Development and Application of New Tools for Studying Cathepsin E and D in MHC II Pathway

Entwicklung und Anwendung neuer Verfahren zur Untersuchung der Beteiligung von Cathepsin E und D am MHC II-Stoffwechsel

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To my family for letting me pursue my dreams

TABLE OF CONTENTS

Abbreviations **1**
Preface **3**

Chapter 1. General introduction **5**
1.1 Overview of cathepsin E 8
1.1.1 Tissue distribution and subcellular localization 9
1.1.2 Activity and specificity 9
1.1.2.1 Active sites 9
1.1.2.2 Specificity 10
1.1.2.3 Assays 10
1.1.2.4 Inhibitors 11
1.1.3 Structural chemistry 12
1.1.4 Regulation of gene expression 12
1.1.5 Processing, maturation and intracellular trafficking of cathepsin E 13
1.1.5.1 Role of propeptide in processing and maturation 13
1.1.5.2 Significance of catalytic activity of CatE for its acid-dependent auto-activation 14
1.1.5.3 ER-retention sequence in maturation and intracellular targeting of cathepsin E 14
1.1.5.4 Role of N-glycosylation of CatE in processing, maturation and intracellular targeting 14
1.1.6 Physiological roles of cathepsin E 15
1.1.6.1 Pathological conditions developing in CatE-deficient mice 15
1.1.6.2 Pathological conditions in which over-expression of CatE is observed 15
1.1.6.3 Potential role in the MHC class II pathway 15
1.2 Overview of cathepsin D 18
1.2.1 Activity and specificity 18

1.2.1.1 Active sites *18*

1.2.1.2 Specificity *18*

1.2.1.3 Assays *18*

1.2.1.4 Inhibitors *19*

1.2.2 Structural chemistry 19

1.2.2.1 Primary structure and proteolytic processing *19*

1.2.2.2 Crystal structure *19*

1.2.3 Regulation of gene expression 20

1.2.4 Processing, maturation and intracellular trafficking of CatD 21

1.2.4.1 Role of glycosylation in intracellular trafficking of CatD *21*

1.2.4.2 Role of CatD-propeptide in lysosomal sorting of CatD *22*

1.2.4.3 Role of catalytic activity and auto-activation of CatD in its maturation *22*

1.2.4.4 Putative β-structure on the surface of Pro-CatD: role in proteolytic maturation of CatD *23*

1.2.5 Physiological roles of cathepsin D 24

1.2.5.1 Pathological conditions developing in CatD-deficient mice *24*

1.2.5.2 Pathological conditions in which over-expression of CatD is observed *24*

1.2.5.3 Potential role in the MHC class II pathway *24*

1.3 Aims of the thesis 27

Chapter 2. A new approach for distinguishing cathepsin E and D activity in antigen processing organelles **29**

2.1 Abstract 29

2.2 Introduction 30

2.3 Experimental procedures 32

2.3.1 Enzymes and chemicals 32

2.3.2 Generation and immobilization of a monospecific CatE antibody 32

2.3.3 ELISA 33

2.3.4 Cell culture 34

2.3.5 Determination of mRNA expression levels for CatE using RT-PCR 34

2.3.6 Subcellular fractionation and Western blot analysis 35

2.3.7 Detection of NAG (N-acetyl-β-D-glucosaminidase) activity 36

2.3.8 Parallel detection of CatE and CatD activity 37

2.3.9 Analytical RP-HPLC 37

2.3.10 MALDI-MS 38

2.4 Results and Discussion 39

2.4.1 Expression of cathepsin E mRNA in different cell lines 39

2.4.2 Determination of antibody specificity 39

2.4.3 Characterization of subcellular fractions 43

2.4.4 Western blot analysis of subcellular fractions from different cell lines used for CatE and CatD determination 43

2.4.5 Specific inhibition of CatE by immunoprecipitation 44

2.4.6 Activity of Cat E and CatD in subcellular fractions of different cell types 45

2.4.7 Analysis of peptide fragments obtained by digestion of the fluorogenic substrate with subcellular fractions, CatE or CatD, using RP-HPLC and MALDI-MS 46

Chapter 3. A novel cell penetrating aspartic protease inhibitor blocks processing and presentation of tetanus toxoid more efficiently than pepstatin A 53

3.1 Abstract 53

3.2 Introduction 54

3.3 Experimental procedures 56

3.3.1 Enzymes and chemicals 56

3.3.2 Synthesis of pepstatin-CPP conjugates 56

3.3.3 Proteinase assay for determination of IC_{50} values for PepA and PepA-CPP conjugates against CatE and CatD 57

3.3.4 Cell culture 57

3.3.5 Cellular uptake of PepA and PepA-CPP conjugates 58

3.3.6 Cell viability assay 58

3.3.7 T cell proliferation assay 59

3.4 Results 59

3.4.1 Synthesis of pepstatin derivatives 59

3.4.2 Inhibitory effect of pepstatin A-CPP conjugates on the enzymatic activity of CatE and CatD 60

3.4.3 Comparative evaluation of cellular uptake of pepstatin A-CPP conjugates 61

3.4.4 Cell viability assay 62

3.4.5 PepA-P reduces tetanus toxoid C-fragment (TTC) specific T cell activation 62

3.5 Discussion 69

Chapter 4. Recombinant cathepsin E has no proteolytic activity at neutral pH 71

4.1 Abstract 71

4.2 Introduction 72

4.3 Experimental procedures 74

4.3.1 Materials 74

4.3.2 Solid-phase peptide synthesis 74

4.3.3 Digestion of peptides and analysis of digestion products using RP-HPLC 75

4.3.4 MALDI-MS 75

4.3.5 FRET-based assay for the determination of cathepsin E cleavage activity 76

4.4 Results and Discussion 76

Chapter 5. Substrate profiling of cathepsin E and D 81

5.1 Abstract 81

5.2 Introduction 82

5.3 Experimental procedures 84

5.3.1 Enzymes and chemicals 84

5.3.2 Solid-phase peptide synthesis 84

5.3.3 Digestion of peptides and analysis of digestion products 85

5.3.4 MALDI-MS 85

5.4 Results and discussion 86

5.4.1 Synthesis of peptide libraries 86

5.4.2 Screening of the peptide libraries for the selection of preferable substrates for cathepsin E or D 89

5.4.3 Effect of incubation time, enzyme concentration and pH and on hydrolysis of selected substrates by cathepsin E and D 94

5.4.4 FRET labeling of the selected substrates 97

Chapter 6. Key findings, conclusions and future perspectives **99**

6.1 Key findings and conclusions 100

6.2 Future perspectives 102

Bibliography **103**

Publications and poster presentations **111**

Liste der akademischen Lehrer **113**

Lebenslauf **114**

Abbreviations

ABTS	Azinodiethyl-benzthiazoline-sulfonate
ACN	Acetonitrile
APC	Antigen presenting cells
BSA	Bovine serum albumin
CatD	Cathepsin D
CatE	Cathepsin E
CF	Cytosolic fraction
CI	Competitive inhibition
CIITA	(MHC) Class II transactivator
CIIV	Class II vesicles
CPP	Cell penetrating peptide
ddH_2O	Distilled and deionized water
DC	Dendritic cells
DMSO	Dimethylsulfoxide
Dnp	Dinitrophenyl
EF	Endosomal fraction
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
Fmoc	N-(9-Fluorenyl) methoxycarbonyl
FRET	Fluorescence resonance energy transfer
HOBt	1-Hydroxybenzotriazol
IC_{50}	Inhibitory concentration (50%)
Ii	Invariant chain
IP	Immunoprecipitation
LF	Lysosomal fraction
LPS	Lipopolysaccharide
MALDI	Matrix-assisted laser desorption ionisation
Mca	(7-methoxycoumarin-4-yl)acetyl
MHC	Major histocompatibility complex
MS	Mass spectrometry
NAG	N-acetyl-β-D-glucosaminidase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PepA	Pepstatin A
RP	Reversed Phase
RP-HPLC	Reversed phase-high performance liquid chromatography
TAPA	Total aspartic proteinase activity
TBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
tBu	Tert.-butyl
TFA	Trifluoroacetic acid

Tris	Tris(hydroxymethyl)aminomethane
TTC	Tetanus toxoid C-fragment
UV	Ultraviolet

For peptide sequences the one- or three-letter amino acid code was used. SI units and standard abbreviations are not explained in the abbreviation list. Figures and tables are numbered for each chapter separately. If not otherwise stated, the mentioned figure and table numbers refer to the figures and tables in the same chapter

.

Preface

Cathepsin E and D are the major aspartic proteases of endolysosomal pathway. The main focus of this PhD thesis was to study biochemical properties and roles of these proteases in MHC class II pathway. To achieve these goals different new tools were designed and characterized because existing ones i.e. the inhibitors or substrates were not able to discriminate between cathepsin E and D. In order to give this PhD dissertation a more structured and intelligible contour it is divided into various chapters. This format should facilitate the readers who are interested in a specific part of the work to find all relevant information easily.

Chapter 1 of this PhD thesis provides the background for the understanding of the work that follows. This chapter has been partly published as a mini-review in *BBRC (2008), 367, 517-522.*

One of the main technical hitches in studying the role and biochemical properties of cathepsin E and D is the lack of specific substrates for either cathepsin. The two enzymes have similar enzymatic properties and it is difficult to distinguish between their activities. **Chapter 2** describes the development of a new assay to differentiate between the activities of cathepsin E and D in endosomal and lysosomal fractions from different cell lines. This chapter has also been published in *The FEBS Journal (2007), 125, 392-9.*

Selective inhibition of enzymes is a valuable tool for investigating their physiological functions. However, aspartic protease inhibitors, including the highly potent pepstatin A (PepA), are inefficiently transported across the cell membrane and thus have limited access to antigen processing compartments. **Chapter 3** describes the synthesis and usefulness of our new cell penetrating inhibitors of aspartic proteases that were synthesized by coupling of PepA to most frequently used cell-penetrating peptides. In this chapter involvement of aspartic proteases in antigen processing pathway is also reported. This chapter has been published in *BBRC (2007), 14;364(2):243-9.*

Isolated cathepsin E is normally most active at acidic pH, but according to some previous reports its activity has also been observed at neutral pH. **Chapter 4** of this thesis deals with this interesting phenomenon. In this study proteolytic potential of recombinant cathepsin E was examined and it was found that recombinant CatE does not have any proteolytic activity at

neutral pH even in the presence of ATP that is known to stabilize this enzyme. This chapter is published in *BBRC (2007), 17;360(1):51-5*.

To deal with the problem of unavailability of specific substrate for cathepsin E and D in a more detailed and comprehensive way, substrate profiling of the two enzymes was performed. This study is described in **Chapter 5**.

The last chapter **(Chapter 6)** summarizes the key findings and conclusion of this PhD dissertation and also gives some future perspectives for research in this field.

All the chapters in this dissertation represent the state-of-the-art when the respective manuscripts were prepared for publication and have been supplemented with the most relevant recent findings.

1

1. General introduction

A related manuscript has been published in

Biochem Biophys Res Commun. 367, 517-522 (2008)

Cathepsins represent a growing family of endocytic proteases with different substrate specificity and tissue distribution. The term “cathepsin” was first introduced in the 1920s . However, the earliest record on "cathepsin" found in *PubMed* dates back to 1934 in the *Journal of Biological Chemistry* [1]. The term “cathepsin” stands for “endosomal/lysosomal proteolytic enzyme”, regardless of the enzyme class. Cathepsins include cysteine proteases, aspartic proteases and serine proteases and are involved in a number of important biological processes such as intracellular protein turnover, immune response and antigen processing, proprotein and hormone activation, remodeling of extracellular matrix and apoptosis [2-5].

Antigen presentation *via* major histocompatibility complex class II (MHC II) is tightly linked with the proteases residing within the endocytic pathway. These proteases participate in two main events in antigen presentation; first, processing of exogenous antigens into small antigenic peptides and second invariant chain (Ii) processing, as the antigen binding groove of class II molecules is blocked by Ii at the time of synthesis, and MHC II molecules can bind to antigenic peptides only after Ii has been degraded [6-9]. Cathepsins which constitute a major portion of this proteolytic system have been found to have essential roles in both, antigen processing and maturation of MHC II molecules.

Role of cysteine cathepsins in MHC class II pathway has been more extensively studied and it is reported that cathepsin B, C, X, H, L and S have a clear function in the immune system, with cathepsins L and S being the only ones with non-redundant roles [10].

