the sequence. The propeptides of the lysosomal cysteine proteinases are generally homologous to the propeptide of papain (Rawlings & Barrett 1994). Although no residue of the propeptide is completely conserved, there are eight residues that are highly conserved in many members of the family. Five of these residues are part of a motif (ERFNIN) that is used to distinguish propeptides in the papain superfamily. The members of the papain superfamily without this motif are all cathepsin B-like enzymes, and Karrer *et al.* (1993) propose that the presence or absence of the motif can be used to distinguish between two subfamilies of the papain superfamily.

Figure 1.1 Comparison of the amino acid sequences of cysteine proteinases.
Alignment of the amino acid sequences of papain and cathepsins B, H, L, and S. Also shown is the sequence of cathepsin O/K (see page 38) The numbering is based on the sequence of papain. Sequences shown are of the mature proteins. The conserved active site cysteine and histidine are boxed.

```
papain          I P E Y V D - - - - W R Q K G - A V T P V K N Q G S C G S 24
cathepsin B     L P A S F D A R E Q W P Q C P - T I K E I R D Q G S C G S
cathepsin H     Y P P S V D - - - - W R K K G N F V S P V K N Q G A C G S
cathepsin L     A P R S V D - - - - W R E K G - Y V T P V K N Q G Q C G S
cathepsin O/K   A P D S V D - - - - Y R K K G - Y V T P V K N Q G Q C G S
cathepsin S     L P D S V D - - - - W R E K G - C V T E V K Y Q G S C G A

papain          C W A F S A V V T I E G I I K I R T G N L N E Y S E Q E L 53
cathepsin B     C W A F G A V E A I S D R I C I H T N A H V S V E V - S A
cathepsin H     C W T F S T T G A L E S A I A I A T G K M L S L A E Q Q L
cathepsin L     C W A F S A T G A L E G Q M F R K T G R L I S L S E Q N L
cathepsin O/K   C W A F S S V G A L E G Q L K K K T G K L L N L S P Q N L
cathepsin S     C W A F S A V G A L E A Q L K L K T G K L V T L S A Q N L

papain          L D C - D - - R R - - S Y G C N G G Y P W S A L Q L V A Q 77
cathepsin B     E D L L T C C G S M C G D G C N G G Y P A E A W N - - - -
cathepsin H     V D C A Q D F N - - - N Y G C Q G G L P S Q A F E - - - -
cathepsin L     V D C - S G P Q G - - N E G C N G G L M D Y A F Q - - - -
cathepsin O/K   V D C V S E - - - - - N D G C G G G Y M T N A F Q - - - -
cathepsin S     V D C S T E K Y G - - N K G C N G G F M T T A F Q - - - -

papain          Y G I H Y - - - - - - - - - R N T Y P Y - E G V Q R Y C R 96
cathepsin B     - - - - F W T R - K G L V S G G L Y E S H V G C R P Y S I
cathepsin H     - - - - Y I L Y N K G I M G E D T Y P Y - Q G K D G Y C K
cathepsin L     - - - - Y V Q D N G G L D S E E S Y P Y - E A T E E S C K
cathepsin O/K   - - - - Y V Q K N R G I D S E D A Y P Y - V G Q E E S C M
cathepsin S     - - - - Y I I D N K G I D E D A S Y P Y - K A M D Q K C Q

papain          S R E K G P Y A A K T D G V R - Q V Q P Y N - E G A L L Y 123
cathepsin B     P P C E - - H H V N G S R P P C T G E G D P T - K C S K I
cathepsin H     F Q S - - - G K A I G F V K D V A N I T I Y D E E A M V E
cathepsin L     Y N P K - - Y S V A N D T G F - V D I P K Q - E K A L M K
cathepsin O/K   Y N P T - - G K A A K C R G Y - R E I P E G N E K A L K R
cathepsin S     Y D S K - - Y R A A T C S K Y - T E L P Y G R E D V L K E
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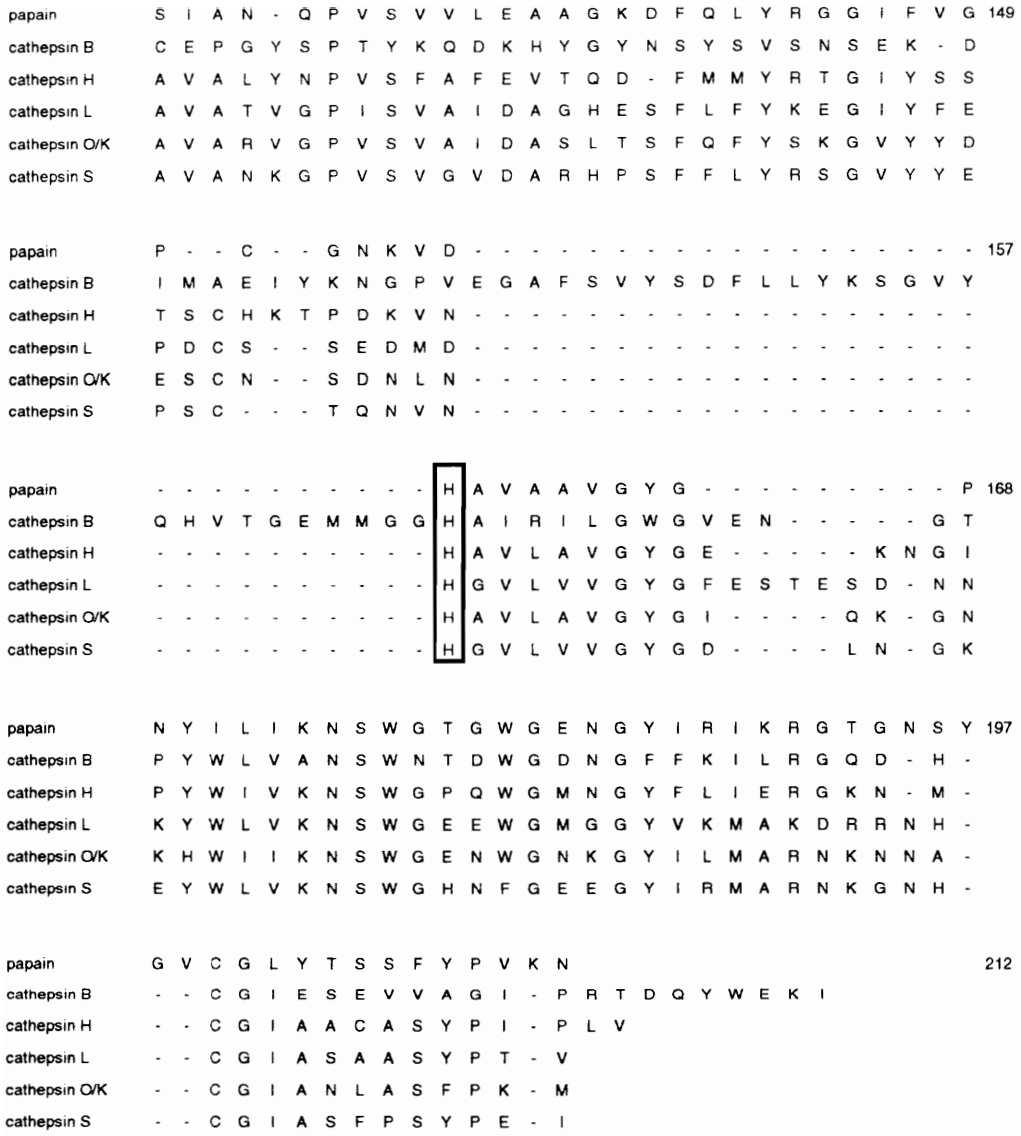

papain S I A N - Q P V S V V L E A A G K D F Q L Y R G G I F V G 149
cathepsin B C E P G Y S P T Y K Q D K H Y G Y N S Y S V S N S E K - D
cathepsin H A V A L Y N P V S F A F E V T Q D - F M M Y R T G I Y S S
cathepsin L A V A T V G P I S V A I D A G H E S F L F Y K E G I Y F E
cathepsin O/K A V A R V G P V S V A I D A S L T S F Q F Y S K G V Y Y D
cathepsin S A V A N K G P V S V G V D A R H P S F F L Y R S G V Y Y E
papain P - - C - - G N K V D - - - - - - - - - - - - - - - - - - - 157
cathepsin B I M A E I Y K N G P V E G A F S V Y S D F L L Y K S G V Y
cathepsin H T S C H K T P D K V N - - - - - - - - - - - - - - - - - -
cathepsin L P D C S - - S E D M D - - - - - - - - - - - - - - - - - -
cathepsin O/K E S C N - - S D N L N - - - - - - - - - - - - - - - - - -
cathepsin S P S C - - - T Q N V N - - - - - - - - - - - - - - - - - -
papain - - - - - - - - - - - H A V A A V G Y G - - - - - - - - - P 168
cathepsin B Q H V T G E M M G G H A I R I L G W G V E N - - - - - G T
cathepsin H - - - - - - - - - - - H A V L A V G Y G E - - - - - K N G I
cathepsin L - - - - - - - - - - - H G V L V V G Y G F E S T E S D - N N
cathepsin O/K - - - - - - - - - - - H A V L A V G Y G I - - - - Q K - G N
cathepsin S - - - - - - - - - - - H G V L V V G Y G D - - - - L N - G K
papain N Y I L I K N S W G T G W G E N G Y I R I K R G T G N S Y 197
cathepsin B P Y W L V A N S W N T D W G D N G F F K I L R G Q D - H -
cathepsin H P Y W I V K N S W G P Q W G M N G Y F L I E R G K N - M -
cathepsin L K Y W L V K N S W G E E W G M G G Y V K M A K D R R N H -
cathepsin O/K K H W I I K N S W G E N W G N K G Y I L M A R N K N N A -
cathepsin S E Y W L V K N S W G H N F G E E G Y I R M A R N K G N H -
papain G V C G L Y T S S F Y P V K N 212
cathepsin B - - C G I E S E V V A G I - P R T D Q Y W E K I
cathepsin H - - C G I A A C A S Y P I - P L V
cathepsin L - - C G I A S A A S Y P T - V
cathepsin O/K - - C G I A N L A S F P K - M
cathepsin S - - C G I A S F P S Y P E - I

Specificity of Cysteine Proteinases

Mechanism of Cysteine Proteinase Activity

The mechanism of action of cysteine proteinases has been determined using the best known of the cysteine proteinases, papain (reviewed in Storer & Ménard 1994). Papain is a plant proteinase found in the latex of unripe papaya fruit (*Carica papaya*); the mechanism will be discussed with reference to papain. Finkle and Smith (1958) determined the involvement of the thiol group of the active site cysteine in catalysis, and it was later demonstrated that a histidine residue was also implicated in enzyme activity. An ion pair is formed between the histidine imidazole group (His 159) and the active site thiol group (Cys 25; Polgár 1973); equilibrium favors the thiolate-imidazolium ion pair over the neutral form of the active site residues (Polgár & Halász 1982). The ion pair is stabilized by the α-helix of which Cys 25 is a part and by hydrogen bonding between His 159 and a conserved asparagine residue, Asn 175 (Rullman *et al.* 1989). After substrate binding, the thiolate ion (S^-) acts as a nucleophile, attacking the carbonyl carbon of the scissile peptide bond and forming an acylated tetrahedral intermediate (Lowe & Williams 1965). The nucleophilic attack is assisted by the binding of the carbonyl oxygen in the oxyanion hole formed by the Cys 25 amine and a glutamine amide group. A proton is then transferred to the substrate amino group from the active site histidine, resulting in the cleavage of the peptide bond, producing a free amine and an acyl-enzyme intermediate. A water molecule then hydrolyzes the acyl group, with His 159 abstracting a proton and the hydroxyl group attacking the carbonyl carbon, forming a tetrahedral intermediate (Szawelski & Wharton 1981). Reformation of the carbonyl bond results in the regeneration of the thiolate-imidazolium ion pair, and, hence, the active configuration of the enzyme (Figure 1.2).

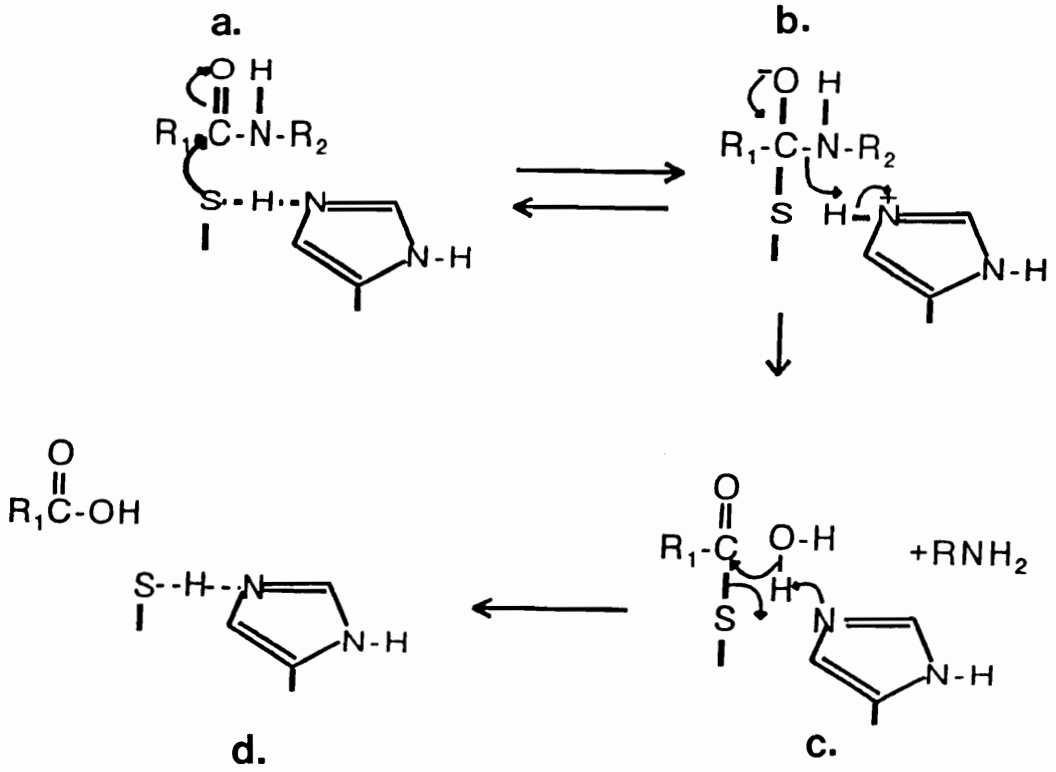


Figure 1.2 Mechanism of action of cysteine proteinases.

Mechanism for the cleavage of peptide bonds by cysteine proteinases (adapted from Mason & Wilcox 1993). See page 12 for a description of the steps of the mechanism.

The crystal structures of two of the cysteine proteinases, papain and cathepsin B, have been solved (Kamphuis *et al.* 1984; Musil *et al.* 1991). The structures are similar, as might be expected from their similarities in primary structure. Both proteins have two domains, the L and R domains, and the domains enclose a water-filled channel. A portion of this channel forms the V-shaped active site cleft containing the active site cysteine and histidine. One side of the active site cleft of cathepsin B is partially blocked by an "occluding loop" not found in papain. Cathepsin B is larger than papain (254 and 212 residues, respectively). The segments conserved between the two proteins tend to be found in central parts of the proteins, including around the active site residues, whereas most of the insertions or deletions are on the surface of the molecules (Musil *et al.* 1991).

Model for the Specificity of Cysteine Proteinases

The lysosomal cysteine proteinases have been differentiated by their activity against small synthetic ester and amide substrates; these substrates have a chromaphoric group on their C-terminal that is released on substrate hydrolysis. Schechter and Berger (1967) proposed a model for the binding of substrate to papain in which the enzyme has a series of up to seven binding pockets on either side of the active site. These pockets, or subsites, correspond to the amino acids on either side of the scissile peptide bond (Figure 1.3). The amino acids are termed P_1 to P_4 N-terminal to the scissile bond, and P_1' to P_3' C-terminal to the scissile bond. The corresponding subsites on the enzyme are S_1 to S_4 and S_1' to S_3'. Using a number of di- and tri-peptide substrates, the nature of the subsites of the lysosomal cysteine proteinases has been elucidated and modeled (Figure 1.4; Xin *et al.* 1992). For example, the S_2 subsites of cathepsins B, L, and S are large, hence they can accommodate bulky side chains such as tyrosine or phenylalanine in the P_2 position. In contrast, cathepsin H has a smaller S_2 subsite and has poor activity against synthetic

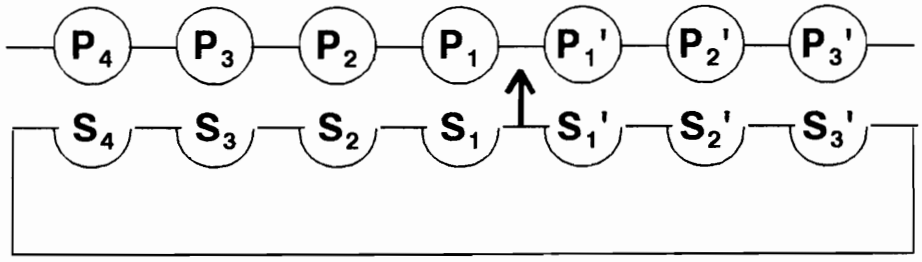


Figure 1.3 Model for the interaction of substrate and proteinase.

Model based on Schechter and Berger (1967) for the interaction of substrate and papain. The arrow denotes the catalytic site and the scissile peptide bond on the substrate. P_1 through P_n refer to the amino acids on the N-terminal side of the scissile bond, and the corresponding binding pockets (or subsites) on the proteinase are termed S_1 through S_n. The amino acids on the C-terminal side of the scissile bond are termed P_1' through P_n', and the corresponding subsites S_1' through S_n'.

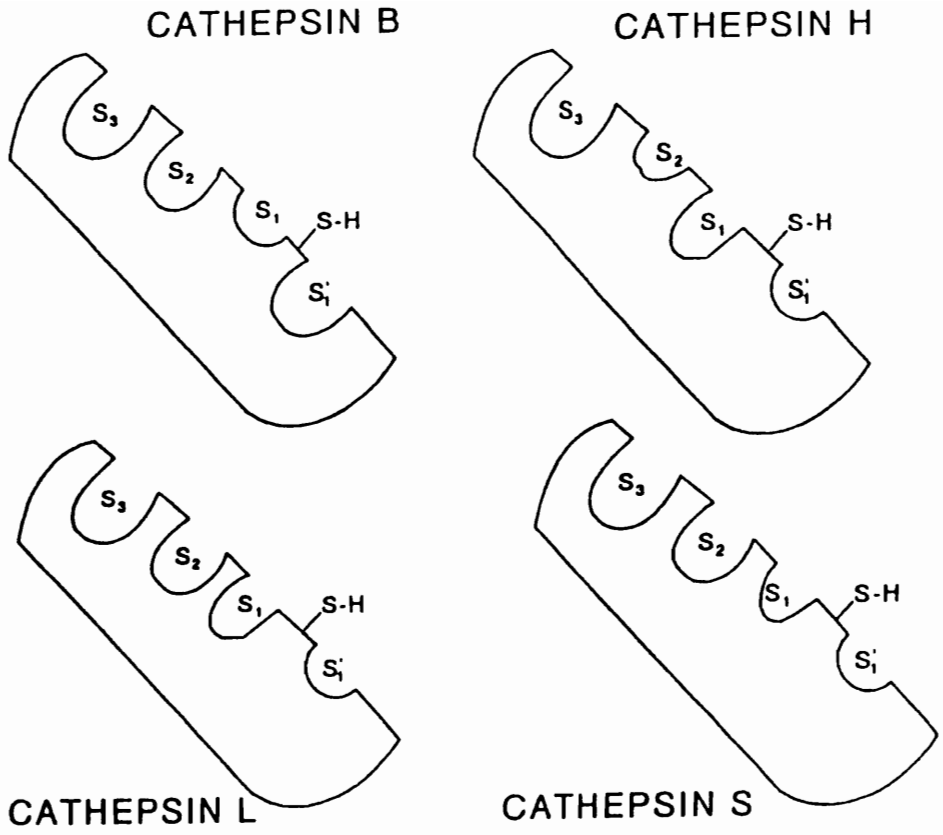


Figure 1.4 Model for the binding pockets of cathepsins B, H, L, and S.
Model of the active sites of cathepsins B, H, L, and S (adapted from Xin *et al.* 1992). The model is based on the ability of the enzymes to accommodate synthetic substrates with bulky amino acid side chains.

substrates with tyrosine or phenylalanine in the P_2 position (Xin *et al.* 1992). The primary determinant of specificity is the S_2 subsite, although other subsites, e.g., the S_1' subsite (Ménard *et al.* 1993), play roles in determining the specificities of the cathepsins and papain.

Using site-directed mutagenesis, the specificity of the cathepsins can be altered. The S_2 subsite in papain and in cathepsins B, L, and S is putatively defined by six residues, three of which are thought to be of primary importance, forming the side walls [residues 133 and 157 (papain numbering)] and end [residue 205 (papain numbering)] of the binding pocket (Brömme *et al.* 1994a). Residues 133 and 157 are hydrophobic amino acids in all four enzymes, but the nature of residue 205 varies among the enzymes in such a way that the substrate preferences at the S_2 subsite may be explained, e.g., an alanine in this position in cathepsin L implies an open pocket that can accept large side chains, whereas a glutamic acid at this location in cathepsin B explains the preference of this enzyme for basic amino acids in P_2 (Khouri *et al.* 1991). When the amino acid in position 205 of cathepsin S was changed by site-directed mutagenesis from phenylalanine to glutamic acid, the activity of the enzyme toward substrates with a basic residue in position P_2 increased 77-fold, to an activity more cathepsin B-like (Brömme *et al.* 1994a).

Endogenous Inhibitors of Cysteine Proteinases

Cystatins

There are a number of endogenous inhibitors in mammalian cells and biological fluids that are responsible for controlling unwanted activity of cysteine proteinases. The members of the cystatin superfamily of cysteine proteinase inhibitors (reviewed in Abrahamson 1994; Turk & Bode 1994) are closely related by amino-acid sequence

(Rawlings & Barrett 1990). The cystatins are reversible competitive inhibitors of cysteine proteinases. The superfamily has been divided into three families; families 1 and 2 consist of low molecular weight inhibitors, while the third family consists of larger proteins. The first family of cystatins, also called the stefins, consists of cystatins A and B. A third member of the family, stefin C, has been reported (Turk *et al.* 1993), but it has not yet been found in human tissues. Family 1 cystatins are single-chain polypeptides, averaging 100 amino acids, with molecular weights of 11-12 kDa; they have no disulfide bonding or glycosylation. They are normally intracellular inhibitors, probably cytosolic, lacking signal sequences, and are strong inhibitors of cathepsins H, L, and S, and papain, and somewhat weaker inhibitors of cathepsin B (Green *et al.* 1984; see Abrahamson 1994).

The second family of cystatins has five members, cystatins C, D, S, SN, and SA, which are single chain polypeptides of 120-122 amino acids and molecular weights of 13-14 kDa. They have two intramolecular disulfide bonds but no glycosylation. Unlike family 1, family 2 cystatins are synthesized with signal sequences and are secreted. They are found in high concentrations in biological fluids, such as saliva, seminal plasma, tears, and cerebrospinal fluid (Abrahamson *et al.* 1986). The three S-type cystatins have very similar primary structures, with about 90% sequence identity with each other (Isemura *et al.* 1991). The family 2 cystatins are strong inhibitors of the lysosomal cathepsins and papain (Balbín *et al.* 1994; see Abrahamson 1994), except for the poor inhibition of cathepsin B by cystatin D (Freije *et al.* 1993). Cystatin C is the most general inhibitor of cysteine proteinases in the cystatin superfamily, binding tightly to all tested enzymes (see Abrahamson 1994).

The kininogens are the third family in the cystatin superfamily. The known human kininogens are L- and H-kininogen, previously called α_1- and α_2-thiol proteinase

inhibitor, and T-kininogen which has been reported in rat (see Barrett *et al.* 1986). The kininogen family members have three cystatin-like domains, two of which are inhibitors of cysteine proteinases (Salvesen *et al.* 1986). Kininogens are glycosylated and have multiple disulfide bonding. Kininogens are secreted from the cell and are found in high concentrations in blood plasma (Abrahamson *et al.* 1986). L-kininogen has 409 amino acids and a molecular weight of 68 kDa. H-kininogen has 628 amino acids, more extensive glycosylation than L-kininogen, and a molecular weigh of 114 kDa. H- and L-kininogen are strong inhibitors of papain and cathepsins H and L, and poorer inhibitors of cathepsin B (Machleidt *et al.* 1986; Salvesen *et al.* 1986).

α_2-Macroglobulin

Cathepsins B, H, and L are also inhibited by α_2-macroglobulin (Mason 1989). α_2-macroglobulin (α_2-M; reviewed by Sottrup-Jensen 1987; Borth 1992) is one of the most abundant serum proteins, comprising up to 10% of total serum protein in humans. α_2-M rapidly inhibits the activity of any proteinase that gets into circulation. α_2-M has an exposed region of 25 amino acids, the "bait" region, that can be cleaved by most proteinases. These proteinases may be either those produced by the host or those produced by other organisms, e.g., venoms or toxins. Cleavage of the bait region by a proteinase produces an immediate conformational change in α_2-M, resulting in the "trapping" of the proteinase. The trapped proteinase is physically constrained by α_2-M, but remains active. Following the conformational change, α_2-M and its bound proteinase are rapidly removed from circulation by receptor-mediated endocytosis and degraded in the endocytic/lysosomal pathway.

Synthetic Inhibitors of Cysteine Proteinases

Several classes of synthetic inhibitors of cysteine proteinases have been developed (reviewed in Rich 1986; Shaw 1990) and used to characterize the active sites and mechanisms of cysteine proteinases. The inhibitors have the basic structure (N-terminus)-peptidyl-(C-terminal reactive group), in which the N-terminal may or may not be blocked and the peptidyl portion consists of at least one amino acid residue. The reactions of these small peptidyl inhibitors fall into two general groups, those that reversibly modify the enzyme and those that covalently modify the enzyme in an irreversible reaction.

Peptide Aldehydes

Peptide aldehydes are transition state analog inhibitors, which are inhibitors that mimic the geometry of the transition state. They have an C-terminal aldehyde (-CHO) and react reversibly with the active site cysteine of cysteine proteinases, forming an hemithioacetal (Lewis & Wolfenden 1977). The best-known of the peptide aldehydes, leupeptin (Acetyl-Leu-Leu-Arg-H) was discovered in culture filtrates from *Streptomyces* (reviewed in Umezawa 1982). Leupeptin has low toxicity (Umezawa 1982) and inhibits cysteine proteinases, including papain (Rich 1986), cathepsin B (Knight 1980), cathepsin L (Mason *et al.* 1985), and calpain (Sasaki *et al.* 1984); it is a poorer inhibitor of cathepsin H (Mason *et al.* 1985). Leupeptin and synthetic peptide aldehyde inhibitors also inhibit serine proteinases (see Powers & Harper 1986), which reduces their usefulness as inhibitors of cysteine proteinases, although their low toxicity is helpful for *in vivo* experiments (Kirschke & Barrett 1987). When intact platelets were incubated with a peptide aldehyde inhibitor of calpain, protein degradation was inhibited, indicating

that the inhibitor was able to penetrate into the cells (Tsujinaka *et al.* 1988); leupeptin, however, does not enter human fibroblasts by diffusion (Wilcox & Mason 1992).

Peptidyl Chloromethane Inhibitors

The peptidyl chloromethanes, also called chloromethyl ketones, have an C-terminal chloromethane group (-CH_2Cl) and act by covalently modifying the enzymes with which they react; they were among the first inhibitors developed for affinity labeling of enzymes. One of the earliest used, TPCK (L-1-tosylamino-2-phenylethyl-chloromethyl ketone), alkylates the active site histidine of the serine proteinase chymotrypsin. TPCK also inactivates cysteine proteinases, for example, papain, by alkylation of the active site cysteine (see Rich 1986). By altering the peptidyl portion of chloromethane inhibitors, their rate of inactivation of cysteine proteinases can be improved (see Rich 1986). Chloromethane inhibitors have been used to identify cellular forms of cysteine proteinases. When cell lysates from several cultured cell lines were incubated with an iodinated chloromethane inhibitor ([^{125}I]Tyr-Ala-Lys-Arg-CH_2Cl), the forms of cathepsin B in the different cell lines could be compared (Docherty *et al.* 1983; Docherty & Phillips 1988). Chloromethyl ketones have limited utility because of their general reactivity with thiol groups (Kirschke & Barrett 1987). In addition, the high reactivity of these inhibitors results in toxicity problems when they are used *in vivo* (Rich 1986).

Peptidyl (Acyloxy)methyl Ketone Inhibitors

Recently-developed inhibitors of cysteine proteinases are the peptidyl (acyloxy)methyl ketones, also called peptidyl (acyloxy)methanes (reviewed in Krantz 1994). They have the general structure Z-peptidyl-CH_2OCOAr, where Ar is a

carboxylate leaving group frequently containing a substituted aromatic hydrocarbon. They are irreversible inhibitors of cysteine proteinases, both *in vitro*, e.g., cathepsins S, L (Brömme *et al*, 1994b), and B (Wagner *et al.* 1994), and interleukin-1β-converting enzyme (ICE; Dolle *et al.* 1995), and *in vivo*, e.g., cathepsin B (Wagner *et al.* 1994). The peptidyl (acyloxy)methanes inactivate cysteine proteinases by alkylating the active site cysteine, but, unlike the chloromethyl ketones, they have low chemical reactivity. Hence they should prove to have low toxicity and to be useful for *in vivo* inhibition of cysteine proteinases. Additionally, they appear to be specific for cysteine proteinases, showing no reactivity with other classes of proteinases (Krantz 1994).

_N_, _O_-Diacyl Hydroxamate Inhibitors

The *N*, *O*-diacyl hydroxamate inhibitors (reviewed in Brömme & Demuth 1994) have the general structure N-peptidyl-NHOCO-R, where the peptidyl portion consists of at least one residue. The R group occupies the S' binding site of the target enzyme, and can be aliphatic, aromatic, amino acid, or peptidyl residue. The inhibitor irreversibly alkylates cysteine proteinases, and the leaving group is proposed to be -OCO-R (Brömme & Demuth 1994). *N*, *O*-diacyl hydroxamate inhibitors rapidly inactivate cathepsins B, L, and S. They also inactivate cathepsin H and the serine proteinases trypsin, thrombin, and plasmin, but at 2-5 orders of magnitude less rapidly than cathepsins B, L, and S (Brömme *et al.* 1993b). Aspartic proteinases and metalloproteinases are not inactivated by the inhibitors. Because of the structure of the *N*, *O*-diacyl hydroxamate inhibitors, it has been proposed that they will be useful tools for exploring the S' subsite specificity of cysteine proteinases (Brömme & Demuth 1994).

Epoxysuccinyl Peptide Inhibitors

The first epoxysuccinyl peptide inhibitor, E-64 [L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane], was isolated from cultures of *Aspergillus japonicus*, purified, and determined to be an inhibitor of cysteine proteinases, including papain, but not of proteinases from other classes (Hanada *et al.* 1978a). The structure of E-64 was determined (Figure 1.5a) and the inhibitor was synthesized (Hanada *et al.* 1978b). An E-64-papain complex has been crystallized (Varughese *et al.* 1989) and the mechanism of enzyme inactivation deduced from the crystal structure. E-64 and most of its derivatives react with the S, and not the S', subsites of cysteine proteinases, irreversibly alkylating the enzymes. The active site cysteine is covalently bound to the C2 carbon of the E-64 oxirane ring, which is opened during the reaction.

A number of derivatives of E-64 have been designed and synthesized (Hanada *et al.* 1978c; Tamai *et al.* 1981; Tamai *et al.* 1986; Gour-Salin *et al.* 1993) in attempts to increase the specificity, inhibitory activity, or membrane permeability of the inhibitors. E-64 or its analogs irreversibly inactivate both lysosomal cysteine proteinases, e.g., cathepsins B (Inaba *et al.* 1979), H (Barrett *et al.* 1982), L (Towatari *et al.* 1978), and S (Kirschke *et al.* 1989), and the cytosolic calpain (McGowan *et al.* 1989).

The E-64 analog CA074 [*N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline] is a selective inhibitor of cathepsin B *in vitro* (Murata *et al.* 1991). CA074 is negatively charged and would not be expected to diffuse into cells, but it has been reported to inhibit cathepsin B *in vivo* (Towatari *et al.* 1991). CA074 has almost no inhibitory activity against cathepsins H, L, or S, or against calpain (Murata *et al.* 1991; Buttle *et al.* 1992). Interestingly, CA074 is believed to bind to cathepsin B with its peptides in the S' subsites of the enzyme (Buttle *et al.* 1992; Gour-Salin *et al.* 1993), unlike the other epoxysuccinyl peptide inhibitors, whose peptides occupy the S subsites.

The E-64 analog, E-64d {*N*-[*N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucyl]-3-methylbutylamine}, once called EST, was first synthesized by Tamai *et al.* (1986). E-64d (Figure 1.5b), unlike E-64, has no charged groups and was predicted to have improved membrane permeability compared to E-64. Evidence for the membrane permeability was provided when human carcinoma A431 cells were incubated in E-64d and the cells were arrested at mitotic metaphase. To determine if calpain II (then called Ca^{2+}-activated neutral protease or CANP) was involved in arresting mitosis, E-64d was radiolabeled with tritium and extracts of cells that had been incubated in the radiolabeled inhibitor were examined by immunoprecipitation, SDS-PAGE, and autoradiography. When the cell extracts were immunoprecipitated with a monoclonal antibody against calpain II, no labeled calpain was detected. However, [^{3}H]E-64d was shown to label a number of other cellular proteins *in vivo* (Shoji-Kasai *et al.* 1988).

When intact human platelets were incubated in E-64d, intracellular calpain activity was inhibited, again demonstrating the membrane-permeability of the inhibitor. A ten-minute incubation of platelets in E-64d prior to their activation blocked the degradation of two proteins normally proteolyzed by calpain (McGowan *et al.* 1989). Human cathepsins B and L were inactivated *in vivo* when cultured human fibroblasts were incubated with E-64d; incubation with E-64 did not affect enzyme activity (Wilcox & Mason 1992).

Peptidyl Diazomethanes: Mechanism of Inhibition of Cysteine Proteinases

The characteristic that distinguishes the peptidyl diazomethane inhibitors (also called peptidyl diazomethyl ketones; reviewed in Shaw 1990; Shaw 1994) is the diazomethane group (-CHN_2) at the C-terminus of the inhibitor. The peptidyl portion of the inhibitors consists of at least one amino-acid residue. Most of the inhibitors have

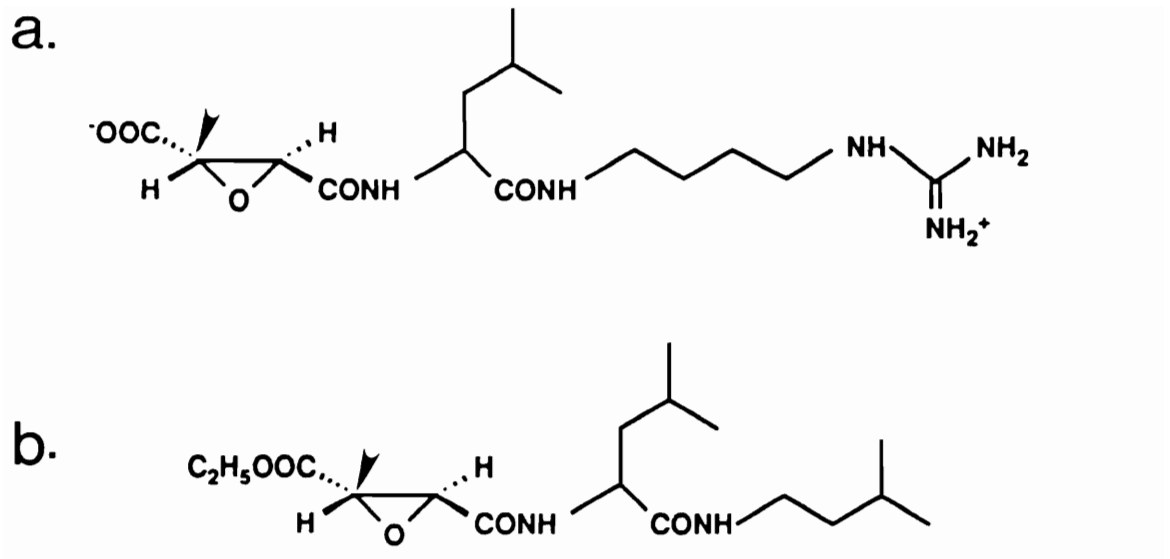


Figure 1.5 Epoxysuccinyl peptide inhibitors.