Involvement of aspartic proteases in MHC II pathway is also reported. Cathepsin E (CatE, E.C. 3.4.23.34) and cathepsin D (CatD, E.C. 3.4.23.5) are two major endolysosomal aspartic proteases and both of these enzymes have been found to play different roles in the MHC class II pathway [11-15]. Cathepsin E and cathepsin D show significant sequence homology and share similar enzymatic properties [16, 17]. However, their different tissue distribution and cellular localization strongly suggest that both enzymes might have different physiological functions. **Table 1** depicts differences and similarities between the two enzymes. In the following sections of this introductory chapter the important features, regulation, biological

aspects and especially possible roles of cathepsin E and D (in MCH II pathway) will be discussed. Furthermore, the principal aims of the presented study are also illustrated.

Table 1. Similarities and differences between cathepsin E and cathepsin D

Characteristics	Cathepsin D	Cathepsin E
pH Range	2-5 [18-20] (pH optimum is around pH 4.0) [19].	1-5.5 [19-21] (pH optimum is around pH 4.0 [19]). CatE is also reported to restore its activity by ATP at pH values above 5.8 [22]. Few studies have reported different cleavage specificity of CatE at neutral pH values [23, 24].However, we have recently reported that recombinant CatE does not exhibit any proteolytic activity at neutral pH and is not stabilized by ATP.
Susceptibility to inhibitors	Pepstatin A	Pepstatin A, *Ascaris* pepsin inhibitor [25, 26]
Substrate Affinity	Prefers aromatic amino acid at P1 and P1´ positions [20], leucine is strongly favored at P1, the hydrophobic requirements are less strict at P2 and P1´ [27]. A charged preferably basic residue is found mostly at positions P2´and P5´; at least 1 basic residue appears to be required by CatD at either position [27].	P1 and P1` positions must be occupied with hydrophobic amino acids with aromatic or aliphatic side chains. Val and Ile residues are not allowed at P1. Position P2´accepts a broad range of amino acids, including charged and polar ones [28]. The presence of proline at the P4 might be important [17].Few studies reported a shift in cleavage specificity at neutral pH with special preference for Arg-Arg bonds [23].However, we have recently reported that recombinant enzyme does not show any proteolytic activity at neutral pH.
Cellular distribution	Widely distributed in almost all mammalian cells [29-32].	Mainly present in cells of the immune system including gastric epithelial cells [33], antigen presenting cells such as lymphocytes [29], microglia [34] and dendritic cells [12]. Not present in resting B-lymphocytes [35].

Table 1 continued.

Characteristics	Cathepsin D	Cathepsin E
Subcellular localization	Lysosome [29-32].	Mature enzyme is localized in endosomes [34]. CatE is also reported to be localized in plasma membranes [36], endoplasmic reticulum and Golgi apparatus [30, 34, 37].
Disease Association		
Pathological condition developed in mice-deficient in CatD/CatE.	Massive intestinal necrosis [38], thromboembolia [38], lymphop-enia [38], and neuronal ceroid lipofuscinosis [39].	Atopic dermatitis like skin lesions [43], increased susceptibility to bacterial infections associated with decreased expression of multiple surface Toll like receptors [44], lysosomal storage disorder [45].
Over expression of the enzyme observed	Prostrate [40], breast [41] and ovarian cancer [42].	Pancreatic ductal adenocarcinoma [46].

1.1 Overview of cathepsin E

Cathepsin E (CatE, EC 3.4.23.34) is an intracellular aspartic protease of the pepsin superfamily. CatE is highly homologous to the analogous aspartic protease cathepsin D. Early reports implicated the presence of an aspartic protease distinct from cathepsin D in vertebrate cells [47]. Different designations for this enzyme have been suggested, such as ***cathepsin D-like proteinase*** [48, 49], ***gastric mucosa non-pepsin acid proteinase*** [50], ***slow moving proteinase*** [51] and ***erythrocyte membrane acid proteinase*** [52]. Later, it was shown that all of these activities were mediated by the same enzyme, which was termed cathepsin E (reviewed in [47]).

In this section the findings regarding the expression, biochemical properties and functions of cathepsin E are summarized and the discrepancies and similarities in the conclusions drawn by different groups are highlighted.

1.1.1 Tissue distribution and subcellular localization

Cathepsin E is mainly present in cells of the immune system, including antigen-presenting cells (APC) such as lymphocytes [29], microglia [34], dendritic cells [12], Langerhans cells [53], interdigitating reticulum cells [53] and human M cells [54]. Cathepsin E is not present in resting B-lymphocytes but is up-regulated late in human B cell activation at both, the mRNA and protein level [35]. It has also been detected in gastric epithelial cells [48] and osteoclasts [36]. Furthermore, tissue-specific distribution of CatE is not the same in different mammalian species, e.g. it has been detected in red blood cells from humans and rats, but not guinea pigs, cattle, goats or pigs [55].

The intracellular localization of cathepsin E also appears to vary according to cell type. In APC, such as microglia [56], dendritic cells [12] and macrophages, CatE is mainly present in endosomal compartments. In contrast, in erythrocytes [52, 57, 58], gastric cells [30, 48, 49, 51], renal proximal tubule cells [30], and osteoclasts [36], it is found in the plasma membrane. CatE is also detected in ER and Golgi complex in different cell types, such as gastric epithelial cells [30, 51], Langerhans cells, interdigitating reticulum cells [53] and human M cells [54].

1.1.2 Activity and specificity

1.1.2.1 Active sites

CatE possesses two homologous domains, each containing the highly conserved tripeptide sequence DTG. These two domains are involved in the formation of the active site [59-61]. There is a high degree of similarity between eukaryotic members of the aspartic protease family, and their DTG sequence domains are virtually identical. The DTG sequence is present in all species with the exception of rabbit CatE, in which the tripeptide sequence near the N-terminal region is replaced by DTV [62]. Liu *et al* synthesized active site mutants of CatE by site-directed mutagenesis to examine the significance of these residues [63]. They found that in mouse CatE, Asp98, Asp283, and Thr284 are indeed critical for catalysis [63].

1.1.2.2 Specificity

Cathepsin E, like cathepsin D, prefers hydrophobic amino acids at the P1 and P1` positions [47]. β-Branched residues, e.g. Val and Ile, are not allowed at P1 [28]. Position P2´ accepts a broad range of amino acids, including charged and polar ones [28]. For CatE, the basic residue e.g. Lys is acceptable at position P2, which is not the case for CatD [64]. The presence of proline at P4 might be important [17].

According to some reports, CatE also retains activity at neutral pH and shows a distinct cleavage specificity [23, 24]. In one of these reports [24], proteolytic activity and cleavage specificity of CatE towards the B chain of oxidized insulin was examined. It was reported that the cleavage specificity changed significantly, with more specific cleavage at pH 7.4 and above, as compared to pH 5.5 and 3.0. At acidic pH, several peptide bonds, especially Phe-X, Tyr-X and Leu-X were cleaved, whereas at pH 7.4 the Glu13-Ala14 bond was selectively cleaved. In a more recent study [23], preferential cleavage of Arg-X and Glu-X bonds at pH 7.4 was reported, with the Arg-Arg bond to be the preferred cleavage site. However, in all studies reporting proteolytic activity of CatE at pH 7.4, the enzyme was isolated either from human gastric mucosa [22-24] or human red blood cells [22]. In our recent study, we investigated proteolytic potential of recombinant cathepsin at neutral pH [65]. Our results fail to reveal any proteolytic activity of recombinant CatE at neutral pH even in the presence of ATP, which is known to stabilize this enzyme (for details see **chapter 4**).

1.1.2.3 Assays

The most common assay for measuring aspartic protease activity in biological samples employs acid-denatured bovine hemoglobin as substrate [52, 66, 67]. Enzymatic reaction liberates trichloroacetic acid-soluble products from hemoglobin which are detected by their absorbance at 280 nm and by the Folin reaction. This method is time-consuming and has the additional disadvantage that it cannot discriminate CatE activity from other aspartic proteases.

Several synthetic chromogenic or fluorogenic substrates have been developed to measure CatE activity [19, 68-70]. These methods are simple and fast, but the described substrates are restricted in their selectivity. Recently, a new selective substrate for cathepsin E based on the

cleavage site sequence of α2-macroglobulin has been described [20]. However, this substrate was also not exclusive for CatE because it had low level of activity for CatD and pepsin as well.

Recently, we have developed a new approach for distinguishing cathepsin E and D activity in subcellular fractions [71]. In this method we made use of a new monospecific CatE antibody and substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 [where Mca is (7-methoxycoumarin-4-yl)acetyl and Dnp is dinitrophenyl]. This substrate is digested by both, cathepsin E and cathepsin D and therefore can be used to detect the total aspartic proteinase activity (TAPA) in biological samples [19]. CatE is depleted from the samples by immunoprecipitation using this new highly specific antibody and the remaining activity is therefore due to CatD (for details see **chapter 2**). The decrease of activity can thus be assigned to CatE. For a schematic representation of the assay, see Figure 2 of chapter 2.

1.1.2.4 Inhibitors

The most widely used and potent inhibitor of CatE is pepstatin A (PepA). Additionally, a wide variety of synthetic peptidomimetic inhibitors has been described that mediate potent CatE inhibition [72, 73]. However, none of these small molecule inhibitors discriminates clearly between cathepsin D and E. A specific inhibitor for CatE, the *Ascaris* pepsin inhibitor, which has no activity against cathepsin D, has been described [26], but is not readily available in quantities sufficient for functional studies because of difficulties in purification. Furthermore, the recombinant inhibitor may have a slightly different inhibitory profile [74].

Although pepstatin A is not specific towards CatE or CatD, it is widely used in cell-based studies aimed at understanding the function of these enzymes; using cells from CatD-deficient mice allows pepstatin to specifically target CatE. However, another limitation of PepA for cell-based studies is that it is inefficiently transported across the cell membrane [75]. To address this problem mannose-pepstatin conjugates were recently developed as targeted inhibitors of antigen processing [12, 75]. These mannosylated conjugates showed higher solubility than pepstatin in water and were efficiently incorporated into the cells via receptor-mediated uptake. This approach is obviously limited to cells carrying mannose receptors.

Our group has synthesized new cell permeable aspartic protease inhibitors that are not specific for any particular cell type. To achieve this, the most frequently employed cell penetrating peptide, namely pAntp(43-58) (penetratin), Tat(49-60), and 9-mer of L-arginine (R9), were synthesized. Pepstatin A was then coupled to these peptides. We found that the bioconjugate PepA-penetratin (PepA-P) was the most efficient cell-permeable aspartic protease inhibitor (for details see **chapter 3**) [76].

1.1.3 Structural chemistry

The crystal structure of CatE has not been solved so far. It is known that the amino terminal portion contains a Cys residue at position 43, which is responsible for disulphide bond formation between the two identical subunits [77]. The homodimeric form is easily converted into a monomeric form exhibiting full catalytic activity under reducing conditions [60, 78]. Additionally, as mentioned above, a highly conserved tripeptide sequence DTG is found in CatE from all species except rabbit [59-62]. Based on available information on CatE and the crystal structures of other aspartic proteases, Kuo-Chen Chou has predicted a three dimensional structure of CatE [79]. Ostermann *et al* have crystallized and solved the structure of an activation intermediate of CatE [80]. They reported that the overall structure resembles intermediate 2 in the proposed activation pathway of aspartic proteases like pepsin C and cathepsin D [80]. The pro-sequence is cleaved from the protease and remains stably attached to the mature enzyme by forming the outermost sixth strand of the interdomain β-sheet. Furthermore, the pro-sequence remains attached to the mature enzyme and the primed binding site is in a closed conformation [80].

1.1.4 Regulation of gene expression

Cathepsin E has a limited tissue distribution. It is apparent that there must be a mechanism that regulates the transcription of the gene encoding CatE so that expression is facilitated only in certain types of cells. The promoter region flanking the procathepsin E gene from human [81] and mouse [61] do not contain a TATA box. Instead, both sequences contain an initiator element [61, 81] which offers an alternative binding site for TFIID (transcription factor IID) to initiate gene transcription [82]. Cook *et al* have reported that regulation of human and mouse

procathepsin E gene expression is not influenced by CpG methylation [83]. Furthermore, transcription of the CatE gene is dependent on the balance between the effects produced by positive-acting tissue-specific transcription factors such as GATA1 and PU1 and the negative influence of the ubiquitous factor YY1 [83].

Additionally, it is also known that CatE transcription is negatively regulated by CIITA (Class II transactivator), a non-DNA-binding transcription factor [84]. The significance of regulation of CatE gene expression by this transcription factor will be discussed later in this chapter.