The structures of the epoxysuccinyl peptide inhibitors E-64 (adapted from Mehdi 1991) (a) E-64 and (b) E-64d. The C2 carbons are indicated by the arrowheads.

blocked N-termini to prevent aminopeptidase sensitivity, yet for the study of the cysteine exopeptidases, inhibitors with an unblocked N-terminus have been used (Angliker *et al.* 1989). When a blocked N-terminus is desired, blocking agents (Figure 1.6) that have been used include t-butyloxycarbonyl (Boc; e.g., Crawford *et al.* 1988), *N*-9-fluorenylmethoxycarbonyl (Fmoc; e.g., Crawford *et al.* 1988), biotin (e.g., Cullen *et al.* 1992), or benzyloxycarbonyl (Z or Cbz; e.g., Mason *et al.* 1989a).

The proposed mechanism by which peptidyl diazomethane inhibitors inactivate cysteine proteinases is shown in Figure 1.7 (Mason & Wilcox 1993). As in substrate binding, the active site thiolate ion acts as a nucleophile and attacks the carbonyl carbon located N-terminal to the diazomethane group, forming a tetrahedral intermediate. A rearrangement then occurs in which the bond to sulfur is transferred from the carbonyl carbon to the methylene carbon of the inhibitor. A proton is then transferred from the active site histidine to the methylene carbon, resulting in the liberation of nitrogen gas and the irreversible alkylation of the enzyme.

It is possible to radioiodinate cysteine proteinases by affinity labeling. Many of the peptidyl diazomethane inhibitors include a tyrosine residue, allowing iodination of the inhibitor, and enabling covalent labeling of active forms of cathepsins both *in vivo* and *in vitro*. In some cases, iodination results in increased inhibitory activity compared to the non-iodinated form. For example, Z-[I]Tyr-Ala-CHN_2 is a ten-fold better inhibitor of cathepsins B and L than is Z-Tyr-Ala-CHN_2 (Crawford *et al.* 1988). In experimental situations in which the presence of a tyrosine residue in the inhibitor is inappropriate, the technique of using a traceable biotinyl group as the N-terminal blocking agent has enabled non-isotopic detection of the inhibitor (Cullen *et al.* 1992; Wikstrom *et al.* 1993).

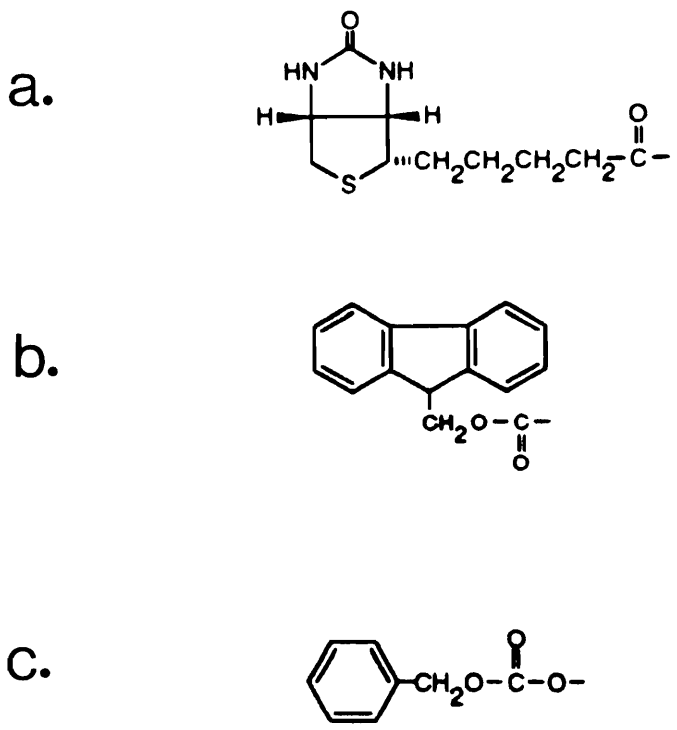


Figure 1.6 N-terminal blocking agents.

Agents used to block the N-termini of peptidyl diazomethane inhibitors. (a) biotin; (b) *N*-9-fluorenylmethoxycarbonyl (Fmoc); (c) benzyloxycarbonyl (Z).

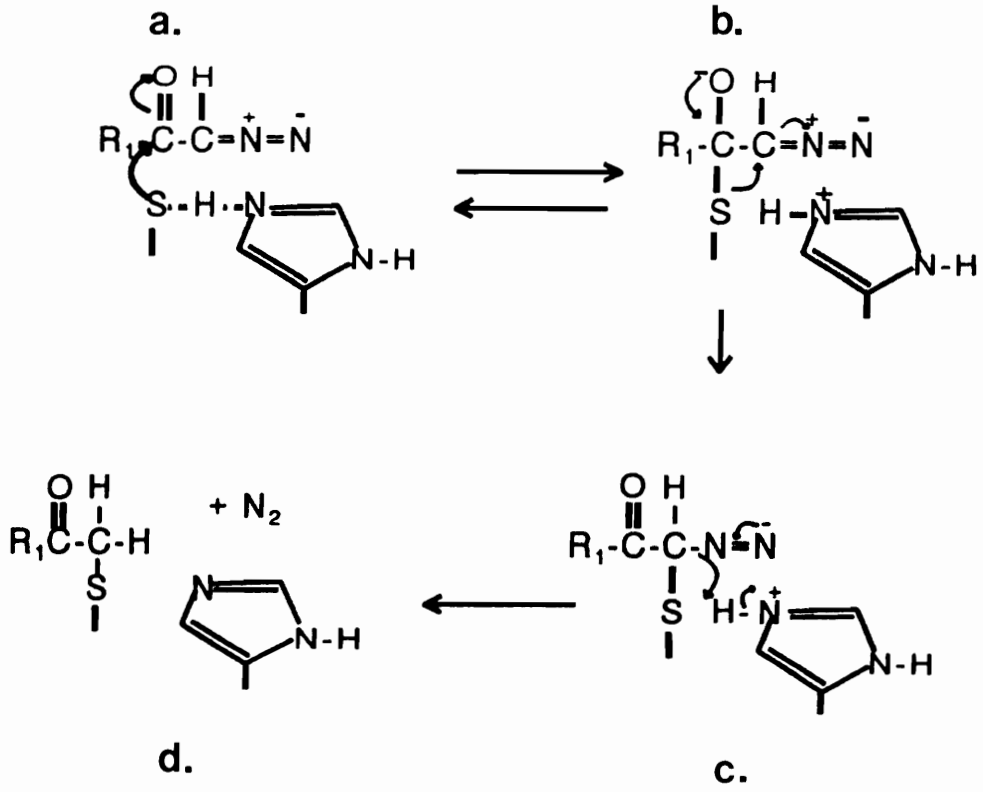


Figure 1.7 Reaction of a cysteine proteinase with a peptidyl diazomethane inhibitor.

Mechanism proposed for the binding of peptidyl diazomethane inhibitors to cysteine proteinases (adapted from Mason & Wilcox 1993). See page 26 for a description of the steps in the reaction.

Peptidyl Diazomethanes: Experimental Use

The peptidyl diazomethanes were first used as inhibitors of cysteine proteinases by Leary *et al.* (1977), who synthesized and used Z-phenylalanyl diazomethane (Z-Phe-CHN_2) and Z-phenylalanylphenylalanyl diazomethane (Z-Phe-Phe-CHN_2, where the first Phe corresponds to the P_2 residue and the second Phe corresponds to the P_1 residue) to inhibit papain. The inhibitors irreversibly inactivate papain, reacting with the active site cysteine residue. Cathepsin B was also inhibited by Z-Phe-CHN_2 and Z-Phe-Phe-CHN_2, and it was proposed that peptidyl diazomethane inhibitors would be useful tools for *in vivo* and *in vitro* studies of the physiological processes in which cathepsin B was implicated (Leary & Shaw 1977).

By varying the peptidyl component of the peptidyl diazomethane inhibitors, Green and Shaw (1981) were able to develop specific inhibitors to other cysteine proteinases. Clostripain, a calcium-dependent cysteine endopeptidase from *Clostridium histolyticum*, was inhibited by Z-Lys-CHN_2, streptopain, a bacterial proteinase from *Streptococcus pyrogenes*, was inhibited by Z-Ala-Phe-Ala-CHN_2, and cathepsin C, a mammalian cysteine exopeptidase now called dipeptidyl peptidase I, was inhibited by NH_2-Gly-Phe-CHN_2. In addition, the peptidyl diazomethane inhibitors were found not to inactivate chymotrypsin (Watanabe *et al.* 1979), cathepsin D, and thermolysin (Green & Shaw 1981), examples of serine, aspartic, and metalloproteinases, respectively, nor do they react with small thiol agents, such as 2-mercaptoethanol or glutathione (Green & Shaw 1981). This lack of reactivity indicated that these inhibitors are specific for cysteine proteinases.

Although peptidyl diazomethanes do not inactivate the serine proteinases chymotrypsin or trypsin, there is evidence that the inhibitor Z-Phe-Arg-CHN_2 does complex with the enzymes, resulting in destruction of the inhibitor (Zumbrunn *et al.*

1988). There have been occasional cases in which peptidyl diazomethane inhibitors have been found to inactivate serine proteinases. Z-Phe-Arg-CHN_2 slowly inactivates the plasma proteinase, kallikrein (Zumbrunn *et al.* 1988), and Z-Ala-Phe-CHN_2 irreversibly inhibits the microbial proteinases, thermitase and subtilisin Carlsberg, by covalently modifying the active site histidine (Ermer *et al.* 1990). The best-documented case is the reaction of prolyl endopeptidase (PE), a cytoplasmic serine proteinase responsible for cleaving bonds on the C-terminal side of proline residues, with acetyl-Ala-Ala-Pro-CHN_2. The reaction is both slow-binding and reversible (Stone *et al.* 1992).

The peptidyl diazomethanes have been used to distinguish between the activities of the cathepsins, providing information about the nature of the binding pockets. For example, Z-Phe-Phe-CHN_2 reacts rapidly with cathepsin L, but reacts very slowly with cathepsin B, and it was concluded that a large hydrophobic residue in P_1 prevented this inhibitor from binding to cathepsin B (Kirschke & Shaw 1981). By synthesizing a series of inhibitors with phenylalanine in the P_2 position and differing residues in the P_1 position, Shaw *et al.* (1983) found a range of reactivities with cathepsin B that varied by 10^4, demonstrating the importance of the size of the P_1 side chain in determining the degree to which peptidyl diazomethanes inhibit cathepsin B. These same inhibitors were tested with cathepsin L. P_1 side chains that resulted in poor inhibition of cathepsin B could be accommodated by the S_1 subsite of cathepsin L (Kirschke *et al.* 1988), again demonstrating the differences between these enzymes.

Peptidyl diazomethane inhibitors have also been used to distinguish between different families of cysteine proteinases. Calpains are cytosolic, calcium-dependent cysteine proteinases of, as yet, unknown function, although they have been implicated in cell division, signal transduction, and platelet activity (see Anagli *et al.* 1991). The inhibitor, t-butyloxycarbonyl-Val-Lys(ε-Z)-Leu-Tyr-CHN_2, inactivates calpain more

rapidly than it does cathepsins L or B, and, when used in combination with other inhibitors, can be used to distinguish calpain (Crawford *et al.* 1988).

Peptidyl diazomethane inhibitors have been used to identify active forms of cysteine proteinases. Mason *et al.* (1989b) incubated mouse fibroblasts in Z-[^{125}I]Tyr-Ala-CHN_2, an inhibitor of cathepsins B and L, and found that iodinated forms of cathepsins L and B appear after 30 minutes and 3 hours of incubation, respectively. The identities of cathepsins B and L were confirmed by immunoprecipitation of the labeled proteins with antisera specific for the two enzymes. The iodinated peptidyl diazomethanes do not label inactive precursors of the cathepsins, as was demonstrated by lack of labeling of procathepsin B in these experiments.

The degree to which the peptidyl diazomethanes diffuse into cells makes them useful for *in vivo* labeling of active forms of cysteine proteinases. It had been proposed that the peptidyl diazomethanes enter cells by pinocytosis (Shaw & Dean 1980), but Wilcox and Mason (1992) demonstrated by incubating purified lysosomes with Z-[^{125}I]Tyr-Ala-CHN_2 that the inhibitors are able to diffuse across a lipid bilayer and label cathepsins B and L. In addition, they showed that the labeling of cathepsins S and B could not be blocked by preincubation of lysosomes or intact cells with leupeptin or the epoxide inhibitors E-64 and E-64c, demonstrating that these inhibitors do not cross membranes by diffusion and must enter cells by pinocytosis. E-64d, however, blocked the labeling, confirming the membrane permeability of this epoxide. In addition, the labeling of cathepsins B and L was inhibited by preincubation of cells or lysosomes with non-iodinated Z-Tyr-Ala-CHN_2.

The Peptidyl Diazomethane Inhibitor Z-Leu-Leu-Tyr-CHN_2

The peptidyl diazomethane, Z-Leu-Leu-Tyr-CHN_2 (Figure 1.8), was originally investigated as an inhibitor of calpain. Calpain has a preference for Leu in the P_2 position (Sasaki *et al.* 1984), and Crawford *et al.* (1988) synthesized a series of sixteen inhibitors with Leu in the P_2 position. They found that Z-Leu-Leu-Tyr-CHN_2 was the most rapid of the inhibitors that inactivated calpain. Blocking the N-terminus was important for rapid inactivation of calpain, as the unblocked Leu-Leu-Tyr-CHN_2 reacted more slowly. In addition to inactivating calpain, Z-Leu-Leu-Tyr-CHN_2 rapidly inactivated cathepsin L, but reacted slowly with cathepsin B. More recently, the biotinylated inhibitor Biot-Aca-Leu-Leu-Tyr-CHN_2 has been synthesized for use in labeling calpain (Wikstrom *et al.* 1993).

Z-Leu-Leu-Tyr-CHN_2 has been used to investigate the functions of cysteine proteinases in human cells. Cultured human platelets undergo structural changes after activation by Ca^{2+} and a calcium ionophore, and activation results in the degradation of certain cytoskeletal proteins. Pre-incubation of platelets in Z-Leu-Leu-Tyr-CHN_2 prior to their activation blocked the protein degradation, although the inhibitor did not prevent other changes, such as an alteration in cell shape (Anagli *et al.* 1991). Incubation of activated and unactivated platelets with Z-Leu-Leu-[^{125}I]Tyr-CHN_2 labeled the active forms of calpain. Peptidyl diazomethanes have also been used to look at changes in the expression of cathepsins expression following differentiation of cells. Labeling of the cultured human cell lines, U-937 and THP-1, with either Z-Leu-Leu-[^{125}I]Tyr-CHN_2 or Z-[^{125}I]Tyr-Ala-CHN_2 before and after the cells were differentiated with phorbol ester (PMA) detected changes in the expression of cathepsins B, L, and S following differentiation (Mountz 1994).

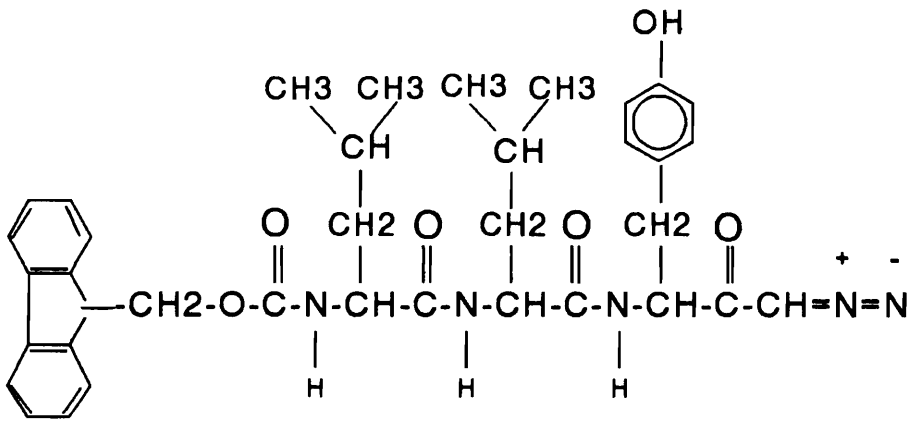


Figure 1.8 The peptidyl diazomethane inhibitor Fmoc-Leu-Leu-Tyr-CHN_2.

In the course of labeling of human cell lines with Z-Leu-Leu-[^{125}I]Tyr-CHN_2, several investigators noted that an additional putative proteinase was being labeled with the inhibitor. Labeling HIFF cells, Wilcox (1990) found a protein of about 22 kDa labeling with Z-Leu-Leu-[^{125}I]Tyr-CHN_2. She concluded that the labeled protein was a cysteine proteinase, as the labeling could be blocked by pre-incubation of the cells with E-64d. The labeled protein did not immunoprecipitate with antiserum against cathepsin L. When human platelets were incubated with Z-Leu-Leu-[^{125}I]Tyr-CHN_2, a band of 23 kDa was labeled (Anagli *et al.* 1991). At the time, the band was attributed to cathepsin L or a calpain fragment, however, later it was suggested that the band was likely due to a cysteine proteinase other than calpain or cathepsin L (Anagli *et al.* 1993).

Physiological Roles of Cysteine Proteinases

Cysteine proteinases fill a variety of intracellular roles. The lysosomal cysteine proteinases, cathepsins B, H, L, and S, will be discussed below. Cytosolic cysteine proteinases have been found, such as the calcium-dependent enzyme calpain, the function of which has yet to be determined. Calpain requires high levels of calcium (Ca^{2+} ions) for activation. A second cysteine proteinase, interleukin-1β-converting enzyme (ICE), also acts in the cytosol, cleaving the precursor form of IL-1β prior to its secretion from monocytes (Thornberry *et al.* 1992). Cysteine proteinases are also found in secretory vesicles, such as prohormone thiol protease (PTP), a 33 kDa enzyme that is responsible for processing the precursor of enkephalin, a peptide neurotransmitter (Krieger & Hook 1991). A putative cysteine proteinase, ER60 endopeptidase, has been proposed as one of the enzymes involved in degradation of proteins within the endoplasmic reticulum (Urade & Kito 1992).

Lysosomal Protein Degradation

Lysosomes are the primary degradative organelles in mammalian cells and have been found in all cell types studied with the exception of erythrocytes. They are membrane-limited organelles with a lumenal pH of 4.5−5.0, responsible both for degradation of exogenous material brought into the cell by endocytosis and for turnover of endogenous macromolecules. Lysosomes may contain over 60 different acid hydrolases (Holtzman 1989). Lysosomes are capable of degrading most cellular molecules, and their enzymes include phosphatases, nucleases, proteinases, lipases, and glycosidases. Of the proteinases detected in lysosomes, only the cysteine and aspartic classes have been found; the lysosomal cysteine proteinases include cathepsin B, H, L, and S.

Specific inhibitors of cysteine proteinases have been used to demonstrate that cysteine proteinases are responsible for a large portion of lysosomal protein degradation. The peptidyl diazomethane inhibitors, Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2, reduced the degradation of both endocytosed and endogenous protein by 50−70% in cultured hepatocytes (Grinde 1983). This loss is equivalent to the reduction of protein degradation seen when hepatocytes are treated with weak bases, for example, methylamine or ammonia. When cells are treated with weak bases, the bases selectively accumulate in lysosomes, resulting in an elevation of lysosomal pH and an inhibition of lysosomal function (Seglen 1983). In macrophages, Z-Phe-Ala-CHN_2 reduced protein turnover by 40%, and Shaw and Dean (1980) concluded that cysteine proteinases play a major role in lysosomal proteolysis.

Antigen Processing

Activation of T cells requires the presentation of antigen fragments on major histocompatibility complex (MHC) class II molecules on the surface of an antigen presenting cell. The T cell receptor recognizes the antigen fragment/MHC complex and secretes cytokines that stimulate the immune response. Lysosomal cysteine proteinases are implicated in the proteolysis of antigen molecules.

The loading of the class II molecule with an antigen fragment occurs in a novel acidic compartment in the endocytic pathway. This class II peptide-loading compartment (CPL) can be distinguished biochemically and functionally from early and late endosomes and from mature lysosomes (Amigorena *et al* 1994; Guagliardi *et al.* 1994; Tulp *et al.* 1994). Receptor-bound antigens enter the cell through endocytosis and are delivered to the CPL from early or late endosomes. Within the early endosome-CPL pathway, the antigen is proteolytically processed into peptides of from 10−30 residues. The intracellular location where the antigen is processed has not yet been defined, but CPLs contain both cathepsin D and cathepsin B (Guagliardi *et al.* 1994).

Cathepsin B can generate known antigenic fragments *in vitro* from recombinant human growth hormone (Bushell *et al.* 1993). When IgG molecules were internalized by human U-937 cells, the proteolytic fragments generated were analyzed by electrophoresis. The fragments were very similar to those generated by the *in vitro* proteolysis of IgG by cathepsin B, suggesting that cathepsin B or a similar cysteine proteinase might be responsible for antigen processing (Santoro *et al.* 1993). Further evidence for the involvement of cathepsin B in antigen processing is the inhibition of a T cell proliferative response by cysteine proteinase inhibitors. When primed mouse splenocytes were challenged with antigen in the presence of E-64d or CA-074, a general

cysteine proteinase inhibitor and a specific inhibitor of cathepsin B, respectively, the responses were suppressed 30−50% (Matsunaga *et al.* 1993).

Bone Resorption

Bone resorption, the degradation of both the mineral and the organic content of bone, occurs in the extracellular matrix of osteoclasts . Osteoclasts (reviewed in Baron 1989) are highly polarized, multinucleated cells that synthesize and secrete lysosomal enzymes. Enzyme secretion is targeted to an apical, ruffled border membrane. The apical domain of the ruffled border is sealed off by attachment of the osteoclast to bone, and the compartment is acidified (Baron *et al.* 1985). Bone resorption in cultured bone was inhibited by the cysteine proteinase inhibitor E-64 (Delaissé *et al.* 1984; Everts *et al.* 1992), suggesting that cysteine proteinases play a major role in this process. Cathepsins B and L were isolated from homogenized bone tissue, and it was proposed that they, among other proteinases, are responsible for bone resorption. Both cathepsins B and L can degrade collagen, the main organic component of bone (Delaissé *et al.* 1991). Using immunohistochemistry, cathepsins B and L were localized in osteoclasts on the surface of bone (Ohsawa *et al.* 1993). Kakegawa *et al.* (1993), however, found that CA074, a epoxide inhibitor specific for cathepsin B, had no effect on bone resorption in cultured rat bone, suggesting that cathepsin L is the primary proteinase responsible for collagen degradation.

However, another cysteine proteinase may play an important role in bone resorption, because a novel gene coding a putative cysteine proteinase has been found in osteoclasts. The gene was detected by screening a cDNA library from rabbit osteoclasts differentially with a cDNA library from rabbit spleen (Tezuka *et al.* 1992). The novel

gene, termed OC-2, is believed to be a member of the papain superfamily. OC-2 was subsequently cloned (Tezuka *et al.* 1994).

More recently, the human homologue of OC-2 has been cloned by two groups (Shi *et al.* 1995; Inaoka *et al.* 1995), who have named the encoded enzymes cathepsin O and cathepsin K, respectively. [This cathepsin O is not the same as another putative cysteine proteinase, also termed cathepsin O, that was coded for by cDNA obtained from a human breast carcinoma (Velasco *et al.* 1994).] The cathepsin K clone was obtained from screening an osteoarthritic hip bone cDNA library with a probe from rabbit OC-2. The clone has not been expressed, but it is predicted to code for a proenzyme and a mature enzyme of molecular weights 35.3 and 23.5 kDa, respectively, without glycosylation. There are two possible glycosylation sites, one in the propeptide and the other in the mature sequence (Inaoka *et al.* 1995). When the deduced sequence was compared to those of the human lysosomal cysteine proteinases, cathepsin K was found to be from 25-48% identical with those sequences (Figure 1.1), with the highest identity with cathepsin S. The sequences around the active site cysteine and histidine show high conservation. These data are in agreement with those of Shi *et al.* (1995). When the cDNA for cathepsin O was transfected into COS cells, it had endoproteinase activity that could be blocked by E-64 (Shi *et al.* 1995).

By Northern blotting, Inaoka *et al.* (1995) found that cathepsin O/K was expressed at high levels in osteoclasts compared to very low levels in other human tissues, and both groups suggest that the proteinase may play an important role in bone resorption (Shi *et al.* 1995; Inaoka *et al.* 1995).

Alzheimer's Disease

Alzheimer's disease (AD; reviewed in Kosik 1994) is a degenerative disease of the nervous system characterized by: 1) loss of neurons in specific areas of the brain; 2) neurofibrillary tangles, composed of highly ordered intraneuronal filaments, in discrete populations of neurons; and 3) protein depositions, known as senile plaques, on the brain. Lysosomal proteinases are implicated in plaque formation.

The primary protein in senile plaques is the 40 residue β-amyloid protein, which is derived from proteolytic degradation of a 100−140 kDa amyloid precursor protein (APP). The non-pathological function of APP is not known. The proteolytic fragments generated from APP depend on the location of the degradation. If the degradation occurs in the secretory pathway, the fragments produced are presumably benign. If proteolysis occurs in the endosomal/lysosomal pathway, the peptides include the β-amyloid protein (Golde *et al.* 1992). β-amyloid is subsequently released from the cell and deposited in extracellular plaques on the surface of the cerebral cortex. Recently, much research has focused on finding the proteinase responsible for the cleavage of APP that produces β-amyloid.

Using immunocytochemistry, Cataldo *et al.* (1990) found high levels of cathepsins B and D in senile plaques from AD brains. The proteinases are enzymatically active and localized to lysosomal dense bodies and granules within the plaques (Cataldo & Nixon 1990). When rat brain was screened for mRNA for cathepsins B, L, and S, it was found the cathepsin B had the highest level of expression and was preferentially expressed in neurons. In brain regions that contain the highest levels of APP, cathepsins B and L are both expressed at high levels, suggesting a role for these proteinases in AD (Petanceska *et al.* 1994).

Tumor Invasion

Tumor invasion of basement membrane occurs during malignant metastasis and involves three steps: 1) attachment of the tumor cell to the extracellular matrix; 2) localized proteolysis of the matrix by enzymes secreted by the tumor cell; and 3) movement of the tumor cell into the proteolyzed area of the matrix (Liotta 1986). Lysosomal cathepsins B and L have been implicated in the proteolytic step in tumor progression, and they have been shown to degrade collagen, elastin, proteoglycan, and laminin, all of which are components of the extracellular matrix (see Trabandt *et al.* 1991). Increased expression and secretion of cathepsin B have been noted in malignant tumors, including breast, colon, gastric and prostate, and increased expression and release of procathepsin L have been observed to accompany malignant transformation of mouse fibroblasts (see Sloane *et al.* 1992).

Cathepsin B is secreted from human breast carcinoma cells but not from normal breast tissue (Poole *et al.* 1978), and increased secretion of cathepsin B is correlated with increased membrane association of cathepsin B in transformed human breast epithelial cells. When non-transformed cells are examined by immunolocalization, cathepsin B is found in lysosomes in the perinuclear area of the cell; in transformed cells, cathepsin B is found at the cell periphery, including within cell processes such as microvilli, in addition to the perinuclear distribution of the enzyme (Sloane *et al.* 1994). In human small cell lung cancer, high expression of cathepsin B was correlated with a high metastatic potential and a shorter survival time (Sukoh *et al.* 1994).

Large amounts of cathepsin L, also known as major excreted protein (MEP), are secreted from malignantly transformed mouse fibroblasts (Gal *et al.* 1985). Cathepsin L, like cathepsin B, shows increased membrane association in human and murine melanomas (Rozhin *et al.* 1989). Chauhan *et al.* (1991) tested 100 human tumor samples

for expression of cathepsin L and found that cancers generally, and kidney, testicular, colon, and lung tumors in particular, express more cathepsin L than do normal tissues, and they suggested that cathepsin L levels could be used as a diagnostic marker for malignancy.

Other Physiological Roles of Lysosomal Cysteine Proteinases

Lysosomal cysteine proteinases are suggested to play roles in other diseases, including emphysema, rheumatoid arthritis, and glomerulonephritis.

Emphysema is characterized by a loss of lung function caused by the degradation of the extracellular connective tissues, particularly degradation of elastin. Cathepsins L and B may both be involved in the degradation. The roles suggested for cathepsin L in emphysema include degradation of elastase, either extracellularly or within lysosomes, and indirect elevation of neutrophil elastase activity (see Mason & Wilcox 1993). Cathepsin B activity has been found in human lung secretions. Procathepsin B is synthesized and secreted from bronchial epithelial cells. The enzyme can be activated extracellularly by neutrophil elastase, which also inactivates cystatin C, an inhibitor of cathepsin B (Burnett *et al.* 1995).

Many of the symptoms of rheumatoid arthritis are a result of the degradation of connective tissues of joints, in particular collagens and proteoglycans. When human fibroblasts from synovial fluid of patients with rheumatoid arthritis were compared to normal fibroblasts, the disease cells had enhanced transcription of cathepsin B (Trabandt *et al.* 1991). Further evidence for the involvement of cysteine proteinases in cartilage degradation in rheumatoid arthritis is the ability of cathepsins B, L, and S to release proteoglycan from cultured cartilage (Buttle & Saklatvala 1992).

Glomerulonephritis is a kidney disease in which there is an increase in the permeability of the glomerular basement membrane, resulting in proteinuria, the increased secretion of plasma proteins into the urine. Both cathepsins B and L are capable of degrading glomerular basement membrane (Baricos *et al.* 1988; Thomas & Davies 1989), but a specific inhibitor of cathepsin L has been shown to reduce proteinuria *in vivo*, suggesting that cathepsin L is the proteinase responsible for the degradation of basement membrane (Baricos *et al.* 1991).

The Present Study

In the course of labeling of human cell lines with Z-Leu-Leu-[^{125}I]Tyr-CHN_2, several investigators noted that an additional proteinase was being labeled with the inhibitor. A putative cysteine proteinase of about 22 kDa was labeled in fibroblasts (Wilcox 1990), and a protein of about 23 kDa was labeled in platelets (Anagli *et al.* 1991). The proteins did not immunoprecipitate with antisera to cathepsin L or calpain, and the investigators could not identify the proteinase.

As none of the known cysteine proteinases that label with Z-Leu-Leu-[^{125}I]Tyr-CHN_2 are of M_r 22−23 kDa, it was decided to investigate this protein, since termed cathepsin Z. As with most lysosomal proteinases, and indeed most proteinases in general, this study focused on the role of cathepsin Z in terms of its activity against synthetic inhibitors. Characterization and purification of cathepsin Z were of the proteinase that had been covalently modified by Z-Leu-Leu-Tyr-CHN_2.

The specific goals of the investigation included development of a procedure for purifying the enzyme from human cells, determination of some of the primary structure of cathepsin Z, and characterization of the labeling of cathepsin Z with Z-Leu-Leu-[^{125}I]Tyr-CHN_2. These characterizations of the enzyme, in addition to determining its

intracellular location and biochemical characteristics, are necessary precursors to understanding the role of cathepsin Z *in vivo*.

Methods and Materials

Materials

Chemicals

General laboratory chemicals, all ACS grade or higher, Concanavalin A-Acrylic Beads, and electrophoresis molecular weight markers (14−66 kDa for SDS-PAGE and 14−545 kDa for native PAGE) were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bis-acrylamide, ammonium persulfate, protein assay reagents, and TEMED were purchased from BioRad Laboratories Inc. (Hercules, CA); Protein A-Sepharose and Protein G-Sepharose from Pharmacia LKB Biotechnology (Piscataway, NJ); prestained molecular weight markers (4−250 kDa) from Novex (San Diego, CA); phosphate-buffered saline (PBS) and cell culture reagents, with the exception of serum, from Gibco BRL Life Technologies (Gaithersburg, MD); and fetal bovine serum (defined) from Hyclone Laboratories (Logan, UT). Metrizamide and sucrose, both analytical grade, were purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). Iodo-beads iodination reagent was purchased from Pierce Chemical Company (Rockford, IL) and sodium [^{125}I]odide from DuPont NEN (Wilmington, DE). Tetrahydrofuran (THF) and methylmorpholine were purchased from Fluka Chemical Corp. (Ronkonkoma, NY); biotin nitrophenol ester and Diazald from Aldrich Chemical Co. (Milwaukee, WI); endoproteinase Glu-C, excision grade, from Calbiochem (La Jolla, CA); and endoglycosidase H from Boehringer Mannheim Corp. (Indianapolis, IN). Reagents for peptide syntheses were purchased from Millipore Corp. (Marlborough, MA). The inhibitor E-64 was purchased from Sigma Chemical Co. (St. Louis, MO). E-64d was a gift from Dr. K. Hanada.

Supplies

Acrodisc syringe filters (0.2 μm), membrane filters (0.45 μm) and AcroCap media filters (0.2 μm) were purchased from Gelman Sciences (Ann Arbor, MI). Immobilon P^{SQ} PVDF membranes were purchased from Millipore Corp. (Marlborough, MA); ProSpin Sample Preparation Cartridges from Applied Biosystems (Foster City, CA); and YM3 membranes from Amicon Inc. (Beverly, MA). Films used were X-omat AR X-ray film from Eastman Kodak Co. (Rochester, NY) and Type 45 positive/negative sheet film from Polaroid Corp. (Cambridge, MA). Electrophoresis Duplicating Paper was also purchased from Eastman Kodak Co. Polypropylene centrifuge tubes (15- and 50-ml), microfuge tubes (1.5-ml), and pipette tips were purchased from Fisher Scientific (Pittsburgh, PA). Sterile tissue culture products (35-, 60-, and 100-mm tissue culture dishes; 24-well multiwell dishes, and 2- and 10-ml pipettes) were purchased from Fisher as well. FPLC columns, Mono Q HR 5/5 and Superose 12, were from Pharmacia LKB Biotechnology (Uppsala, Sweden).

Peptides were synthesized in the lab on a Milligen/Biosearch 9600 Peptide Synthesizer (Millipore Corp., Bedford, MA). N-terminal amino acid sequence analysis was performed by the Center for Biotechnology in the Biology Department at VPI & SU on an Applied Biosystems Model 477A Protein Sequencer (Foster City, CA). Database searches and sequence alignments were done using Lasergene software (DNASTAR, Inc., Madison, WI).

Biologicals

Human cultured cell lines U-937, A549, HeLa, THP-1, Hep G2, Wil2NS, and Raji were purchased from America Type Culture Collection (ATCC; Rockville, MD). The primary cultured cell line used was the U-937 line, established by Sundström and

Nilsson (1976). Rabbit anti-cathepsin S was a gift from Dr. Heidrun Kirschke and sheep anti-cathepsin B from Dr. David Buttle. Rabbit anti-cathepsin L had been prepared in the laboratory as previously described (Mason 1986b).

Methods

Preparation of Solutions

All solutions were prepared using distilled water. The pH was adjusted prior to bringing the solution to final volume. Solutions were filtered using a 0.45 μm filter under vacuum prior to storage. Storage conditions depended on the solution. Solutions were stored at room temperature (~15−25°C), in the refrigerator at 4°, or in the freezer at −20°C. If needed, the solutions were protected from light by storage in dark bottles.

Cell Culture Methods

Preparation of Cell Culture Materials

Bottles and other glassware used for cell culture were washed, rinsed with distilled water, and allowed to dry. Bottles, coverslips, pipette tips, and other supplies were sterilized in a steam autoclave for 20 min with a 10 min drying time. Other items, such as tissue culture dishes, pipettes, and multiwell plates, were purchased as sterile products.

Cell Culture Reagents

Cells were cultured in RPMI-1640 or DMEM (see Table 2.1). The media were prepared either in 1 or 10 L lots using powdered media. Ninety percent of the final volume of distilled water was placed in a flask with a stirbar. The powdered medium was added slowly with stirring. When the medium was in solution, sodium bicarbonate

was added, 2 g per L of medium. The pH of the solution was adjusted to 7.4 by addition of 1 M hydrochloric acid. The medium was brought to the final volume with distilled water and sterilized by filtration through 0.2 μm bottletop filters into sterile bottles. Media was stored at 4°C.

RPMI-1640 or DMEM were supplemented with 10% fetal bovine serum (FBS). The serum had endotoxin levels below 0.1 EU per ml; higher levels were cytotoxic to U-937 cells. The serum arrived frozen and was thawed at 4°C. It was heat-inactivated by placing the bottles in a 56°C water bath for 15 min, swirling the bottles every 2−3 min. Immediately after heat-inactivation, the serum was placed in a freezer. The serum was stored at −20°C for long-term storage; short-term storage was at 4°C. In addition, β-mercaptoethanol (2-mercaptoethanol) was added to RPMI-1640 to a final concentration of 5×10^{-5} M. A stock solution of 5×10^{-3} M was prepared by adding 35 μl of β-mercaptoethanol to 100 ml of distilled water. The solution was sterilized by filtration through a 2 μm filter and stored protected from light at 4°C. RPMI-1640 containing 10% FBS and 5×10^{-5} M β-mercaptoethanol is termed complete medium.