1.1.5 Processing, maturation and intracellular trafficking of cathepsin E

Like many other aspartic proteases, CatE is synthesized as a zymogen which is catalytically inactive towards its natural substrates at neutral pH and which auto-activates in an acidic environment to generate the mature enzyme *via* an intermediate. The processing events of pro-cathepsin E include the removal of propeptide, glycosylation and formation of a disulphide bond between two cysteine residues at the NH_2-terminal region to yield a homodimer. CatE is synthesized as a 46 kDa precursor and is later converted into the 42 kDa mature form. In this section, we will discuss the molecular basis of processing and intracellular trafficking of CatE.

1.1.5.1 Role of propeptide in processing and maturation

CatE is synthesized as an inactive precursor and is activated by proteolytic removal of N-terminal propeptide; the process is triggered by acidic pH. The unique structural characteristics of propeptide are conserved in all species [59, 60, 85, 86]. The propeptide is composed of 40 amino acid residues and is highly positively charged. Therefore, it is hypothesized that the propeptide is bound to active CatE mainly through electrostatic interactions.

It has been reported that the propeptide of CatE plays an important role in the correct folding, maturation and targeting of this protein to its final destination [87]. Tsukuba *et al*. have constructed CatE mutants lacking the propeptide (Leu23-Phe58). They reported that this mutant protein was neither processed not matured and was found mostly in the ER, in comparison to the wild type which was mainly located in endosomes [88].

1.1.5.2 Significance of catalytic activity of CatE for its acid-dependent auto-activation

Procathepsin E is readily auto-activated upon brief incubation at an acidic pH and transforms to the mature form. The active site residues (Asp98, Asp283, and Thr284) are critical for catalysis, as mentioned before in this review [63]. Tsukuba *et al* published that, active site mutants of CatE, in which one or both aspartic acid residues were substituted with alanine, not only lacked catalytic activity, but were also unable to mature, thus remain stably as a 46 kDa precursor [88]. This indicates that catalytic activity is essential for the processing and maturation of this enzyme.

1.1.5.3 ER-retention sequence in maturation and intracellular targeting of cathepsin E

Finley and Kornfeld reported that amino acids 1-48 of mature cathepsin E are important for its retention in the ER [89]. Tsukuba *et al* constructed a mutant lacking most of this putative ER-retention sequence, which was not converted into a mature form of the enzyme, was rapidly degraded in the cells and could not be detected in the endosomes [88]. This indicates that the putative ER-retention sequence is required for correct folding, processing, maturation and targeting of CatE to endosomes.

1.1.5.4 Role of N-glycosylation of CatE in processing, maturation and intracellular targeting

CatE is known to be N-glycosylated with high-mannose and/or complex oligosaccharides in the native state. In APC such as microglia and macrophages, the mature form of CatE is localized in endosomal compartments and is N-glycosylated mainly with complex-type oligosaccharides [34]. CatE from erythrocyte membranes (human and rat) [58] and thymocytes (rat) [90] is also N-glycosylated with complex-type oligosaccharides, while the enzyme from the spleen (rat) [59] and stomach (rat) [91] has high mannose-type oligosaccharides. This is interesting in view of the fact that CatE shows different subcellular localization in different cell types, as discussed previously in this review, and it has been suggested that the nature of its oligosaccharide chains may be cell-specific or may vary with its cellular localization.

Yasuda *et al* constructed an N-glycosylation mutant (by changing the N residue to Q and D at position 73 and 305 in potential glycosylation sites of rat CatE) [92]. It was found that this

mutant was less stable to temperature and pH than glycosylated CatE, although its catalytic properties were equivalent to wild type. Tsukuba *et al* reported that N-glycosylation mutants were stably retained in cells but were not processed to the mature form and were exclusively confined to the ER [88]. Hence, N-glycosylation of CatE seems to play an important role in processing, maturation and trafficking of CatE to its final destination, but might not be essential for correct folding of the enzyme.

1.1.6 Physiological roles of cathepsin E

1.1.6.1 Pathological conditions developing in CatE-deficient mice

CatE-deficient mice are found to develop atopic dermatitis-like skin lesions [43]. It was reported that they also show an increased susceptibility to bacterial infection associated with decreased expression of multiple cell surface Toll-like receptors [44]. According to a recent study [45], CatE deficiency induces a novel form of lysosomal storage disorder characterized by accumulation of lysosomal membrane sialoglycoproteins and the elevation of lysosomal pH in macrophages.

1.1.6.2 Pathological conditions in which over-expression of CatE is observed

CatE is expressed in pancreatic ductal adenocarcinoma [77], and its presence in pancreatic juice may be a diagnostic marker for this cancer [93]. Increased levels of CatE in neurons and glial cells of aged rats are suggested to be related to neuronal degeneration and reactivation of glial cells during the normal aging process of the brain [94].

1.1.6.3 Potential role in the MHC class II pathway

Several lines of evidence suggest that cathepsin E plays a role in antigen processing *via* the MHC class II pathway. CatE is detectable in APC such as Langerhans and interdigitating dendritic cells [53] and its expression is up-regulated on activation of human B cells and B cell lines [35]. Moreover, a peptide derived from CatE was found to be associated with HLA-DR molecules in human EBV-transformed B cells [95], suggesting that it is found at the site of assembly of peptides and MHC class II complexes.

On the basis of studies utilizing specific inhibitors and cathepsin-deficient mice, CatE has been implicated in antigen processing. The first study to report involvement of cathepsin E in antigen processing was carried out by Bennett *et al* [13]. This study made use of a CatE-specific *Ascaris* inhibitor and demonstrated that CatE is essential for processing of ovalbumin by murine B cells, whereas, presentation of synthetic OVA-derived peptides corresponding to the epitope was not affected by the specific CatE inhibitor. Similar results were obtained when the role of CatE in antigen presentation by microglia [56] and dendritic cells [12] derived from CatD-deficient mice was examined. CatE was found to be involved in processing of ovalbumin but not in the presentation of OVA-derived peptides.

An immunological role for CatE in microglia was further supported by the observation that its expression at the RNA level is enhanced after treatment with IFN-γ while expression of CatD, CatB, CatL and CatS remains constant [56]. Microglia also show strong pepstatin A-sensitive antigen presenting ability for both, native OVA and OVA-peptides only after IFN-γ treatment. Because IFN-γ enhances only CatE expression, it can be suggested that CatE plays a major role in antigen processing. IFN-γ-activated peripheral macrophages also showed similar results [56]. The association of CatE expression and activity with IFN-γ treatment in microglia indicates the involvement of CatE in the MHC class II pathway, because IFN-γ is known to induce MHC class II expression as well as co-stimulatory molecules required for T-helper cell activation [96, 97]. Moreover, in microglia and dendritic cells, CatE is present in the early endosomes and partially in lysosomes while CatD has purely lysosomal localization [12, 56]. This indicates that localization of CatE is far more appropriate for processing of exogenous antigens than CatD, because exogenous antigens are first processed in early endosomes after endocytosis.

An interesting report by Yee *et al* [84] provided the first link between immune-specific regulation and Ag-processing by CatE. They observed that CatE expression is negatively regulated by the MHC class II transactivator (CIITA), a non-DNA-binding transcription factor which activates and is required for the expression of MHC II and other genes related to antigen presentation [98, 99]. CIITA is known to modulate immune responses also by repressing the transcription of other genes including IL4 [100], collagen α2 [101] and Fas ligand [102]. It was reported that CIITA-deficient murine and human B cells express higher amounts of CatE than

wild-type B cells, whereas cells overexpressing CIITA showed decreased CatE mRNA, protein and proteolytic activity [84]. This negative regulation of CatE expression by CIITA was very specific towards CatE and did not affect CatD. This regulation of CatE by CIITA might either inhibit or promote Ag-processing and presentation. If the latter, then production of antigenic epitopes by CatE could be down-regulated as part of an activation-induced shift from Ag-processing to Ag-presentation [84]. Alternatively, it is possible that CatE cleaves productive antigens, such as Ag-MHC complexes or chaperones, and therefore it is down-regulated by CIITA. This explanation is supported by the fact that CatE cleaves human α2-macroglubulin, a capture protein that promotes internalization and degradation of target proteins [103].

Most of the above-mentioned reports point towards the involvement of CatE in antigen processing but not in the presentation of the synthesized antigenic peptides [12, 13, 56]. Thus, the general conclusion from these findings is that inhibition of CatE does not alter the number of MHC molecules at the cell surface which are able to bind processed peptides, and that inhibition of CatE might also have no effect on invariant chain processing because if the Ii processing is blocked, loading of MHC molecules with processed peptides is also blocked [104].

However, there are several discrepant reports showing that aspartic protease inhibitors effectively prevent the processing of the invariant chain [105]. CatE could be involved in the initial cleavage of the invariant chain, in which an unidentified endosomal aspartic protease has been implicated [105, 106]. Although CatE is absent from the classical late endosomal class II loading compartments [12], which contain cathepsin S, and in which invariant chain processing is thought to take place, it is possible that the invariant chain traffics transiently *via* an early endosomal compartment *en route* to the class II-loading pathway.

1.2 Overview of cathepsin D

Cathepsin D (CatD, EC 3.4.23.5) is an intracellular aspartic protease of the pepsin superfamily. CatD is found in almost all mammalian cells and has a typical lysosomal localization [29, 30]. Early isolations of cathepsin D showed various different forms which differed in size, number of associated polypeptides and iso-electric point but, had similar activities [107]. Later on, site-directed covalent modifications demonstrated that CatD had a similar active centre as pepsin, confirming its identity as an aspartic protease [108, 109]. It was hypothesized that the two-chain forms were derived from the single polypeptide forms by proteolysis [110]. This is confirmed by the determination of N-terminal sequences of different forms of pig CatD [111]. Isolation of a cDNA clone [112] and of the gene [113], detailed studies of its biosynthesis [114], activation [115], substrate specificity [116] and determination of crystal structure [117, 118] makes cathepsin D one of the best studied lysosomal proteinases (reviewed in [119]).

1.2.1 Activity and specificity

1.2.1.1 Active Sites

Cathepsin D contains highly conserved active site residues (DTG) for the aspartic protease family located at residues 97-99 and 295-297 for human cathepsin D [79].

1.2.1.2 Specificity

Cathepsin D prefers aromatic amino acids at P1 and P1´ positions [20]. At P1, leucine is strongly favoured, at P2 and P1´ hydrophobic requirements are less strict [27]. A charged preferably basic residue is found mostly at P2´and P5´positions; at least 1 basic residue appears to be required by CatD at either position [27].

1.2.1.3 Assays

Conventional assays for measuring aspartic protease activity in biological samples employs acid-denatured bovine hemoglobin as substrate [52, 66, 67] . The most common assays for measuring aspartic protease activity and our new approach for distinguishing CatE and CatD activity are described in section 1.1.2.3 and **chapter 2** of this thesis.

1.2.1.4 Inhibitors

Cathepsin D is inhibited potently by pepstatin A. Synthetic peptides containing statine derivatives inhibit CatD to varying degrees but none of them has a Ki values as low as PepA [72, 120]. Several compounds screened for inhibition of HIV-1 protease also inhibit CatD [121]. Apart from that some non-peptide inhibitors have been identified, but they are also not as potent as PepA [122]. The specificity of these for CatD and other related aspartic proteases has not been carefully examined and is an important area for future work.

The limitations of using PepA in different studies and the efforts to overcome these limitations, by us and other groups have been discussed in section 1.1.2.4 and **chapter 3** of this thesis.

1.2.2 Structural chemistry

1.2.2.1 Primary structure and proteolytic processing

Human CatD is synthesized as a precursor comprising 412 amino acids [112] and is termed as "preprocathepsin D". The precursor contains an N-terminal secretion signal peptide which is 20 residues in human [123] and is cleaved during translocation across the membrane to generate an inactive procathepsin D. The procathepsin undergoes autocatalytic cleavage and eventually become mature double-chain form. The mature form is made up of N-terminal light chain of 14 kDa and C-terminal heavy chain of 34 kDa linked by non-covalent interactions [124].

Cathepsin D is a glycoprotein with two N-linked oligosaccharides, one each in the light and heavy chains. These oligosaccharides are normally modified by phosphorylation of sixth position of mannose during biosynthesis [125]. Glycosylation is not necessary for folding [126] or enzyme activity [127] but is required for targeting of the enzyme [127]. The multiplicity of CatD isoforms purified from tissue extracts can be explained by proteolytic processing and carbohydrate modifications which produce molecules of differing isoelectric points [119].