Adherent cells were removed from their substrate using 0.05% trypsin, 0.53 mM EDTA. The trypsin solution was purchased in 100 ml bottles, thawed, and 10 ml aliquots placed in 15 ml tubes. Long-term storage of the aliquots was at −20°C; short-term storage was at 4°C.

A combined antibiotic and antimycotic solution was used for some tissue culture. The 100x solution contained 10,000 units penicillin, 10,000 μg streptomycin, and 25 μg amphotericin B per ml; the solution was used at 1x concentration. The solution was purchased in 20 ml bottles and stored at −20°C until use.

Cell Culture

Suspension cell lines (see Table 2.1) were maintained routinely in 60-mm plastic tissue culture dishes in RPMI-1640, 5 x 10^{-5} M β-mercaptoethanol, supplemented with 10% FBS (complete medium). Cells were grown in an atmosphere of 5% CO_2 in a humidified incubator at 37°C. All cell counts were done using a Neubauer haemacytometer. Twice a week, the cells were diluted into fresh medium at three concentrations. Usually, the cells in a dish containing approximately 1 x 10^6 cells per ml were resuspended, and ~5 x 10^4, 1.5 x 10^5, and 2.5 x 10^5 cells (1, 3, and 5 drops of cell suspension) seeded into 4 ml fresh medium.

Table 2.1 Human Cultured Cell Lines Used.

Cell Line	Source	Growth	Medium
U-937	histiocytic lymphoma	suspension	RPMI
A549	lung carcinoma	adherent	RPMI
HeLa	epitheloid carcinoma	adherent	DMEM
Hep G2	hepatocellular carcinoma	adherent	RPMI
THP-1	acute monocytic lymphoma	suspension	RPMI
Wil2-NS	lymphoblastoma	suspension	RPMI
Raji	Burkitt lymphoma	suspension	RPMI

Adherent cell lines (see Table 2.1) were maintained in complete medium in a similar manner, but the cells were resuspended using trypsin and EDTA. The medium was removed from a 60-mm tissue culture dish near confluence (~1 x 10^6 cells per ml) and the cells were rinsed once with 1 ml of 0.05% trypsin, 0.53 mM EDTA to remove the serum. One ml of trypsin was added to the dish, and the dish placed in the incubator at 37°C for 5 min. One ml of complete medium was then added to the dish and the cells

were resuspended. The cells were seeded into 4 ml of complete medium as above, adding ~1.0 x 10^5, 3.0 x 10^5, and 5.0 x 10^5 cells (1, 3, and 5 drops of cell suspension) per dish.

U-937 cells were grown on a larger scale in 100-mm plastic tissue culture dishes. usually, 1 ml of cells (~1 x 10^6 cells) was seeded into 15 ml of complete medium and allowed to grow to a final concentration of 1.0−1.5 x 10^6 cells per ml. For large scale production of U-937 cells, 500 ml stirrer bottles were used. Complete medium (~550 ml) and 1x antibiotic/antimycotic were placed in the bottles, and U-937 cells seeded into the bottles at ~5 x 10^5 cells per ml. The bottles were sealed and placed on a magnetic stirring platform in a 37°C oven for 3−4 d, until the cell concentration reached 1−2 x 10^6 cells per ml. The cells were counted and harvested by pelleting them in 200-ml glass bottles in a Beckmann TJ-6 centrifuge at ~2000 x g for 10 min at room temperature. The medium was discarded and the cell pellets washed twice in phosphate-buffered saline (PBS). The cell pellet was frozen at −20°C for use in future experiments.

Frozen Cell Storage

Stocks of the cell lines used were stored in liquid nitrogen. Cells were cultured until they reached log phase growth. Freezing medium was prepared by supplementing RPMI-1640 with 20% FBS, and 10% dimethyl sulfoxide (DMSO). Cells were pelleted by centrifugation in a Sorvall RT6000 centrifuge at ~1000 x g for 5 min at room temperature, counted, and resuspended in freezing medium to give a final concentration of 1.0 x 10^6 cells per ml. The cells were aliquotted into freezing vials, 1 ml per vial, and the vials placed at in an insulated box at −80°C. After 16 h the vials were transferred to liquid nitrogen for long-term storage.

Cells were thawed by placing a vial in a 37°C water bath and agitating the vial for approximately 1 min. The cells were gently resuspended and transferred to a 15-ml centrifuge tube containing 10 ml warmed complete medium. The cells were pelleted as above and the supernatant discarded. The cells were resuspended in 4 ml complete medium and cultured in a 60-mm tissue culture dish.

In vivo Labeling of Cultured Cells with Iodinated Inhibitors

Suspension culture cells in log phase growth were pelleted by centrifugation and washed twice in serum-free growth medium. Cells were counted and 1 x 10^6 cells cultured in 1 ml of serum-free medium either in 35- or 60-mm tissue culture dishes or in 24-well tissue culture plates. Adherent cells were cultured one day prior to the experiment at 5 x 10^5 cells per dish or well. At the time of the experiment, adherent cells were washed twice in serum-free medium by adding 1-2 ml of medium to the well and carefully removing it by pipette. Cells were incubated in serum-free medium for 1 h at 37°C in a humidified CO_2 incubator. Iodinated inhibitor was added to the cells, routinely to a final concentration of 2 μM, and the cells were incubated for 3 h at 37°C. Suspension cells were harvested by pipette and adherent cells were harvested either by scraping the well or dish with a rubber policeman or by incubating the cells for 5 min at 37°C in 0.05% trypsin, 0.53 mM EDTA. Cells were transferred to 1.5-ml microfuge tubes and pelleted by centifugation in an Eppendorf Model 5415 C microfuge at ~1300 x g for 5 min at room temperature. The supernatant was discarded and the cells washed twice in PBS. The cell pellets were resuspended in sample buffer and analyzed by SDS-PAGE and autoradiography.

Cell Lysis

Cells were lysed by repetitively freezing and thawing them. The cell pellet was resuspended in the desired buffer, which might or might not contain detergents, and the suspended cells placed either in a microfuge tube or in a 15-ml centrifuge tube. The tube was placed in a bath of dry ice and methanol for 5−10 min. The tube was then placed in a 37°C water bath for 10 min. The sample was vortexed briefly, then the cycle was repeated twice.

Differentiation of U-937 Cells with Phorbol Ester

U-937 cells were induced to differentiate with phorbol 12-myristate 13-acetate (PMA). PMA was stored in microfuge tubes at −20°C, with 0.01 μmoles/tube. PMA was resuspended by the addition of 200 μl acetone, giving a stock of 50 μM. The PMA stock was diluted 1:1000 for use, making the final PMA concentration 500 nM. The U-937 cells were plated at 1−2 x 10^5 cells/ml in complete medium, the PMA was added, and the cells were allowed to differentiate for 2, 24, or 72 h. After differentiation, the cells were labeled with iodinated inhibitor, harvested, counted, and analyzed by SDS-PAGE. Cells in the 72-h incubations were fed after 48 h by the addition of complete media.

Isolation of Lysosomes

A subcellular fraction enriched for lysosomes was isolated from suspension cells using the procedure of Storrie and Madden (1990). All steps were performed at 4°C, and all low speed centrifugations were in a Beckmann TJ-6 centrifuge. Samples of material from each stage were saved for analysis. Cells were harvested by pelleting them at ~2000 x g for 10 min. The cell pellet was washed once in 5 ml of PBS and twice in 0.25

M sucrose. The cells were resuspended in 2 ml of 0.25 M sucrose, placed in a 15-ml nitrogen cavitation bomb, and pressurized to 30 psi for 15 min. The cell suspension was collected while the bomb was still pressurized, and the cells were homogenized with 4 strokes of a Potter-Elvehjem homogenizer. A postnuclear supernatant was prepared by pelleting nuclei and unbroken cells at 1200 x g in a conical-bottomed centrifuge tube. The pellet was washed twice with 0.25 M sucrose and the washes were added to the supernatant.

Lysosomes were isolated with sequential density gradients. The first gradient consisted of 2 ml of 35% metrizamide, 2 ml of 17% metrizamide, and 5 ml of 6% Percoll. The ~6 ml of postnuclear supernatant was placed on top of the gradient and the gradient was centrifuged using a SW-28 rotor for 30 min at 50,000 x g. Lysosomes should be enriched at the interface of 6% Percoll and 17% metrizamide. This interface was removed in a volume of 1.2 ml, mixed with 0.875 ml of 80% metrizamide, and placed at the bottom of the second gradient, which consisted of 2 ml of 17% metrizamide, 2 ml of 5% metrizamide, and 5 ml of 0.25 M sucrose. The gradient was centrifuged in a SW-28 rotor for 30 min at 50,000 x g. Lysosomes should be enriched at the interface of 5% metrizamide and 17% metrizamide. This interface was collected and all samples were analyzed by SDS-PAGE and autoradiography.

Altering Lysosomal pH with Ammonium Chloride

To raise the pH within lysosomes, U-937 cells were incubated in a weak base solution. U-937 cells in log phase growth were washed in serum-free media and plated in 24-well dishes at 2 x 10^6 cells in 0.5 ml of serum-free media per well. A 1 M stock of NH_4Cl was prepared, and added to the wells to give final concentrations of 0, 8, 16, and 32 mM. The cells were incubated for 1 h at 37°C. Z-Leu-Leu-[^{125}I]Tyr-CHN_2 was

added to the wells at 5 μM final concentration and the cells were incubated for 3 h at 37° C. The cells were harvested, washed 3x in PBS, and analyzed by SDS-PAGE and autoradiography, loading 8 x 10^5 cells/lane.

Electrophoresis of Proteins

Routine Electrophoretic Methods

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed routinely on a BioRad Mini-Protean II apparatus using a discontinuous buffer system based on the procedure of Laemmli (1970). For most analyses, the separating and stacking gels were 12.5% and 4.5% acrylamide, respectively. The stock solution of 30.8% acrylamide was 30% acrylamide and 0.8% *N*, *N*'-methylene bisacrylamide (bis), prepared by adding 30 g of acrylamide and 0.8 g of bis to 50 ml of distilled water and stirring until dissolved. The solution was brought to 100 ml, filtered, and stored at 4°C in a dark bottle; acrylamide solutions were stored for no more that 6 weeks. SDS and ammonium persulfate (APS) stocks were 10% solutions. Small aliquots (0.5 ml) of APS stock were stored at −20°C. When the gel was being prepared, an aliquot was thawed and used for both the stacking and separating gels. Excess thawed APS stock was discarded. The buffers, 0.375 M Tris-HCL, pH 8.8, and 0.125 M Tris-HCL, pH 6.8, for the separating and stacking gels, respectively, were prepared as 4x stocks. Fifty percent glycerol was prepared by mixing equal volumes of glycerol and distilled water, filtering, and storing in a dark bottle at room temperature. Gel formulations are given in Appendix 1, Table A.1.

The pre-cleaned glass plates were rinsed with methanol and dried, and the gel casting apparatus assembled. The components of the separating gel solution, with the exception of TEMED and APS, were placed in a flask and mixed gently. The TEMED

and APS were added, and the solution mixed and pipetted into the apparatus, allowing 1 cm below the length of the comb for the stacking gel. The gel was gently overlaid with water-saturated butanol and allowed to polymerized for 1 h. After the separating gel had polymerized, the butanol was removed, the surface of the gel rinsed with distilled water, and the comb inserted. The stacking gel was prepared as above, and the solution pipetted into the apparatus and allowed to polymerize.

Samples were resuspended in sample buffer (8 M urea, 12.5% glycerol, 5% SDS, 5 mM EDTA, 0.01% bromphenol blue, and 250 mM Tris-HCl, pH 6.8); unless otherwise noted, the sample buffer contained 2.5% β-mercaptoethanol. Samples were heated to 95 °C for 3 min. The gel running buffer was 0.05 M Tris, 0.375 M glycine, 0.1 % SDS, pH 8.3. After the samples were loaded, 10 mA constant current was applied to the gel until the tracking dye reached the bottom of the gel. The gel was removed from the apparatus, fixed, and stained, either with Coomassie or by silver staining (see below). After destaining, the gel was photographed using Polaroid positive/negative film, dried onto filter paper, and analyzed by autoradiography. For autoradiography, the dried gel was placed in a Kodak exposure cassette with a phosphor intensifying screen and a piece of X-ray film, and the sealed cassette placed at -80°C for from 6 h to 2 weeks. The X-ray film was developed for 3$-$5 min, depending on developer temperature, fixed, washed, and dried. Autoradiographs were photographed using Electrophoresis Duplicating Paper.

To quantify the amount of radioisotope associated with a labeled protein, two methods were used. First, the area of a dried gel corresponding to a band observed by autoradiography was excised from the gel and counted on a gamma counter. To determine the background in each lane, similarly-sized slices were excised from each lane from areas of the lane with no apparent labeled proteins. The samples were counted

for 5−60 min. To correct for background, the background counts were subtracted from the counts associated with the labeled proteins.

The second method used for quantifying the amount of radioisotope associated with a labeled protein was to analyze the autoradiograph to determine the density of the bands. This was done using a Molecular Dynamics Personal Densitometer and ImageQuant software (Sunnyvale, CA). The autoradiograph was scanned and the image digitized. The labeled bands were closely framed by a rectangular box. When multiple lanes were of interest on the same gel, the same-sized box was used for each band. For background density, a box of the same size was used to delimit an area in an unlabeled portion of each lane. For quantification, the background density was set, and volume of the band of interest was integrated (corrected for lane background). Background was always determined from the same lane as the band of interest.

SDS-PAGE for Low Molecular Weight Proteins

To examine low molecular weight proteins, the gel system described by Okajima *et al.* (1993) was used. The stacking gel was 4.5% acrylamide and identical to that described above. The separating gel was 20% acrylamide, lacked glycerol, and contained twice the routine concentration of Tris. The buffer for the separating gel was 0.75 M Tris-HCL, pH 8.85, prepared as a 4x stock. Gel formulations are given in Appendix 1, Table A.2. The electrophoresis was performed at 10 mA constant current. After electrophoresis, the gel was processed as for routine SDS-PAGE.

Staining Gels for Protein

Slab gels were stained for protein either with Coomassie blue or by silver staining. All steps in both methods were performed at room temperature on a shaker

platform. For silver staining, which can detect as little as 10 ng of protein, the gel was fixed overnight in 40% methanol, 10% trichloroacetic acid, and then fixed 2x for 15 min in 10% ethanol, 5% acetic acid (Garfin 1990). The trichloroacetic acid fixation is recommended to maximize fixation of low molecular weight proteins. The gel was incubated in freshly made oxidizer solution (0.034 M potassium dichromate, 0.0032 N nitric acid) for 15 min. The gel was washed 4x for 5 min in water, or until the yellow coloration had completely disappeared. The gel was then soaked in freshly made 0.012 M silver nitrate for 15 min, followed by washing for 2 min in water. The color was developed by incubating the gel in 0.28 M sodium carbonate, 1.85% paraformaldehyde for 1−5 min. Color development was stopped by incubating the gel in 5% acetic acid. For subsequent staining with Coomassie blue, the silver staining was removed by incubating the gel with Kodak fixer until no color remained. After washing with water, the gel could be stained with Coomassie blue.

Gels were routinely stained with Coomassie Blue (0.02% Coomassie Brilliant Blue G-250 in 7.5% acetic acid, 50% methanol) for 30 min; the stain should detect proteins down to low microgram concentrations. Gels were destained in 7.5% acetic acid, 5% methanol for from 1 h to overnight.

Molecular Weight Determinations

For routine 12.5% SDS-PAGE, molecular weight markers with a 14.1−66 kDa range were used. For 20% gels, prestained molecular markers with a 4−250 kDa range were used; the lower molecular weight proteins in this solution were 4, 6, 17, and 22 kDa. Molecular weight determinations were made by measuring the migration of the protein standards from the gel origin. The migrations (x-axis) were plotted against the molecular weights of the standard proteins (y-axis) on a semilog graph, a line fitted to the

points, and an equation derived for the line. The migration of the unknown (x) was measured on the film or dried gel, and the molecular weight of the unknown (y) calculated from the equation of the line. All molecular weights are given in kilodaltons (kDa) unless otherwise noted.

Preparatory Electrophoresis

Preparative electrophoresis was performed using a BioRad Model 491 Prep Cell and a 37 mm ID gel tube. The separating gel was 10 cm in height and 14% acrylamide, and was prepared following the BioRad protocols. The stock solution of 30% acrylamide was 29.2% acrylamide and 0.8% bis. The Tris-HCl gel buffers were the same as those used for routine SDS-PAGE, pH 8.8 and 0.375 M for the separating gel and pH 6.8 and 0.125 M for the stacking gel, prepared as 4x stocks. APS stock was that used for routine SDS-PAGE. Gel formulations are given in Appendix 1, Table A.3. For the separating gel, the acrylamide solution, water, and buffer were mixed in a sidearm flask and degassed for 15 min under vacuum. The TEMED and APS were gently mixed into the solution and the gel was poured, using a 10-ml pipette, to ~3 cm. At that point, the apparatus was tapped to remove trapped air bubbles from the gel. The gel was then poured to 10.5 cm in height. The gel was overlaid with ~2 ml of water-saturated butanol and allowed to polymerize for 2 h, with cooling. After polymerization, the gel height was 10 cm.

After the 2 h polymerization period, the cooling was stopped. The butanol was removed from the gel and replaced with 5 ml of 0.375 M Tris-HCl, pH 8.8. The gel was covered and left at room temperature for 2−4 days. Allowing the polymerization to continue for ≥2 d improved the resolving power of the gel.

The stacking gel was 2 cm in height and 4% acrylamide. The gel solution was prepared the same way as the separating gel, following the formulations in Appendix 1, Table A.3. The solution was degassed for 15 min prior to adding the catalyst. The overlay was removed from the separating gel surface, the stacking gel poured to a height of 2.5 cm, and the gel overlaid with water-saturated butanol. After a 2 h polymerization period, the gel was ready to run. The butanol was removed and the surface of the gel rinsed with distilled water.

The gel running buffer and the elution buffer were the same solution, consisting of 0.192 M glycine, 0.025 M Tris, 0.1% SDS, pH 8.3, which was prepared as a 10x stock. For use, the stock was diluted 1:10 in distilled water; ~6 L were used for each Prep Cell run.

The sample was prepared as for routine electrophoresis, by diluting it 2x in sample buffer containing β-mercaptoethanol. Cytochrome c (2−5 mg) was added to the sample; the visible red band from the cytochrome c served as a marker, as did the tracking dye, and helped to determine when to begin collecting fractions. The two markers also allowed comparisons to be made between prep cell runs. The sample was heated to 95°C for 3 min.

When the gel was to be run, the lower buffer chamber was filled with ~3.5 L of running buffer that had been degassed under vacuum for $\geq$15 min. The dialysis membrane, with a molecular weight cut off of 6 kDa, and the support frits had been pre-soaked in running buffer. The gel apparatus was assembled and the elution chamber filled with buffer; ~50 ml of buffer were placed in the upper buffer chamber. Using a 50-cc syringe, air was removed from around the elution membrane. The sample was placed on the surface of the gel using a syringe and a Teflon tube. The upper buffer

chamber was filled with running buffer and the buffer recirculation pump started, running at ~100 ml/min. The gel was run at 12 W constant power for 2−3 d.

When the bromphenol blue neared the bottom of the gel, fraction collection was started using a peristaltic pump set at 1 ml/min. Four ml fractions were collected in 5-ml borosilicate glass tubes. Fractions were transferred to 5-ml scintillation vials, and the fractions counted on a gamma counter. The elution profile was graphed, with volume eluted on the x-axis and cpm on the y-axis.

Determining the Molecular Weight of a Protein Using Native PAGE

To calculate the molecular weight of a protein under non-denaturing conditions, the sample and molecular weight standards were run on a series of non-SDS gels (Bollag & Edelstein 1991). The acrylamide concentrations of the gels were 5, 6, 7, 8, 9, and 10%, and they were prepared using routine PAGE reagents. See Appendix 1, Table A.4, for gel formulations. The sample and standards were suspended in sample buffer that did not contain SDS or β-mercaptoethanol and they were not heated to 95°C prior to loading. The gel running buffer was 0.025 M Tris, 0.192 M, pH 8.3. The gels were run, stained with Coomassie Blue, destained, dried, and subjected to autoradiography.

Molecular weight determinations were made by measuring the migration of the standards, the sample, and the dye front on each gel. From these measurements, the relative mobility (R_f) was calculated for each band [R_f = (protein migration ÷ dye front migration)]. For each standard protein and the sample, the R_f was plotted in the form $\{100\ [\log (R_f \times 100)]\}$ on the y-axis and acrylamide concentration of the gel on the x-axis. The equation of the line from each standard was determined. The slope, in the form (−slope), of each line was plotted on the y-axis against the molecular weight of each standard on the x-axis on a log/log graph and the equation of this line was

determined. The molecular weight of the unknown sample was calculated by substituting the slope of the line derived from the R_f of the unknown sample into the equation derived for the standard proteins.

Electroblotting of Proteins

Proteins were transferred from polyacrylamide slab gels to polyvinylidene fluoride (PVDF) membranes in a BioRad Mini Transblot Cell, using a method adapted from Wilson and Yuan (1989). A piece of PVDF membrane, cut to the size of the gel, was pre-wet with methanol for 30 s. The membrane, fiber pads, and filter papers were soaked for 30 min in blotting buffer (10 mM CAPS, 10% methanol, pH 11.0). The SDS-PAGE gel was removed from the gel apparatus, rinsed for 5 min in blotting buffer, and placed within the blotting sandwich in apposition with the PVDF membrane. The sandwich was placed in the gel box and electroblotted overnight at 30 V constant voltage.

After electroblotting, the membrane was rinsed in water for 5 min and then stained for 5 min with 0.1% Coomassie Brilliant Blue R-250, 40% methanol, 1% acetic acid. The membrane was destained for 5−10 min with 50% methanol, 10% acetic acid, changing the destain every 30−60 s. After the membrane had dried, bands were excised to be used for N-terminal sequence analysis. The gel was routinely stained for protein to confirm that the transfer had been completed.

Column Chromatography

Affinity Chromatography

Affinity chromatography was performed using concanavalin-A immobilized on acrylic beads. Concanavalin A binds proteins containing α-D-mannopyranosyl residues, e.g., N-linked glycoproteins. The chromatography medium was resuspended in ConA

buffer (100 mM sodium acetate, 200 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 6.5; see Appendix 2, Table A.5, for preparation) and allowed to swell for 16 h at room temperature. A 2-ml column was poured, washed extensively with ConA elution buffer (0.5 M methyl α-D-mannopyranoside in ConA buffer), and re-equilibrated in ConA buffer. The sample was diluted 1:1 in ConA buffer, acidified to pH 6.5, and loaded on the column. After the column was washed extensively, the bound glycoproteins were eluted with ConA elution buffer. One ml fractions were collected and assessed for isotope content. The elution profile was graphed, with volume eluted on the x-axis and cpm on the y-axis.

Ion Exchange Chromatography

Ion exchange chromatography was performed on a Pharmacia FPLC using a 1 ml Mono Q anion exchange column. The column was equilibrated in 20 mM Tris-HCl, pH 8.5. The sample, also in that buffer, was loaded on the column at 0.5 ml/min. Material eluting from the column was monitored for protein content by reading the absorbance of the sample at 280 nm. Material that did not bind the column was collected and saved. The column was washed until no more protein came off. The protein bound to the column was eluted with 0.5 M NaCl, 20 mM Tris-HCl, pH 8.5; 0.5-ml fractions were collected and assessed for isotope content. The elution profile was graphed, with volume eluted on the x-axis and cpm and protein concentrations on the y-axes.

Gel Filtration Chromatography: Sephadex G-100

Gel filtration chromatography was performed using Sephadex G-100 Superfine resin. The resin was allowed to swell for 72 h at room temperature in column buffer (20 mM Tris-HCl, pH 8.5, 400 mM NaCl, 0.1% Brij 35). The column had an ID of 1 cm and

was poured to a height of 90 cm. All samples were run using a peristaltic pump set for ~15 ml/h; 1-ml fractions were collected. To determine the void volume of the column, a sample of blue dextran was run. The elution volume was determined by reading the absorbance of the fractions at 595 nm on a Hitachi Model U-2000 Spectrophotometer. Samples of bovine serum albumin (BSA) and cytochrome c were run on the column to determine the elution volume for proteins of known molecular weight. The elution volumes were determined by reading the absorbance of the fractions at 280 nm, and A_{280} was graphed against ml eluted. Fractions collected from runs of experimental samples were assessed for isotope content, the elution profile graphed, with volume eluted on the x-axis and cpm and protein concentrations on the y-axes, and the molecular weight estimated by comparison of the elution volume to that of BSA and cytochrome c. The samples containing isotope were further analyzed by SDS-PAGE.

Gel Filtration Chromatography: Superose 12 on FPLC

Gel filtration chromatography was also performed using a Superose 12 column on a Pharmacia FPLC. The 12-ml column was equilibrated in 20 mM sodium acetate, 200 mM NaCl, pH 5.5, and the column flow rate was 0.5 ml/min. Samples were loaded using a 100 μl superloop. The molecular weight of standard proteins and the void volume of the column were determined as described for Sephadex G-100 gel filtration (see above). The collected fractions (0.5 ml) were also analyzed as described for Sephadex G-100 gel filtration.

General Methods

Measurement of Radioactivity

The amount of [^{125}I] in samples was determined by counting the samples in a LKB Wallac Model 1282 CompuGamma gamma counter. The counting window was set to include the full range of energy detectable by the machine, from 10−2000 KeV. Disintegrations per minute (dpm) were calculated by multiplying the counts per minute (cpm) by the efficiency of the machine.

Concentration of Protein Samples

Two methods were used to concentrate dilute protein solutions. For samples with volumes <1−2 ml, the protein was precipitated with deoxycholic acid (DOC) and trichloroacetic acid (TCA), adapted from the procedures of Peterson (1983) and Mahuran *et al.* (1983). Stock solutions of 1% DOC and 50% TCA were prepared. DOC was added to the sample to give a final concentration of 150 μg/ml and the sample was incubated on ice for from 30 min to 1 h. Ice cold 50% TCA was added to the sample to give a final concentration of 10% TCA, and the sample was incubated on ice for 1 h. The pellet was pelleted by centrifugation at ~12000 x g in an Eppendorf Model 5415C microfuge for 5 min and the supernatant discarded. The pellet was washed once in 1 ml ice cold acetone and the wash discarded. The pellet was then resuspended in sample buffer for analysis.

To concentrate dilute protein samples of greater volume, 10-ml and 200-ml Amicon stirred cell concentrators were used with YM3 dialysis membranes. YM3 membranes have a molecular weight cutoff of 3 kDa. The stirred cell was pressurized to ~50 psi with N_2 gas and the solution concentrated to the desired volume.

Protein Determination

All protein determinations were performed using the microassay procedure of the BioRad Protein Assay, which is based on the method of Bradford (1976). Several dilutions of the sample and serial dilutions of a protein standard (routinely from 1−20 μg of BSA) were made in water, each in 200 μl total volume, and placed in 1.5-ml disposable polystyrene cuvettes. The dye reagent was diluted 1:4 in water and 800 μl of the diluted reagent added to each cuvette. The cuvettes were covered with Parafilm, and the contents mixed by inversion. After a color development time $\geq$5 min, the absorbance at 595 nm was measured on a Hitachi Model U-2000 Spectrophotometer. A standard curve was plotted, with μg of protein on the x-axis and A_{595} on the y-axis. From the line derived, the protein concentrations of the unknowns were calculated.

Endoglycosidase H Digestion of Samples

Endoglycosidase H hydrolyzes N-linked high mannose oligosaccharides from glycoproteins. The enzyme was used to determine the extent of glycosylation on protein samples. Labeled cell pellets were resuspended in Endo H buffer (100 mM citrate, 0.1% SDS, 0.2% β-mercaptoethanol, 1 mM PMSF, pH 5.6) and the samples were heated to 95 °C for 2 min. After the samples had cooled, 5 mU of endoglycosidase H (in 5 μl) was added to a sample; as a control, 5 μl of buffer was added to a second sample. The samples were incubated overnight at 37°C and then analyzed by SDS-PAGE.

V8 Digestion of Samples

V8 (endoproteinase Glu-C) cleaves peptide bonds on the C-terminal side of glutamatic acid, and, under some conditions, aspartic acid residues. The proteinase was used to digest protein samples for N-terminal sequence analysis. V8, with a specific

activity of 32 units/mg, was suspended in sample buffer (50 mM Tris-HCl, 20% glycerol, 0.1% SDS, 0.2% bromphenol blue, pH 6.8), giving a stock concentration of 80 ng/μl. The protein sample was diluted 1:1 with V8 solution, giving a final V8 concentration of 40 ng/μl, and incubated at 37°C for 2 h. A control sample was incubated in the absence of V8. The digests were analyzed by SDS-PAGE for low molecular weight samples, following which the proteins were electroblotted to a PVDF membrane. After staining, bands were excised for N-terminal sequence analysis.

Immunoprecipitation of Samples

Protein samples were immunoprecipitated with antisera to cathepsins B, L, and S. All incubations were performed in 1.5-ml microfuge tubes on a rotary mixer at room temperature. The antisera to cathepsins L and S were both made in rabbits to native cathepsins, whereas the antiserum to cathepsin B was made in sheep to denatured cathepsin B. The 100-μl protein samples were first pre-cleared to remove any non-specific interactions with the reagents by incubating them for 2 h in 10 μl pre-immune rabbit serum and 100 μl Protein A-Sepharose slurry (cathepsins L and S) or 10 μl pre-immune sheep serum and 100 μl Protein G-Sepharose slurry (cathepsin B). The samples were pelleted at ~2500 x g in an Eppendorf Model 5415C microfuge and the supernatants transferred to new microfuge tubes. Two μl of 1% SDS was added to the cathepsin B sample; the sample was heated to 95°C for 5 min and allowed to cool. The samples were then immunoprecipitated by incubating them for 2 h in 10 μl rabbit anti-cathepsin and 100 μl Protein A-Sepharose slurry (cathepsins L and S) or 10 μl sheep anti-cathepsin and 100 μl Protein G-Sepharose slurry (cathepsin B). In addition, control samples were incubated in the presence of Protein A- or G-Sepharose, but in the absence of specific antiserum. The immunoprecipitates were pelleted at ~2500 x g in a

microfuge, the supernatants transferred to new tubes, and the pellets washed 3x in PBS. The protein in the supernatants was precipitated with DOC and TCA, the precipitates and the pellets suspended in sample buffer, and the samples analyzed by SDS-PAGE.

Synthesis of the Inhibitor Fmoc-Leu-Leu-Tyr-CHN_2

Preparation of Diazomethane

Diazomethane (CH_2N_2) was synthesized from *N*-methyl-*N*-nitroso-4-toluenesulfonamide (Diazald) following the manufacturer's instructions. Five grams of Diazald were dissolved in 45 ml ethyl ether and the solution placed in a separatory funnel. The separatory funnel was placed above a round-bottomed flask containing 5 g of potassium hydroxide, 8 ml water, and 10 ml of 95% ethanol. The round-bottomed flask was placed in a 65−70°C water bath. The Diazald solution and then 10 ml diethyl ether were added dropwise to the flask over 30 min. Concurrently, the reaction mix was distilled, and the distillate (ethereal diazomethane) collected in a flask that was placed in a dry ice/acetone bath. The reaction should yield ~16.6 mmoles of diazomethane, which was stored at −20°C overnight.

Diazomethylation of Peptide

The N-blocked tripeptide was diazomethylated using a procedure adapted from Shaw and Green (1981). The tripeptide Fmoc-Leu-Leu-Tyr-OH was synthesized on a Milligen/Biosearch 9600 Peptide Synthesizer. To prepare the acid chloride of the peptide, 1 mmole of peptide was dissolved in 5 ml tetrahydrofuran in a flask, and the flask was purged with nitrogen gas. *N*-methylmorpholine (0.8 mmoles) was added to the flask, and the flask was placed in a dry ice/acetone bath (−20°C). Isobutyl chloroformate (0.8 mmoles) was added to the flask, and the flask incubated for 15 min at

−20°C. To diazomethylate the acid chloride, the flask was warmed to 0°C in an ice bath, 2.5 mmoles of diazomethane in ethyl ether were added, and the flask was incubated at 0°C for 15 min.

Purification and Assay of Inhibitor

The solvent was removed from the reaction mix by rotary evaporation and the inhibitor was resuspended in 80% acetonitrile in 50 mM ammonium acetate. The inhibitor was purified by HPLC using a Waters RCM 25 x 100 Bondapack C-18 column, eluting with a 20−100% acetonitrile gradient. Five-ml fractions were collected and the absorbance of the eluant was monitored at A_{260}. To determine which fractions contained the diazomethylated inhibitor, 25 μl of each fraction were tested to determine if they would inhibit the activity of sheep cathepsin L against the synthetic substrate Z-Phe-Arg-NHMec (Barrett & Kirschke 1981). The fractions with inhibitory activity were pooled and the pool titrated against sheep cathepsin L. The inhibitor was aliquotted into microfuge tubes, 100 nmoles/tube, lyophilized, and stored at −20°C.

Radiolabeling of Peptidyl Diazomethane Inhibitors

Peptidyl diazomethane inhibitors were radioiodinated on tyrosine residues using sodium [^{125}I]odide and Iodo-beads iodination reagent. The iodination reagent in Iodo-beads is *N*-chloro-benzenesulfonamide, sodium salt, immobilized on polystyrene beads. One Iodo-bead was washed in labeling buffer (100 mM sodium phosphate, pH 7.0) for 5 min, dried with a Kimwipe, and placed in a 5-ml borosilicate glass tube. To the tube was added 100 μl of labeling buffer and 2 mCi (in 20 μl) of sodium [^{125}I]odide. The tube was incubated at room temperature for 5 min. The peptidyl diazomethane inhibitor, 100 nmoles of either Fmoc-Leu-Leu-Tyr-CHN_2 or Fmoc-Tyr-Ala-CHN_2, was resuspended in

100 μl of acetonitrile and added to the tube, and the solution was incubated at room temperature for 10 min. The labeling mixture was diluted by the addition of 780 μl of 50% acetonitrile in labeling buffer, and the labeled inhibitor was aliquotted into microfuge tubes, 2−10 nmoles/tube, and stored at −20°C.

Results

Occurrence of Cathepsin Z in Human Cell Culture Lines

To determine if Cathepsin Z, a 22 kDa protein that labels with the peptidyl diazomethane inhibitor Z-Leu-Leu-[^{125}I]Tyr-CHN_2, was widely expressed in human cells, a number of cultured cell lines were screened with the inhibitor. U-937, A549, THP-1, Wil2NS, Raji, HeLa, and Hep G2 cells (2 x 10^6 cells) were washed 2x in serum-free media and incubated for 1 h at 37°C in 1 ml of serum-free media. The iodinated inhibitor Z-Leu-Leu-[^{125}I]Tyr-CHN_2 was added to the cells to a final concentration of 2 μM and the cells were incubated for 3 h at 37°C. After the labeling period, the cells were harvested, washed 3x in PBS, and resuspended in sample buffer containing β-mercaptoethanol. The samples were analyzed by SDS-PAGE and autoradiography, with 2 x 10^5 cells loaded per lane.