1.2.2.2 Crystal structure

Baldwin *et al* described crystal structure of human liver cathepsin D at 2.5Å resolution [117] and Metcalf *et al* reported the structure of human spleen CatD at 3.0 Å resolution [118]. These structures are in general agreement with each other and previous computationally derived

models of CatD based on the structures of other aspartic proteases [128]. Metcalf *et al* reported that two related 170 residue domains (mostly β sheet) lie on either side of the deep 30 Å long active site cleft [118]. Each domain contributes an active site aspartate at the centre of the cleft and each contains a single carbohydrate group and two disulphide bonds. The lysosomal targeting region of cathepsin D defined by previous expression studies [129] is located in well defined electron density on the surface of the molecules. This region includes the putative binding site of the *cis*-Golgi phosphotransferase which is responsible for the initial sorting step for soluble proteins destined for lysosomes by phosphorylating the carbohydrates on these molecules [118]. Carbohydrate density is visible at both expected positions on the cathepsin D molecules and, at the best defined position, four sugar residues extend towards the lysosomal targeting region [118].

1.2.3 Regulation of gene expression

Eukaryotic gene expression is controlled by both, proximal and distal elements, generally located in the 5` upstream region of the gene [130]. Many class II gene promoters contain a TATA box, which binds to the transcription factor IID and thus define the transcription initiation site for that gene. Genes having such promoters are termed as facultative or regulated genes. In contrast the promoter region of the house keeping genes lack a recognizable TATA box but contain multiple GC boxes which are putative binding sites for transcription factors Sp1 [131]. The promoter of cathepsin D has a mixed structure and it shows both of these features. Transcription of CatD is initiated at five major transcription sites (TSSI to –V) spanning 52 base pairs [132]. It has been shown that in human breast cancer cell lines, estrogens stimulate transcription of CatD [133]. The promoter of CatD directs two types of transcription initiations; TATA-dependent transcription starting about 28 bp downstream from the TATA box and TATA-independent transcription initiating at the other initiation sites upstream of the TATA box possibly directed by GC boxes and SP1 factor as in many housekeeping genes [132]. Estrogens stimulate TATA-dependent transcription and therefore not only increase transcription but also affect the pattern of initiation, with TATA-dependent transcription predominating. Therefore, in stimulated conditions, CatD mRNAs preferentially have short 5`-untranslated sequences, whereas in basal conditions where TATA-independent

transcription is dominant, the proportion of longer CatD mRNAs is increased. Since the CatD gene is controlled by a mixed promoter this gene has the advantage of being both constitutively expressed from TATA-independent start sites and overexpressed in some physiological conditions such as development or tissue modeling [132].

1.2.4 Processing, maturation and intracellular trafficking of CatD

Cathepsin D is synthesized on the rough endoplasmic reticulum as a pre-pro-enzyme that undergoes several proteolytic steps during biosynthesis to produce the mature form. Following initial co-translational removal of the signal peptide to generate the inactive pro-enzyme, sugars are attached at two N-linked glycosylation sites and the pro-enzyme is transported to Golgi apparatus. This 52 kDa pro-CatD is tagged for mannose-6-phospahate (M-6-P) receptors and is targeted to lysosomes. In lysosomal compartments, the peptide backbone of human pro-CatD undergoes a two-step maturation process: in the first step the pro-piece of 44 amino acids is removed to yield an active intermediate 48 kDa single-chain molecule. In the second step, this intermediate is further processed to generate double-chain mature form (reviewed in [119]). In this section these posttranslational modification and processing events and the factors affecting these events are discussed.

1.2.4.1 Role of glycosylation in intracellular trafficking of CatD

Normal cellular life of CatD is characterized by a special feature i.e. its strict localization within acidic lysosomal compartments. Transport of Pro-CatD from Golgi complex to downstream acidic compartments is mainly mediated by receptors specific for mannose-6-phosphate [134] groups and also by interaction of Pro-CatD with prosaposin or other unknown molecules [135].

Since glycosylation is essential in M-6-P pathway, N-linked oligosaccharides play an important role in the biosynthesis of pro-CatD. The two N-linked oligosaccharides (one each in the light and heavy chain) of CatD are normally modified by phosphorylation of sixth position of mannoses during biosynthesis [125]. CatD sequences from different species show substantial amino acid sequence identity, including conservation of the N-linked glycosylation site at residue 70 (human CatD numbering [112]). Human procathepsin D carries two N-linked

glycosylation sites at asparagine residues 70 and 199, widely separated on the surface of the folded protein. Fortenberry *et al* [126] created monoglycosylated procathepsin D molecules by site-directed mutagenesis of the individual glycosylation sites. The expressed proteins were stable, targeted to the lysosome, and partially secreted into the medium. But the mutant in which both glycosylation sites were eliminated the expressed proteins were stable but most were not secreted and targeted poorly to the lysosome [126] .

1.2.4.2 Role of CatD-propeptide in lysosomal sorting of CatD

Conner [136] studied the function of the propeptide of procathepsin D in sorting to the lysosome using a cathepsin D deletion mutant lacking the propeptide, and using a chimeric cDNA encoding the cathepsin D propeptide fused to the secretory protein alpha-lactalbumin. The deletion mutant was glycosylated but was rapidly degraded and did not acquire an active conformation. Thus, the propeptide appeared to be necessary for correct folding. The chimeric protein was glycosylated and secreted. The coincidence of complex oligosaccharide modification and secretion of the chimeric protein suggested that it was slowly released from the endoplasmic reticulum and rapidly passed through the cell to the extracellular compartment. This indicates that the propeptide is necessary for folding of cathepsin D but, was not sufficient to direct a secretory protein to the lysosomes. In a more recent study by Yasuda *etal* [87] it was reported that propeptide for CatD is essential for the correct folding, activation and delivery of the protein in lysosomes

1.2.4.3 Role of catalytic activity and auto-activation of CatD in its maturation

Once segregated into the lysosomal compartments, the pro-CatD undergoes several proteolytic processing events [119]. The general mechanism proposed for the processing and activation of the 52 kDa pro-CatD was a combination of partial auto-activation generating a 51 kDa pseudo-CatD which is followed by proteolytic cleavage by a lysosomal cysteine or aspartic protease generating a 48 kDa intermediate and finally a double-chain mature species [138]. In a recent study, it has been shown that cellular CatD is processed in a manner independent of its catalytic function and that auto-activation is not a required step [138]. Moreover, it is reported that

cysteine proteases are required for the maturation of CatD, specifically the enzymes cathepsin B and L are shown to be involved in the process [138].

1.2.4.4 Putative β-structure on the surface of Pro-CatD: Role in proteolytic maturation of CatD

The final processing step of CatD from single to two-chain form has also been extensively studied. Comparison of primary structure of CatD with other aspartic proteases demonstrates an insertion in the region that is cleaved to generate a two-chain form [119]. The two-chain enzyme lacks a short segment of the insertion that is excised following cleavage. Molecular modeling of this region suggests that it forms a putative β-structure (β-hairpin loop) on the surface of human pro-CatD and thus would be disposed to proteolysis in the lysosomes where the cleavage occurs [139]. Yonezawa *et al* have identified this sequence in the stretch P^{159}CQSASSASAL^{169} and suggested that seven (i.e. S^{162}ASSASA^{168}) of these 11 residues are removed during single to two-chain maturation process [139].

Previously this region was suggested to play a role in stabilization and targeting of the single-chain CatD to the processing compartments [139, 140]. The deletion of this region forming β-hairpin loop impaired the formation of the double-chain CatD and substantially affected the stability of the protein [141]. Therefore, it was not possible to study its folding and targeting to lysosomes. However, in a recent study by Follo *et al* a new approach was used to mutagenize the region of the amino acids comprising the β-hairpin loop [124]. These new mutants were unable to be converted into the two-chain form but they do not compromise the folding and stability of the protein. These mutants reach the lysosomes and are stable as single-chain polypeptide. Moreover, they bear high-mannose type sugars, bind to pepstatin and are enzymatically active [124]. Thus, in contrast to previous reports, this new study indicated that the mutagenesis of this proteolytic processing region did not affect the stability or the folding and enzymatic activity of CatD despite the structural impairment of single to double-chain processing [124]. Furthermore it shows that the processing loop does not contain signals for lysosomal targeting as suggested previously [139, 141].

1.2.5 Physiological roles of cathepsin D

1.2.5.1 Pathological conditions developing in CatD-deficient mice

CatD-deficient mice have been shown to develop pathological conditions like massive intestinal necrosis [38], thromboembolia [38], lymphopenia [38], and neuronal ceroid lipofuscinosis [39].

1.2.5.2 Pathological conditions in which over-expression of CatD is observed

Cathepsin D is found in elevated levels in many types of cancers such as breast cancer [41], prostrate cancer [40] and ovarian cancer [42]. The prognostic value remains controversial.

1.2.5.3 Potential role in the MHC class II pathway

Cathepsin D is the major aspartic protease of the lysosomal compartment. Several studies have demonstrated the ability of cathepsin D to release T cell epitopes from protein antigens [11, 15, 142, 143] and some have reported the association of cathepsin D with MHC class II antigen presentation [11, 144, 145].

CatD generates antigenic peptides from ovalbumin (OVA) [11], hen egg lysozyme [143], and sperm whale myoglobin [142]. It is involved in processing and presentation of human serum albumin by murine peritoneal macrophages and veiled cells [145]. According to one study [144], CatD is necessary for the processing of ovalbumin. Additionally, it is localized to the multivesicular endosomes known as MIIC or CIIV [146, 147] that indicates the role of the enzyme in both antigen and invariant chain processing.

In contrast, some reports stated that CatD may not be that important in antigen processing. Deussing *et al* [148] analyzed the ability of splenocytes from CatD-deficient mice to present various antigens to murine T cell hybridomas. They reported that the absence of CatD did not impair the presentation of epitopes from ovalbumin, hen egg lysozyme, myelin basic protein and pigeon cytochrome C [148]. Nishioku *et al* [56] reported that microglia prepared from cathepsin D-deficient mice retained the ability to present ovalbumin-derived antigenic peptides.

A more recent report from Chain *et al* [12] also demonstrated ovalbumin processing and presentation in dendritic cells derived from CatD-deficient mice. They have also observed that CatD deficiency does not affect antigen processing or presentation ability of the cells.

An important limitation of all these studies mentioned above indicating that, CatD is not involved in antigen presentation, is that it was not clear whether or not the antigens tested were the substrates for CatD. In a recent study by Moss *et al* [149], this issue was taken into consideration and the involvement of CatD in processing and presentation of the model antigen myoglobin was tested. *In vitro* digestions of myoglobin with lysosomal fractions isolated from antigen presenting cells (APCs) showed that myoglobin is digested into a set of discrete products by aspartic proteases [142, 149, 150]. Moreover, myoglobin-specific T cell hybridomas are stimulated by CatD-digested myoglobin, but not by untreated protein, suggesting that CatD plays a role in myoglobin processing [149].

But in dendritic cells, derived from CatD-deficient mice presentation of two different myoglobin T cell epitopes is enhanced rather than hindered [149]. This contradiction in results can be explained by the finding that myoglobin processing activity persists in lysosomes of CatD-deficient dendritic cells. Thus, this interesting report [149] suggested that a low level of aspartic protease activity is required for processing of myoglobin, but a higher level of activity becomes destructive.

Deussing *et al* [148] reported that presentation of some antigens was enhanced in splenocytes lacking CatD. Similarly, Chain *et al* [12] noticed that DCs from CatD-deficient mice occasionally showed a slight increase in ovalbumin processing. Several explanations can be given to elucidate this effect of aspartic protease concentration on antigen presentation. For example, as suggested by Moss *et al* [149], it might reflect relative preferability for cleavage sites within myoglobin. At low protease concentration only the most proteolytically-susceptible sites, which appear to lie outside the T cell epitopes, will be cleaved [149]. At higher protease concentrations, less-favoured sites might also be targeted, and if these sites are present within T cell epitopes, antigen presentation might be impaired. This explanation was favoured by the fact that CatE and CatD cleavage sites were observed in A3 epitope (102-118) [149].

Secondly, it is also observed that MHCII may preferentially capture large processed antigen fragments [151]. Thus higher levels of protease activity may generate antigenic peptides with a less favourable size [149]. Some of the studies discussed earlier in this chapter have also implied that aspartic proteases in general are not involved in antigen presentation as pepstatin A has not affected presentation of processed peptides [13, 56], whereas antigen processing was pepstatin A-sensitive.

Chain *et al* [12] reported that DCs derived from CatD-deficient mice were capable of antigen processing as well as presentation. Furthermore, it has also been reported that microglia isolated from CatD-deficient mice retained ability for antigen presentation [56]. Consistently, some other studies that utilized peripheral APCs derived from CatD-deficient mice indicated that CatD is not involved in degradation of invariant chain [148, 152]. Riese *et al* also described that cathepsin S, but not cathepsin B or D, is necessary for the digestion of Ii from MHC II molecules [153].