All cell lines had a labeled band of ~22 kDa, albeit of varying intensities (Figure 3.1). In four of the cell lines, U-937, A549, Wil2NS, and Raji, the 22 kDa band was the most intensely labeled protein. In two of these cell lines, U-937 and A549, the 22 kDa band was the strongly labeled band, although both also had a second labeled band at ~31 kDa; this band was subsequently named CP31. In addition, the U-937 cells had a faintly labeled band at 24 kDa. In many ensuing experiments, a labeled band was also seen at ~45 kDa; this band was subsequently named CP45. The U-937 cell line was chosen for further investigation of the 22 kDa protein based on several factors. First, U-937 cells grow in suspension culture, simplifying the growth of large numbers of cells. Second, undifferentiated U-937 cells are known not to express cathepsin L, which would also label with Z-Leu-Leu-[^{125}I]Tyr-CHN_2. Finally, the 22 kDa protein was the predominant

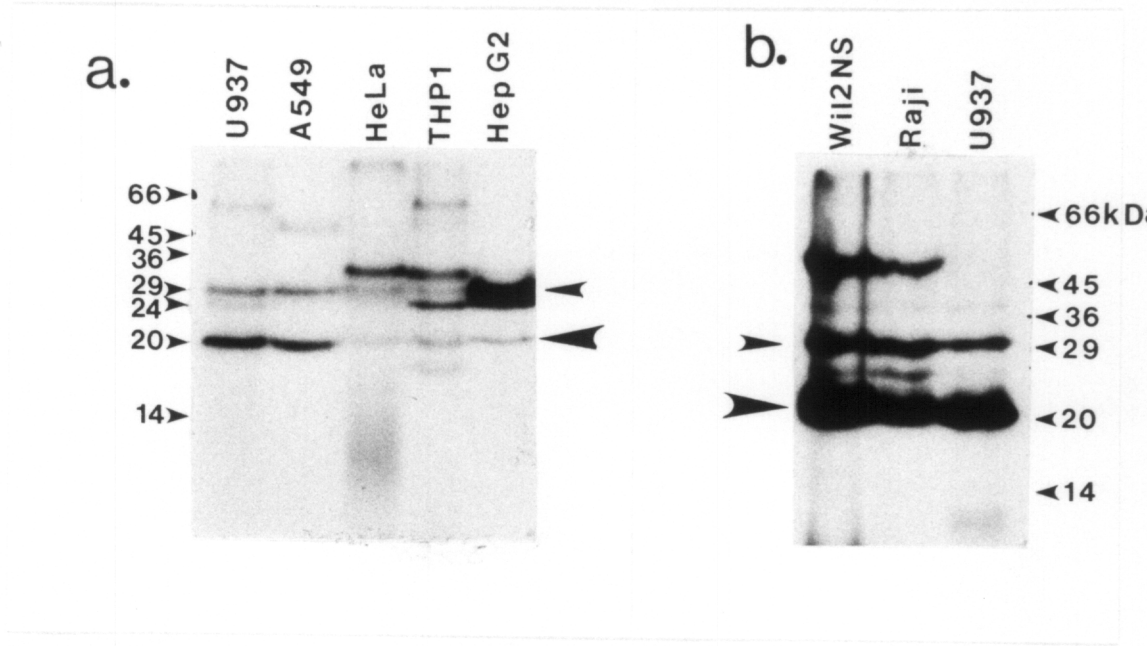


Figure 3.1 Labeling of human cultured cell lines with Z-Leu-Leu-[^{125}I]Tyr-CHN_2.

Autoradiographs of two SDS-PAGE gels of human cultured cells labeled for 3 h with 2 μM Z-Leu-Leu-[^{125}I]Tyr-CHN_2. Cells were incubated in 2 μM inhibitor for 3 h at 37°C and the samples analyzed by SDS-PAGE and autoradiography, loading 2 x 10^5 cells/lane. Large arrowheads indicate cathepsin Z, small arrowhead indicates CP31. (a) Lane 1: U-937; Lane 2: A549; Lane 3: HeLa; Lane 4: THP-1; Lane 5: Hep G2. (b) Lane 1: Wil2NS; Lane 2: Raji; Lane 3: U-937.

species labeled with Z-Leu-Leu-[^{125}I]Tyr-CHN$_2$ in U-937 cells. The 22 kDa protein was considered to be a putative cysteine proteinase and it was named cathepsin Z.

Characterization of the Labeling of U-937 Cells with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$: Time Dependence

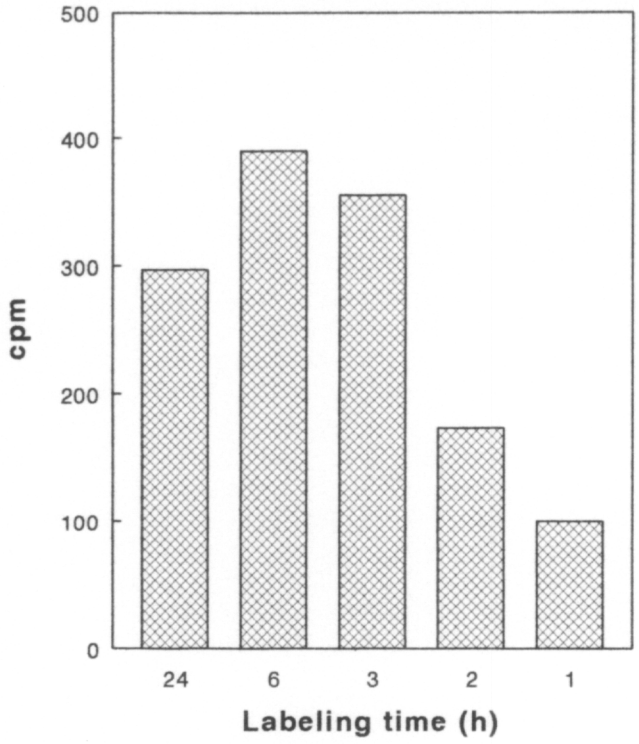
To determine if the extent of labeling of cathepsin Z in human cells was dependent on the time the cells were incubated in inhibitor, U-937 cells were labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$ for 1−24 h at 37°C in a humidified CO_2 incubator. U-937 cells in log phase growth were washed 2x in serum-free media and plated in 10 wells of a 24-well tissue culture dish, 5 x 10^5 cells in 0.5 ml serum-free media per well. The cells were incubated for 1 h at 37°C. Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$ was added to the wells at a final concentration of 2 μM. Inhibitor was added to 2 wells at time 0 h, 18 h, 21 h, 22 h, and 23 h, providing incubation times in iodinated inhibitor of 24, 6, 3, 2, and 1 hours. After the 24-h labeling period, the cells were harvested, washed 3x in PBS, and analyzed by SDS-PAGE and autoradiography, with 2 x 10^5 cells loaded per lane. Samples from all 10 wells were analyzed, providing data from 2 samples at each time point. The cathepsin Z bands were excised from the dried gel and counted on a gamma counter. To determine background levels in each lane, 2 similarly-sized slices from unlabeled portions of the same lane were excised and counted. To determine the counts associated with cathepsin Z from each time point, the background counts from each lane were averaged, and the average was subtracted from the counts for cathepsin Z from that lane.

The labeling of cathepsin Z showed time-dependence (Figure 3.2). In the time periods tested, maximal labeling of cathepsin Z was seen with a 6-h incubation in Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$, although the 3-h incubation gave almost equivalent labeling

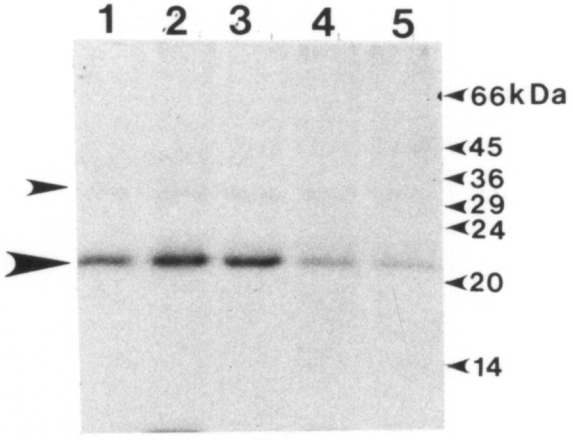
Figure 3.2 The effect of incubation time in Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 on the labeling of cathepsin Z.

U-937 cells were incubated in Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 for 1, 2, 3, 6, and 24 h at a 2 μM final inhibitor concentration. The samples were analyzed by SDS-PAGE and autoradiography, loading 2 x 10^5 cells/lane. Labeled cathepsin Z bands were excised from the dried gel and counted in a gamma counter. The counts were corrected for background (see text). (a) A graph showing the effect of incubation time in Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 on the labeling of cathepsin Z. (b) Autoradiograph of the gel. Large arrowhead indicates cathepsin Z; small arrowhead denotes CP31. Lane 1: 24 h; Lane 2: 6 h; Lane 3: 3 h; Lane 4: 2 h; Lane 5: 1 h.

a.


500
400
300
200
100
0
cpm
24
6
3
2
1
Labeling time (h)

b.


1
2
3
4
5
66kDa
45
36
29
24
20
14

(Figure 3.2). The labeling increased between 1 and 3 h. Labeling dropped ~25% in the 24-h incubation sample, perhaps due to degradation of the iodinated inhibitor over time. The protein levels were determined by examination of Coomassie staining to be approximately equivalent in all samples (data not shown). In addition to cathepsin Z, the 31 kDa protein, CP31, was also labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2, and the labeling followed the same pattern as the labeling of cathepsin Z (Figure 3.2b).

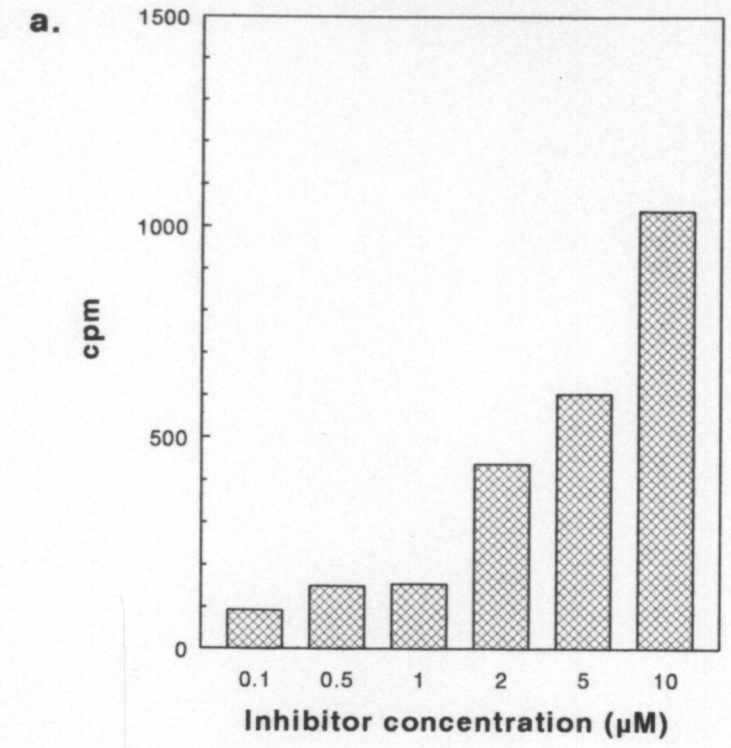
Characterization of the Labeling of U-937 Cells with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2: Concentration Dependence

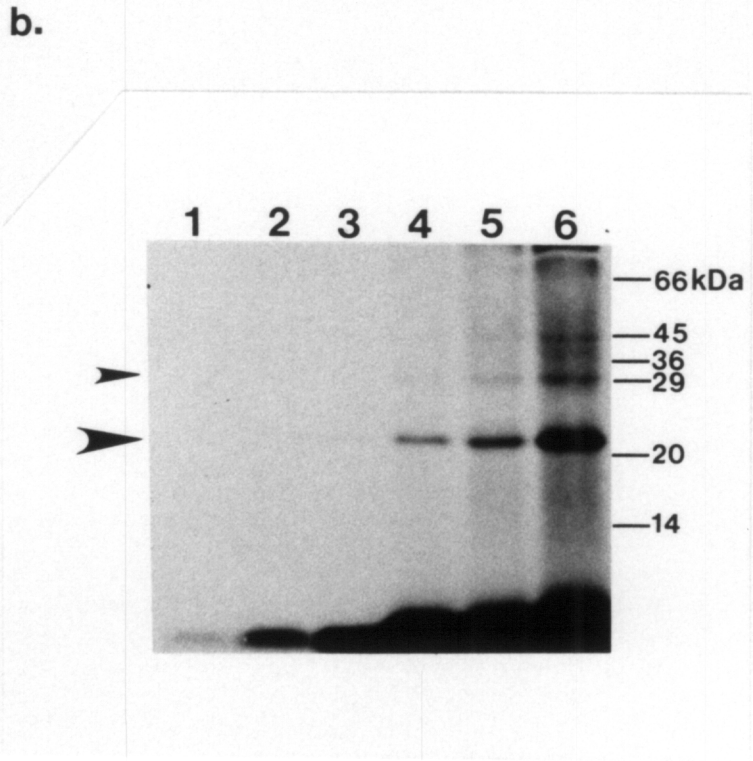
To determine if the extent of labeling of cathepsin Z in human cells was dependent on inhibitor concentration, U-937 cells were labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 at concentrations ranging from 0.1−10 μM. U-937 cells in log phase growth were washed 2x in serum-free media and plated in 6 wells of a 24-well tissue culture dish, 5 x 10^5 cells in 0.25 ml serum-free media per well. The cells were incubated for 1 h at 37°C. Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 was added to the wells at 0.1, 0.5, 1, 2, 5, and 10 μM and the cells were incubated for 3 h at 37°C. The cells were harvested, washed 3x in PBS, and analyzed by SDS-PAGE and autoradiography, with 1 x 10^5 cells loaded per lane. The cathepsin Z bands were excised from the dried gel and counted on a gamma counter. To determine background levels in each lane, a similarly-sized slice from an unlabeled portion of the same lane was excised and counted. To determine the counts associated with cathepsin Z from each time point, the background counts from each lane were subtracted from the counts for cathepsin Z from that lane.

The labeling of cathepsin Z showed concentration-dependence (Figure 3.3). The least labeling of cathepsin Z was seen with 0.1 μM concentration of Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2, and the extent of labeling increased with increasing inhibitor

Figure 3.3 The effect of the concentration of Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 on the labeling of cathepsin Z.

U-937 cells were incubated in Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 at 0.1, 0.5, 1, 2, 5, and 10 μM final concentration and the samples were analyzed by SDS-PAGE and autoradiography, loading 1 x 10^5 cells/lane. Labeled cathepsin Z bands were excised from the dried gel and counted in a gamma counter. The counts were corrected for background (see text). (a) A graph showing the effect of the concentration of Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 on the labeling of cathepsin Z. (b) Autoradiograph of the gel. Large arrowhead indicates cathepsin Z; small arrowhead denotes CP31. Lane 1: 0.1 μM; Lane 2: 0.5 μM; Lane 3: 1 μM; Lane 4: 2 μM; Lane 5: 5 μM; Lane 6: 10 μM.


a.
1500
1000
500
0
cpm
0.1
0.5
1
2
5
10
Inhibitor concentration (μM)


b.
1
2
3
4
5
6
66kDa
45
36
29
20
14

concentration (Figure 3.3a). The background in the lanes also increased with increasing inhibitor concentration (Figure 3.3b). The labeling of CP31 also showed concentration dependence (Figure 3.3b).

Characterization of the Labeling of U-937 Cells with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$: Reversibility

Because Stone *et al.* (1992) found that a peptidyl diazomethane inhibitor bound to serine proteinases in a slow-binding and reversible manner, the reversibility of the inhibition of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$ was tested. U-937 cells were labeled and then incubated in non-iodinated inhibitor. If the labeling of cathepsin Z with inhibitor were reversible, a reduction in the amount of labeled cathepsin Z should be seen with increasing time in non-iodinated inhibitor.

U-937 cells were washed in serum-free media and plated in a 24-well dish at 1 x 10^6 cells in 0.5 ml per well. After a 1 h incubation at 37°C, 4 μM Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$ was added and the cells were incubated for 3 h. The media was removed from the wells, and fresh serum-free media added to each well. Non-iodinated Fmoc-Leu-Leu-Tyr-CHN$_2$ was added to 2 wells at a final concentration of 8 μM. To 2 other wells, the solvent equivalent without inhibitor was added. After 2 h incubation at 37°C, 2 wells were harvested, one of each experimental condition. After a further 2-h incubation, the final 2 wells were harvested. The cells were washed 3x in PBS and analyzed by SDS-PAGE and autoradiography.

There was no reduction in labeling of cathepsin Z when labeled U-937 cells were post-incubated in non-iodinated Fmoc-Leu-Leu-Tyr-CHN$_2$ for either 2 or 4 h (Figure 3.4). There is an increase in labeling in both of the 4 h samples over the 2 h samples. This is probably due to the continued presence of iodinated inhibitor both within the cells

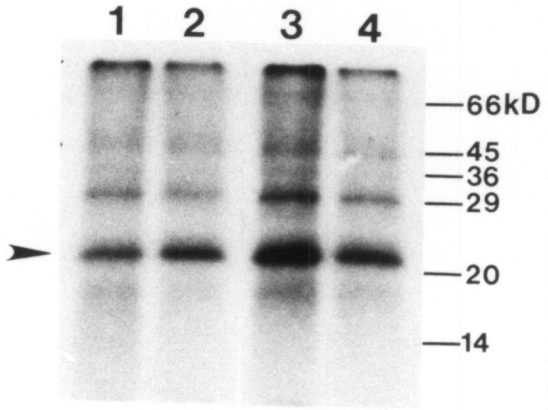


Figure 3.4 Reversibility of labeling of cathepsin Z.

The labeling of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 is not reversed by post-incubating labeled U-937 cells with non-iodinated Fmoc-Leu-Leu-Tyr-CHN_2. U-937 cells were labeled and then incubated in the presence (Lanes 1 & 3) or absence (Lane 2 & 4) of a 2-fold excess of non-iodinated inhibitor for 2 h (Lanes 1 & 2) or 4 h (Lanes 3 & 4). The samples were analyzed by SDS-PAGE and autoradiography, loading 2 x 10^5 cells/lane. Arrowhead indicates cathepsin Z.

and in the culture media. Although the labeling media was removed prior to adding the non-iodinated inhibitor, the cells were not washed. The results of this experiment imply that the binding of Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 to cathepsin Z is not reversible.

Inhibition of Cathepsin Z Labeling: Non-iodinated Inhibitor

To determine if the labeling of cathepsin Z with Z-Leu-Leu-[^{125}I]Tyr-CHN_2 was specific, U-937 cells were pre-incubated in non-iodinated Z-Leu-Leu-Tyr-CHN_2 prior to incubation in iodinated inhibitor. U-937 cells in log phase growth were washed 2x in serum-free media and plated in 2 wells of a 24-well dish, 1 x 10^6 cells in 1 ml serum-free media in each well. The cells were incubated for 1 h at 37°C. Non-iodinated Z-Leu-Leu-Tyr-CHN_2 was added to one well of cells at 10 μM final concentration and the cells were incubated for 1 h at 37°C. Z-Leu-Leu-[^{125}I]Tyr-CHN_2 was then added to the both wells at a final concentration of 1 μM, and the cells were incubated for 3 h at 37°C. The cells were harvested, washed 3x in PBS, and analyzed by SDS-PAGE and autoradiography, with 1 x 10^5 cells loaded per lane.

Labeling of cathepsin Z in U-937 cells by Z-Leu-Leu-[^{125}I]Tyr-CHN_2 was completely blocked by the pre-incubation of the cells in non-iodinated inhibitor (Figure 3.5a). The labeling of CP31 was completely blocked as well. The experiment was later repeated using Fmoc-blocked iodinated and non-iodinated inhibitor, with the same results (data not shown). This result indicates that the labeling of these 2 proteins with Z-Leu-Leu-[^{125}I]Tyr-CHN_2 is a specific reaction of the inhibitor with the proteinases.

The experiment was repeated with A549 cells. A549 cell were plated in complete media the day preceding the experiment at 5 x 10^5 cells in 2 wells of a 24-well dish. On the following day, the cells were rinsed 2x in serum-free media and incubated for 1 h at 37°C. Non-iodinated Fmoc-Leu-Leu-Tyr-CHN_2 was added to one well of cells at 2 μM

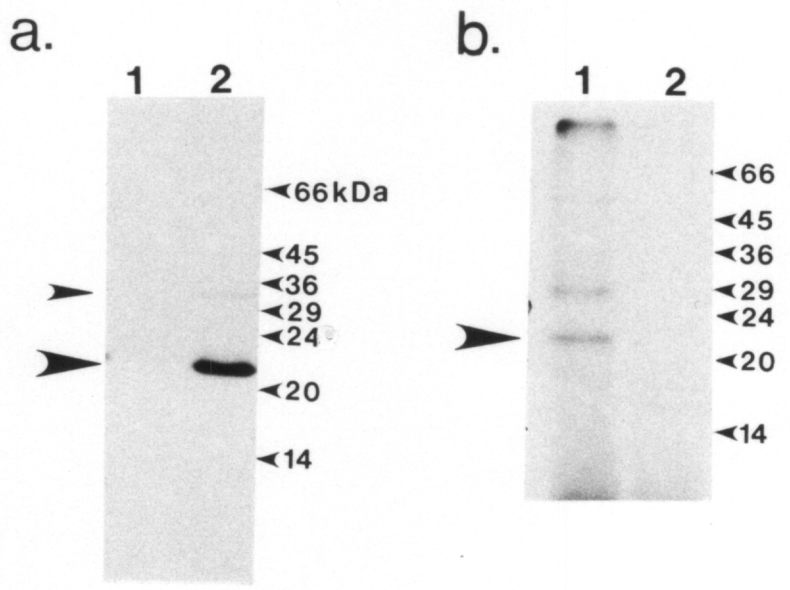


Figure 3.5 Inhibition of labeling by non-iodinated Z-Leu-Leu-Tyr-CHN_2.

Autoradiographs of U-937 and A549 cells that were pre-incubated in non-iodinated inhibitor prior to their labeling with N-blocked-Leu-Leu-[^{125}I]Tyr-CHN_2. U-937 cells were preincubated in 10 μM Z-Leu-Leu-Tyr-CHN_2 for 1 h and and A549 cells were preincubated in 4 μM Fmoc-Leu-Leu-Tyr-CHN_2 for 3 h. The cells were then incubated for 3 h in 2 μM N-blocked-Leu-Leu-[^{125}I]Tyr-CHN_2, and the samples analyzed by SDS-PAGE and autoradiography, with 1 x 10^5 cells loaded per lane. Large arrowhead indicates cathepsin Z; small arrowhead denotes CP31. (a) Lane 1: U-937 cells pre-incubated in Z-Leu-Leu-Tyr-CHN_2; Lane 2: U-937 cells with no pre-incubation in Z-Leu-Leu-Tyr-CHN_2. (b) Lane 1: A549 cells with no pre-incubation in Fmoc-Leu-Leu-Tyr-CHN_2; Lane 2: A549 cells pre-incubated in Fmoc-Leu-Leu-Tyr-CHN_2.

final concentration and the cells were incubated for 3 h at 37°C. Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 was added to both wells at 4 μM final concentration and the cells were incubated at 37°C for a further 3 h. The cells were harvested, washed 3x in PBS, and analyzed by SDS-PAGE and autoradiography, with 1 x 10^5 cells loaded per lane.

As with U-937 cells, the labeling of cathepsin Z and CP31 with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 in A549 cells was completely inhibited by the pre-incubation of the cells in non-iodinated Fmoc-Leu-Leu-Tyr-CHN_2 (Figure 3.5b).

Inhibition of Cathepsin Z Labeling: E-64d

To determine if the labeling of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 could be inhibited by a membrane-permeable inhibitor of cysteine proteinases, U-937 cells were pre-incubated in 20 μM E-64d. E-64d is an epoxysuccinyl inhibitor, a membrane-permeable analog of E-64, and a specific inhibitor of cysteine proteinases. All incubations were done at 37°C. U-937 cells were plated in complete media in 5 wells in a 24-well dish at 5 x 10^5 cells/well. For the 24-h pre-incubation time point, E-64d was added to 1 well. Nineteen and 21 h later, E-64d was added to 2 wells for the 5- and 3-h time points. Two h later, the cells in all 5 wells were washed 2x in serum-free media. Four of the wells were plated in 20 μM E-64d in serum-free media, and the fifth well was plated in serum-free media alone. After a 1 h incubation, Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 was added to each well to a final concentration of 2 μM and the cells were incubated for 3 h. The cells were harvested, washed 3x in PBS, and analyzed by SDS-PAGE and autoradiography, with 1 x 10^5 cells loaded per lane.

The labeling of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 in U-937 cells was inhibited by pre-incubating the cells in the membrane-permeable cysteine proteinase

inhibitor E-64d. Maximal inhibition of 80% was seen with a 24-h pre-incubation in E-64d (Figure 3.6a). The inhibition by E-64d was time-dependent.

The experiment was repeating using A549 cells to determine the extent which E-64d inhibited labeling of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 in a second cell line. The experiment was performed as with the U-937 cells, except for the omission of a 24-h pre-incubation time point. E-64d inhibited the labeling of cathepsin Z less extensively in A549 cells than in U-937 cells (Figure 3.6b), reaching 66% inhibition after 5 h pre-incubation in E-64d.

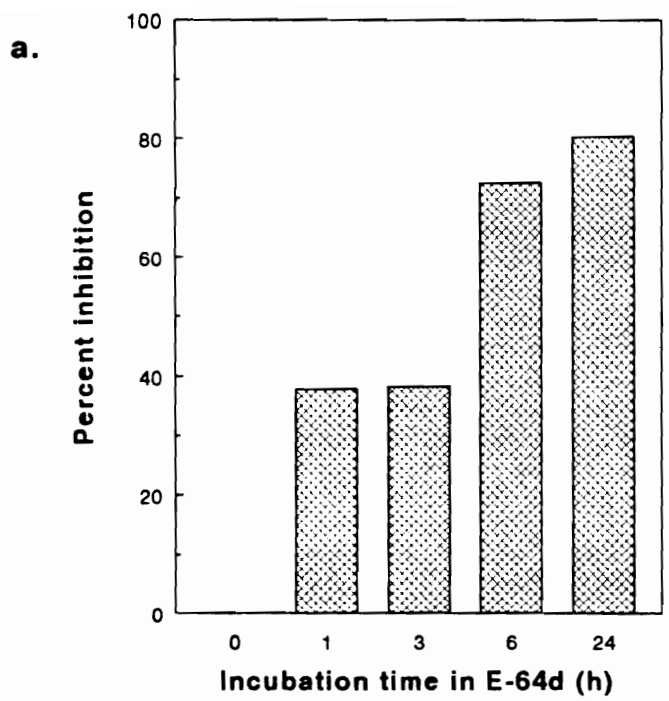
Expression of Cathepsin Z after Differentiation of U-937 Cells

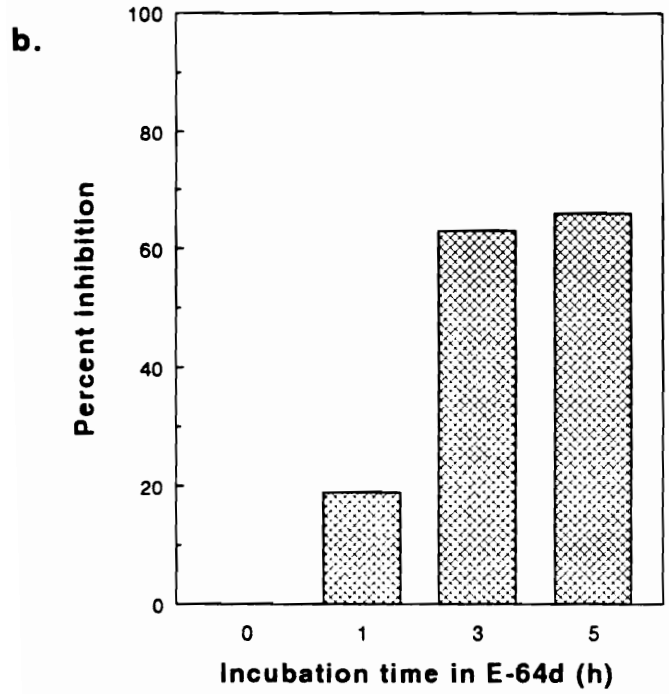
U-937 cells are monocytic cells that can be induced to terminal differentiation into macrophage-like cells by phorbol esters. To determine if the expression of cathepsin Z was affected by differentiation of U-937 cells, cells were treated with PMA and labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. The cells were also labeled with Fmoc-[^{125}I]Tyr-Ala-CHN_2 as a control. When U-937 cells are differentiated with PMA, their expression of cathepsin B, which is labeled by Fmoc-[^{125}I]Tyr-Ala-CHN_2, increases considerably. Before differentiation, very little cathepsin B is detectable, but after differentiation there is strong expression of a 33 kDa proteinase that corresponds to the single-chain form of cathepsin B.

U-937 cells were plated in 60-mm dishes at 1 x 10^6 cells in 5 ml of complete media. PMA was added to four of the six dishes at 500 nM final concentration. The other 2 dishes were no-PMA controls. The cells were placed in a CO_2 incubator at 37°C. After 2 h, the cells from the no-PMA control plates were washed 2x in serum-free media and plated into 2 wells of a 24-well tissue dish in 1 ml of serum-free media. After a 1 h incubation at 37°C, Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 and Fmoc-[^{125}I]Tyr-Ala-CHN_2 were

Figure 3.6 Inhibition of labeling by E-64d.

U-937 and A549 cells were preincubated in 20 μM E-64d prior to labeling with 2 μM Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. U-937 cells were pre-incubated for 24, 6, 3, and 1 h in E-64d, and A549 cells were pre-incubated for 5, 3, and 1 h in E-64d. Both cell lines were labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 for 3 h. Samples were analyzed by SDS-PAGE and autoradiography, with 1 x 10^5 cells loaded per lane. (a) A graph showing the effect of the preincubation of U-937 cells in E-64d prior to labeling with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. (b) A graph showing the effect of the preincubation of A549 cells in E-64d prior to labeling with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2.


a.
100
80
60
40
20
0
Percent Inhibition
0
1
3
6
24
Incubation time in E-64d (h)


b.
100
80
60
40
20
0
Percent Inhibition
0
1
3
5
Incubation time in E-64d (h)

each added to one of the wells and the cells were incubated 3 h at 37°C. The cells were harvested, washed 3x in PBS, and the cell pellets frozen at −20°C. After 24 h in PMA, two more dishes of cells were washed and labeled with each inhibitor, as above, and again after 72 h in PMA. All the cell pellets were analyzed by SDS-PAGE and autoradiography, loading 2 x 10^5 cells/lane. After autoradiography, the cathepsin Z and cathepsin B bands were excised from the gel and counted on a gamma counter, as described previously.

The expression of cathepsin Z in U-937 cells is not affected by differentiation of the cells with PMA (Figure 3.7). Although there is a slight increase (~1.3-fold) in expression of cathepsin Z after 72 h in PMA, it is probably due to experimental variability, and is minimal when compared to the 11.5-fold increase in the expression of cathepsin B (Figure 3.7). There was no increase in the expression of CP31 after differentiation (data not shown).

Extent of Glycosylation of Cathepsin Z

To determine the extent of N-linked glycosylation of cathepsin Z, U-937 cells that had been labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 were incubated in the presence of endoglycosidase H, an enzyme that hydrolyzes N-linked high mannose oligosaccharides from glycoproteins. U-937 cells (1.2 x 10^6 cells) that had been labeled for 3 h with 5 μM Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 were resuspended in Endo H buffer (see Methods) and heated to 95°C for 2 min. The sample was divided between 2 microfuge tubes and cooled. Endoglycosidase H (5 mU in 5 μl of Endo H buffer) was added to one aliquot, and 5 μl of Endo H buffer to the other. Both samples were incubated 16 h at 37°C. Sample buffer was added to each, and the samples were analyzed by SDS-PAGE and autoradiography, loading 1 x 10^5 cells/lane.

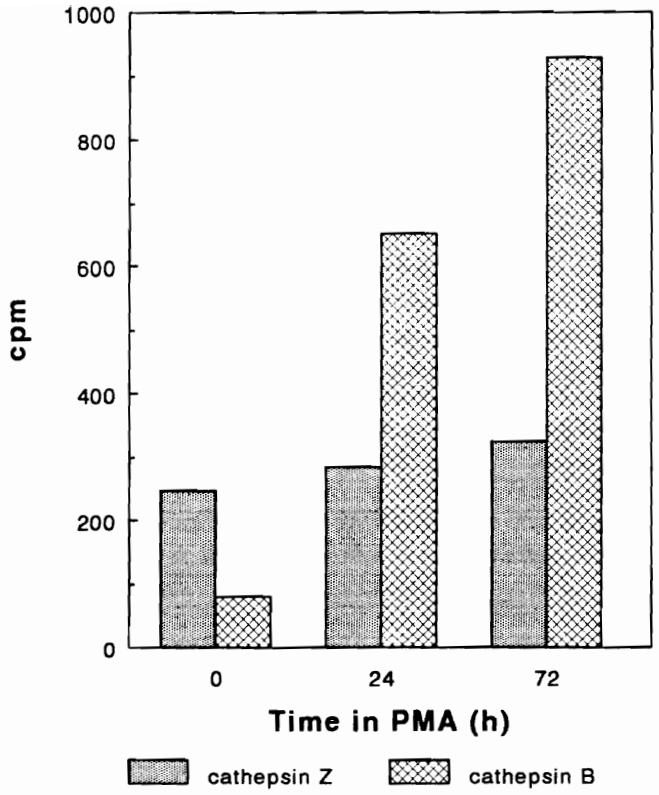


Figure 3.7 Effect of differentiation on the expression of cathepsin Z.

Graphic representation of the effect of differentiation of U-937 cells with phorbol ester on the expressions of cathepsin Z and cathepsin B. U-937 cells were incubated in 500 nM PMA for 0, 24, and 72 h, then labeled for 3 h with either 2 μM Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 or 2 μM Fmoc-[^{125}I]Tyr-Ala-CHN_2. Samples were analyzed by SDS-PAGE and autoradiography, loading 2 x 10^5 cells/lane. Labeled bands were excised from the gel and counted in a gamma counter.

Cathepsin Z has N-linked glycosylation that accounts for ~3.1 kDa of its molecular weight (Figure 3.8). The calculated molecular weight of cathepsin Z from SDS-PAGE is 22.4 kDa, and after incubation in endoglycosidase H, the molecular weight is 19.3 kDa. Since the average molecular weight of an N-linked oligosaccharide side chain is ~1.5 kDa, the 3.1 kDa corresponds to 2 N-linked high mannose side chains on cathepsin Z.

Determination of the Native Molecular Weight of Cathepsin Z

The molecular weight of cathepsin Z determined from SDS-PAGE gels was 22.4 kDa. The samples used in those determinations were heated to 95°C in the presence of β -mercaptoethanol, so the protein would be reduced. A number of approaches were tried to determine the native molecular weight of cathepsin Z.

Non-reduced Cathepsin Z Analyzed by SDS-PAGE

To determine the non-reduced molecular weight of Cathepsin Z, U-937 cells (4.2 x 10^7 cells) were labeled with 2.5 μM Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. These cells were used for this experiment and for native PAGE (see below) Two aliquots of the labeled cells (1.0 x 10^6 cells/aliquot) were resuspended in sample buffer either with or without 5% β-mercaptoethanol, the samples were heated to 95°C and analyzed by SDS-PAGE and autoradiography. To determine the extent of reduction by β-mercaptoethanol within the gel, minus-β-mercaptoethanol samples were placed in wells both adjacent to the plus-β-mercaptoethanol sample and separated from the plus-β-mercaptoethanol sample by 4 wells.

The non-reduced cathepsin Z has a molecular weight of ~45-47 kDa, compared to a molecular weight of ~22.4 kDa for the reduced protein (Figure 3.9). The ~45 kDa band was the only band detected in the non-reduced sample. In the reduced sample, CP31 was

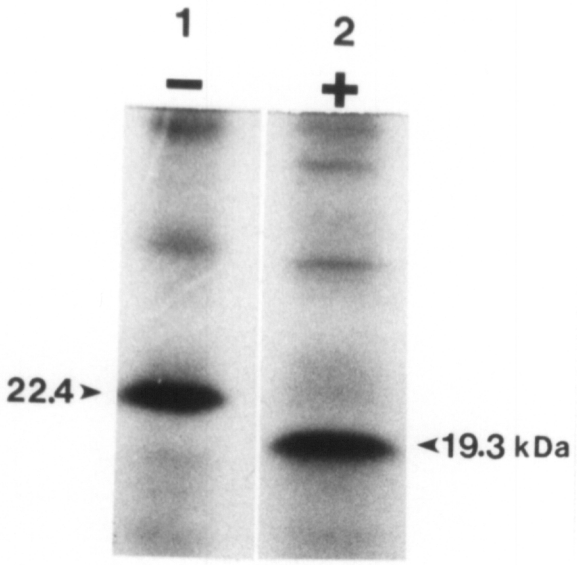


Figure 3.8 Effect of endoglycosidase H on cathepsin Z.

Autoradiograph of U-937 cells that were incubated in 5 μM Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2, then resuspended in Endo H buffer (see Methods) and heated to 95°C for 2 min. The sample was divided into 2 aliquots and 5 mU endoglycosidase H added to one aliquot. Both aliquots were incubated 16 h at 37°C and analyzed by SDS-PAGE and autoradiography, loading 1 x 10^5 cells/lane. Lane 1: U-937 cells − endoglycosidase H; Lane 2: U-937 cells + endoglycosidase H.

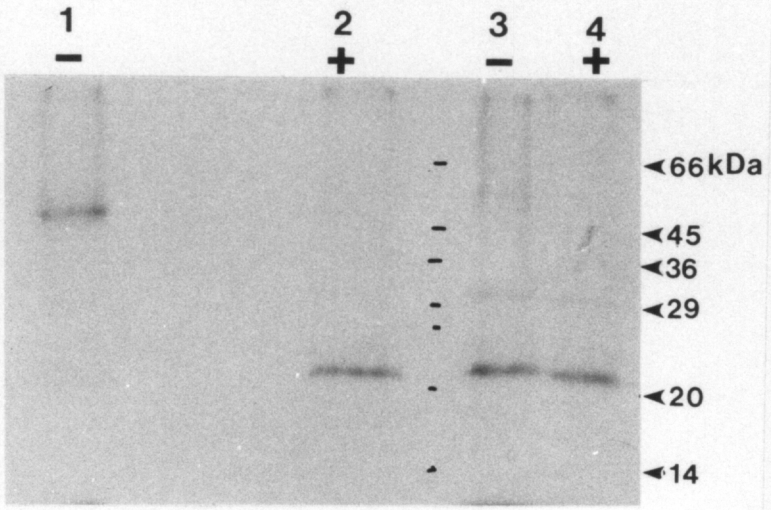


Figure 3.9 Molecular weight of cathepsin Z: SDS-PAGE and β-mercaptoethanol.

Autoradiograph of U-937 cells labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 and analyzed by SDS-PAGE in the presence or absence of β-Mercaptoethanol. Lanes 1 and 3: Minus-β-Mercaptoethanol; Lanes 2 and 4: Plus-β-Mercaptoethanol.

also present, albeit at levels much lower than cathepsin Z. β-mercaptoethanol diffused from well-to-well. The minus-β-mercaptoethanol sample placed in the well adjacent to a plus-β-mercaptoethanol sample was completely reduced (Figure 3.9, Lanes 3 and 4).

Subsequently, the experiment was repeated using a 10% SDS-PAGE gel and high range molecular weight markers. The non-reduced molecular weight of cathepsin Z was calculated to be 46.1 kDa (data not shown).

Molecular Weight Determination with Non-SDS Gels

To determine the native molecular weight of cathepsin Z, U-937 cells labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 (see previous experiment) were analyzed by native (non-SDS) polyacrylamide gel electrophoresis. A cell pellet (1 x 10^7 cells) was resuspended in sample buffer without SDS or β-mercaptoethanol. The cells were lysed by freezing and thawing (see Methods). The insoluble material in the sample was pelleted by centrifugation and the supernatant transferred to a new tube. The sample was analyzed on a series of non-denaturing gels with acrylamide concentrations of 5, 6, 7, 8, 9, and 10%. Five protein standards were analyzed on the same gels. Carbonic anhydrase has 3 bands, due to charge isomers. Urease runs as a trimer and a hexamer, and BSA as a monomer and dimer. Lactalbumin and chicken egg albumin run as single bands. This provided 8 bands for analysis. The gels were stained, destained, dried, and subjected to autoradiography.

The autoradiographs showed 2 labeled bands in each sample lane, a sharp band of high molecular weight and a diffuse band of lower molecular weight (Figure 3.10). The R_f values for each for these bands (Figure 3.11a) and the standard proteins (e.g., Figure 3.11b) were determined and plotted against the percentage acrylamide in each gel (see

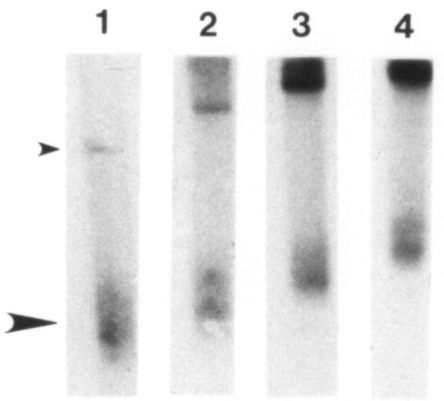
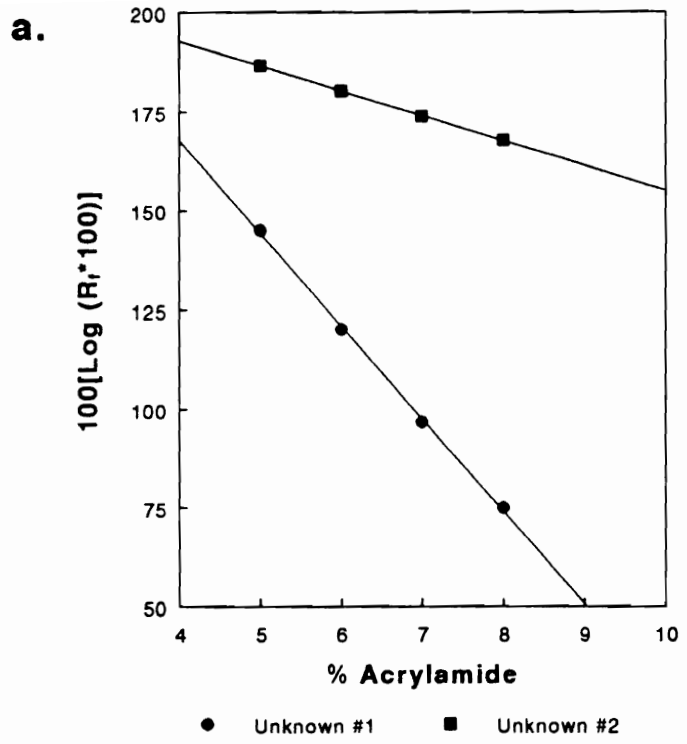


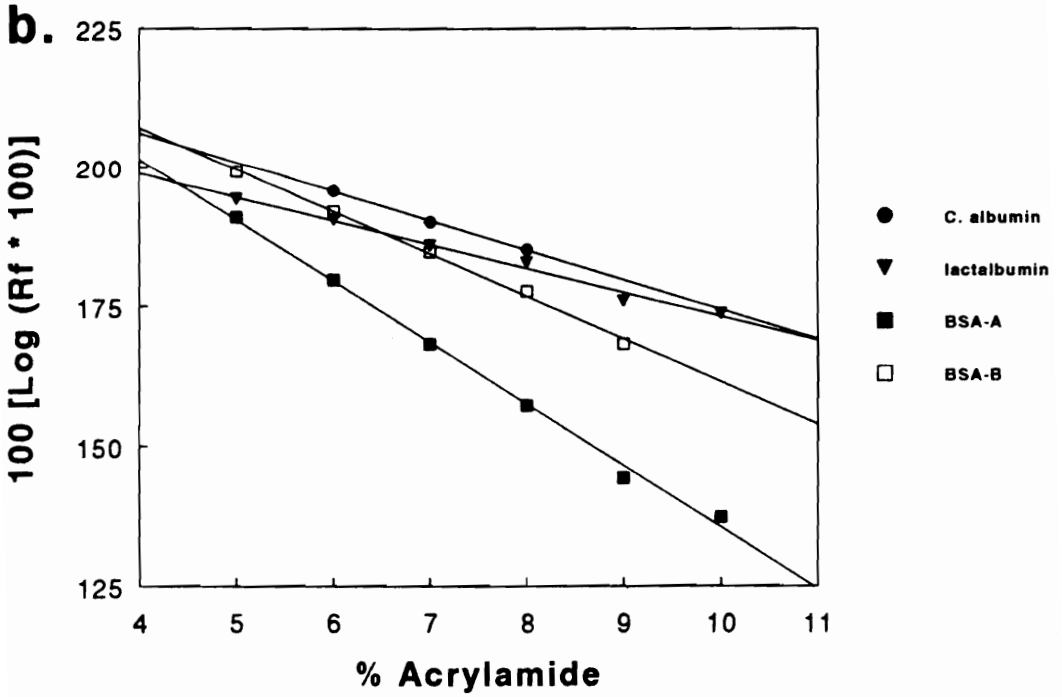
Figure 3.10 Native PAGE analysis of cathepsin Z.

Autoradiographs of U-937 cells labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 and lysed. The soluble fraction was analyzed on a series of native polyacrylamide gels in the absence of SDS and β-mercaptoethanol (see Methods). Lane 1: 5% acrylamide; Lane 2: 6% acrylamide; Lane 3: 7% acrylamide; Lane 4: 8% acrylamide. Large arrowhead indicates diffuse band. Small arrowhead indicates the sharp band.

Figure 3.11 Native gels: graphs of R_f against percentage acrylamide.

Graphs of the migration of proteins against the percentage acrylamide in native gels. The R_f of each protein band was determined and plotted, in the form 100 [log(R_f * 100)], against acrylamide percentage of each gel. (a) Graph of the two unknown proteins. Unknown 1 is the sharp, high molecular weight band. Unknown 2 is the diffuse, lower molecular weight band. (b) Graph of some of the standard proteins run on the native gels. The four shown are chicken albumin, lactalbumin, and the monomeric and dimeric forms of BSA. Not shown are the three charge isomers of carbonic anhydrase and the trimeric and hexameric forms of urease.


a.
200
175
150
125
100
75
50
100[Log (R,*100)]
4
5
6
7
8
9
10
% Acrylamide
Unknown #1
Unknown #2


b.
225
200
175
150
125
100 [Log (Rf * 100)]
4
5
6
7
8
9
10
11
% Acrylamide
C. albumin
lactalbumin
BSA-A
BSA-B

Methods). The slopes of each of these lines were determined and the slopes of the standards were plotted against their molecular weights (Figure 3.12).

The equation of the line derived from the molecular weights was determined (Figure 3.12). The molecular weights of the 2 unknown proteins were determined by substituting their slopes (Figure 3.11a) into this equation. The molecular weights calculated were 800 kDa for the sharp band and 44 kDa for the diffuse band. The 44 kDa protein is probably the same as the 45-47 kDa protein seen by SDS-PAGE in the absence of β-mercaptoethanol. The band in the native gel was quite diffuse, and it was difficult to get an accurate measurement for the migration of the band. The measurement was taken at the densest region of the band.

To determine the reduced molecular weight of these two unknown proteins observed on autoradiographs of native gels, more U-937 cells (from the same labeled cell preparation as above) were lysed and the soluble protein run on a 6% native gel. The lane containing the cell extract was sliced into $1-2$ mm slices. The slices were transferred to microfuge tubes and the protein eluted from the slices by incubating them for 16 h at 37°C in sample buffer containing β-mercaptoethanol and SDS. The samples were counted on a gamma counter, and the protein was analyzed by SDS-PAGE and autoradiography. The material remaining in the tubes were counted as well, and it was determined that the recovery rate of the labeled protein was 40-50%.

Most cathepsin Z was in the area of the gel that corresponded to the the diffuse band that calculated to 44 kDa (Figure 3.13), although minor amounts could be seen in all gel slices except for those in the bottom 10 mm of the gel. Interestingly, the 24-kDa cathepsin S was found exclusively in the high molecular weight band, indicating that native cathepsin S is associated with a large complex in U-937 cells.

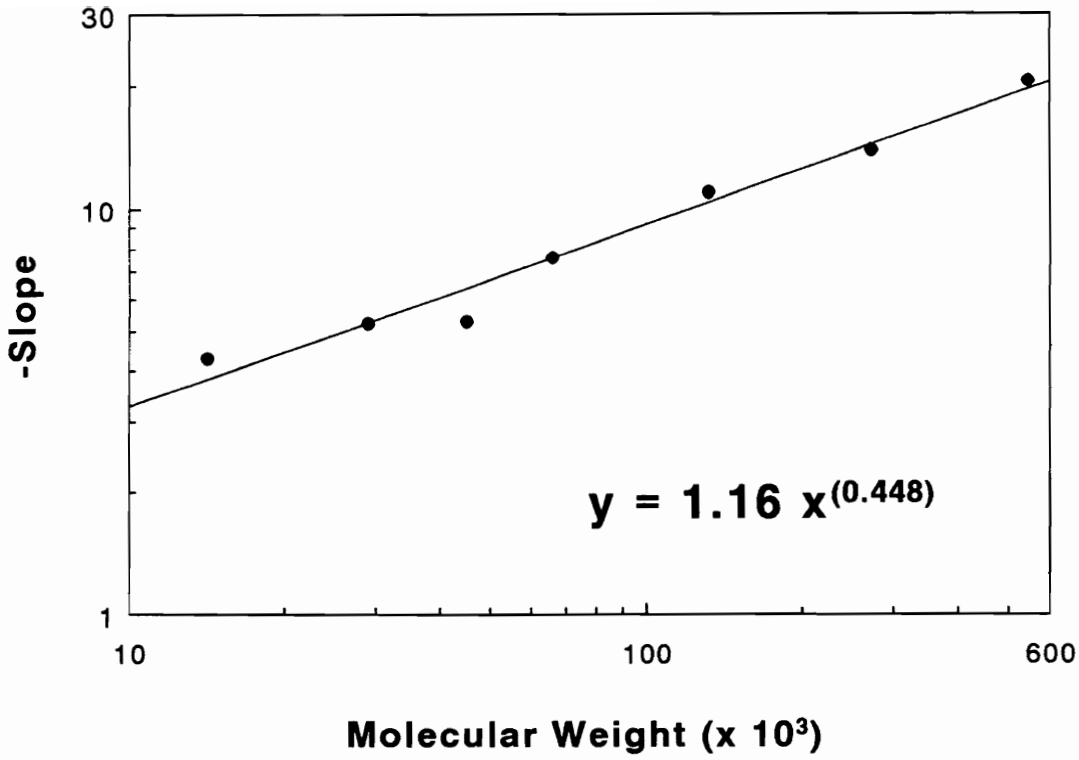
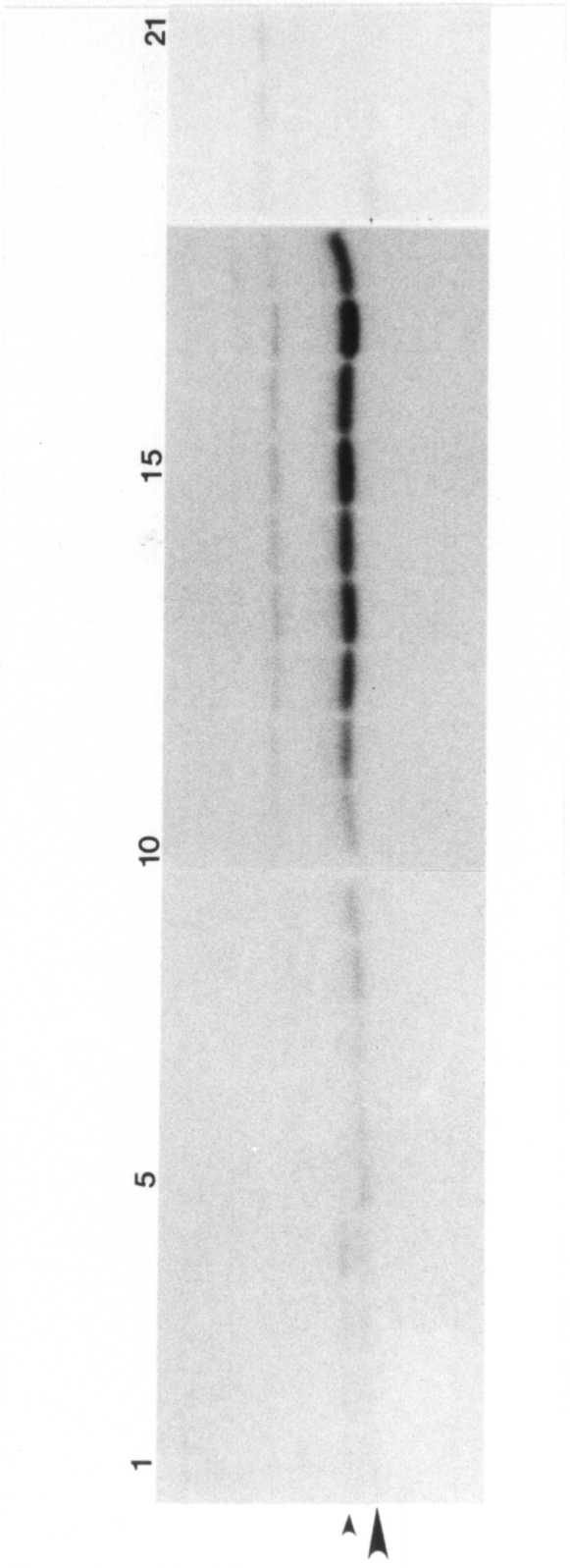


Figure 3.12 Native gels: graph of the (−)slope against molecular weights of the standards.

Equation of the line and graph of the slopes determined for each standard protein against the molecular weights of the standards. The slopes, plotted in the form −slope on the y-axis, were determined from lines shown in Figure 3.10b, plus the lines of the protein standards not shown in the figure (urease and carbonic anhydrase). The equation of the line was used to determine the native molecular weights of the unknowns.

Figure 3.13 Analysis of labeled cathepsin Z by native and SDS-PAGE.

U-937 cells that had been labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 were analyzed on a 6% native PAGE as described above. After electrophoresis, the gel lane contained the sample was cut into slices, the protein was eluted from the gel slices, and the eluted protein analyzed by SDS-PAGE in the presence of β-mercaptoethanol. Lane 1 contains the sample eluted from the top gel slice, Lane 24 contains the sample eluted from the bottom gel slice, etc. Large arrowhead indicates cathepsin Z. Small arrowhead indicates cathepsin S.


21
15
10
5
1

Molecular Weight Determination with Gel Filtration Chromatography

Two methods were tested for estimation of the native molecular weight of cathepsin Z using gel filtration chromatography. The first entailed use of a FPLC Superose 12 column equilibrated in 20 mM sodium acetate, 200 mM NaCl, pH 5.5. Superose is a cross-linked agarose matrix that is used for gel filtration. First, BSA and cytochrome c were run on the column as molecular weight standards. Then partially purified cathepsin Z that was labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 (in 100 μl) was loaded on the column. The eluted fractions were counted on a gamma counter and the protein in the fractions was precipitated with DOC and TCA. The samples were resuspended in sample buffer without β-mercaptoethanol and analyzed by SDS-PAGE and autoradiography.

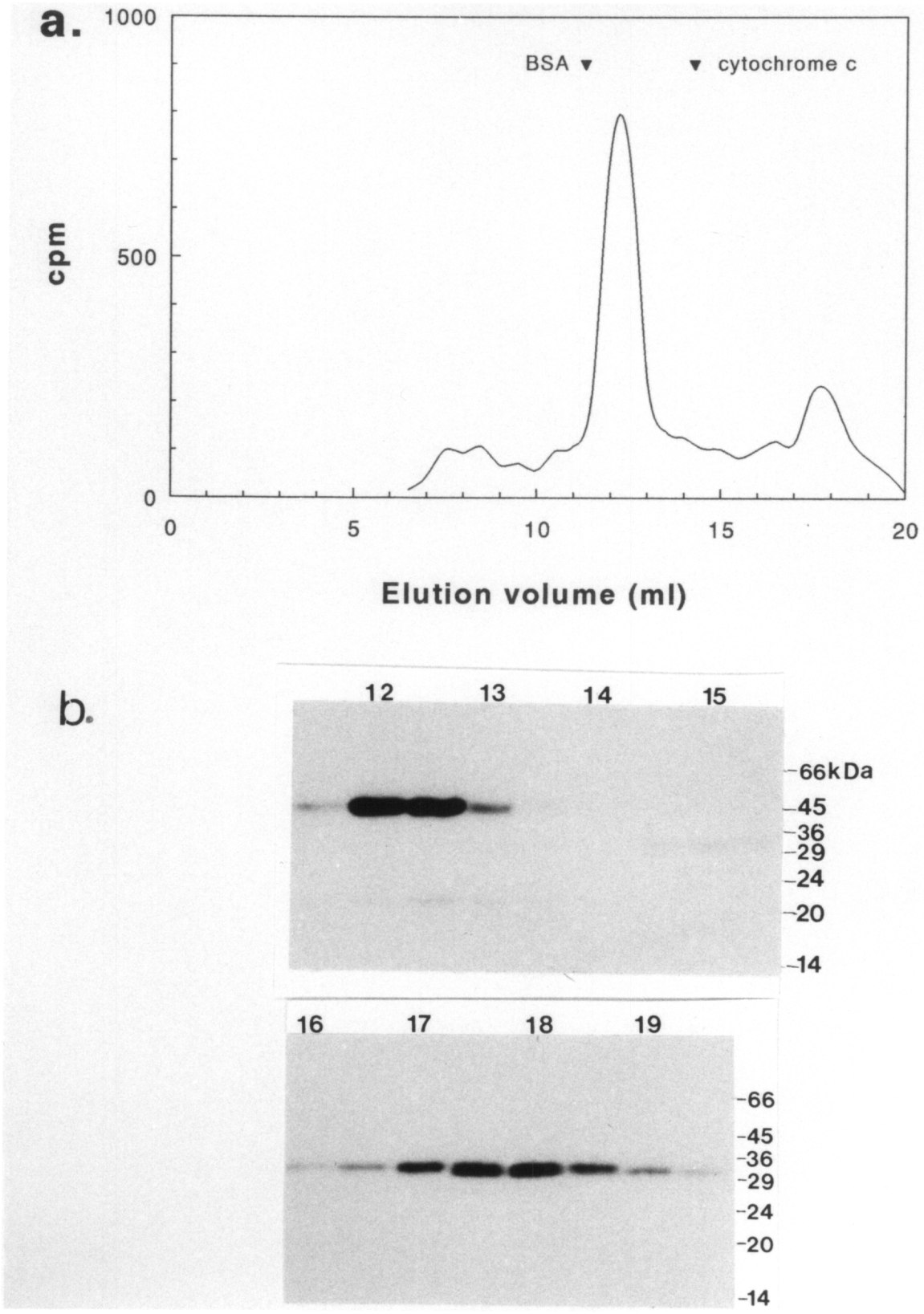
A large peak of labeled protein eluted from the Superose 12 column at a position that corresponded to a molecular weight of 46 in Z, at a calculated molecular weight of 45.4 kDa, in the fractions corresponding to this peak (Figure 3.14b).

A second interesting result from this experiment was that CP31 did not co-elute with the other labeled species from the Superose 12 column. A broad peak of labeled protein eluted from the column at an unexpected position, beyond the total column volume (Figure 3.14a). A likely interpretation of this result is that some labeled proteins were retarded due to interactions with the agarose matrix. When these samples were analyzed, this peak of labeled protein consisted of a ~33 kDa protein exclusively (Figure 3.14b). Although this molecular weight is slightly high for CP31, a lack of reducing agent in the samples could account for this difference.

Subsequent attempts to determine the native molecular weight of cathepsin Z by gel filtration utilized Sephadex G-100 Superfine resin; Sephadex is crosslinked dextran.

Figure 3.14 Molecular weight determination: gel filtration using Superose 12.

Cathepsin Z labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 subjected to gel filtration chromatography on an FPLC Superose 12 column. Eluted fractions were counted in a gamma counter. The protein was precipitated and analyzed by SDS-PAGE and autoradiography. (a) Elution profile of Superose 12 column, showing volume eluted on the x-axis and cpm on the y-axis. The elution volumes of BSA and cytochrome c are also shown. (b) Autoradiograph of fractions eluted, showing from 11.5 to 20 ml elution volume.


a.
1000
500
0
cpm
BSA ▼
▼ cytochrome c
0
5
10
15
20
Elution volume (ml)
b.
12
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14
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66kDa
45
36
29
24
20
14
16
17
18
19
66
45
36
29
24
20
14

Unlike the Superose experiments, in the Sephadex experiments all proteins eluted within the column volume. Very little new information was gathered from these experiments, but they did confirm that CP31 does not co-migrate with cathepsin Z (data not shown). Precise molecular weights could not be determined using a Sephadex column due to diffusion within the column, but CP31 eluted from the column after cathepsin Z.

Subcellular Localization of Cathepsin Z

Subcellular Fractionation of U-937 Cells

To determine the subcellular localization of cathepsin Z, a subcellular fractionation was performed on U-937 cells that had been labeled with Z-Leu-Leu-[^{125}I]Tyr-CHN_2 and a fraction enriched for lysosomes was isolated. U-937 cells (3.2 x 10^8 cells) that had been cultured in 100-mm dishes were washed 2x in serum-free media and incubated for 1 h in serum-free media at 37°C. Z-Leu-Leu-[^{125}I]Tyr-CHN_2 was added to the cells at a final concentration of 0.8 μM and the cells were incubated for 3 h at 37°C. All subsequent steps were performed at 4°C and samples were saved from each step and stored at -20°C. The cells were harvested by centrifugation and the cell pellets resuspended in monovalent PBS, pooled, and pelleted. The cells were washed 2x in 0.25 M sucrose, resuspended in 1.5 ml of 0.25 M sucrose, and placed in a N_2 cavitation bomb. The bomb was pressured to 30 psi for 15 min; the contents were then released, with the bomb still pressurized. The cells were homogenized on a motor-driven homogenizer using a Teflon pestle and transferred to a centrifuge tube.

The cells were checked microscopically to determine the extent of lysis. The cells were only partially lysed, but the experiment was continued, as intact cells should pellet with the nuclei. A postnuclear supernatant (PNS) was prepared by pelleting the nuclei and intact cells by centrifugation. The PNS was transferred to a new tube, the pellet

washed 2x in 0.25 M sucrose, and the washes added to the PNS pool. A fraction enriched in lysosomes was purified by sequential discontinuous density gradients (see Methods). Protein determination were performed on all samples from the lysosome preparation. The protein in 10 μg of each sample was precipitated with TCA, resuspended in sample buffer, and analyzed by SDS-PAGE and autoradiography. Areas of the dried gel corresponding to the labeled proteins were excised from the gel and counted on a gamma counter.

Cathepsin Z was enriched in the fractions in which lysosomes should also be enriched. Figure 3.15a shows the autoradiograph of the gel. Lysosomes should be enriched in: (1) Lane 3: PNS; (2) Lane 5: the interface of 6% Percoll and 17% metrizamide from Gradient 1; and (3) Lane 8: the interface of 5% and 17% metrizamide from Gradient 2. Cathepsin Z, at 22 kDa, is also enriched in these fractions (Figure 3.15). The other labeled proteins that are enriched in the lysosomal fraction have molecular weights of 24, 31, and ~45-50 kDa (Figure 3.15a). The 31and ~45−50 kDa bands are CP31 and CP45, respectively. The 24 kDa band is cathepsin S, a small amount of which is expressed in U-937 cells (see below). Under normal labeling conditions, i.e., labeling ~1 x 10^6 U-937 cells with 2 μM Z-Leu-Leu-[^{125}I]Tyr-CHN$_2$ and analyzing ~1 x 10^5 cells by SDS-PAGE and autoradiography, labeled cathepsin S either is not detected or is seen at very low levels.

The enrichment of cathepsin S, a known lysosomal cysteine proteinase, in fractions that should be enriched for lysosomes confirms that lysosomes are enriched in these fractions. The enrichment of cathepsin Z mirrors that of cathepsin S in these fractions (Figure 3.15b), thus it is probable that cathepsin Z is also a lysosomal cysteine proteinase.

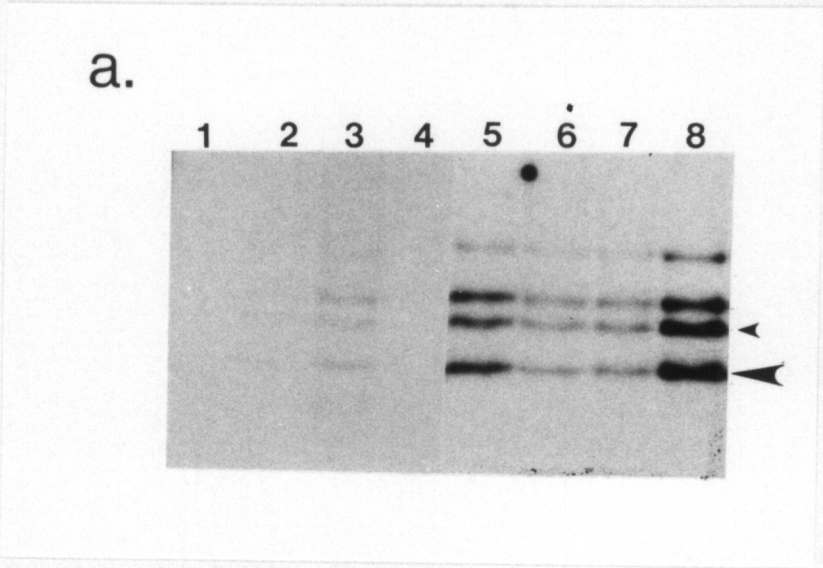
Figure 3.15 Subcellular localization of cathepsin Z.

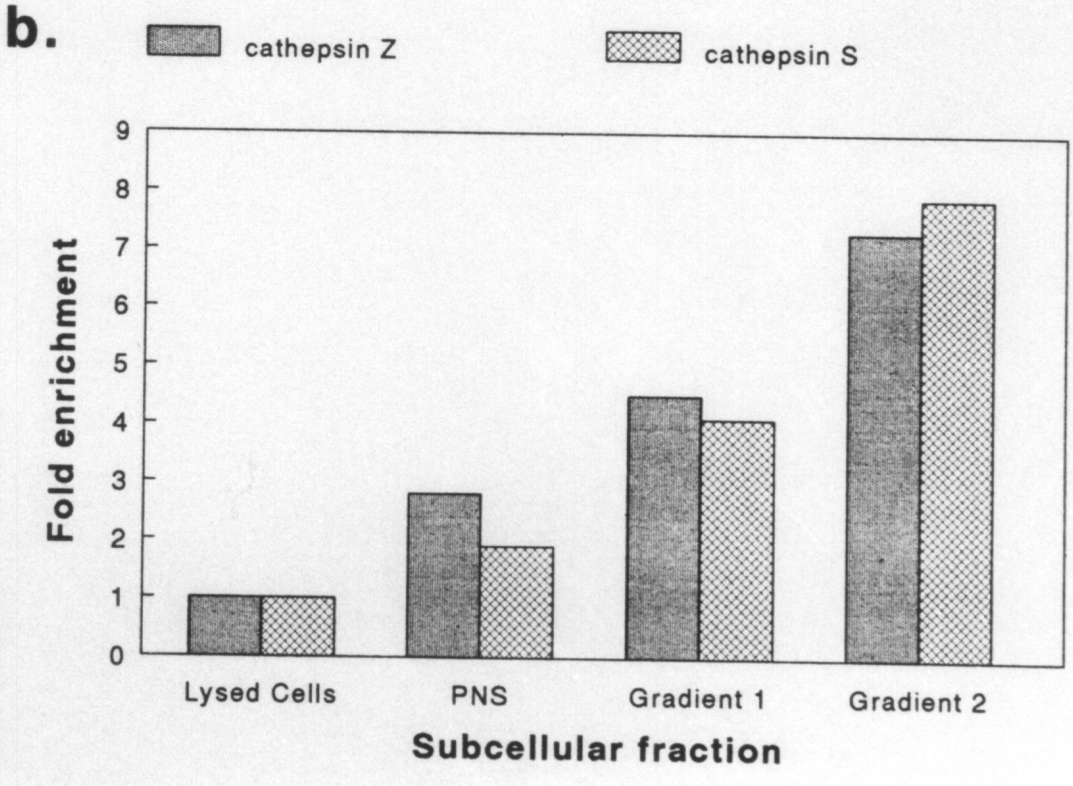
U-937 cells (2 x 10^8 cells) were labeled with Z-Leu-Leu-[^{125}I]Tyr-CHN_2, harvested, and a postnuclear supernatant (PNS) prepared (see Methods). The PNS was fractionated using sequential discontinuous density gradients and a lysosomal fraction isolated. Fractions were saved from each step of the fractionation, and 10 μg of each sample analyzed by SDS-PAGE and autoradiography.

(a) Autoradiograph of the samples. Lysosomes should be enriched in Lanes 3, 5, and 8.

- Lane 1: Lysed cells
- Lane 2: Nuclear pellet
- Lane 3: PNS
- Lane 4: Gradient 1 top
- Lane 5: Gradient 1- 6% Percoll/35% metrizamide interface
- Lane 6: Gradient 1- 17/35 % metrizamide interface
- Lane 7: Gradient 1- bottom
- Lane 8: Gradient 2- 5/17% metrizamide interface

(b) Graphic representation of the enrichment of cathepsin Z and cathepsin S in the lysosomal fractions. Gradient 1 corresponds to Lane 5 and gradient 2 corresponds to Lane 8.


a.
1
2
3
4
5
6
7
8


b.
cathepsin Z
cathepsin S
Fold enrichment
0
1
2
3
4
5
6
7
8
9
Lysed Cells
PNS
Gradient 1
Gradient 2
Subcellular fraction

Labeling of Cathepsin Z with Z-Leu-Leu-[^{125}I]Tyr-CHN$_2$ after Elevation of Lysosomal pH

To determine if the labeling of cathepsin Z was affected by elevated lysosomal pH, U-937 cells were incubated in various concentrations of NH_4Cl prior to labeling. When cells are incubated in media containing weak base amines, the bases accumulate in lysosomes and raise lysosomal pH from <5 to 6−6.5 (reviewed in Seglen 1983). NH_4Cl at 10 mM is known to affect lysosomal pH in U-937 cells (Lindmark *et al.* 1994). U-937 cells in log phase growth were washed in serum-free media and plated in 24-well dishes at 2 x 10^6 cells in 0.5 ml of serum-free media per well. NH_4Cl added to the wells to give final concentrations of 0, 8, 16, and 32 mM. The cells were incubated for 1 h at 37°C. Z-Leu-Leu-[^{125}I]Tyr-CHN$_2$ was added to the wells at 5 μM final concentration and the cells were incubated for 3 h at 37°C. The cells were harvested, washed 3x in PBS, and analyzed by SDS-PAGE and autoradiography, loading 8 x 10^5 cells/lane.

There was no decrease in the labeling of cathepsin Z or CP31 with Z-Leu-Leu-[^{125}I]Tyr-CHN$_2$ when the cells were pre-incubated in 8 mM NH_4Cl (Figure 3.16). There was, however, a 56% decrease in labeling of cathepsin Z with 16 mM NH_4Cl, but this reduction may be due to a toxicity effect from the ammonia. Higher concentrations of NH_4Cl, e.g., 32 mM, were clearly cytotoxic. This result does not preclude the lysosomal localization for cathepsin Z; it may be that cathepsin Z has a non-acidic pH optimum and hence is not strongly effected by an elevation in lysosomal pH. Cathepsin H, for example, has a pH optimum of 6.8 (see Mason and Wilcox 1993)

Attempts to Immunoprecipitate Cathepsin Z

To determine if cathepsin Z was recognized by antisera to other cysteinyl cathepsins, attempts were made to immunoprecipitate labeled cathepsin Z with antisera to cathepsins B, L, and S. U-937 cells were labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$.

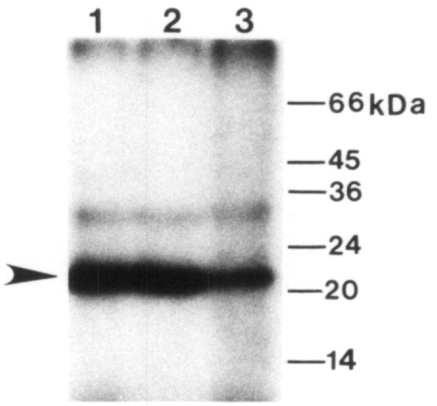


Figure 3.16 Labeling cathepsin Z with Z-Leu-Leu-[^{125}I]Tyr-CHN$_2$ after elevation of lysosomal pH.

U-937 cells were pre-incubated for 1 h in NH_4Cl at 0, 8, and 16 mM prior to labeling with 5 μM Z-Leu-Leu-[^{125}I]Tyr-CHN$_2$ for 3 h. Samples were analyzed by SDS-PAGE and autoradiography. Arrowhead indicated cathepsin Z. Lane 1: 0 NH_4Cl; Lane 2: 8 mM NH_4Cl; Lane 3: 16 mM NH_4Cl.

The cell pellet was resuspended in PBS, the cells were lysed, and the insoluble material pelleted. The soluble fraction was divided into aliquots and pre-cleared of non-specific reactivity with the reagents by incubation in pre-immune serum (either sheep or rabbit) and either Protein-A or -G Sepharose. The supernatants from the pre-cleared samples were transferred to new tubes. The proteins in the samples to be incubated with antiserum to cathepsin B were denatured by adding SDS and heating to 95°C for 5 min, as the sheep anti-cathepsin B was prepared to denatured enzyme. Antiserum was added to each sample, and either Protein-G Sepharose (for cathepsin B) or Protein-A Sepharose (for cathepsins L and S, the antisera to which were prepared to native proteins in rabbits). Control samples received pre-immune rabbit or sheep serum and either Protein-A or -G Sepharose. The samples were incubated on a rotary mixer for 2 h at room temperature. The precipitates were pelleted by centrifugation, and the supernatants transferred to new tubes. The protein in the supernatants was precipitated with DOC and TCA. The pellets (the immunoprecipitates) were washed 3x with PBS. These pellets, the pellets from the pre-clear step, and the precipitated protein from the supernatants were analyzed by SDS-PAGE and autoradiography.