However, there are several discrepant reports showing that aspartic protease inhibitors effectively prevented the processing of invariant chain [14, 105, 154]. According to one study [144], CatD is necessary for degradation of Ii from the MHC class II alpha beta heterodimer in endosomes in order to express functional MHC class II molecules, that will then bind to antigenic peptides. Zhang *et al* [14] has reported that CatD is involved in degradation of invariant chain in ovalbumin-immunized mice.

1.3 Aims of the thesis

The present work aims to contribute to the understanding of differences in biochemical characteristics of cathepsin E and D and their possible functional role in MHC II pathway. More specifically, the project intended to develop and characterize new tools for studying these proteases that are very similar in their enzymatic characteristics.

The specific aims were as follows

- Synthesis and characterization of monospecific antibodies for cathepsin E.
- To develop an assay for distinct measurement of cathepsin E and D and utilization of this assay in measuring the activity of these enzymes in endosomal and lysosomal fractions of different antigen presenting cells.
- To improve the accessibility of pepstatin A (PepA, highly potent aspartic protease inhibitor) to antigen processing compartments by conjugating it with well-known cell penetrating peptides (CPPs). Once the cell-penetrating ability of different PepA-CPP conjugates is evaluated, exploitation of these conjugates in antigen presentation assays.
- To study the proteolytic activity of cathepsin E at different pH values, as distinct cleavage specificities of CatE at neutral and acidic pH have been reported in some previous studies.
- To study in detail the substrate specificity of cathepsin E and D and selection of the specific substrate for either enzyme. Utilization of these substrates in monitoring the activity of CatE and CatD in different biological samples.

2

2 A new approach for distinguishing cathepsin E and D activity in antigen processing organelles

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2.1 Abstract

Cathepsin E (CatE) and D (CatD) are the major aspartic proteinases in the endolysosomal pathway. These proteinases exhibit similar specificity and therefore it is difficult to distinguish between them since known substrates are not exclusively specific for one proteinase. Here, a substrate-based assay is presented, which is highly relevant for immunological investigations since it detects both, CatE and CatD in antigen-processing organelles. Therefore it could be used to study the involvement of these proteinases in protein degradation and the processing of invariant chain. An assay combining a new monospecific CatE antibody and the substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 [where Mca is (7-methoxycoumarin-4-yl)acetyl and Dnp is dinitrophenyl] is presented. This substrate is digested by both proteinases and therefore can be used to detect the total aspartic proteinase activity (TAPA) in biological samples. After depletion of CatE by immunoprecipitation, the remaining activity is due to CatD and the decrease of activity can be assigned to CatE. The activity of CatE and CatD in cytosolic, endosomal and lysosomal fractions of B cells, dendritic cells and

human keratinocytes was determined. Our data clearly indicate that CatE activity is mainly located in endosomal and that of CatD in lysosomal compartments. Hence, this assay can also be used for characterization of subcellular fractions using CatE as an endosomal marker, whereas CatD is a well-known lysosomal marker. Moreover, the highest TAPA (total aspartic protease activity) was detected in dendritic cells and the lowest in B cells. The presented assay exhibits a lower detection limit compared to common antibody-based methods without lacking specificity.

2.2 Introduction

Cathepsin E and D are the major intracellular aspartic proteinases. They have similar enzymatic properties, e.g. susceptibility to various proteinase inhibitors such as pepstatin A and similar substrate preferences as both prefer bulky hydrophobic amino acids at P1 and P1` positions [155]. Additionally, both enzymes have approximately the same acidic pH optimum towards various protein substrates such as hemoglobin [57, 67].

However, these enzymes have different tissue distribution and cellular localization suggesting that both enzymes might have more specific physiological functions. CatE is a non-lysosomal proteinase with a limited distribution in certain cell types, including gastric epithelial cells [33], but mainly present in cells of the immune system, such as macrophages [29], lymphocytes [29], microglia [34] and dendritic cells [12]. It is reported to be localized in different cellular compartments, such as plasma membranes [36], endosomal structures [34], endoplasmic reticulum and Golgi apparatus [30, 34, 37]. In contrast, CatD is a typical lysosomal enzyme widely distributed in almost all mammalian cells [29-32].

Recent studies with CatE- and CatD-deficient mice have provided an additional evidence of the association of these enzymes with different physiological effects. CatD-deficient mice developed massive intestinal necrosis [38], thromboembolia [38], lymphopenia [38], and neuronal ceroid lipofuscinosis [39]. Cathepsin E-deficient mice are found to develop atopic dermatitis-like skin lesions [43]. It was reported recently that CatE-deficient mice show an increased susceptibility to bacterial infection associated with decreased expression of multiple cell surface Toll-like receptors [44]. According to a most recent study [45], CatE deficiency

induces a novel form of lysosomal storage disorder showing the accumulation of lysosomal membrane sialoglycoproteins and the elevation of lysosomal pH in macrophages.

CatD has also been suggested to play a role in determining the metastatic potential of several types of cancer; high levels of CatD have been found in prostate [40], breast [41] and ovarian cancer [42]. CatE is expressed in pancreatic ductal adenocarcinoma [77], and its presence in pancreatic juice is reported to be a diagnostic marker for this cancer [93]. Increased levels of CatE in neurons and glial cells of aged rats are suggested to be related to neuronal degeneration and reactivation of glial cells during the normal aging process of the brain [94].

CatE and CatD are found to play an important role in the MHC class II pathway. CatD is reported to be involved in processing of MHC II-associated invariant chain [14], in antigen processing and presentation [15, 145]. CatE is also reported to be involved in antigen processing by B cells [13, 35] microglia [56] and murine dendritic cells [12].

Several studies have been conducted to determine the subcellular localization of CatE and CatD in different cell types, but there are few reports regarding the activity of these enzymes in antigen processing-relevant organelles [29, 156]. Previous reports have described highly selective substrates for aspartic proteinases, but none of the described substrates is exclusively specific for CatE or CatD [19, 20, 156]. In most of the studies additional methods or inhibitors are used to measure the specific activity of CatE or CatD. For example, to determine the CatD activity specifically, a CatD digest and pull-down assay was described [156]. Other studies have utilized a specific inhibitor for CatE, the *Ascaris* pepsin inhibitor, which inhibits pepsins and CatE [26], but does not affect other types of aspartic proteinases including CatD [19, 157]. This inhibitor was originally isolated from the round worm *Ascaris* lumbricoides [25]. However, it is not commercially available.

In the present study, CatE and CatD activity was determined in subcellular fractions (lysosomal, endosomal and cytosolic) of antigen-presenting cells. For measuring TAPA (total aspartic proteinase activity) in biological samples, the previously described peptide substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 [19] was used which is digested by both, CatE and CatD. It is an intramolecularly-quenched fluorogenic peptide

derivative in which the fluorescent signal of the fluorophore Mca is quenched by the chromophoric residue Dnp. After cleavage of the peptide, the quenching efficiency is decreased resulting in an increase in fluorescence. The activity determined in subcellular fractions was completely inhibited by pepstatin A. Therefore, this activity can be only attributed to aspartic proteinases and represents TAPA. For the specific determination of CatE and CatD activity, CatE was specifically depleted by immunoprecipitation. The remaining activity is due to CatD, and the decrease of activity is assigned to CatE. This approach allows the specific and highly sensitive measurement of both, CatE and CatD activities in biological samples.

2.3 Experimental procedures

2.3.1 Enzymes and chemicals

CatD (bovine kidney) was purchased from Calbiochem (Darmstadt, Germany) and stored as a 300 U/ml stock solution in 0.1 M citrate buffer, pH 4.5, at -20 °C. CatE was purchased from R&D systems (Wiesbaden, Germany) and stored as a 0.1 mg/ml stock solution in 50 mM citrate buffer, pH 6.5, containing 150 mM NaCl at -20 °C. Pepstatin A (Calbiochem) was dissolved in methanol. Activated CH Sepharose 4B was purchased from Amersham Biosciences (Munich, Germany). The substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2 [19] was obtained from Bachem (Weil am Rhein, Germany).

2.3.2 Generation and immobilization of a monospecific CatE antibody

The antigenic peptide SRFQPSQSSTYSQPG (CatE 118-132) was selected from the protein sequence using the laser gene software (DNASTAR, Madison, WI) and controlled for specificity to CatD. It was synthesized as a single peptide and as a multiple peptide antigen (MAP) peptide $(SRFQPSQSSTYSQPG)_8$-[70]4-$(Lys)_2$-Lys-Gly-OH using standard Fmoc/tBu [158] chemistry on a multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany). The peptides were purified using RP-HPLC and the identity was confirmed using ESI-MS. Peptide purities were determined *via* analytical RP-HPLC and proved to be higher than 90%. The single peptide was coupled to keyhole limpet hemocycanin (KLH) using the

glutardialdehyde method. The antiserum was obtained after repeated immunization of a rabbit with a 1:1 mixture of the peptide-KLH-conjugate and the MAP. This antiserum was purified by affinity chromatography on a CH-activated Sepharose 4B column (Amersham Biosciences) containing the peptide immobilized *via* a stable peptide bond. Peptide immobilization was performed as described by the manufacturer. The antiserum was applied on the column at 0.5ml/min and recycled overnight. The column was washed with 20 column volumes of PBS (Gibco). Elution was performed with 10 volumes of 0.1M glycine/HCl (pH 2.5). Antibody-containing fractions were immediately neutralized with 1M Tris/HCl (pH 8.5) and then concentrated on a 20 kD membrane. The resulting antibody was retested by ELISA and showed the expected specificity to the peptide epitopes and the CatE protein, but a complete negative reaction against CatD. The purified monospecific antibody was immobilized on CH-activated sepharose as described by the manufacturer. After coupling for 3 hours at room temperature, the gel was deactivated with 0.1M Tris-HCl, pH 8.0, for 2 hours at room temperature. To block any remaining active sites, the material was further incubated with 5% bovine serum albumin for additional 2 hours. After washing with PBS, the immobilized antibody was stored in PBS containing 0.02% (w/v) NaN_3 at 4°C.

2.3.3 ELISA

The wells of microtiter plates (Nunc Brand Products, MaxiSorb surface, Wiesbaden, Germany) were coated with CatE (10ng), CatD (10ng) or the peptide SRFQPSQSSTYSQPG (1ng) in PBS in a final volume of 100 µl/well at 4°C overnight. The plates were washed three times with 200 µl of washing buffer (PBS/0.05% Tween 20, pH 7.0) and blocked with blocking buffer (PBS/0.05% Tween 20, pH 7.0, containing 2% BSA) for 2 hr at 37°C. After washing, the plates were treated for 1 h at 37°C with our monospecific CatE antibody (diluted in PBS/0.05% Tween 20, pH 7.0, containing 0.5% BSA) or commercial CatD antibody. After washing, the plates were incubated with HRP-conjugated goat anti-rabbit Ig (Dianova, Hamburg, Germany; 1:5000 diluted in PBS/0.05% Tween 20/0.5% BSA). 100 µl/well of ABTS (azinodiethyl-benzthiazoline-sulfonate)/H_2O_2 in substrate buffer (citrate buffer 100mM, pH 4.5) was added and the colour development analyzed at a wavelength of 405 nm.

For competitive inhibition ELISA, the antiserum was pre-incubated with different concentrations of CatE or CatD (40 min, room temperature) and then used as primary antibodies for standard ELISA for detecting the antigenic peptide SRFQPSQSSTYSQPG (0.1µg/well).

2.3.4 Cell culture

The EBV-transformed human B cell line WT100 and the immortalized human keratinocyte cell line HaCaT were cultured in RPMI 1640 medium (Gibco Life Technologies, Paisly, United Kingdom) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS, Gibco), penicillin (final concentration: 100 U/ml, Gibco) and streptomycin (final concentration: 0.1 mg/ml, Gibco) at 37 °C in tissue culture flasks (Nunc, Wiesbaden, Germany).

PBMC (peripheral blood mononuclear cells) were isolated by Ficoll/Paque (PAA Laboratories Pasching Austria) density gradient centrifugation of heparinized blood obtained from buffy coats. Isolated PBMC were plated (1x 10^8 cells/8 ml/ flask) into 75 cm^2 Cellstar tissue culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) in RPMI 1640 (Gibco Life Technologies) under the same culture conditions as for WT100 and HaCaT. After 1.5 hour of incubation at 37°C, non-adherent cells were removed and adherent cells were cultured in complete culture medium supplemented with GM-CSF (Leukomax; Sandoz, Basel, Switzerland) and IL-4 (R&D systems) for six days as described previously [159]. This resulted in a cell population consisting of approximately 70% DCs (data not shown) as determined by flow cytometry (BD FACSCalibur, Heidelberg, Germany).

2.3.5 Determination of mRNA expression levels for CatE using RT-PCR

RNA was extracted from DCs, WT100 and HaCaT cells using the TRIazol reagent as described by the manufacturer (Invitrogen, Karlsruhe, Germany). Reverse transcription of 2 µg total RNA was initialized by 200 U of Superscript II reverse transcriptase (Invitrogen), 4 µl synthesis buffer (5-fold concentrated, Invitrogen), 2.5 µl random primers (10 mM, Promega, Mannheim, Germany), 1 µl dithiothreitol (100 mM, Invitrogen), 1 µl dNTP mix (10 mM, Promega) and 0.5 µl rRNAsin (Promega) in a final volume of 20 µl. After incubation at room temperature for 10 min, the reaction mixtures were set to 42 °C for 1 h. Then amplification was carried out adding

5 µl generated cDNA to 45 µl reaction mixture (11.0 % (v/v) 10-fold PCR buffer (Roche, Basel, Switzerland), 3.3 % (v/v) of both primers (5'-CATGATGGAATTACGTT-3' and 5'GAATGATCCAGGTACAGCAT-3') 10 µM each (Operon Technologies Alameda, California, USA), 2.2 % (v/v) dNTP mix (10 mM, Promega) in molecular grade water and 1.1 % (v/v) Taq DNA polymerase (Roche) and running 35 cycles each for 35 sec at 94 °C, 30 sec at 50 °C and 60 sec at 72 °C. Single PCR amplicons were analysed using agarose gel electrophoresis.

2.3.6 Subcellular fractionation and Western blot analysis

Cell fractionation was performed as previously described by Schroter et al [160]. Briefly, 4×10^7 - 8×10^7 cells were harvested, resuspended in 1.5 ml fractionation buffer, (10 mM Tris buffer, 250 mM sucrose, pH 6.8), and then homogenized using a cell cracker (HGM Lab Equipments, Heidelberg, Germany). Then debris was separated by centrifugation at 8000 xg for 10 min. Mitochondria and the endolysosomal fractions were separated by ultra-centrifugation at 100,000 xg for 5 min (Beckman TL100 ultracentrifuge, Palo Alto, USA). Finally, lysosomes were separated from endosomes by hypotonic lysis with ddH_2O (approx. 2.5-fold of the pellet volume for keratinocytes and DCs, and 5-fold of the pellet volume for B cells) and centrifugation at 100,000 xg for 5 min. Lysosomal material was released in the supernatant, endosomes remained in the pellet. Total protein content was determined according to Bradford [161]. For schematic representation of the protocol see **Figure 1**.

Subcellular fractions were separated by SDS-polyacrylamide gel electrophoresis (50 µg total protein per lane) on a 12 % separating gel and transferred to a PVDF-membrane (Amersham Biosciences, Freiburg, Germany). Membranes were then blocked for 1 h using TBST (0.15 M NaCl, 10 mM Tris, 0.05 % (v/v) Tween 20, pH 8.0) containing 10 % (v/v) Roti® Block (Roth, Karlsruhe, Germany). Rabbit anti-human CatD antibody (Calbiochem) was diluted 1:5000 and rabbit anti-human CatE antibody was diluted 1:2000. Western blots were developed according to the ECL protocol of Amersham Biosciences.

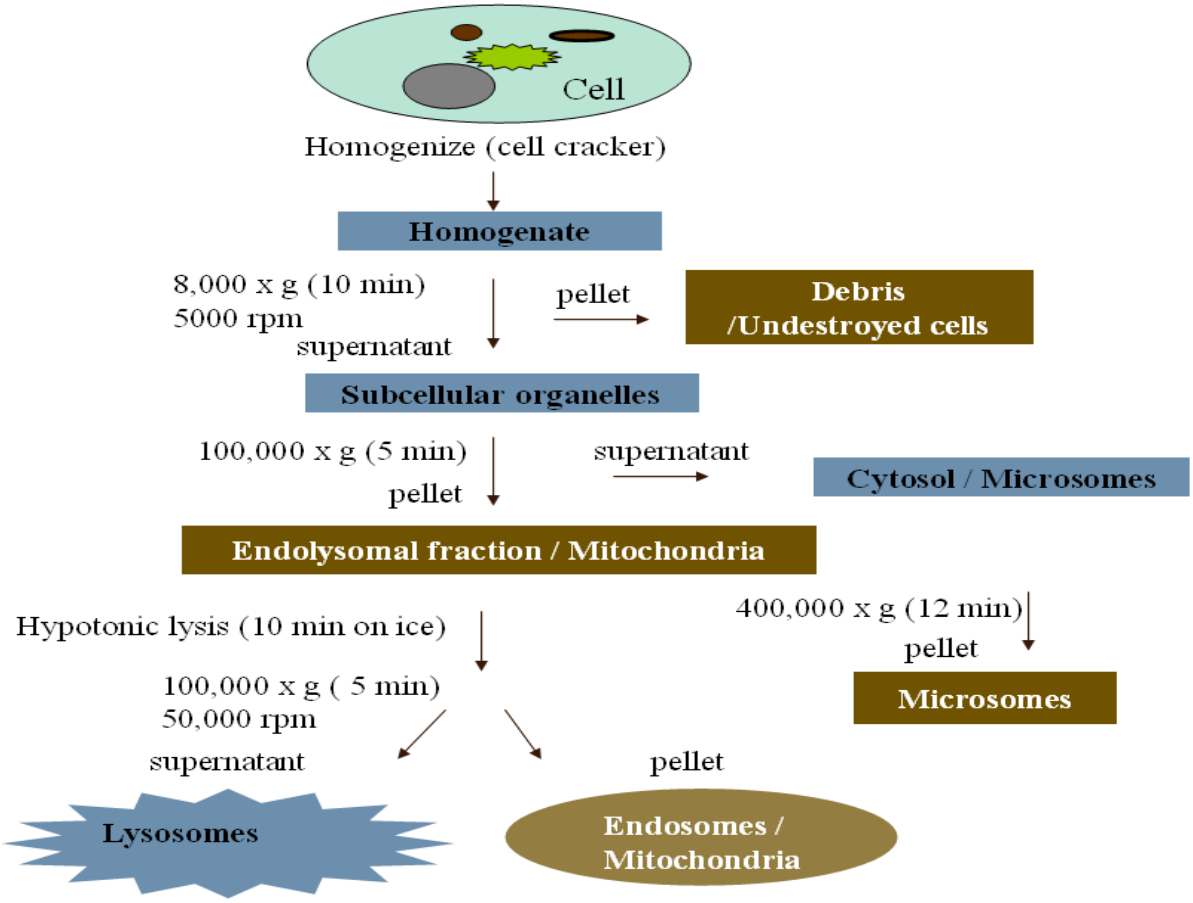

Figure 1: Schematic representation of subcellular fractionation.

2.3.7 Detection of NAG (N-acetyl-β-D-glucosaminidase) activity

NAG activity was measured as described by Schmid *et al* [162]. Briefly, 1µg protein from each fraction was added to 100 µL of 0.1M citrate buffer, pH 5, containing 0.8 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma, Deisenhofen Germany) and 0.1% Triton X-100. Fluorescence (λex = 360 nm, λem. = 465 nm) was measured every 5 min at 37° C using a fluorescence reader (Tecan Spectra Fluor, Crailsheim, Germany). NAG activity was determined by linear regression using a minimum of seven measurement points.

2.3.8 Parallel detection of CatE and CatD activity

TAPA (total aspartic proteinase activity) and specific catalytic activities of CatE and CatD were determined fluorometrically by hydrolysis of the substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2. Appropriate amounts of CatE, CatD or subcellular fraction (20µg of total protein) were added to 80µl digestion buffer (50mM sodium acetate buffer pH 4.0) and the reaction was started by addition of 1µl substrate solution (stock solution 2mM in DMSO). Progress of fluorescent product formation was recorded using a fluorescence reader (Tecan Spectra Fluor) on kinetic mode at 37°C (λ_{ex} =340, λ_{em} = 405). Activities were determined by linear regression analysis using a minimum of five measurement points. All the experiments were done in triplicates yielding TAPA i.e. CatE and CatD activity. Aspartic proteinase activity could be completely inhibited using 1 µl of a 1 mM pepstatin A solution in methanol (1 µl methanol showed no inhibitory effect).

For the specific determination of CatE activity samples were subjected to immunoprecipitation of CatE prior to the above described assay. 20 µg of total protein from each subcellular fraction was incubated with 20µl of monospecific CatE antibody immobilized on a CH-activated sepharose at 4°C overnight.

In this way, the measured increase in fluorescence intensity is exclusively caused by CatD. The difference between total aspartic proteinase and CatD activity can be assigned to CatE activity. For schematic representation of the assay see **Figure 2**.

2.3.9 Analytical RP-HPLC

1 µl of the fluorogenic peptide substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 (1 mM in DMSO) was incubated at 37 °C in 80 µl digestion buffer (50 mM sodium acetate buffer, pH 4.0) containing the appropriate amount of CatE, CatD or a subcellular fraction (with or without pepstatin A treatment or after immunoprecipitation (IP) of CatE). The reaction was terminated by boiling the samples for 5 min at 98°C. 5µl of the reaction mixture was separated by analytical RP-HPLC using a C8 column (150 × 2 mm, Reprosil 100, Dr. Maisch GmbH, Tuebingen, Germany) with the following solvent systems:

(A) 0.055 % (v/v) TFA in water and (B) 0.05 % (v/v) TFA in 80 % (v/v) ACN in water. Elution was performed using a linear gradient from 5 % to 80 % B within 35 min. Fluorescence detection was carried out at $\lambda_{em.}$= 350 and λ_{ex} = 450 . Appropriate fractions were collected and analysed by MALDI-MS.

2.3.1 MALDI-MS

0.5 µl of each RP-HPLC fraction was mixed with 0.5 µl DHB-matrix (10 mg/ml (w/v) 2,5-dihydroxybenzoic acid in 60 % (v/v) ethanol containing 0.1 % (v/v) TFA) and applied on a gold target for MALDI-MS using a MALDI time-of-flight system (Reflex IV, serial number: 26159.00007, Bruker Daltonics, Bremen, Germany). Signals were generated by accumulating 120 – 210 laser shots. Raw data were analyzed using the software Flex Analysis 2.4 (Bruker Daltonics).

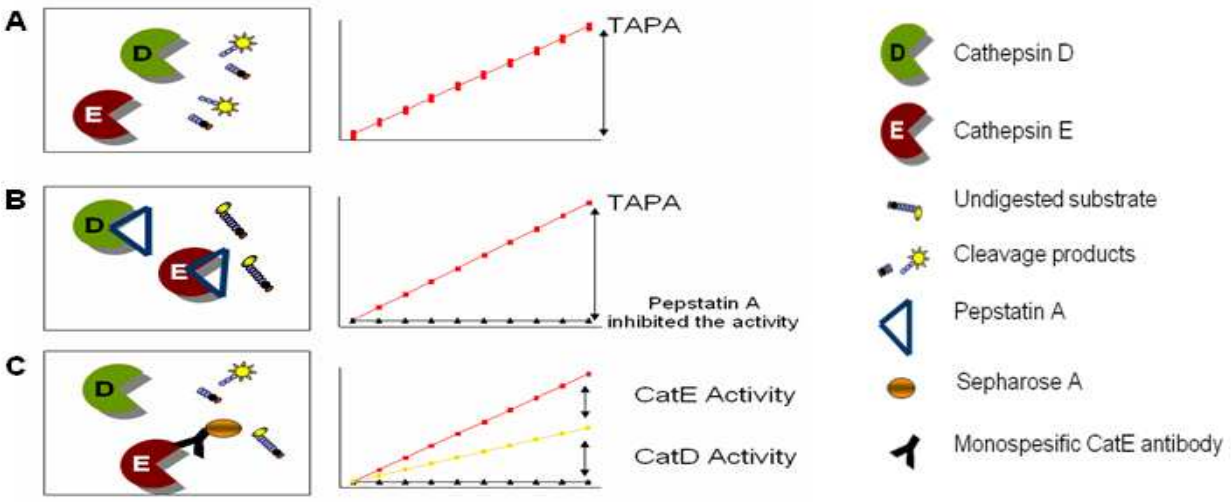

Figure 2. Schematic representation of assay to distinguish between CatE and D activity. (A) *TAPA (total aspartic proteinase activity) was measured by hydrolysis of fluorogenic substrate* Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2. ***(B)*** *The observed activity was pepstatin A (PepA) sensitive hence was only due to aspartic proteases.* ***(C)*** *Samples were subjected to immunoprecipitation (IP) using our monospecific antibody before above described assay. Now increase in fluorescence intensity is exclusively caused by CatD. The difference between total aspartic proteinase and CatD activity can be assigned to CatE activity.*

2.4 Results and Discussion

2.4.1 Expression of cathepsin E mRNA in different cell lines

To determine the expression of CatE at the mRNA level in different cell lines, RT-PCR was performed using RNA extracted from DCs (monocyte derived human dendritic cells), WT100 (EBV-transformed B-cell line) and HaCaT (immortalized human keratinocyte cell line). PCR products from the cell lines were analyzed by gel electrophoresis and found to contain a band of the expected size (241bp) (**Figure 3**.). As these cell lines were found to be positive for CatE mRNA they were used for the determination of enzymatic activity of CatE and CatD. Previous studies have also shown that murine dendritic cells [12] as well as another EBV-transformed B-cell line (Fc7) are positive for CatE mRNA [35].