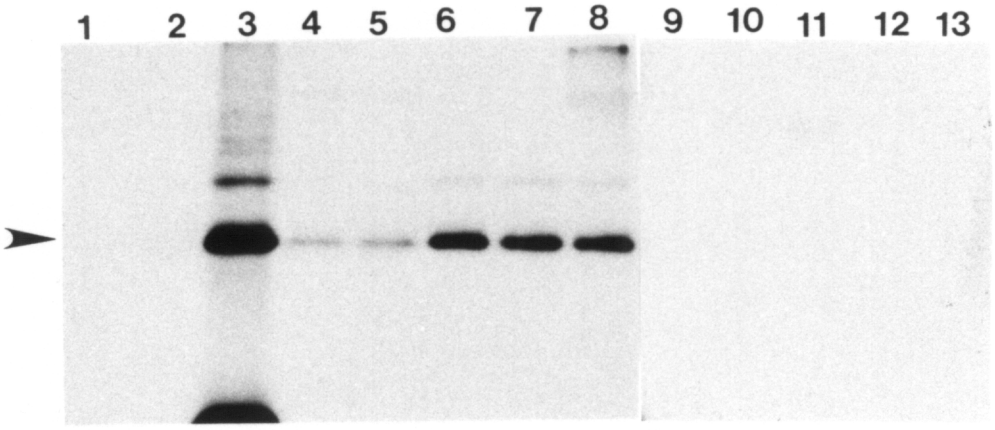
There was no cathepsin Z immunoprecipitated by antisera to cathepsins B, L, or S (Figure 3.17). CP31 was not immunoprecipitated either. Labeled cathepsin Z and CP31 were present in the supernatants. Less labeled protein is apparent in the supernatants from anti-cathepsins B and L, but this was probably experimental error (loss of protein during the precipitation of the supernatants). No labeled protein was precipitated in the pre-clear step.

Figure 3.17 Cathepsin Z is not recognized by antisera to cathepsins B, L, or S.

To determine if cathepsin Z was recognized by antisera to other cathepsins, lysed U-937 cells that had been labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 were incubated in Protein-A or -G Sepharose and antisera to cathepsins B, L, or S. Precipitates and supernatants were analyzed by SDS-PAGE and autoradiography. Lane 3 is an intact cell control. Arrowhead indicates cathepsin Z.

Lane	Sample	Antiserum	Sepharose
1	P	none	Protein-A
2	P	none	Protein-G
4	S	α-cathepsin B	Protein-G
5	S	α-cathepsin L	Protein-A
6	S	α-cathepsin S	Protein-A
7	S	none	Protein-A
8	S	none	Protein-G
9	P	α-cathepsin B	Protein-G
10	P	α-cathepsin L	Protein-A
11	P	α-cathepsin S	Protein-A
12	P	none	Protein-A
13	P	none	Protein-G

P: pellet; S: supernatant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Purification of Cathepsin Z

Cathepsin Z was purified from U-937 cells. Because there is as yet no *in vitro* activity assay for cathepsin Z, enzyme that had been trace labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 was used for developing a purification procedure.

Initial Cell Preparation

For purification of cathepsin Z, large numbers ($2-5 \times 10^9$) of U-937 cells were grown in spinner bottles in complete media. The cells were pelleted, washed 2x in PBS, and, if needed, the pellets stored at −20°C. U-937 cells, usually ~$1-2 \times 10^8$ cells, were labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 using routine procedures. The unlabeled cell pellet was resuspended in 5−20 ml (depending on cell number) of 20 mM Tris-HCl, pH 8.5, and the labeled cell pellet in 1−2 ml of the same buffer. The cells were pooled, lysed by freezing and thawing, and the insoluble material pelleted by centrifugation at 100,000 x g for 1 h at 4°C. The supernatant was transferred to a superloop for FPLC. If the supernatant was cloudy (due to disturbing the pelleted material), it was first filtered on a 0.2 μm filter.

Ion Exchange Chromatography

Cathepsin Z binds to Mono Q anion exchange resin at pH 8.5, and this characteristic was exploited in the purification procedure. A 1-ml Mono Q HR 5/5 anion exchange column was cleaned using 2 M NaCl, 2 M NaOH, 75% acetic acid, and methanol, as described by Pharmacia. The column was equilibrated in 20 mM Tris-HCl, pH 8.5, with the flow rate set at 1 ml/min. Using a superloop, the sample was loaded on the column; the column flow rate might need to be reduced to 0.5 ml/min during the loading step. Material that did not bind the column was collected and saved. The

column was washed in the same buffer until protein ceased eluting off the column, determined by monitoring the absorbance of the eluate at 280 nm. The protein bound to the column was eluted with 0.5 M NaCl, 20 mM Tris-HCl, pH 8.5. The column flow rate was 0.5 ml/min and 1-ml fractions were collected. The absorbance of the eluate was monitored at 280 nm, and the samples counted on a gamma counter.

The chromatographs obtained from the Mono Q anion exchange column have an interesting feature. The protein, as determined by absorption at 280 nm, elutes from the column within 10 ml, but the isotope elutes from the column in three peaks (Figure 3.18). The third peak of radioactivity was always the largest, and the second peak the smallest. To determine which of these peaks contained cathepsin Z, protein in the fractions was precipitated with DOC and TCA, and the samples analyzed by SDS-PAGE and autoradiography. Only the first peak of isotope, the peak that corresponds to the protein peak, contained cathepsin Z, CP31, and CP45 (data not shown). Almost all the label proteins were in three 1-ml fractions (fractions 2, 3, and 4 in Figure 3.18), although the peak did trail off for another 6−8 ml. No peak of iodinated protein was found that corresponded to either the second or third peak of isotope. These peaks are probably iodinated inhibitor and free [^{125}I], neither of which would appear on the autoradiography due to the experimental conditions.

When the column is stripped with 1M NaCl, there is a peak of protein eluted that is approximately the same size as the peak eluted with 0.5 M NaCl, but there is almost no isotope associated with this peak (data not shown).

This purification step served an important function. Cathepsin S is expressed, albeit at levels much lower than cathepsin Z, in U-937 cells. Cathepsin S, however, should not bind to Mono Q at pH 8.5. To confirm that cathepsin S did not bind to Mono Q, the material that did not bind the column was immunoprecipitated with antiserum to

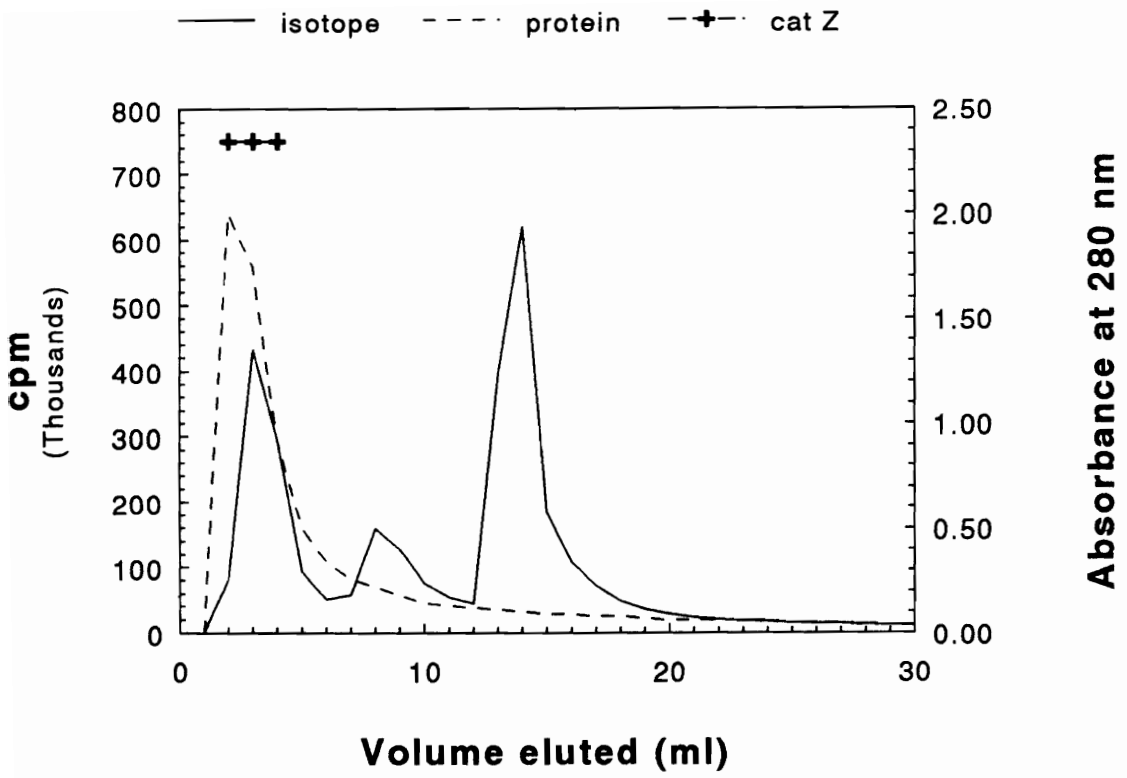


Figure 3.18 Chromatograph from Mono Q anion exchange column.

Mono Q anion exchange chromatography is the first chromatographic step in the purification of cathepsin Z. The column is equilibrated in 20 mM Tris-HCl, pH 8.5. The sample loaded on the column was U-937 cells that had been labeled with Z-Leu-Leu-[^{125}I]Tyr-CHN_2, lysed, and clarified. Elution is with 0.5 M NaCl, 20 mM Tris-HCl, pH 8.5. The plus marks (+) denote the fractions which a subsequent experiment determined contained the majority of cathepsin Z.

cathepsin S. A 1-ml aliquot of the material was incubated for 3 h at room temperature with rabbit anti-cathepsin S and Protein-A Sepharose. A second aliquot was incubated with Protein-A Sepharose and rabbit anti-multicatalytic proteinase (MCP). Both immunoprecipitates were washed 2x in PBS and resuspended in sample buffer. As a control, a 5-ml aliquot of the material was precipitated with TCA. The 3 samples were analyzed by SDS-PAGE and autoradiography.

Labeled cathepsin S was immunoprecipitated from the material that did not bind to Mono Q at pH 8.5 (Figure 3.19). No cathepsin S was immunoprecipitated with antiserum to MCP. A labeled protein that was the same molecular weight as the immunoprecipitated cathepsin S was precipitated with TCA from the material. The intensity of the band of the putative cathepsin S was less than that of the immunoprecipitated material, even though more starting material was used. The reduced amount is was probably due to poor precipitation of a dilute sample with TCA; DOC was not used in this precipitation.

Affinity Chromatography with Concanavalin A

As a glycoprotein, cathepsin Z binds to concanavalin A (ConA), making it possible to use affinity chromatography as a step in the purification. Because of fears that cathepsin Z might bind non-specifically to agarose, concanavalin A bound to acrylic beads was used. A 2-ml concanavalin A column was equilibrated in ConA buffer (100 mM sodium acetate, 200 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 6.5). The sample eluted from the Mono Q column was diluted 1:1 in ConA buffer and the pH adjusted to 6.5. The sample was loaded on the column, and the column washed extensively in ConA buffer. The proteins bound to concanavalin A were eluted in 1-ml fractions with 0.5 M methyl α-D-mannopyranoside in ConA buffer. During elution, the

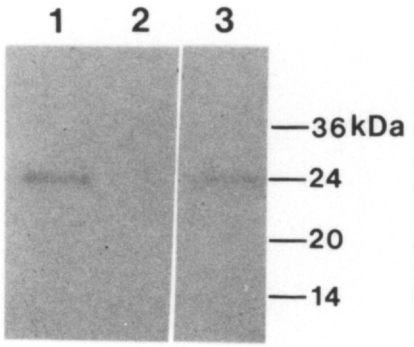


Figure 3.19 Cathepsin S does not bind to Mono Q at pH 8.5.

Cathepsin S was immunoprecipitated from material that did not bind a Mono Q anion exchange column at pH 8.5. The same labeled protein was precipitated from the material with TCA. A control antiserum to multicatalytic proteinase (MCP) did not precipitate cathepsin S. Lane 1: Anti-cathepsin S immunoprecipitate; Lane 2: Anti-MCP; Lane 3: TCA precipitate.

column was incubated in elution buffer for from 2-16 h, after which further 1-ml fractions were eluted. The fractions were counted on a gamma counter.

Cathepsin Z binds to concanavalin A, confirming that it has N-linked glycosylation, and the protein is eluted from concanavalin A by 0.5 M methyl α-D-mannopyranoside (Figure 3.20). CP31 and CP45 also bind to concanavalin A and are eluted with 0.5 M methyl α-D-mannopyranoside, proving that they too have N-linked glycosylation. Increasing the molarity of the elution buffer to 1 M methyl α-D-mannopyranoside did not improve recovery of labeled glycoproteins.

This purification step served to remove another labeled protein from the preparation. The labeled protein is intermediate in molecular weight, as determined by SDS-PAGE, between cathepsins Z and S, i.e., ~23 kDa. This protein was not observed in experiments in which <1 x 10^7 U-937 cells were labeled with 2 μM inhibitor. During larger-scale purification procedures, however, it was sometimes observed. Fortunately, the protein did not bind to concanavalin A, and was removed from the preparation by this step (Figure 3.21).

Preparatory Electrophoresis

Preparatory SDS-polyacrylamide gel electrophoresis using a BioRad Prep Cell proved to be an excellent technique for separating cathepsin Z from other proteins. When U-937 cells labeled with Z-Leu-Leu-[^{125}I]Tyr-CHN_2 were resuspended in sample buffer, heated to 95°C for 3 min, and run on a 14% separatory gel with a 4% stacking gel, there was complete separation of labeled proteins (Figure 3.22). Hence, preparatory electrophoresis was used as the final stage in the purification of cathepsin Z.

The sample eluted from concanavalin A was concentrated, equilibrated in 0.5 M Tris-HCl, pH 6.8, and diluted 1:1 with sample buffer. A small amount of cytochrome c

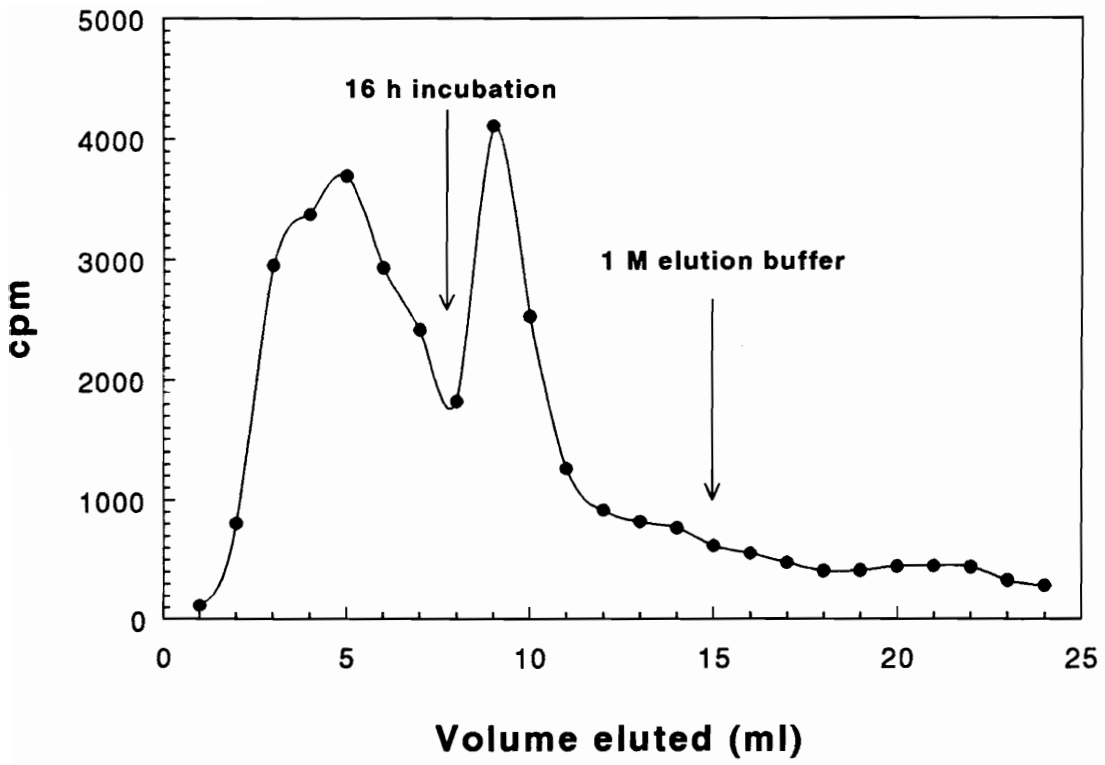


Figure 3.20 Chromatograph from concanavalin A column.

Cathepsin Z binds to concanavalin A, and this was the second chromatographic step in its purification. Protein eluted from Mono Q was loaded on a concanavalin A-acrylic bead column, and bound glycoproteins eluted with 0.5 M methyl α-D-mannopyranoside. During the elution, the column was incubated 16 h in elution buffer and then further fractions were eluted. Eluting the column in 1 M methyl α-D-mannopyranoside resulted in the elution of no additional labeled protein.

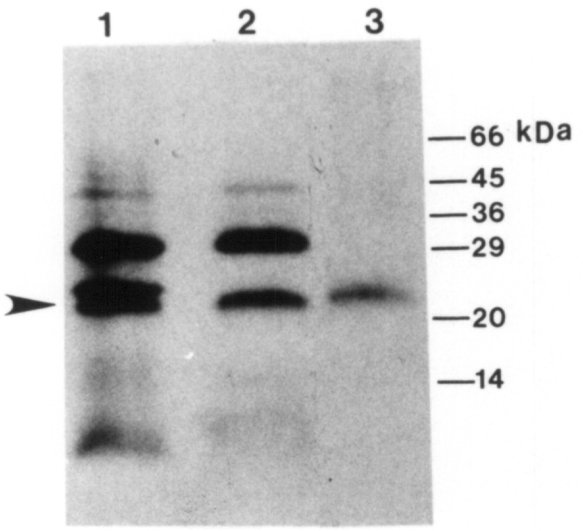


Figure 3.21 Purification of cathepsin Z using affinity chromatography.

Protein eluted from Mono Q was loaded on a concanavalin A-acrylic bead column, and bound glycoproteins eluted with 0.5 M methyl α-D-mannopyranoside. Samples of the material eluted from Mono Q and concanavalin A columns were analyzed by SDS-PAGE and autoradiography. Arrowhead indicates cathepsin Z. Lane 1: Eluent from Mono Q column, total protein load 50 μg; Lane 2: Eluent from concanavalin A column, total protein load 33 μg; Lane 3: purified cathepsin Z, total protein load 2.8 μg.

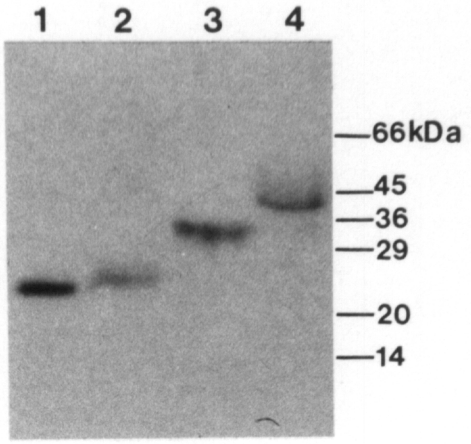


Figure 3.22 Separation of proteins labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$ by preparatory electrophoresis.

U-937 cells that had been labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$ were suspended in sample buffer, heated to 95°C for 3 min, and the proteins separated by preparatory electrophoresis using a BioRad Prep Cell. The separating and stacking gels were 14% and 4% acrylamide, respectively. Electrophoresis was at 12 W constant power, elution at 1 ml/min and fraction collection at 1 ml/min. The collected fractions were counted in a gamma counter and the peaks of isotope pooled, concentrated, and analyzed by SDS-PAGE and autoradiography. There is no overlap between the sample pools. Lane 1: cathepsin Z; Lane 2: either cathepsin S or the unglycosylated 23 kDa protein; Lane 3: CP31; Lane 4: CP45.

was added to the sample, and the sample was heated to 95°C for 3 min. The gel had been prepared previously (see Methods). The separating and stacking gels were 14% and 4% acrylamide, respectively. Samples were eluted at 1 ml/min and 4-ml fractions collected. The fractions were counted on a gamma counter.

The elution profile from preparatory electrophoresis had at least 2 peaks of isotope (Figure 3.23). The first peak co-eluted with bromphenol blue, and was probably due to free iodinated inhibitor or free [^{125}I]. The second peak was cathepsin Z. The third and fourth peaks, which were much smaller, corresponded to CP31 and CP45. The cathepsin Z purified by preparatory electrophoresis was uncontaminated by other proteins to the limits of detection by Coomassie blue staining, silver staining (Figure 3.24), and N-terminal sequence analysis (see below).

Attempts to purify CP31 from the same preparations proved unsuccessful, however. The difficulty was that concanavalin A leaches from the concanavalin A column. Concanavalin A is a tetramer of four 27 kDa subunits, and the monomeric concanavalin A was not separated from CP 31 by the 14% Prep Cell. Stability of the concanavalin A tetramer is promoted by the presence of divalent cations in buffers and by buffer pH greater that 6. Below pH 6, the concanavalin A tetramer dissociates. The buffers used always included divalent cations, and buffer pH was raised to 6.5 in an attempt to increase concanavalin A stability. At pH 6.5, concanavalin A did not leach from the column at levels that were detected by silver staining (data not shown).

Determining the Yield of Cathepsin Z

A yield table for purified cathepsin Z was prepared (Table 3.1). Cathepsin Z was purified from 4.5 x 10^9 U-937 cells mixed with 1 x 10^8 cells that had been labeled with

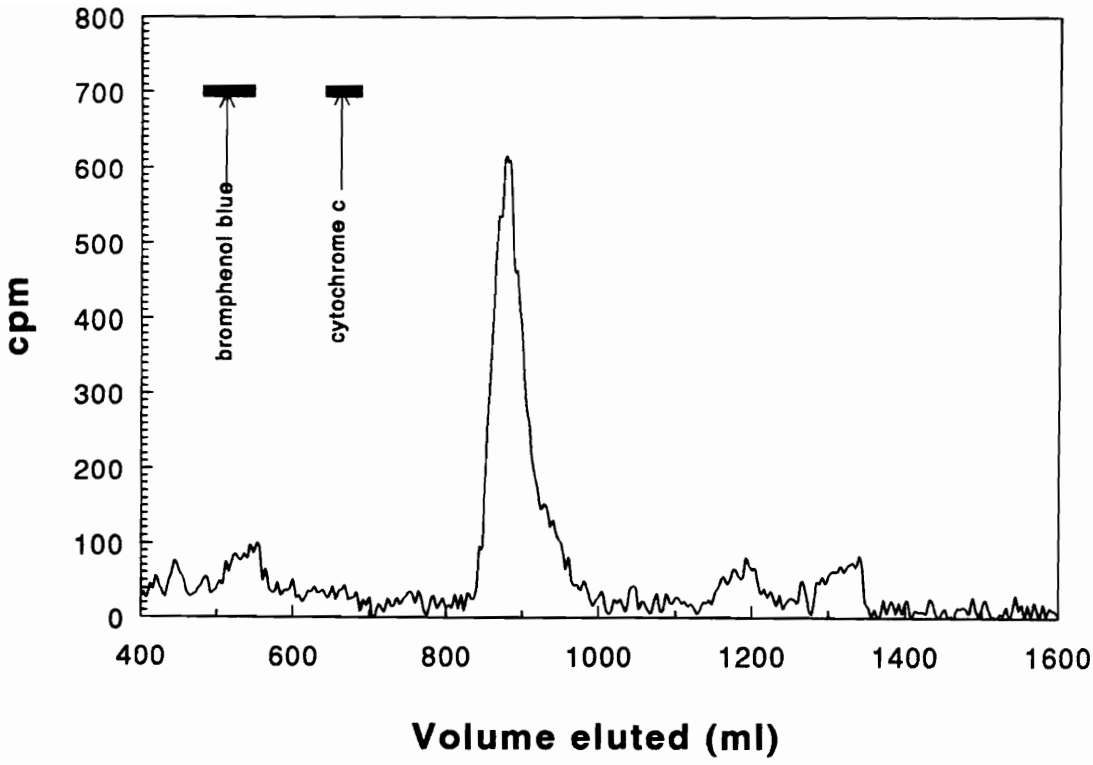
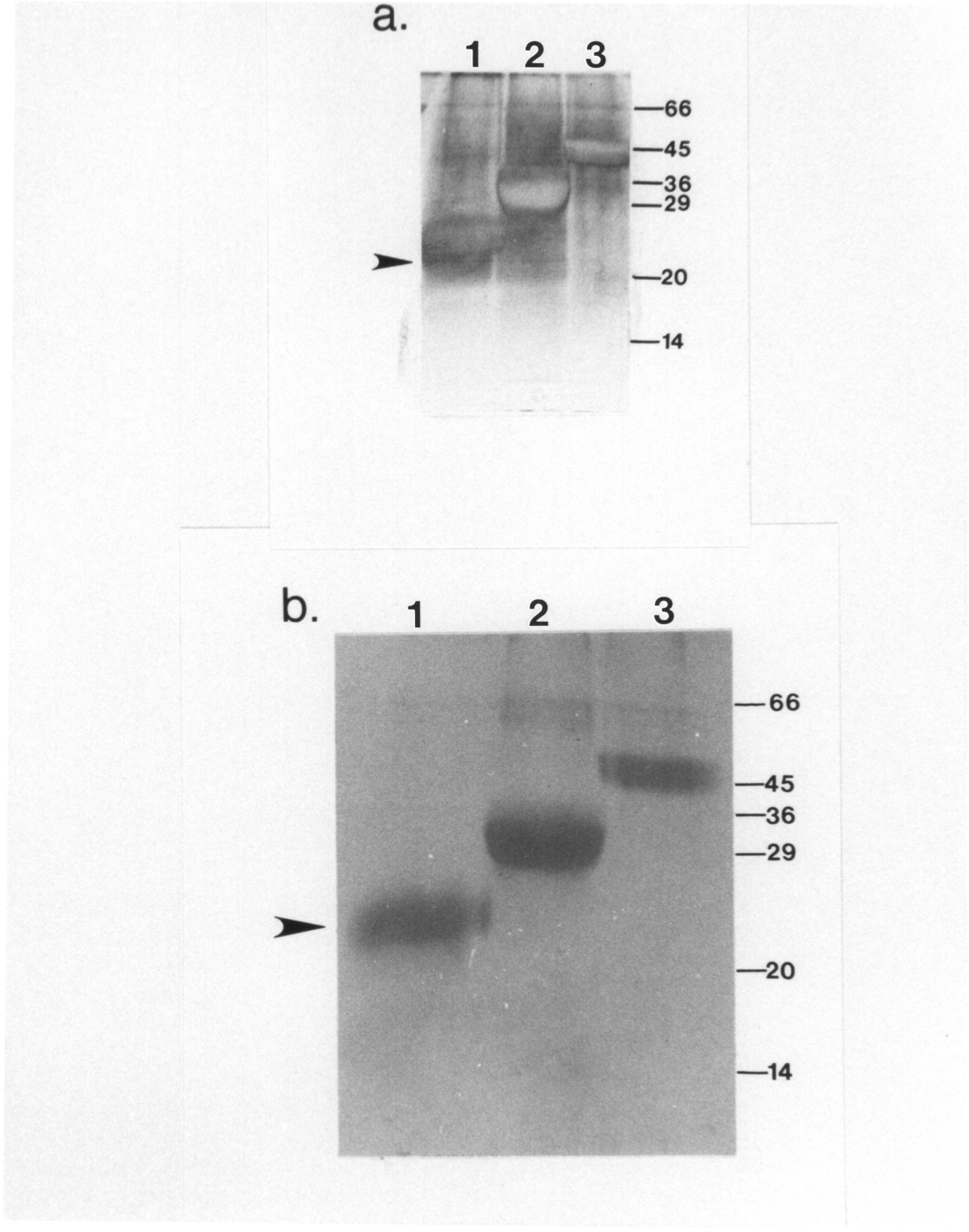


Figure 3.23 Elution of labeled proteins from Prep Cell

Sample of an elution profile from preparatory electrophoresis, the final step in the purification of cathepsin Z. Protein eluted from concanavalin A was loaded on a 14% separatory gel with a 4% stacking gel. The large peak of isotope at ~900 ml is cathepsin Z. In this run, the free isotope/labeled inhibitor peak, at ~550 ml, was unusually small. The fractions containing bromphenol blue and cytochrome C are also noted.

Figure 3.24. Purity of cathepsin Z as demonstrated by staining with Coomassie blue and silver.

Cathepsin Z was purified from 5 x 10^9 U-937 cells that had been trace labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. The purification utilized anion exchange chromatography, affinity chromatography, and preparatory SDS-PAGE. The three peaks of radioisotope that had been eluted from the Prep Cell were pooled, concentrated, and precipitated with DOC and TCA. The samples were analyzed by SDS-PAGE; 12% of the purified, reduced cathepsin Z was loaded on the gel, which was stained sequentially with silver and Coomassie blue. The N-terminal sequence of cathepsin Z was later determined from this preparation. Arrowheads indicated reduced cathepsin Z. (a) Coomassie blue staining of purified cathepsin Z. (b) Silver staining of purified cathepsin Z. Lane 1: cathepsin Z; Lane 2: CP31; Lane 3: CP45.


a.
1
2
3
66
45
36
29
20
14
b.
1
2
3
66
45
36
29
20
14

Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. The purification was as described above, and samples were saved at each step of the purification.

The amount of protein in each of these samples was determined and 5 μg of each were analyzed by SDS-PAGE and autoradiography (Figure 3.25). The labeled bands were excised from the gel and counted on a gamma counter. Because a known amount of protein was loaded per lane, the isotope associated with the cathepsin Z band could be used to calculate cathepsin Z counts per μg of total protein. Knowing the total amount (mg) of protein in each sample, the total amount of cathepsin Z could be estimated, as total counts associated with cathepsin Z. This figure was used to determine the yield of cathepsin Z, and the isotope per μg of total protein was used to determine the fold purification.

Table 3.1 Yield of cathepsin Z.

	Total Protein (mg)	Cathepsin Z counts/μg protein	Total Cathepsin Z counts	% Yield	Fold Purification
Lysed Cells	141	1.3	183300	100	
Soluble fraction	121	1.4	169400	92.4	1.1
Mono Q Pool	39.2	1.9	74480	40.6	1.5
Con A Pool	0.82	24.5	20090	11	18.8
Cathepsin Z	0.32	39.9	12770	7	31

N-terminal Sequence of Cathepsin Z

The N-terminal sequence of cathepsin Z was determined. Cathepsin Z was purified from 5 x 10^9 U-937 cells; the protein was pure to the limits of detection of silver staining (Figure 3.24). Protein purified from ~1.2 x 10^9 cells (24% of the preparation) was deposited on a PVDF membrane using a ProSpin Sample Preparation Cartridge. N-

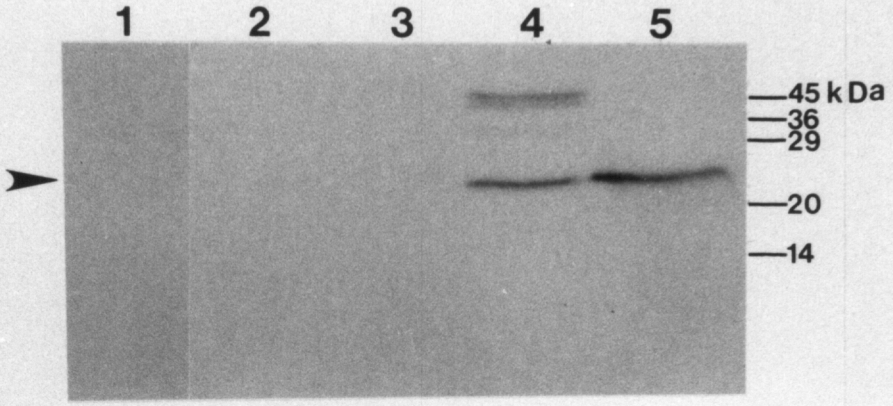


Figure 3.25 Purification of cathepsin Z.

Autoradiograph of stages in the purification of cathepsin Z. Five μg of protein from each stage were analyzed by SDS-PAGE and autoradiography. No labeled protein was detected until purification on concanavalin A. Arrowhead indicates cathepsin Z. Lane 1: lysed cells; Lane 2: soluble fraction of lysed cells; Lane 3: Mono Q pool; Lane 4: concanavalin A pool; Lane 5: cathepsin Z purified by preparatory SDS-PAGE.

terminal sequence analysis was performed. The procedure used for sequencing does not detect cysteinyl residues. The identities of 20 amino acids were determined. These were:

^{1}G-W-P-W-F-K-T-Y-L-D ^{11}L-L-G-T-I-K-F-Q-N-H.

The non-redundant (nr) protein database was searched for similarities between the N-terminus of cathepsin Z and previously sequenced proteins. The nr database is a compilation of the Swiss-Prot, PDB, and PIR databases in which identical sequences have been merged, producing a database without redundancies. The searches utilized the BLAST algorithm. BLAST looks for regions of similarity, i.e., it finds the best alignments for portions of the query sequence rather than the entire sequence; the alignments generated are ungapped. The substitution matrix used in the search was BLOSUM62. No sequences matched the N-terminal of cathepsin Z. The highest score obtained in the search was for a bacterial endoglucanase precursor, but it did not have a significant P-value (at a 95% confidence level).

Further Sequencing Data from Cathepsin Z

In order to determine internal amino acid sequence from cathepsin Z, the protein was digested with endoproteinase Glu-C (V8). Preliminary experiments showed that incubating cathepsin Z for 3 h at 37°in V8 digested the protein (data not shown). The undigested control protein from a preliminary experiment was subjected to N-terminal sequence analysis, and the identities of 7 of the first 10 N-terminal amino acids of cathepsin Z were confirmed. The sequence obtained was ^{1}A-W-P-W-F-x-T-Y-L-W ; the sixth amino acid could not be identified.

Cathepsin Z was purified from 5.9 x 10^{9} U-937 cells that had been trace labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. One quarter of the preparation (from 1.5 x 10^{9}

cells) was digested with V8 for 3 h at 37°C. The digest was run on a SDS-PAGE gel and blotted to PVDF, loading digested cathepsin Z from 2.6 x 10^8 cells per lane. There were 9 lanes of digest and one of molecular weight markers. The membrane was stained for protein with Coomassie blue, and protein bands excised for N-terminal sequence analysis.

There were 7 stained bands visible in each digest lane (Figure 3.26). One of these bands, at ~12.1 kDa, proved to be a V8 fragment from self-digestion of the enzyme. N-terminal sequence was obtained from 3 of the other bands. The sequenced proteins had molecular weights of 15.1, 6.6, and 4 kDa. Isotope was associated only with the 4 kDa fragment. The N-terminal sequences of the 15.1 and 6.6 kDa fragments were G-I-W-D-I-D-W-S-W-G-G and S-I-L-V-T-T-Y-W-G-G-W, respectively.

These two sequences were appended to the N-terminal sequence of cathepsin Z in the order N-terminus/15.1 kDa/ 6.6 kDa or N-terminus/6.6 kDa/15.1 kDa. The resulting sequences were aligned with members of the papain superfamily of cysteine proteinases. Since V8 acts C-terminal to aspartic or glutamic residues, either a D or E was placed at the N-terminal of each of the fragments. The sequences are shown with aspartic acid only, but both amino acids were used. The sequences thus constructed were:

^{1}GWPWFKTYLD ^{11}LLGTIKFQNH ^{21}DSILVTTYWG ^{31}GWDGIWDIDW ^{41}SWGG
^{1}GWPWFKTYLD ^{11}LLGTIKFQNH ^{21}DGIWDIDWSW ^{31}GGDSILVTTY ^{41}WGGW

The alignments were done using the Lasergene MEGALIGN program, utilizing the Jotun Hein method and a weight table derived from a PAM250 matrix. Default settings were used for all other options. Artificially-created gaps, i.e., gaps within one of the 3 sequences from cathepsin Z, were closed manually. Both of the above sequences aligned

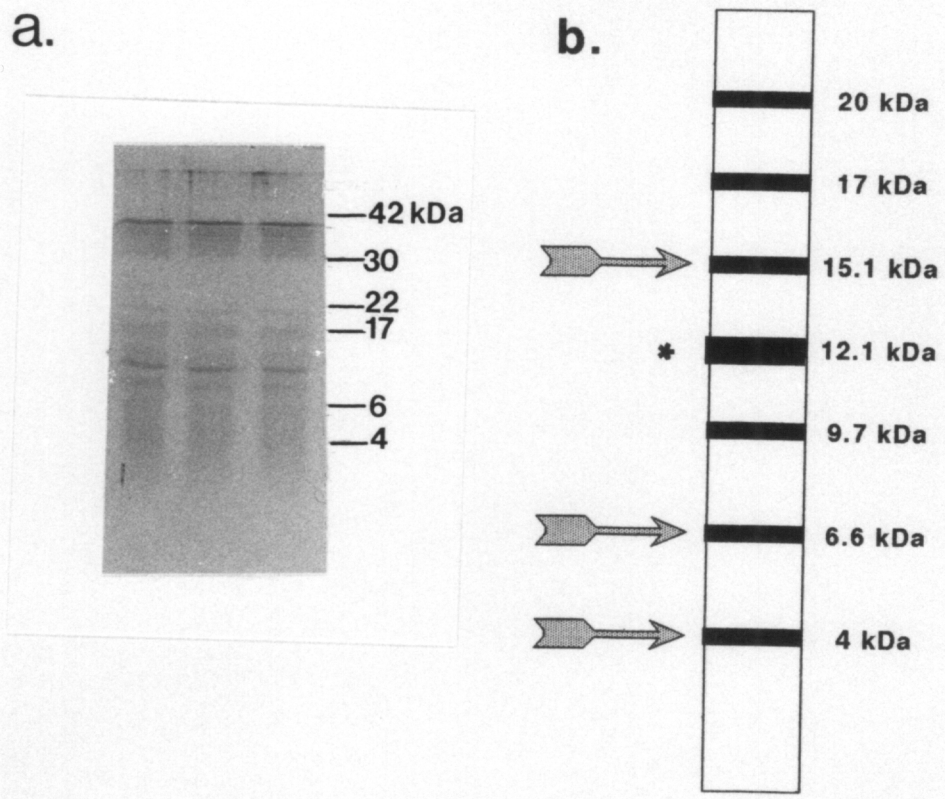


Figure 3.26 V8 digest of purified cathepsin Z.

Purified cathepsin Z was digested with V8 and run on an SDS-PAGE gel; the sample was loaded in nine lanes and molecular weight markers in the tenth lane. The proteins were then blotted on to PVDF membrane and the membrane was stained with Coomassie blue. Bands were excised from the membrane and subjected to N-terminal sequence analysis. For each sample, protein from multiple lanes (3−5 lanes) were loaded on the sequencer. (a) Coomassie blue staining of cathepsin Z digested with V8. (b) Graphic representation of a lane from the V8 digest of cathepsin Z, showing the calculated molecular weight of each fragment. Arrows indicate cathepsin Z bands from which N-terminal sequence was obtained. Asterick indicates band from V8.

with papain and cathepsin L in such a way that the N-terminal amino acid of cathepsin Z aligned with the active site cysteine (see below). The C-terminal region of the second of the sequences (N-terminus/16.6 kDa/6.6 kDa) also aligned with papain and cathepsin L (Figure 3.27), hence the sequence

^{1}GWPWFKTYLDLLGTIKFQNH...DGIWDIDWSWGG...DSILVTTYWGGW

probably contains the correct ordering of the fragments.

If the alignment of proteinases shown in Figure 3.27 is correct, then fragments of papain and cathepsin L generated by cleavage at the N-termini of the alignments should produce fragments with molecular weights similar to those produced by the V8 digest of cathepsin Z. The fragments of cathepsin L and papain were computer-generated using the Lasergene EDITSEQ program, and the molecular weights of the fragments were calculated using the Lasergene PROTEAN program. The calculated molecular weights were from the amino acid sequences only; molecular weight due to glycosylation would not be included.

The fragment molecular weights from cathepsin L and papain were similar to the molecular weights of the cathepsin Z fragments (Table 3.2). The cathepsin Z molecular weights were consistently larger that the weights of cathepsin L and papain fragments.

Table 3.2 Molecular weights (daltons) of cathepsin Z, two fragments generated from cathepsin Z, and the equivalent fragments of cathepsin L and papain.

	cathepsin Z	**cathepsin L**	**papain**
From cathepsin Z N-terminus	22400	21774	20828
V8 fragment 1	15100	12350	11295
V8 fragment 2	6600	4547	4935

Figure 3.27 Comparison of the amino acid sequences of papain and cathepsins L and Z.

Alignment of the amino acid sequences of cathepsins L and Z and papain. The numbers are based on the sequence of papain. Sequences of papain and cathepsin L are of the mature proteins.

```
papain      I P E Y V D W R Q K G A V T P V K N Q G S C G S C W A - F 28
cathepsin L A P R S V D W R E K G Y V T P V K N Q G Q C G S C W A - F
cathepsin Z                                               G W P W F

papain      S A V V T I E G I I K I R T G N L N E Y S E Q E L L D C D 57
cathepsin L S A T G A L E G Q M F R K T G R L I S L S E Q N L V D C S
cathepsin Z K T Y L D L L G T I K F Q N H

papain      - - R R S Y G C N G G Y P W S A L Q L V A Q Y G I H Y - - 82
cathepsin L G P Q G N E G C N G G - - - - - - - - L M D Y A F Q Y V Q
cathepsin Z

papain      - - - - - - - R N T Y P Y E G V Q R Y C R S R E K G P Y A 104
cathepsin L D N G G L D S E E S Y P Y E A T E E S C K Y N P K - - Y S
cathepsin Z

papain      A K T D G V R Q V Q P Y N - E G A L L Y S I A N - Q P V S 131
cathepsin L V A N D T G F V D I P K Q - E K A L M K A V A T V G P I S
cathepsin Z       D G I W - D I D W S W G G

papain      V V L E A A G K D F Q L Y R G G I F V G P - - C G N K V D 158
cathepsin L V A I D A G H E S F L F Y K E G I Y F E P D C S S E D M D
cathepsin Z

papain      H A V A A V G Y G - - - - - - - - - P N Y I L I K N S W G T 179
cathepsin L H G V L V V G Y G F E S T E S D N N K Y W L V K N S W G E
cathepsin Z                                   E S I L V T T Y W G -

papain      G W G E N G Y I R I K R G T G N S Y G V C G L Y T S S F Y 208
cathepsin L E W G M G G Y V K M A K D R R N H - - - C G I A S A A S Y
cathepsin Z G W

papain      P V K N                                               212
cathepsin L P T - V
cathepsin Z
```

As stated above, the proposed alignment places the N-terminal amino acid of cathepsin Z in alignment with the active site cysteine of cathepsin L and papain. The N-terminal amino acid of cathepsin Z was identified as either glycine (original sequencing) or alanine (second sequencing). It is possible, however, that neither of these is the correct residue. Determining the identity of the first residue sequenced presents more difficulty than later residues, because there is only one residue with which to compare it, i.e., the chromatograph from the first residue can be compared only to the chromatograph from the second residue, whereas chromatographs from later residues can be compared to chromatographs from both the preceding and the following residues. In addition, the sequencing method precluded the detection of cysteine residues.

The 4 kDa fragment of cathepsin Z provided additional evidence in support of the proposed alignment. There was very little sequence information gained from the 4 kDa fragment; only the amino acids in positions 2 and 4 were tentatively identified as tryptophans. This could be significant in light of the fact that the 4 kDa fragment was the only one generated that had isotope associated with it. The sequence x-W-x-W is the same as the N-terminal of cathepsin Z, implying that this fragment was generated by V8 cleavage ~40 residues from the N-terminal. If the inhibitor has covalently modified cathepsin Z at the active site cysteine, as occurs with all other members of the papain superfamily, isotope would be associated only with cathepsin Z fragments that include the N-terminal. This will be discussed below.

Discussion

Characterization of Cathepsin Z

Catalytic Mechanism of Cathepsin Z

Cathepsin Z is a cysteine proteinase. The catalytic mechanism was identified by the covalent modification of the enzyme by the peptidyl diazomethane inhibitor Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2, a specific inhibitor of cysteine proteinases. The mechanism was confirmed by the blocking of the inhibition by E-64d, an epoxysuccinyl peptide inhibitor of cysteine proteinases. There are no documented examples of E-64d inhibition of any other class of proteinase.

Subcellular Localization of Cathepsin Z

Cathepsin Z is a lysosomal cysteine proteinase. When U-937 cells were fractionated, cathepsin Z was enriched in a lysosomal fraction. In addition, cathepsin Z co-fractionated with cathepsin S, a known lysosomal cysteine proteinase.

Cathepsin Z has ~3 kDa of N-linked glycosylation, as demonstrated by binding to concanavalin A and by sensitivity to endoglycosidase H. Concanavalin A is a lectin that binds glycoproteins that contain α-linked mannose, such as N-linked glycoproteins. Endoglycosidase H hydrolyzes N-linked high mannose oligosaccharides from glycoproteins. N-linked high mannose oligosaccharides are added to proteins in the endoplasmic reticulum and subsequently processed in the endoplasmic reticulum and the Golgi apparatus.

Amino Acid Sequence of Cathepsin Z: N-terminus

Determining the N-terminal sequence of cathepsin Z provided supporting evidence for the proposal that cathepsin Z is a cysteine proteinase. In addition, the results of the sequencing of cathepsin Z suggest that it is a member of the papain superfamily. The N-terminal amino acids aligned with papain and human cathepsin L (Figure 3.27). The alignment is interesting for several reasons. In this alignment, the first amino acid of cathepsin Z aligns with the twenty-fifth amino acid of papain and cathepsin L. There are two possible ways of explaining this. First, cathepsin Z may have a truncated N-terminus compared to other members of the papain superfamily. Because lysosomal cysteine proteinases are synthesized as pre-pro-proteins, this would mean that there is a deletion in the sequence of cathepsin Z. The more likely explanation for the alignment is that purified cathepsin Z has a clip immediately preceding the first amino acid of the N-terminal sequence. The clip could be have been made at some point during the purification process, or it could be present in the protein *in situ*. When the protein is denatured for preparatory electrophoresis, the clipped portion would be lost.

The second interesting facet of the alignment of cathepsin Z with papain and cathepsin L is that the twenty-fifth amino acid of papain and cathepsin L is the active site cysteine. The N-terminal amino acid of cathepsin Z was identified as glycine (or alanine), but the sequencing procedure could not detect cysteine residues. It is possible that the first residue of cathepsin Z is a mis-identified cysteine. If cathepsin Z is a catalytically active cysteine proteinase of the papain superfamily, then this must be so, as the active site cysteine is conserved in all known members of the superfamily that retain peptidase activity (Rawlings & Barrett 1994).

A second possibility considered was that Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 was modifying the active site histidine. This histidine modification had been observed in the

inactivation of the serine proteinases, subtilisin Carlsberg and thermitase, by the Z-Ala-Ala-Phe-CHN_2 (Ermer *et al.* 1990). However, evidence obtained from the N-terminal sequence of a V8 fragment of cathepsin Z did not support this theory, and suggested that the active site cysteine was the N-terminal amino acid of the sequenced cathepsin Z. If the first amino acid of cathepsin Z is the active site cysteine, a fraction of the cysteine residues should be covalently modified by the radiolabeled inhibitor. The only V8 digest product to be radiolabeled was a 4 kDa fragment with the N-terminal sequence x-W-x-W. This is the same sequence as the N-terminal of the purified cathepsin Z. None of the other V8 digest products, including those that include the putative active site histidine, were radiolabeled.

Determining if the N-terminal amino acid of the purified cathepsin Z is in fact the modified active site cysteine could be accomplished by purifying enzyme that has been labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 and sequentially removing and collecting the first several N-terminal amino acids. This can be done manually using the Edman degradation reaction, extracting the modified peptide after each reaction (Yarwood 1989). If the N-terminal amino acid is the modified active site cysteine, all of the radioisotope should be associated with the first amino acid.

Amino Acid Sequence of Cathepsin Z: Internal Sequence

The N-termini of two other V8 digest products from cathepsin Z were sequenced, and the amino acid sequences aligned with cathepsin L and papain (Figure 3.27). The proposed alignment is supported by the molecular weights of the sequenced fragments (Table 3.2). If cathepsin Z is a lysosomal cysteine proteinase related to the papain superfamily, then the molecular weights of the fragments from the three proteins should be similar. They are similar, although the molecular weights derived from cathepsin Z

are consistently larger than those from papain and cathepsin L. This could be accounted for by the fact that the molecular weights of the cathepsin Z fragments were derived from SDS-PAGE and would include any amino acid modifications such as glycosylation, whereas the molecular weights of the cathepsin L and papain fragments were calculated from their amino acid sequences and would not include glycosylation. Cathepsin Z has ~3 kDa of oligosaccharide, so if 1.5 or 3 kDa, representing one or two oligosaccharide moieties, respectively, were removed from each cathepsin Z fragment, the molecular weights would be quite close.

Labeling Characteristics of Cathepsin Z

Labeling of Cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$

The inhibition of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$ is time-dependent. Labeled cathepsin Z is apparent after U-937 cells are incubated in 2 μM inhibitor for 1 h, and the extent of labeling increases with increasing incubation time up to 6 h. The extent of labeling declined slightly between 6 and 24 h. Interim incubation times were not examined to determine if a 6-h incubation period resulted in maximal labeling of cathepsin Z. The inhibition of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$ is also concentration-dependent. The extent of labeling increases with increasing inhibitor concentration. Labeling of cathepsin Z is seen after a 3-h incubation in 0.1 μM inhibitor, and the extent of labeling has not reached a plateau after a 3-h incubation in 10 μM Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$. The inhibition of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$ is irreversible. If labeled cells are incubated in non-iodinated inhibitor, there is no reduction in the extent of labeling, so radiolabeled inhibitor cannot be removed by competition.

Estimates of the Rate of Reactivity of Cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2

Although the kinetic parameters of cathepsin Z cannot be directly determined at this time, estimates can be made by comparing the reactivity of cathepsin Z with the reactivity of other cysteine proteinases with peptidyl diazomethane inhibitors. For example, the rate constants for the inactivation of cathepsins L and B *in vitro* have been determined with diazomethane inhibitors (Table 4.1).

Table 4.1 Rate constants for the inhibition of cathepsins B and L.

Inhibitor	Cathepsin L Rate constant (M^{-1} s^{-1})	Cathepsin B Rate constant (M^{-1} s^{-1})
Z-Tyr-Ala-CHN_2	176600 ± 24000	1180 ± 80
Z-[I]Tyr-Ala-CHN_2	1128000 ± 112000	27800 ± 2480
Z-Leu-Leu-Tyr-CHN_2	1500000 ± 82000	1300 ± 90

Data from Crawford *et al.* (1988).

Data are also available for the kinetics of inactivation of cathepsins B and L *in vivo*. The labeling of cathepsins B and L *in vivo* is time-dependent up to 3 h at a 0.1 μM inhibitor concentration of Z-[^{125}I]Tyr-Ala-CHN_2; no additional labeling was seen after a 24-h incubation in inhibitor. Cathepsin L was completely labeled after a 3-h incubation in 0.1 μM inhibitor, but labeling of cathepsin B is concentration-dependent up to 1 μM inhibitor (Mason *et al.* 1989b). The *in vivo* labeling of cathepsin Z by Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2, however, is not saturated by 10 μM inhibitor, implying that the rate of inactivation of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 is less than that of cathepsins B and L with Z-[^{125}I]Tyr-Ala-CHN_2. If a 3-h incubation of cathepsin B in 1 μ M inhibitor saturates the enzyme, and cathepsin Z is not saturated after a 3-h incubation

in 10 μM inhibitor, it is likely that the rate of inactivation of cathepsin Z is at least ten-fold less than that of cathepsin B. Since the rate of inactivation of cathepsin B *in vitro* is 27800 $\pm$ 2480 M^{-1} s^{-1}, an estimate for the rate of inactivation of cathepsin Z would be less than 2700 M^{-1} s^{-1}. Similarly, the rate of inactivation of cathepsin Z compared to cathepsin L is at least 100-fold less, or <11280 M^{-1} s^{-1}, as a 3-h incubation in 0.1 μM inhibitor saturates cathepsin L.

The labeling of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 is not directly comparable to the labeling of cathepsins B and L with Z-[^{125}I]Tyr-Ala-CHN_2, however. The inhibitors are different, and structural differences between Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 and Z-[^{125}I]Tyr-Ala-CHN_2 may well affect their permeability. In addition, the experiments that examined the *in vivo* labeling of cathepsins B and L with Z-[^{125}I]Tyr-Ala-CHN_2 were done in cultured fibroblasts; permeability of the inhibitor may vary between cell types. Cathepsin L labeled in human fibroblasts (HIFF cells) when the cells were incubated for 3 h with 0.1 μM Z-Leu-Leu-[^{125}I]Tyr-CHN_2, the same inhibitor used to identify cathepsin Z in this study (Wilcox 1990). Cathepsin Z labels very poorly under those conditions in U-937 cells.

In summary, the inactivation of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 *in vivo* is slower than the *in vivo* inactivation of cathepsins B and L with Z-[^{125}I]Tyr-Ala-CHN_2. It is probably that the rate constant for cathepsin Z is 10- to 100-fold less than the rate constants for cathepsin B and L, respectively; from this, one can derive a range of 2500$-$12000 M^{-1} s^{-1} for the rate constant for the *in vivo* inactivation of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2.

Inhibition of Labeling of Cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$ by E-64d

The labeling of cathepsin Z with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$, is inhibited by E-64d, a specific inhibitor of cysteine proteinases. The inhibition of labeling shows time-dependence up to 6-h The inhibition of labeling by E-64d was not complete; maximal inhibition observed in U-937 cells was 80% after a 24-h pre-incubation in E-64d. In A549 cells, 66% inhibition was seen after a 5-h pre-incubation. One possible reason for the less than complete inhibition by E-64d of the labeling of cathepsin Z is that it is probable that E-64d has a relatively slower rate of inactivation for cathepsin Z than it does for other cysteinyl cathepsins. Wilcox (1990) showed that a 1-h pre-incubation in 10 μM E-64d completely blocked labeling of cathepsin L with Z-Leu-Leu-[^{125}I]Tyr-CHN$_2$ in human fibroblasts. Because complete inhibition of labeling of cathepsin Z was not observed in either U-937 cells or A549 cells, it is not likely that the slow rate of inactivation is cell line-specific; it is more probable that it is enzyme-specific, although there may be some variability in membrane permeability among cell lines.

Purification of Cathepsin Z

Cathepsin Z was purified from U-937 cells that had been trace labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$. Five proteins were labeled by the inhibitor, but the majority of the labeled protein was cathepsin Z. CP31 was labeled to a much lesser extent, and trace amounts of cathepsin S and a non-glycosylated ~23 kDa protein were also labeled. For the purification, the cells were first lysed and the insoluble material removed by centrifugation. The first chromatographic step in the purification was ion exchange chromatography, during which cathepsin S was removed from the preparation. Most of the free inhibitor and free isotope were also removed in this step. Affinity

chromatography using concanavalin A separated the N-linked glycoproteins from the remainder of the proteins eluted from the ion exchange column. The 23 kDa non-glycosylated protein did not bind to concanavalin A, so it was removed at this step.

The final stage in the purification of cathepsin Z was preparatory SDS-polyacrylamide electrophoresis. Preparatory electrophoresis proved to be a very useful tool for separating proteins with similar molecular weights. Preliminary experiments showed that it was possible to get clear separation of proteins separated in molecular weight by only 1−2 kDa. The pool of cathepsin Z obtained from preparatory electrophoresis was pure by all detection methods available, including silver staining and N-terminal sequence analysis.

The purification of cathepsin Z will be simplified when an *in vitro* assay is developed for the proteinase. Early attempts to detect the enzyme in lysed cells were unsuccessful. U-937 cells proved to be difficult to lyse using detergents (Appendix 5). When cells that had been lysed by freezing and thawing were probed with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2, using *in vitro* assay methods that are widely used for other cathepsins, no cathepsin Z activity was detected.

Assessing the Amount of Cathepsin Z in U-937 Cells

Yield of Purified Cathepsin Z

A yield table was constructed for the purification of cathepsin Z (Table 3.1). Since no *in vitro* activity assay was available, the amount of radioisotope associated with cathepsin Z at each stage of the purification was used as an activity estimate. This estimate was used to determine that the yield of purified cathepsin Z was 7%. The amount of radioisotope per microgram of total protein was used to calculate the fold-purification of cathepsin Z.

The purification factor of 31-fold determined in the yield table is unlikely to be an accurate number. The implication would be that cathepsin Z comprises 3.2% of the cellular protein of U-937 cells; 31-fold purification means that cathepsin Z represents 1/31 of cellular protein, or 3.2%. Protein staining, among other results, indicates that this percentage probably is too high. Two previous purifications from which yield tables were constructed produced similar figures, so the problems are probably inherent in the procedures used to construct the yield table.

There are two areas of the yield table in which there may be inaccuracies. First, using the amount of isotope associated with a band on a gel as an estimation for the amount of cathepsin Z present in each step of the purification is not an adequate substitute for an *in vitro* activity assay. It appears particularly inaccurate at the early stages of the purification. Cathepsin Z represents such a small amount of cellular protein that enough protein cannot be loaded on SDS-PAGE to get an adequate number of counts associated with the cathepsin Z band.

The results of this study were that there are 31 μg of protein per 10^6 U-937 cells. Literature values indicate that macrophages have 80 μg of protein per 10^6 cells (Steinman *et al.* 1976), and recent unpublished data suggest that U-937 cells have 60.8 ± 3.4 μg of protein per 10^6 cells (B. Storrie & Y. Li, personal communication). The 31-μg figure may be understated. My yield table indicated 31 μg of protein per 10^6 lysed cells, and 26 μg per 10^6 cells after the insoluble material is removed, meaning that the insoluble fraction represented only 16% of the cellular protein. After centrifugation of the lysed cells, the pellet was substantial. Although the pellet volume was never measured, it comprised at least a third of the original packed cell volume, so 16% appears to be an unreasonably low value. If the value of 31 μg of protein per 10^6 U-937 cells is understated, then one possible explanation for the disparity is poor quantification of

protein in the lysed cell samples. The lysed cell samples had considerable amounts of aggregated cellular material and the protein determinations may not have been made on representative samples.

If the initial protein determination was understated by a factor of two, the re-calculated purification factor would be 61-fold. This would mean that cathepsin Z comprises 1.6% of the total cellular protein. Unfortunately, it is not possible to re-assess the accuracy of the radioisotope associated with cathepsin Z in the same manner.

Estimate of Cathepsin Z in U-937 Cells: Method 1

Based on the purification of cathepsin Z represented by the yield table, an estimation of the concentration of cathepsin Z in the lysosomes of U-937 cells can be calculated. The yield of cathepsin Z was 0.32 mg; this was a 7% yield. This means that at the beginning of the preparation there were 4.6 mg of cathepsin Z in 4.6 x 10^9 U-937 cells (0.32 mg/0.07 = 4.6 mg). The approximate volume of 4.6 x 10^9 U-937 cells is 2.5 ml, so there are 1.84 g of cathepsin Z in 1 liter of U-937 cells. The molecular weight of cathepsin Z is 22.4 kDa, so the molarity of cathepsin Z in U-937 cells 8.2 x 10^{-5} M or 0.08 mM.

To determine the concentration of cathepsin Z within lysosomes, it is necessary to use an estimate for the percentage of cell volume that is lysosomal. Holtzman (1989) states that lysosomes occupy 2−10% of cell volume in macrophages. Using an estimate of 5% of cell volume for lysosomes, if cathepsin Z is 0.08 mM in whole cells, then the concentration of cathepsin Z in U-937 lysosomes is 1.6 mM (0.08 mM/0.05 = 1.6 mM).

Estimate of Cathepsin Z in U-937 Cells: Method 2

Very similar results can be obtained by comparing the labeling of cathepsin Z with the labeling of other cysteine proteinases. When U-937 cells are induced to differentiate with phorbol ester, intracellular cathepsin B levels increase, as measured by the amount of cathepsin B labeled with Fmoc-[^{125}I]Tyr-Ala-CHN_2. The same effect on the expression of cathepsin B is seen when THP-1 cells are induced to differentiate, and the amount of cathepsin B expressed is very similar in differentiated U-937 and THP-1 cells (Mountz 1994). The concentration of cathepsin B in the lysosomes of differentiated THP-1 cells is 0.8 mM (R.W. Mason, personal communication), and the concentration of cathepsin B is probably similar in lysosomes of differentiated U-937 cells.

The concentration of cathepsin Z in differentiated and non-differentiated U-937 cells is comparable to the concentration of cathepsin B in differentiated U-937 cells. The intensity of the cathepsin Z band on an autoradiograph from differentiated U-937 cells is one third than that of cathepsin B (data not shown), but the cathepsin Z was labeled with a sub-saturating (2 μM) concentration of Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. If the U-937 cells had been labeled with a saturating inhibitor concentration (i.e., >10 μM), the labeling of cathepsin Z would have been greater. Hence, by comparing the labeling of cathepsin Z with that of cathepsin B, one can estimate a lysosomal concentration of 0.3–0.8 mM for cathepsin Z in U-937 cells. This estimate is similar to the 1.6 mM calculated above and is within the range of concentrations in lysosomes that have been determined for other lysosomal cathepsins. For example, cathepsins D and B have been calculated to comprise 10.6% and 11.8%, respectively, of lysosomal protein in hepatocyte lysosomes, and their concentrations in lysosomes have been calculated to be 0.78 and 1.54 mM (Dean & Barrett 1976). Cathepsin L is found at concentrations of 0.028 and 0.7 mM in

lysosomes from undifferentiated and differentiated THP-1 cells, respectively (R.W. Mason, personal communication).

Other Characteristics of Cathepsin Z

Molecular Weight of Native Cathepsin Z

The non-reduced molecular weight of cathepsin Z was been calculated to be 46.1 kDa, 45.4 kDa (non-reduced SDS-PAGE), 43.6 kDa (native PAGE), and 45.5 kDa (gel filtration).

The technique of using a series of non-SDS gels of varying acrylamide concentrations to determine the native molecular weight of cathepsin Z worked quite well. Even using mini-gels, when values derived from the migration of each standard were plotted against the percentage acrylamide, the linear fit for each had a high R-value. Despite the difficulty with the diffuse band from cathepsin Z, the lines derived for the unknowns also had high R-values.

The lane-to-lane diffusion of β-mercaptoethanol within polyacrylamide slab gels led to incorrect calculations of the molecular weight of non-reduced cathepsin Z early in this study. "Non-reduced" samples of cathepsin Z, those lacking β-mercaptoethanol in the sample buffer, were placed in lanes next to reduced molecular weight markers, and the molecular weights determined for cathepsin Z were identical to those seen in the presence of reducing agent. Subsequently it was determined that 2.5% β-mercaptoethanol in 25 μl of sample buffer would diffuse into the adjoining lane in a 10% SDS-PAGE of 1-mm thickness, but it did not diffuse into the second lane distant.

pH Stability of Cathepsin Z

The labeling of cathepsin Z with Z-Leu-Leu-[^{125}I]Tyr-CHN_2 was not affected by incubation of U-937 cells in 10 mM ammonium chloride. Raising the lysosomal pH from <5 to 6−6.5 with weak base amines has been shown to inhibit protein degradation up to 75% in hepatocytes (Seglen & Gordon 1980). Since many lysosomal enzymes have pH optima in the acidic range, their activity is reduced at elevated lysosomal pH. This reduction in activity has been used as an indication that a proteinase is lysosomal.

Since cathepsin Z is a lysosomal cysteine proteinase with activity unaffected by elevated lysosomal pH, probably cathepsin Z has broad pH stability. Some other lysosomal cysteine proteinases exhibit activity at a wide pH range. For example, the pH optimum of cathepsin H against synthetic and protein substrates *in vitro* is 6.5−6.8 (see Kirschke & Barrett 1987), so presumably its activity would not be negatively affected by raising the lysosomal pH to 6−6.5. Cathepsin S has also been shown to have activity against elastin *in vitro* from pH 4−8 (Xin *et al.* 1992).

The Structure of Cathepsin Z

A line of evidence leads to the proposal that the ~45−46 kDa form of cathepsin Z is a tetramer, consisting of two catalytically active, covalently-linked domains, each of which has 22.4 and 2.6 kDa subunits. The covalent linkage is demonstrated by SDS-PAGE analysis in the presence or absence of reducing agent. In the absence of reducing agent, the molecular weight of cathepsin Z is ~45−46 kDa; in the presence of reducing agent, the molecular weight of cathepsin Z is ~22.4 kDa. This indicates that the domains must be linked by one or more disulfide bonds.

Each catalytic domain of cathepsin Z must contain the 22.4 kDa subunit purified in this study. In addition, I propose the presence of a ~2.6 kDa subunit in each catalytic

domain, a subunit consisting of the 24 amino acids synthesized N-terminal to the active site cysteine of cathepsin Z. The 2.6-kDa estimate is based on the molecular weights of amino acids 1−24 of cathepsin L and papain (2662 and 2619 daltons, respectively). If cathepsin Z is a cysteine proteinase of the papain superfamily, as indicated by the sequence data, then it is unlikely that the missing N-terminal amino acids are no longer a part of the proteinase. Crystallography studies of papain and cathepsin B have shown that several of the amino acids N-terminal to the active site cysteine comprise part of the active site cleft in these enzymes (Kamphuis *et al.* 1984; Musil *et al.* 1991). In papain, Gln 19 and Gly 23 are believed to stabilize the position of the active site cysteine through hydrogen bonding (Kamphuis *et al.* 1984).

When labeled U-937 cells were analyzed by SDS-PAGE and autoradiography, only the 22.4 kDa subunit and the 46 kDa form of cathepsin Z were covalently modified by the peptidyl diazomethane inhibitor. This result suggests either that the domains that form the holoenzyme are identical, i.e. that the enzyme has an $(\alpha\beta)_2$ structure, or that the domains are unlike and only one domain has catalytic activity. Another option, that native cathepsin Z has two unlike domains, both with catalytic activity, does not fit the data. In that case, multiple labeled proteins would have been observed by autoradiography. Further, if cathepsin Z consisted of two domains close in molecular weight but with only one possessing catalytic activity, then contamination of the purified 22.4 kDa subunit by the other non-catalytic domain might be expected. During the purification process, the native protein was not reduced until it was prepared for preparatory electrophoresis, so both domains would have been loaded on the gel. No contamination was observed; the reduced 22.4 kDa subunit was pure, as confirmed by the N-terminal sequence analysis.

The molecular weights of the two domains of cathepsin Z should total ~46 kDa, the molecular weight estimated for the holoenzyme by native PAGE and chromatography. The molecular weight of the purified subunit, the α subunit, was calculated by SDS-PAGE to be ~22.4. If the β subunit consists of similar amino acids to those found in the N-terminus of cathepsin B and papain, then the subunit molecular weight should be ~2.6 kDa. Hence, the molecular weight of the $(\alpha\beta)_2$ enzyme can be calculated to be 50 kDa. This is slightly higher that the molecular weight determined experimentally, but that does not invalidate the proposed structure for cathepsin Z. If one allows for a 5% error in the experiment values, that would give a range of 21.3−23.5 kDa for the α subunit, and, consequently, a range of 47.8−52.2 kDa can then be calculated for the $(\alpha\beta)_2$ enzyme. Allowing for a 5% error in the molecular weight estimate for the native enzyme gives a range of 43.7−48.3 kDa.

If, as proposed, cathepsin Z is a tetramer with two catalytic domains, it would not be the first cysteine proteinase with that composition. Interleukin-1β-converting enzyme (ICE) is believed to be a homodimer of two catalytically active domains (Walker *et al.* 1994). ICE is synthesized as a 45 kDa propeptide that is subsequently processed proteolytically, perhaps through an autocatalytic mechanism, to subunits of 20 and 10 kDa. The interactions between the subunits are non-covalent. The active site cysteine is in the 20 kDa subunit, but both subunits are required for activity (Thornberry *et al.* 1992). The crystal structure of ICE showed that active enzyme was actually a homodimer; each domain consisted of a 20 and a 10 kDa subunit (Walker *et al.* 1994). Unlike cathepsin Z, however, the association between the domains is non-covalent. This is not surprising, since the cytoplasmic ICE exists in a reducing environment.

To confirm that cathepsin Z is an $(\alpha\beta)_2$ tetramer, cathepsin Z that has been labeled by Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 must be purified without being reduced (see

Appendix 5). The purified protein could then be analyzed by reducing SDS-PAGE, and the gels stained for protein. Purified reduced cathepsin Z was easily detectable by Coomassie blue and silver staining, so presumably protein staining should detect subunits of cathepsin Z. If cathepsin Z is indeed a tetramer of two domains, each domain consisting of a 22.4 and a 2.6 kDa subunit, only bands at those molecular weights should be detected. In either case, subsequent autoradiography should show only a single band of 22.4 kDa radiolabeled by the inhibitor.

Effect of Differentiation on the Expression of Cathepsin Z

U-937 cells can be induced to terminal differentiation by a variety of factors, including phorbol esters, vitamin D3, retinoic acid, γ-interferon, and tumor necrosis factor (see Hay *et al.* 1992). Upon differentiation with phorbol ester, U-937 cells become more macrophage-like and exhibit increased rates of phagocytosis and degradation of extracellular material (Ward *et al.* 1990). Concurrent with the increased phagocytosis, the cells have increased expression of cathepsin B (Ward *et al.* 1990) and cathepsin S (Mountz 1994). The expression of cathepsin Z is not affected by differentiation of U-937 cells with phorbol ester. This observation suggests that cathepsin Z is responsible for degradation of proteins in normal cellular functions, i.e., a housekeeping proteinase, and is not specifically a macrophage protein. Cathepsins B and S, however, have very low levels of expression in undifferentiated U-937 cells, but greatly increased expression after differentiation, implying that they are not involved in intracellular protein degradation as a part of normal cellular function.

Further evidence that cathepsin Z is not specifically a macrophage proteinase is found in the comparison of cathepsin Z expression in U-937 and THP-1 cells. THP-1 cells are also monocytic in origin, and can be induced to differentiate to macrophage-like

characteristics. It has been suggested that THP-1 cells are more macrophage-like than U-937 cells (Mountz 1994). Undifferentiated THP-1 cells express cathepsins B, L, and S constituitively. The expression of cathepsin Z in undifferentiated THP-1 cells, however, is considerably less than the expression in U-937 cells (Figure 3.1), indicating that high levels of cathepsin Z are not expressed constituitively in pro-macrophagic cells.

Distribution of Cathepsin Z in Human Cultured Cell Lines

Cathepsin Z was detected in the seven human cell lines examined in this study. In addition, it has been observed in human platelets (Anagli *et al.* 1991) and fibroblastic HIFF cells (Wilcox 1990). Because the cell lines in which cathepsin Z has been detected are derived from a variety of tissue types, it is probable that cathepsin Z is expressed in most, if not all, human cells, albeit to varying degrees. The extent of expression of cathepsin Z varied in the cell lines examined in this study. However, the contribution of lysosomes to total cellular volume varies among cell types (0.5% to >10%; Holtzman 1989), so it is not unexpected that the expression of cathepsin Z also varies.

Other Proteins Labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN$_2$

Attempted Purification of CP31

Several attempts were made during the course of this study to purify CP31, none of which were successful. An attempt to sequence the N-terminal of what was believed to be purified CP31 resulted in N-terminal sequencing of concanavalin A. Monomeric concanavalin A has a molecular weight of 27 kDa and, by preparatory SDS-polyacrylamide gel electrophoresis using a 14% acrylamide separating gel, the monomeric concanavalin A co-eluted with CP31. (The 14% gel is optimal for the purification of cathepsin Z.) This problem could be solved in several ways. First, the

stability of concanavalin A on the affinity chromatography column could be improved. This was attempted with limited success. At pH 6.5, concanavalin A did not leach from the column at levels that were detectable by silver staining. However, as the column aged, concanavalin A again began leaching from the column. Hence, using a column for a limited period, perhaps only once or twice, should solve the problem. The second, and more promising approach, would be to optimize the percentage acrylamide in the preparatory electrophoresis step to purify CP31. A higher percentage of acrylamide should allow the separation of concanavalin A monomer and CP31. Clean separation of proteins of 27 and 31 kDa is easily within the capacity of preparatory electrophoresis. After preparatory electrophoresis, it would be necessary to confirm the purity of CP31 by N-terminal sequence analysis. Assessing purity by protein staining on a slab gel, regardless of the sensitivity of the stain, has proven to be inadequate. Only a single stained band was been seen by protein staining and by autoradiography when the "purified" CP31 was contaminated with concanavalin A.