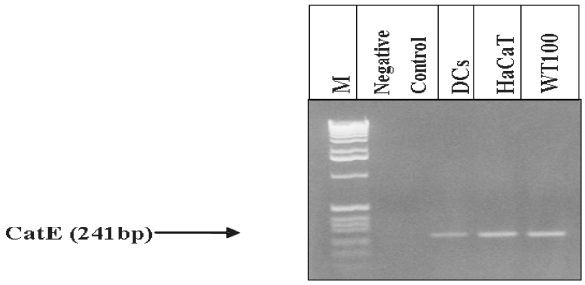

Figure. 3. CatE expression at mRNA level in different cell lines. *Total RNA was extracted from HaCaT, WT100 and DCs. Equal amounts of total RNA (2µg) from each sample were used for RT-PCR. After reverse transcription, specific primers for human CatE were used to amplify CatE cDNA.*

2.4.2 Determination of antibody specificity

The monospecific antibody for cathepsin E was raised against the antigenic peptide SRFQPSQSSTYSQPG (CatE 118-132). This peptide was selected from the CatE sequence using laser gene software (DNASTAR, Madison, WI) for antigenicity and surface probability **(Figure 4A).** BLAST tool analysis showed that the selected peptide sequence does not exhibit significant homology with sequences in cathepsin D or any other known protein therefore it is

specifically present in cathepsin E. **Figure 4B**. shows sequence alignment of cathepsin E and cathepsin D.

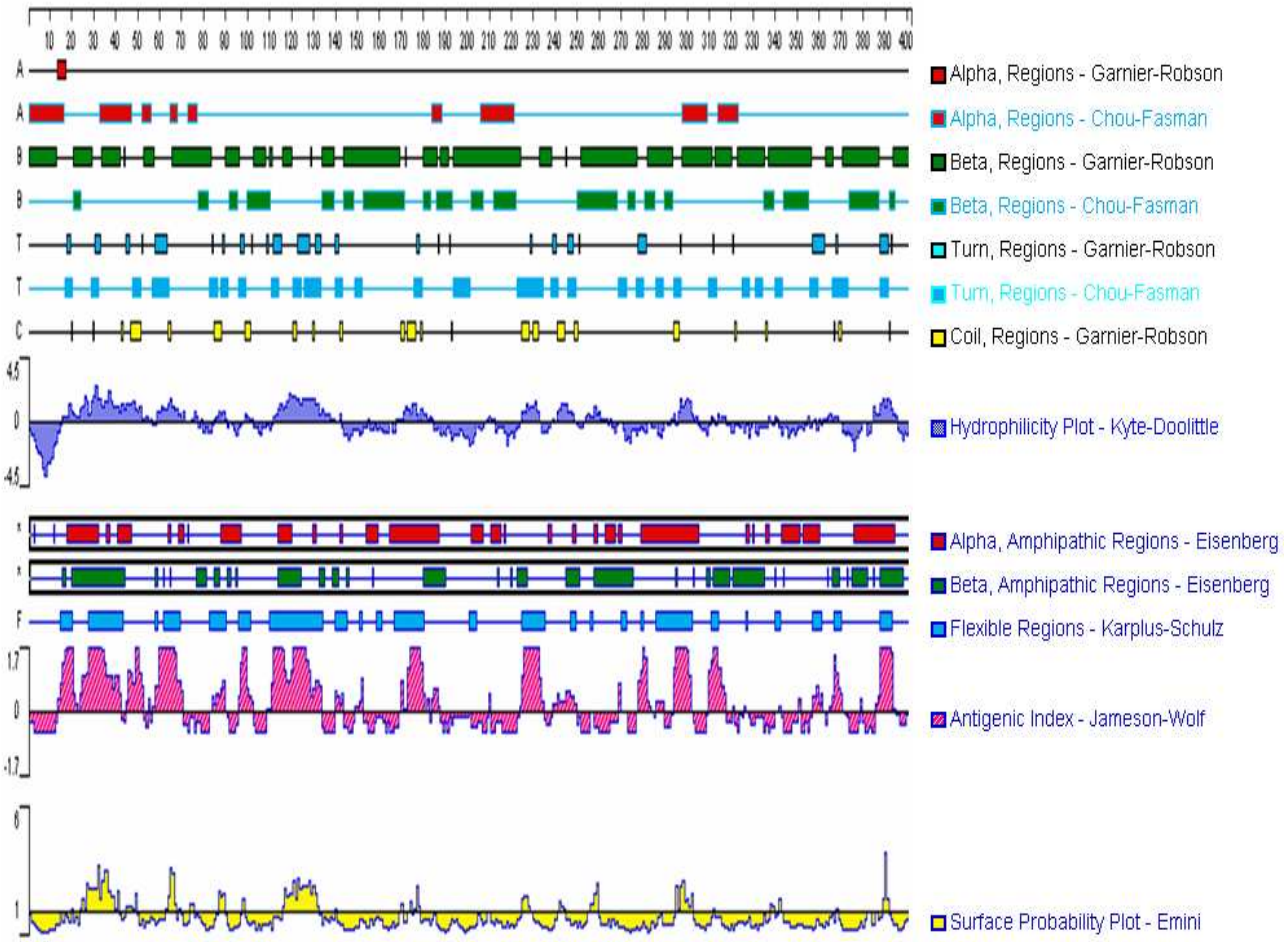

Figure. 4A. Prediction of antigenic peptides for generation of monospecific antibody against cathepsin E. *Amino acid sequence of CatE was obtained from National Center for Biotechnology Information Protein database and subjected to analysis of secondary structure, hydrophilicity profile, and antigenicity using the program Protean of DNAStar software (DNAStar, Inc., Madison, WI). The polypeptide composed of 15 amino acids SRFQPSQSSTYSQPG (CatE 118-132) was selected as an antigenic peptide for raising CatE antibodies.*

The antiserum obtained was further purified by affinity chromatography on CH-activated sepharose containing the peptide SRFQPSQSSTYSQPG immobilized via stable peptide bonds.

The results of indirect ELISA (**Figure 4C**) showed that the antibody specifically recognized CatE and the antigenic peptide SRFQPSQSSTYSQPG used to generate the antibody and gave a complete negative reaction towards CatD.

To determine the specificity and cross reactivity of resulting CatE antibody, indirect ELISA, competitive inhibition ELISA (CI-ELISA) and Western blot analysis were performed.

CI-ELISA was performed to further enhance the specificity of the antibody. The antibody was pre-incubated with different concentrations of CatE and CatD before performing the standard ELISA for detecting the antigenic peptide SRFQPSQSSTYSQPG. Pre-incubation of cathepsin E with the antibody exhibited a dose-dependant inhibition of antibody binding (IC_{50} value: 48.6 ng; **Figure. 4D**). Increasing concentration of cathepsin D did not affect antibody binding. This experiment shows that CatE specifically binds to the monospecific antibody in a free system. Western Blot analysis also confirms that the monospecific antibody specifically recognizes CatE and not CatD (data no shown).

```
CatE:  68  EPLINYLDMEYFGTISIGSPPQNFTVIFDTGSSNLWVPSVYCT--SPACKTHSRFQPSQS 125
           E L NY+D +Y+G I IG+PPQ FTV+FDTGSSNLWVPS++C     AC  H ++   +S
CatD:  69  EVLKNYMDAQYYGEIGIGTPPQCFTVVFDTGSSNLWVPSIHCKLLDIACWIHHKYNSDKS 128

CatE: 126 STYSQPGQSFSIQYGTGSLSGIIGADQVS------AFATQVEGLTVVGQQFGESVTEPGQ 179
           STY + G SF I YG+GSLSG +  D VS       + A+ + G+ V  Q FGE+  +PG
CatD: 129 STYVKNGTSFDIHYGSGSLSGYLSQDTVSVPCQSASSASALGGVKVERQVFGEATKQPGI 188
```

Figure 4B. Sequence alignment of cathepsin E and cathepsin D*. The alignment was performed using conventional BLAST search engine. Only the small region of CatE containing the sequence SRFQPSQSSTYSQPG (antigenic peptide, CatE 118-132, which was used for generating monospecific antibodies) was included during the BLAST operation (sequence is seen underlined in the figure). This peptide was selected from the CatE sequence using laser gene software (DNASTAR, Madison, WI) for antigenicity and surface probability. BLAST tool analysis showed that the selected peptide sequence does not exhibit significant homology with sequences in cathepsin D or any other known protein, therefore it is specifically present in cathepsin E.*

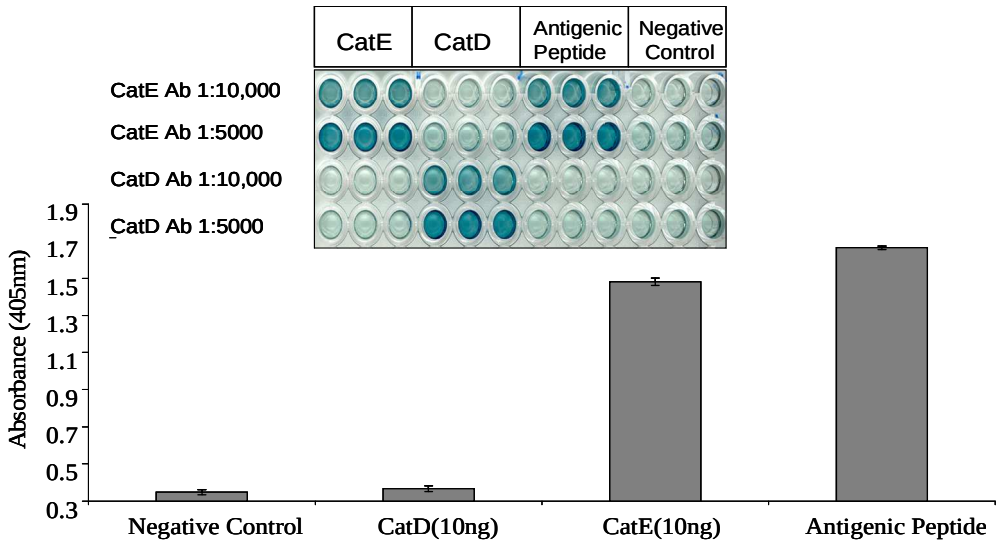

Figure 4C. Determination of specificity of monospecific antibody (raised against SRFQPSQSSTYSQPG) by indirect ELISA. *The purified monospecific antibody specifically recognized CatE (10ng) and the antigenic peptide (SRFQPSQSSTYSQPG), and gave a complete negative reaction towards same amount of CatD (10ng). Values are means ± standard deviations, n = 3 (Insertion: 10 ng of CatE and CatD, and 1ng of antigenic peptide were incubated on ELISA plate, CatE and CatD antibodies were used for the detection at dilutions of 1:10000 and 1:5000).*

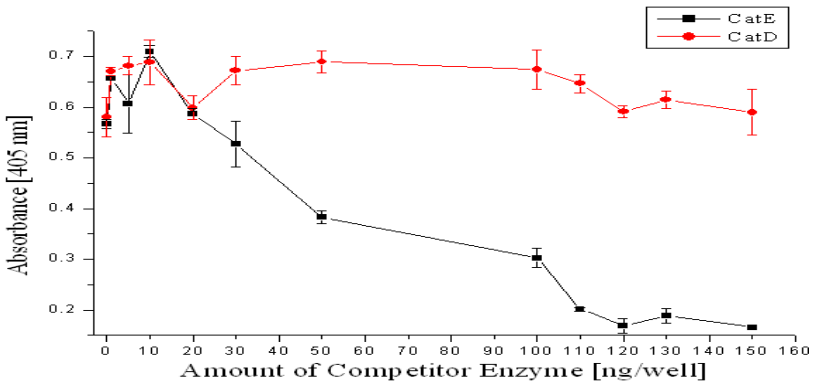

Figure 4D. Competitive Inhibition of antibody (raised against SRFQPSQSSTYSQPG) binding to SRFQPSQSSTYSQPG-coated plates by CatE*. Immunoplates were coated with antigenic peptide SRFQPSQSSTYSQPG (0.1µg/well). Monospesific antibodies were pre-incubated with different concentrations of CatE or CatD, before standard ELISA. ELISA was performed as described in material and methods. The increasing concentration of cathepsin E caused inhibition of antibody binding giving the IC_{50} value of 48.6 ng. Whereas, the same concentrations of CatD had no effect on antibody binding. Data points are means ± standard deviations, n = 2*

2.4.3 Characterization of subcellular fractions

To control the quality of subcellular fractions, N-acetyl-β-D-glucosaminidase (NAG) activity was determined, since it is a wide-spread and well-established marker for endosomal/lysosomal compartments [162]. **Table 1** shows the activity of NAG in subcellular fractions of different cell lines. As expected, all cell lines showed highest NAG activity in lysosomal fractions (LFs) and lower in endosomal fractions (EFs). Cytosolic fractions (CFs) had very low NAG activity.