What is CP31?

CP31 may be unrelated to cathepsin Z, except that it is a lysosomal cysteine proteinase. CP31 is covalently modified by Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2, and the labeling is both time- and concentration-dependent. Labeled CP31 is detected after a 3-h incubation in $0.1-10$ μM inhibitor, and labeling is seen after a 1-h incubation in 2 μM inhibitor. The labeling is blocked by pre-incubation in 20 μM E-64d, an epoxysuccinyl peptide inhibitor of cysteine proteinases. When labeled U-937 cells were subjected to subcellular fractionation, CP31 was found in a lysosomal fraction. In addition, CP31 co-fractionated with the lysosomal cysteine proteinases cathepsins S and Z. CP31 has N-

linked oligosaccharides, as indicated by its binding to concanavalin A and susceptibility to endoglycosidase H.

There were other similarities between CP31 and cathepsin Z. Like cathepsin Z, CP31 was seen in all human cell lines that were incubated in Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. Expression of CP31 seemed similar to that of cathepsin Z in the different cell lines; in other words, CP31 was strongly expressed in the same cell lines that have strong expression of cathepsin Z. CP31 also co-eluted from anion exchange with cathepsin Z. Like cathepsin Z, CP31 expression in U-937 cells was not affected by differentiation of the cells with phorbol ester.

Unlike cathepsin Z, however, CP31 labeled with Z-Leu-Leu-[^{125}I]Tyr-CHN_2 in intact and, to a lesser extent, lysed purified lysosomes (data not shown). In addition, CP31 did not co-elute with cathepsin Z on gel filtration chromatography. CP31 eluted from Superose 12 in a broad peak beyond the total column volume, indicating that the elution of CP31 was probably retarded by non-specific interactions with the agarose matrix of the Superose 12 column. CP31 did not co-elute with cathepsin Z from a Sephadex gel filtration column either, although both proteins eluted within the column volume.

The identity of CP31 is, as yet, unknown. It shares characteristics with lysosomal cysteine proteinases, e.g., glycosylation, subcellular localization, and inhibition with peptidyl diazomethanes and E-64d. CP31 has significant differences from cathepsin Z, however, such as its behavior on gel filtration chromatography. More importantly, CP31 labels in isolated lysosomes. It may be that CP31 is yet another unidentified cysteine proteinase. If CP31 can be purified from U-937 cells, N-terminal sequence analysis should determine if CP31 is a novel cysteine proteinase.

Native Cathepsin S is Found in a High Molecular Weight Complex in U-937 Cells

An unexpected result from using native gels to determine the native molecular weight of cathepsin Z was that native cathepsin S is associated with a large complex (~800 kDa) in undifferentiated U-937 cells. Cathepsin S is expressed at low levels in undifferentiated U-937 cells, and it would be interesting to see if the association persists in differentiated U-937 cells, in which the expression of cathepsin S is greatly increased.

Relevance of Cathepsin Z

This work has established that cathepsin Z is a major lysosomal cysteine proteinase. In undifferentiated U-937 cells, cathepsin Z appears to be the major cysteine proteinase in lysosomes. In these cells, cathepsin L is not expressed at detectable levels, and cathepsins B and S are expressed at levels far below that of cathepsin Z.

The lysosomal cathepsins are the predominant cysteine proteinases in the cell, although there are cysteine proteinases found in other subcellular compartments, e.g., the cytosolic calpain. In addition, there are other putative non-lysosomal cysteine proteinases under investigation, e.g., interleukin-1β-converting enzyme in the cytosol (Thornberry *et al.* 1992) and ER60 in the endoplasmic reticulum (Urade & Kito 1992). Among the lysosomal cysteine proteinases, cathepsin B was first described in 1941 (Fruton *et al.* 1941), and the lysosomal cathepsins H, L, and S have subsequently been described and characterized (reviewed in Mason & Wilcox 1993). The lysosomal cysteine proteinases are believed to be responsible for as much as 50−70% of intracellular protein degradation (Grinde 1983). This is similar to the amount of inhibition of protein degradation observed when cells are treated with weak bases (Seglen 1983).

The characterization of cathepsin Z is important for several reasons. First, cathepsin Z is a major cysteine proteinase that has wide distribution in human cells. Cathepsin Z was detected in all human cell lines examined. Second, many physiological functions, both normal and pathological, are being assigned to cysteine proteinases, for example, roles in bone resorption, Alzheimer's disease, cancer metastasis, and emphysema. Many of the assignments are based on blockage of the functions of cysteine proteinases by inhibitors of cysteine proteinases, e.g., inhibition of protein turnover by peptidyl diazomethane inhibitors (Grinde 1983) or the prevention of a T cell proliferative response by E-64d or CA074 (Matsunaga *et al.* 1993). If, in fact, these inhibitors are not specific and can affect the catalytic activity of other cysteine proteinases, then the assignments are premature. This argument is particularly relevant to the studies in which the function of calpain has been investigated using Z-Leu-Leu-[^{125}I]Tyr-CHN_2. Incubation of human platelets in Z-Leu-Leu-Tyr-CHN_2 prevented the degradation of certain cytoskeletal proteins, and the degradation was attributed to calpain (Anagli *et al.* 1991). Since Z-Leu-Leu-Tyr-CHN_2 also inhibits the activity of cathepsin Z, perhaps the functions attributed to calpain have been misassigned.

As long as novel cysteine proteinases continue to be identified, studies that utilize "specific" inhibitors of cysteine proteinases must include a determination that the inhibitor is indeed specific. This determination is accomplished by establishing which proteinases are labeled by the inhibitor *in vivo*. It was in two studies of this nature that cathepsin Z was first noticed (Wilcox 1990; Anagli *et al.* 1991).

Although much information on cathepsin Z has been gained, much more work needs to be done to gain a full understanding of the proteinase. Two areas are of particular importance. First, there is a strong need to develop a method to purify active cathepsin Z. Once active enzyme has been isolated from cells, many more studies will be

possible. Synthetic inhibitors could then be used to characterize the binding pockets of cathepsin Z, and accurate kinetic parameters could be determined. Perhaps the key to purification may be as simple as switching to a different cell line for the investigation. Although U-937 cells had many useful characteristics that justified their use in this study, there were other cultured cell lines, e.g., Raji and Wil2NS, that also had high levels of expression of cathepsin Z. If a less rigorous method of cell lysis were used, it might make isolation of active enzyme feasible.

The second area of investigation that would be particularly useful would be the development of an inhibitor of cathepsin Z that does not react with calpain or other lysosomal cathepsins. Fmoc-Leu-Leu-Tyr-CHN_2 also inhibits cathepsins S and L and calpain, so the inhibitor is of limited use for defining the intracellular functions of any of the proteinases. Its utility in this study was made possible by the cell line-specific levels of expression of cathepsins L and S; U-937 cells do not express detectable levels of cathepsin L, cathepsin S expression is quite low, and calpain requires high concentrations of Ca^{2+} and a calcium ionophore for activation. To investigate cathepsin Z activity in cell lines that express cathepsins L and S, a more specific inhibitor is needed.

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Appendix 1. Gel Formulations

Table A.1 Gel formulations for routine SDS-PAGE.

Ingredient	12.5% Separating Gel	4.5% Stacking Gel
distilled water	2.4 ml	2.2 ml
1.5 M Tris-HCl, pH 8.8	2.8 ml	
0.5 M Tris-HCl, pH 6.8		0.94 ml
30.8% acrylamide solution	4.7 ml	0.56 ml
50% glycerol	1.15 ml	
10% SDS	0.112 ml	37.5 μl
TEMED	7.5 μl	3.75 μl
10% APS	75 μl	50 μl

Table A.2 Gel formulations for SDS-PAGE for low molecular weight proteins.

Ingredient	20% Separating Gel	4.5% Stacking Gel
distilled water	0.827 ml	2.93 ml
3.0 M Tris-HCl, pH 8.85	2.5 ml	
0.5 M Tris-HCl, pH 6.8		1.25 ml
30.8% acrylamide solution	6.5 ml	0.73 ml
10% SDS	0.1 ml	37.5 μl
TEMED	6.6 μl	7.5 μl
10% APS	66 μl	50 μl

Table A.3 Gel formulations for preparatory electrophoresis.

Ingredient	14% Separating Gel	4% Stacking Gel
distilled water	25.2 ml	18.3 ml
1.5 M Tris-HCl, pH 8.8	22.5 ml	
0.5 M Tris-HCl, pH 6.8		7.5 ml
30% acrylamide solution	42.0 ml	4.0 ml
TEMED	22.5 μl	30 μl
10% APS	225 μl	150 μl

Table A.4 Gel formulations for native PAGE.

Ingredients	5%	6%	7%	8%	9%	10%	3% stacker
30.8% acrylamide	1.62 ml	1.95 ml	2.27 ml	2.60 ml	2.92 ml	3.25 ml	0.487 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	
0.5 M Tris-HCl, pH 6.8							1.25 ml
water	4.81 ml	4.48 ml	4.16 ml	3.83 ml	3.51 ml	3.18 ml	3.19 ml
50% glycerol	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml	
10% APS	66 μl	66 μl	66 μl	66 μl	66 μl	66 μl	50 μl
TEMED	6.6 μl	6.6 μl	6.6 μl	6.6 μl	6.6 μl	6.6 μl	5 μl

Appendix 2. Buffer for Concanavalin A Affinity Chromatography

Table A.5 Preparation of buffer.

Concentration Desired	Stock	To make 500 ml
100 mM acetate	1 M acetic acid	50 ml
200 mM sodium chloride		5.84 g
1 mM manganese chloride	1 M $MnCl_2$	0.5 ml
1 mM magnesium chloride	1 M $MgCl_2$	0.5 ml
1 mM calcium chloride	1 M $CaCl_2$	0.5 ml

To prepare the buffer, the acetic acid and sodium chloride are added to 400 ml distilled water, and the pH was adjusted to 6.5 with 1 M NaOH. The other salts were added the solution brought to 500 ml final volume. If the pH was adjusted after the addition of the magnesium chloride, the solution changed color and a solid precipitate was formed.

Appendix 3: Synthesis of the Inhibitor Biotin-Leu-Leu-Tyr-CHN_2

Biotin-Leu-Leu-Tyr-CHN_2 was prepared by a method adapted from Walker *et al.* (1992) and Green and Shaw (1981).

Biotinylation of Peptide

One gram of the tripeptide Fmoc-Leu-Leu-Tyr was synthesized on resin on a Milligen/Biosearch 9600 Peptide Synthesizer. This should be 0.36 mmoles, assuming 100% recovery. The peptide-resin was washed 3x with dimethyl formamide (DMF). The N-terminal Fmoc group was removed from the N-terminal of the peptide by incubating the peptide-resin with 30% piperidine in DMF for 1 h at room temperature. The peptide-resin was then washed 5x with DMF and 2x with methanol and dried under nitrogen gas.

One mmole (365 mg) of biotin nitrophenol and 230 mg of 1-hydroxy-benzotiazole (HObt) were dissolved in 40 ml DMF. (HObt catalyzes the biotinylation reaction.) This solution was added to the peptide-resin, and the flask was placed on a rotary mixer for 1 h at room temperature. The resin was washed 5x in DMF and 10x in methanol, then dried under nitrogen gas.

The biotinylated peptide was cleaved from the resin by incubation for 45 min at room temperature in 2 ml of 1% dichloromethane, 5% thioanisole, and 94% trifluoroacetic acid. The resin slurry was filtered and washed with TFA on a sintered glass funnel; the filtrate contained the free biotin-peptide. The solvent was removed by rotary evaporation, and the biotin-peptide was dried under vacuum over KOH overnight.

Preparation of Diazomethane

Diazomethane (CH_2N_2) was synthesized from *N*-methyl-*N*-nitroso-4-toluenesulfonamide (Diazald). Five grams of Diazald were placed in 45 ml ethyl ether in a separatory funnel. The separatory funnel was placed above a round-bottomed flask containing 5 g of potassium hydroxide, 8 ml water, and 10 ml of 95% ethanol, and the round-bottomed flask was placed in a 65−70°C water bath. The Diazald solution and then 10 ml of diethyl ether were added dropwise to the flask over 30 min. The reaction mix was distilled, and the distillate (diazomethane) collected in a flask that was placed in a dry ice/acetone bath. The reaction should yield ~16.6 mmoles of diazomethane, which was stored at −20°C overnight.

Diazomethylation of Peptide

To prepare the acid chloride of the peptide, the biotin-peptide was dissolved in 1.8 ml tetrahydrofuran; the peptide was very soluble. The solution was placed in a flask and the flask was purged with nitrogen gas. *N*-methylmorpholine (0.29 mmoles) was added to the flask, and the flask was placed in a dry ice/ acetone bath (−20°C). Isobutyl chloroformate (0.29 mmoles) was added to the flask, and the flask incubated for 15 min at −20°C. To diazomethylate the acid chloride, the flask was warmed to 0°C in an ice bath, 1.2 mmoles of diazomethane in ethyl ether were added, and the flask was incubated at 0°C for 15 min. When the diazomethane was added, a white, gummy precipitate appeared.

Purification and Assay of Inhibitor

The solvent was removed by rotary evaporation and the inhibitor resuspended in 80% acetonitrile in 50 mM ammonium acetate. One half of the inhibitor preparation was

purified by HPLC using a Waters RCM 25 x 100 Bondapack C-18 column, eluting with a 20-100% acetonitrile gradient. Five ml fractions were collected and the absorbance of the eluant was monitored at A_{260} (Figure A.1).

To determine which fractions contained the diazomethylated inhibitor, each fraction (10 μl) was tested to determine if it would inhibit the activity of sheep cathepsin L against the synthetic substrate Z-Phe-Arg-NHMec. Two peaks of inhibition were found (Figure A.2), centering on fractions 40 and 50. These fractions correspond to elution with 50% and 60% acetonitrile. These peaks should be biotinylated and non-biotinylated peptidyl diazomethane.

To determine if either of the peaks contained biotin, the inhibitor fractions were pre-incubated in avidin bound to acrylic beads, and the beads pelleted to remove the biotin-avidin complexes from the solution. Ten μl of each fraction were incubated either in 20 μl of avidin slurry or in 20 μl of buffer overnight at 4°C. The samples were then assayed as described above to determine if inhibitor activity of either of the peaks had been removed by the avidin pre-incubation.

There was a reduction in the inhibitory activity of the diazomethylated peptide in Fractions 46−48 (Figure A.3). This corresponds to elution at 56-58% acetonitrile. This meant that the avidin pre-incubation had removed some of the inhibitor, and hence that the inhibitor that was removed was biotinylated. The three fractions were pooled and titrated against cathepsin L. The yield was determined to be 12.8 mmoles. Since the synthesis was started with 360 mmoles of peptide, and only half of the preparation was purified on HPLC, the yield was 7.2%. The biotinylated inhibitor was aliquotted into microfuge tubes at 1 nmole/tube, lyophilized, and stored at −20°C.

Experimental Use of Biotin-Leu-Leu-Tyr-CHN_2: *in vivo* Labeling

The biotin-Leu-Leu-Tyr-CHN_2 was tested in several preliminary experiments. U-937 cells were incubated in 2 μM biotin-Leu-Leu-Tyr-CHN_2 plus 2 μM Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 following normal labeling procedures. Control cells were incubated in 4 μM Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2. The labeled cells were analyzed by SDS-PAGE and autoradiography.

Normal cathepsin Z and CP31 labeling was seen in the control lane, but there was no labeling of cathepsin Z or CP31 with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 in the cells that had been incubated with biotin-Leu-Leu-Tyr-CHN_2 (data not shown). There are several possible explanations for this result. First, the biotinylated inhibitor may diffuse into U-937 cells more rapidly than the Fmoc inhibitor, or the biotinylated inhibitor might be a more rapid inhibitor of cathepsin Z and CP31 than the Fmoc inhibitor. Another possibility is that the solvent (acetonitrile) interfered with inhibitor permeability or inhibitory activity. A solvent control was not included in the experiment.

If the lack of labeling with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 is a result of blocking by the biotinylated inhibitor, then biotinylated cathepsin Z should be detectable. Samples from the above labeling were run on SDS-PAGE, electroblotted to nitrocellulose membrane, and probed with alkaline phosphatase-conjugated strepavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Color was developed with Sigma *Fast* BCIP/NBT. The membrane was then analyzed by autoradiography. No biotinylated cathepsin Z was detected with the strepavidin probe (data not shown), nor was there radiolabeled cathepsin Z in the biotin lane on the autoradiograph. Normal cathepsin Z labeling was observed in the control lane of the autoradiograph.

It is possible that cathepsin Z has been labeled by the biotinylated inhibitor, but the assay methods employed are not be sensitive enough to detect it. More work needs to

be done to determine the limits of detection of biotin-Leu-Leu-Tyr-CHN_2. A second question to be answered is whether the observed lack of labeling with Z-Leu-Leu-[^{125}I]Tyr-CHN_2 when cells are co-incubated with biotin-Leu-Leu-Tyr-CHN_2 is a result of blocking by the biotinylated inhibitor.

Experimental Use of Biotin-Leu-Leu-Tyr-CHN_2: Immunofluorescence Assays

Preliminary immunofluorescence assays were not successful. Since adherent cells, as opposed to the more rounded, suspension cells, provide better photomicroscopic images, A549 cells were used for these experiments. The cells were cultured on 12-mm glass coverslips, and labeled for 3 h with biotin-Leu-Leu-Tyr-CHN_2 using standard labeling procedures. The experiment design was to fix the cells with paraformaldehyde and probe with Texas red-conjugated strepavidin. Unfortunately, by the end of the 3-h labeling period, the cells were rounded and peeling off the coverslips. This could have been due to a solvent effect. A second possibility is the 4-h incubation in serum-free media during the labeling procedure; A549 cells may not adhere as well to glass as they do to plastic. These possibilities will have to be investigated.

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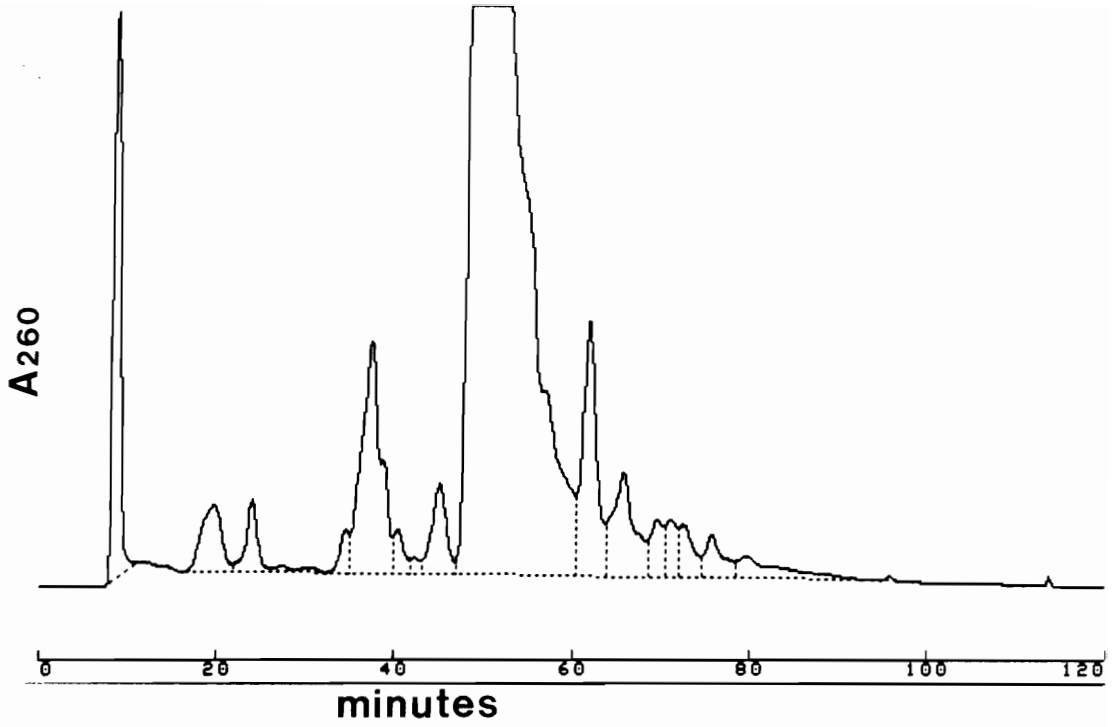


Figure A.1 Purification of biotin-Leu-Leu-Tyr-CHN_2 by HPLC.

Chromatography from C-18 HPLC column of the absorbance at 260 nm. Biotinylated Leu-Leu-Tyr-CHN_2 was resuspended in 80% acetonitrile in 50 mM ammonium acetate and chromatographed on a Waters RCM 25 x 100 Bondapack C-18 column on HPLC. The inhibitor was eluted with a 20-100% acetonitrile gradient, and 5 ml fractions were collected.

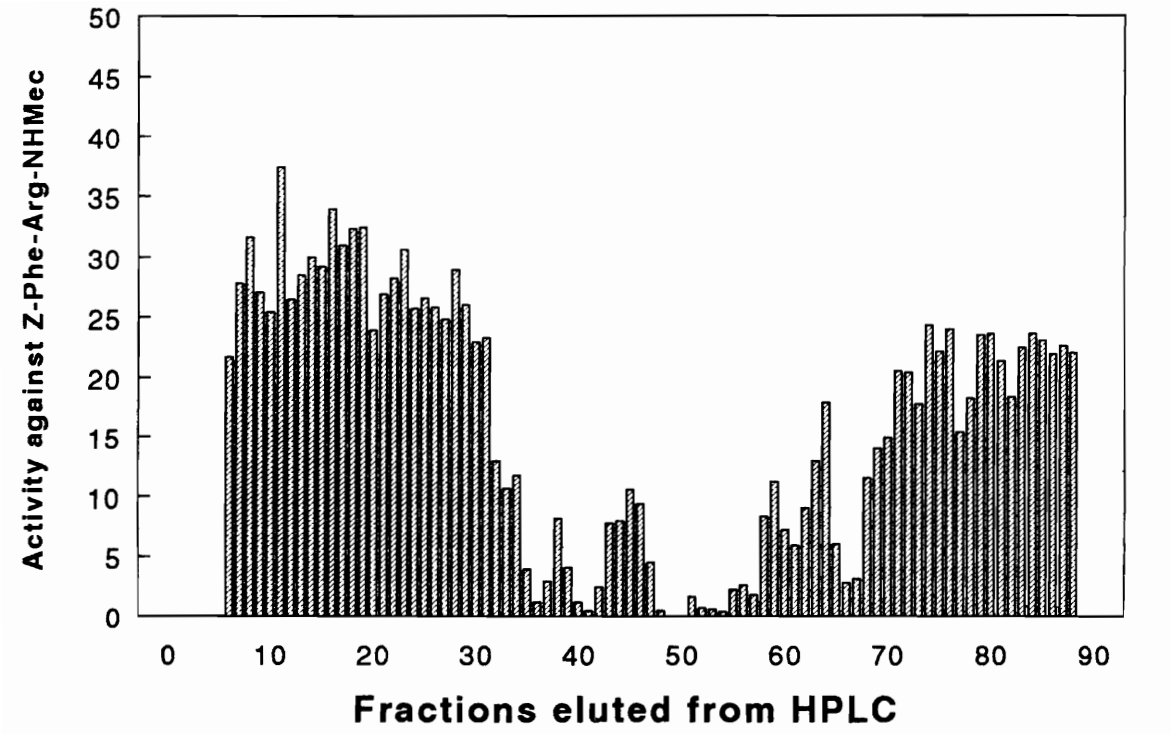


Figure A.2 Assay of biotin-Leu-Leu-Tyr-CHN_2 for inhibitory activity.

Inhibition of cathepsin L activity against Z-Phe-Arg-NHMec by fractions eluted from the HPLC C-18 column. Ten μl of each 5 ml fraction was assayed. The percentage acetonitrile with which each fraction eluted is equal to the fraction number + 10.

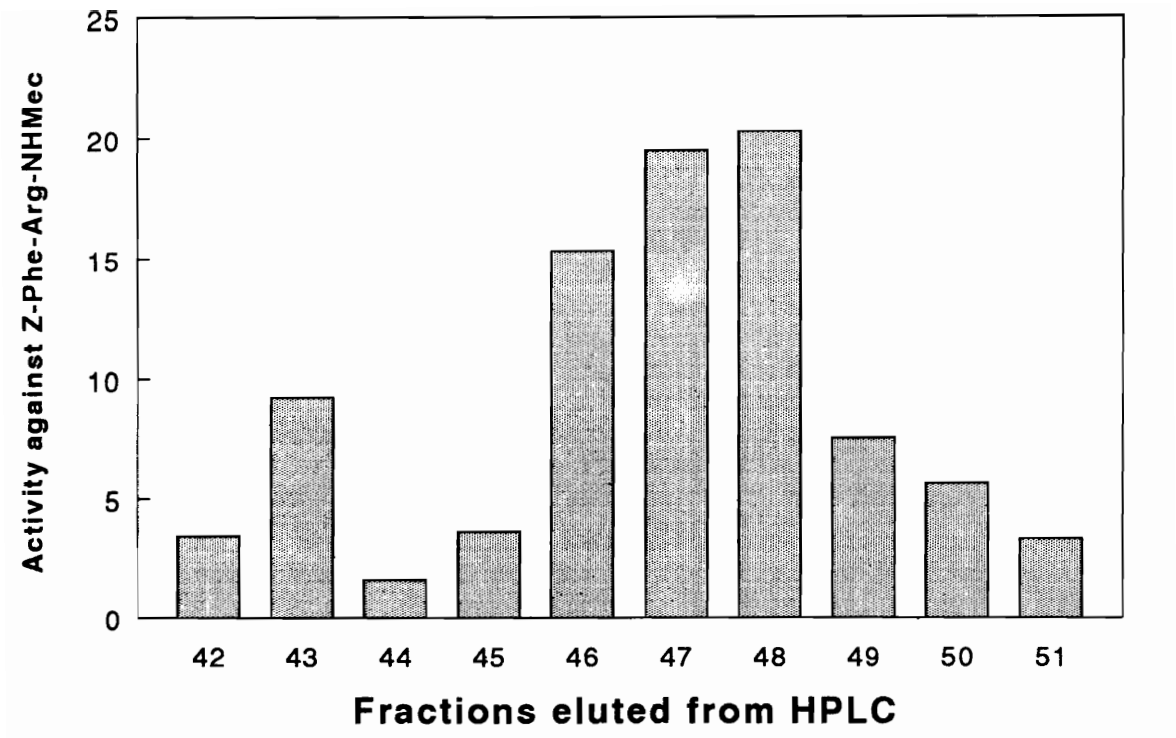


Figure A.3 Assay of fractions with inhibitory activity for biotin content.

Reduction of the inhibition of cathepsin L activity against Z-Phe-Arg-NHMec by biotin-Leu-Leu-Tyr-CHN_2 in samples that were pre-incubated with avidin bound to acrylic beads. Ten μl of each 5 ml fraction was assayed. Graph is of the difference in activity in the presence or absence of avidin pre-incubation.

Appendix 4. Solubilization of Proteins from U-937 Cells and Subsequent *in vitro* Labeling Attempts

Lysis of U-937 Cells

Early efforts of this investigation concentrated on labeling cathepsin Z in U-937 cells that had been lysed. It was determined that concentrations of non-ionic detergents that are normally used to solubilize proteins had very little, if any, affect on the solubility of U-937 proteins. U-937 cells were washed 2x in serum-free media and incubated for 1 h in serum-free media. The cells were pelleted by centrifugation and aliquotted into microfuge tubes at 1 x 10^6 cells/tube. The cells were resuspended in 1 ml of assay activation buffer (20 mM sodium acetate, pH 5.5, 1 mM EDTA, 4 mM DTT), either with or without detergent. The detergents used were Triton X-100 (0.5% and 1.0%), NP40 (0.5% and 1.0%), and Tween 20 (0.1% and 0.5%). Two samples had no detergent in their buffer.

The tubes were vortexed and incubated for 1 h at 37°C. One of the no detergent tubes was treated to 3 cycles of freezing and thawing, using a bath of dry ice and methanol and a 37°C water bath. The insoluble material was pelleted by centrifugation at 12,000 x g in a microfuge. The supernatants were removed. The protein in the pellets and the supernatants was precipitated with TCA and analyzed by SDS-PAGE.

Protein staining of the gels with Coomassie blue demonstrated that the presence of detergents in activation buffer had virtually no effect on the solubilization of proteins in U-937 cells (Figure A.4). Almost all the protein was associated with the non-solubilized fraction. The samples that contained detergents (Lanes 2−7) were indistinguishable from the sample that lacked detergent (Lane 1). Freezing and thawing of the U-937 cells served to solubilize more that 50% of the protein (Lane 8) and was used subsequently for cell lysis.

Labeling of Lysed U-937 Cells with Z-Leu-Leu-[^{125}I]Tyr-CHN_2

U-937 cells suspended in activation buffer and lysed by freezing and thawing as described above. The lysed cells were then incubated in 2 μM Z-Leu-Leu-[^{125}I]Tyr-CHN_2 for 1 h at 37°C. The insoluble material was pelleted by centrifugation and the soluble protein precipitated with TCA. Both the samples were analyzed by SDS-PAGE and autoradiography. The autoradiograph showed no labeling of cathepsin Z in either fraction.

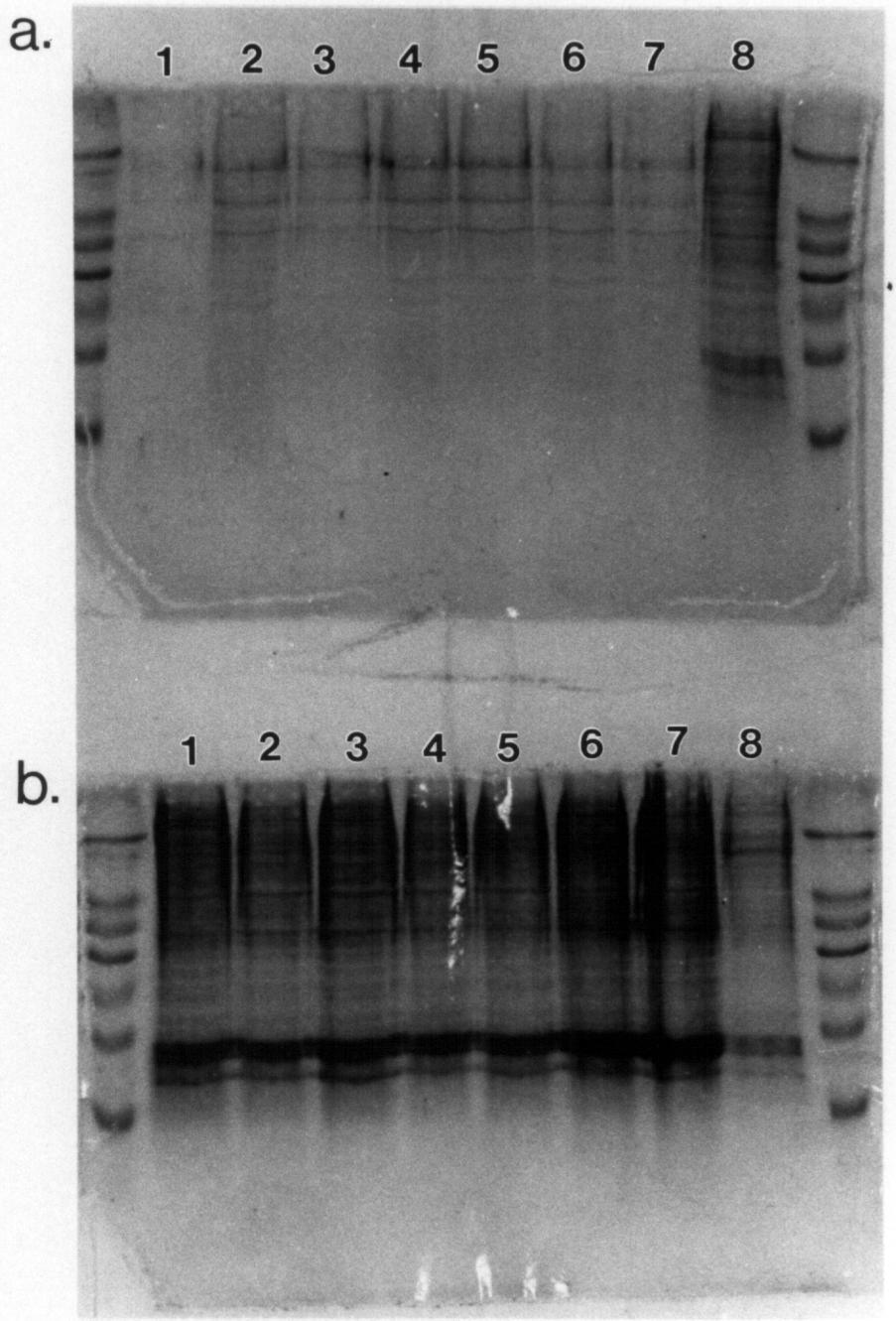
A fraction enriched for lysosomes was isolated from 2 x 10^9 U-937 cells. A postnuclear supernatant (PNS) was prepared following the method of (Storrie & Madden 1990). The PNS was centrifuged at 11,000 x g for 30 min at 4°C. The pellet should include mitochondria and lysosomes. Both intact and lysed lysosomes were incubated in Z-Leu-Leu-[^{125}I]Tyr-CHN_2 for 1 h at 37°C. Lysosomes were lysed by pre-incubating them in 1% Triton for 45 min at 37°C. The samples were analyzed by SDS-PAGE and autoradiography. The autoradiograph showed no labeling of cathepsin Z in either intact or lysed lysosomes. CP31, however, did label with the inhibitor in intact and, to a lesser extent, in lysed lysosomes (data not shown).

References

Storrie, B., and Madden, E.A. 1990. Isolation of subcellular organelles. Meth. Enzymol. **182**: 203-225.

Figure A.4 Solubilization of proteins from U-937 cells.

Proteins from U-937 cells proved difficult to solubilize. U-937 cells were suspended in 20 mM sodium acetate, pH 5.5, 1 mM EDTA, 4 mM DTT, in the presence or absence of non-ionic detergent. The samples were vortexed and incubated for 1 h at 37°C. One sample lacking detergent was subjected to 3 cycles of freezing and thawing. The samples were pelleted, and the supernatants removed. The protein in the both the pellets and the supernatants was precipitated with TCA and analyzed by SDS-PAGE. Gel a: the protein from the supernatants; Gel b: protein from the pellets. Lane 1: No detergent; Lane 2: 0.5% Tween 20; Lane 3: 0.1% Tween 20; Lane 4: 1% NP-40; Lane 5: 0.5% Tween 20; Lane 6: 1% Triton 100-X; Lane 7: 0.5% Triton X-100; Lane 8: cells that were frozen and thawed.


a.
1 2 3 4 5 6 7 8
b.
1 2 3 4 5 6 7 8

Appendix 5: Ideas for the Purification of Native Cathepsin Z

It should be possible to purify native cathepsin Z from U-937 cells by methods similar to those used to purify reduced cathepsin Z. There are no reducing steps in the purification of cathepsin Z prior to preparatory electrophoresis. When U-937 cells that had been labeled with Fmoc-Leu-Leu-[^{125}I]Tyr-CHN_2 were suspended in sample buffer that did not contain β-mercaptoethanol and analyzed by SDS-PAGE and autoradiography, cathepsin Z ran as a single labeled band of ~46 kDa. If glycoproteins purified by concanavalin A affinity chromatography are resuspended in sample buffer without β-mercaptoethanol and separated by preparatory electrophoresis, it should be possible to obtain purified native cathepsin Z. The percentage acrylamide of the separating gel would have to be optimized for separating proteins with molecular weights of ~45−47 kDa. As with CP31, it would be necessary to confirm the purity of the native cathepsin Z with N-terminal sequence analysis.

Vita

Kristi Roberts DeCourcy was born in Roanoke, Virginia, in 1955. She attended St. John's College, Annapolis, Maryland, and received her Bachelor of Arts in Biology from the State University of New York, Purchase, New York, in 1980. She worked as a laboratory specialist in the Department of Microbiology at the University of Virginia, Charlottesville, Virginia, for five years, and then resumed her studies in the Department of Biology at Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Under the guidance of Dr. T.A. Jenssen, she investigated the behavior of the green anole *Anolis carolinensis*. She received her Master of Science in Biology in 1991. The same year, she entered the doctoral program in the Department of Biochemistry at Virginia Polytechnic Institute and State University, and did her research under the sponsorship of Dr. R.W. Mason, investigating a cysteine proteinase found in human cells. She received her Doctor of Philosophy in Biochemistry and Anaerobic Microbiology in 1995. Kristi DeCourcy married Jacob E. Waller, Jr., in 1989. They reside in Christiansburg, Virginia.