Table 1. NAG activity in subcellular fractions (fluorescence /min /µg protein) of different cell lines. *Activities were determined by linear regression analysis taking at least seven measurement points. Values depicted are means ± SD (n = 3)*

Cell line	Subcellular fraction	NAG activity (fluorescence /min/µg protein)
HaCaT	Cytosolic (CF)	0.5 ± 0.05
	Lysosomal (LF)	15.2 ± 0.3
	Endosomal (EF)	7.0 ± 0.37
WT100	Cytosolic (CF)	0.4 ± 0.02
	Lysosomal (LF)	9.6 ± 0.1
	Endosomal (EF)	2.2 ± 0.07
DCs	Cytosolic (CF)	0.7 ± 0.07
	Lysosomal (LF)	25.2 ± 0.07
	Endosomal (EF)	8.0 ± 0.2

2.4.4 Western blot analysis of subcellular fractions from different cell lines used for CatE and CatD determination

For immunochemical determination of subcellular localization of CatE and CatD, Western blot analysis was performed. No CatE was recovered from any subcellular fraction of WT100. Endosomal fraction of DCs and HaCaT contained a significant larger amount of CatE than the

respective lysosomal fractions, but no CatE was found in the cytosolic fractions of any of the cell lines (**Figure 5**.). As expected, higher amounts of CatD were detectable in lysosomal fractions. No CatD was detected by Western blotting in the cytosolic fraction of any of the three cell types (**Figure 5**.).

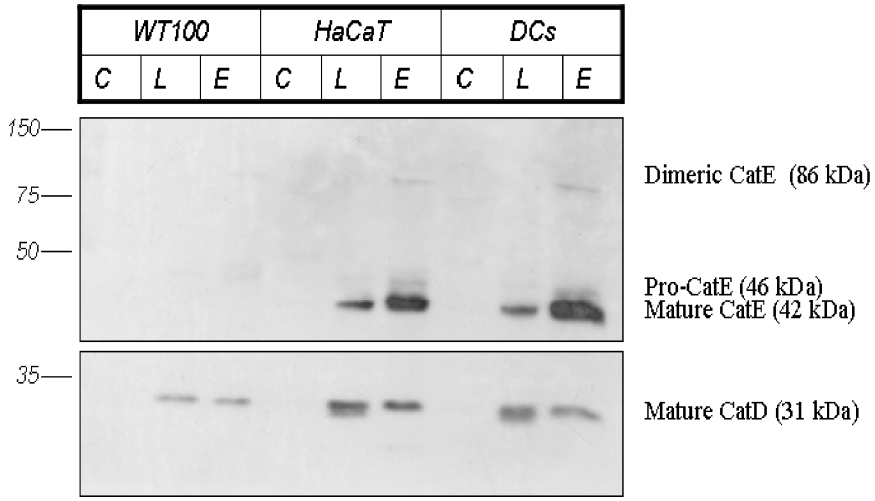

Figure 5. CatE and CatD expression at protein level in antigen processing relevant organelles of different cell lines. *Equal amounts of total protein (50 μg) from each sample were applied for SDS-PAGE followed by Western blot analysis. Representative immunoblots with the monospecific CatE antibody and reprobe of the same blot with the CatD antibody are shown. (C; Cytosolic fraction, L; Lysosomal fraction, E; Endosomal Fraction).*

2.4.5 Specific inhibition of CatE by immunoprecipitation

In order to determine the specificity of our immobilized CatE antibody in depleting CatE from the samples, it was tested with CatE and CatD. CatE (recombinant) was completely immunoprecipitated by the antibody against Cat E (**Figure 6A**), whereas it had almost no effect on CatD activity (**Figure 6B**.). This approach for depleting proteinase activity from complex biological samples is flexible and can be used for other proteinases as well.

A **B**

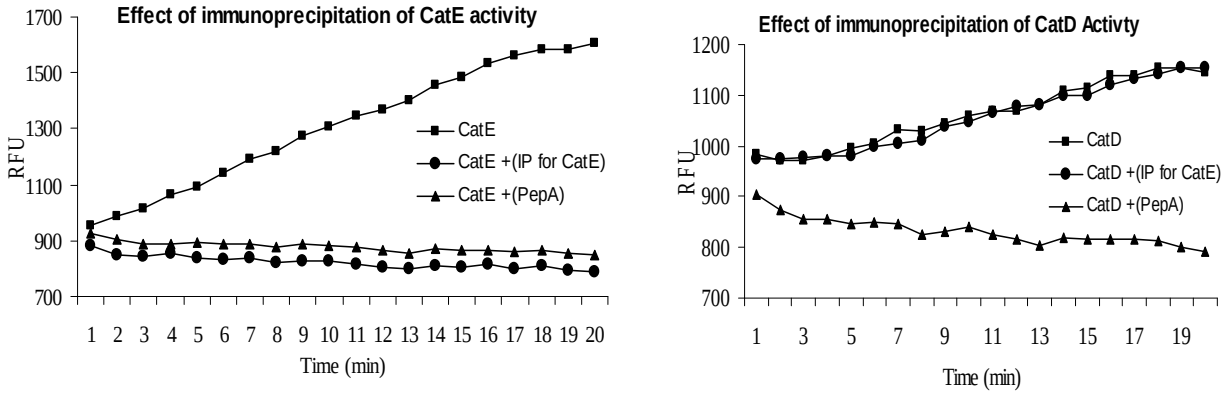

Figure 6. Effect of immunoprecipitation (IP) of CatE and pepstatin A treatment on (A) CatE and (B) CatD activities. (A) *(■) Hydrolysis of the fluorogenic peptide substrate* Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 *(1µM) by 10 ng of CatE in 50mM sodium acetate buffer (pH 4) at 37°C. (▲) Incubation with pepstatin A for 15 min at 37°C prior to hydrolysis inhibited the activity of CatE completely. (●) Immunoprecipitation (IP) of CatE prior to hydrolysis reaction also completely inhibited the activity of CatE.* ***(B)*** *(■) Hydrolysis of the fluorogenic peptide substrate (1µM) by 10 ng of CatD in 50mM sodium acetate buffer (pH 4) at 37°C. (▲) Incubation with pepstatin (1µM) A for 15 min at 37°C prior to hydrolysis reaction inhibited the activity of CatD completely (●) IP of CatE prior to hydrolysis has no effect on CatD activity, hence IP was specific towards CatE only.*

2.4.6 Activity of Cat E and CatD in subcellular fractions of different cell types

The activity of CatE and CatD was determined in subcellular fractions of different cell types using a combination of the peptide substrate, aspartic proteinase inhibitor (pepstatin A) and depletion of CatE by immunoprecipitation. Activities were determined by linear regression using a minimum of five measurement points as described in Materials and Methods. The activity in all subcellular fractions of these different cell types was completely inhibited when the samples were pre-incubated with pepstatin A (TAPA).

For differential measurements of CatE and CatD activity, samples were subjected to IP (immunoprecipitation) of CatE. The decrease in activity after IP is attributed to CatE and the remaining activity is assigned to CatD. As expected, the highest CatD activity was determined for lysosomal fractions of all the three cell types tested [156]. In contrast, CatE activity was

mainly detected in endosomal fractions as indicated in **Table 2** and **Figure 7 A-C**. A low level of CatD activity was determined for endosomal fractions of all three cell types. In HaCaT and DCs a low level of CatE activity was found in lysosomal fractions. In the EBV-transformed B cell line (WT 100) an almost equal level of CatE and CatD activity was found in the lysosomal fraction, most likely due to overlapping subcellular fractions. Cytosolic fractions of all three cell types showed very low CatE activity, but no CatD activity. Moreover, the overall activity in subcellular fractions of the three cell types tested varied substantially, as did CatE and CatD activity. DCs showed highest while WT100 showed lowest overall activity.

As shown in **Table 2**, endosomal fractions (EFs) of HaCaT showed about 5.5fold larger CatE activity as compared to the corresponding fractions of WT100, whereas DCs (EFs) showed around 19 times larger CatE activity than WT100 (EFs). **Table 2** also demonstrates that lysosomal fractions (LFs) of HaCaT had 7.2 times higher CatD activity than LFs of WT100 and LFs from DCs had around 16.6 times higher CatD activity than LFs of WT100.

2.4.7 Analysis of peptide fragments obtained by digestion of the fluorogenic substrate with subcellular fractions, CatE or CatD, using RP-HPLC and MALDI-MS

To further confirm that the activity measured in subcellular fractions by the fluorescence assay was only due to aspartic proteinases, the peptide substrate was digested by CatE, CatD or subcellular fractions (as described in Experimental Procedures). The peptide fragments thus generated were separated by RP-HPLC using fluorescence detection (λ_{ex} =350, λ_{em} = 450) and identified by MALDI-MS. This method allowed detection of only N-terminal fragments containing the fluorophore Mca.

Figure 8A shows the chromatogram of the undigested peptide substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 as a negative control. The fluorescence signal is quenched due to resonance energy transfer between the fluorophore and the quencher group. **Figure 8B** depicts the results of digestion of the substrate with CatE, leading to only one cleavage product, because only the Phe-Phe bond is susceptible to cleavage by CatE or CatD [19]. The peak with a retention time of 25.54 min corresponds to fragment Mca-Gly-Lys-Pro-

Ile-Leu-Phe as analyzed by mass spectrometry (**Table 3**). **Figure 8C** shows digestion of the substrate with CatD giving a profile similar to that of CatE, i.e. only one peak is visible with the same retention time. However, when digested with the lysosomal fraction (**Figure. 8E**.) of HaCaT, an additional peak with the retention time of 22.87 min was observed. Digestion of substrate with the endosomal fraction (**Figure. 8F**.) of HaCaT gave similar RP-HPLC-profile as the lysosomal fraction.

Digestion of the substrate with LF (lysosomal fraction) and EF (Endosomal fraction) (**Figure. 8H.** and **I.**) was completely inhibited by pepstatin A confirming that the activity observed in our assay was solely due to aspartic proteinases. The additional peak observed after digestion of substrate with LF and EF (**Figure 8E** and **F**) was a C-terminal truncated peptide (Mca-Gly-Lys-Pro-Ile-Leu) as analyzed by MALDI-MS (**Table 3**). This carboxypeptidase activity can only occur after aspartic proteinases have created cleavage products, as the undigested substrate contains a protecting D-Arg residue at the C-terminus.

Substrate digestion by the lysosomal fraction (**Figure 8K**) after IP of CatE had almost no effect on the RP-HPLC profile. This indicates that the activity observed in LF was mainly due to CatD. Digestion by the endosomal fraction (**Figure 8L**) was inhibited after IP of CatE indicating that the activity in the endosomal fraction was primarily CatE activity. The cytosolic fraction (CF) had not indicated any cleavage; hence no activity of CatE and CatD was observed by RP-HPLC. This coincides with the results from the fluorescence assay by which only very low level activity in CF was determined. Digestion of substrate with subcellular fractions of DCs and WT100 also gave similar profiles in RP-HPLC (data not shown).

In conclusion, the combination of methods described here facilitates the specific and parallel measurement of CatE and CatD activity in antigen-processing organelles. The data clearly show that our approach for detection of CatE and CatD is more sensitive than immunodetection by Western blot analysis. It allows detection of CatE activity in subcellular fractions of WT100, compared to Western blot analysis, where CatE was not detectable. With this assay it was also possible to discriminate between CatD activity in endosomal and lysosomal fractions, whereas the difference between the amounts of CatD in these fractions was not significant when detected by Western blot.

Theses experimental conditions are also more specific compared to previously described assays, because specificity of detection was not only based on the peptide sequence but was markedly increased by the use of monospecific antibody, utilized for depletion of CatE. This type of assay is flexible and can be used for discriminating activity of other proteinases with similar enzymatic properties.

This approach distinguishes between the activities of enzymatically similar proteinases CatE and CatD and therefore can be used in studies aimed at understanding the involvement these enzymes in antigen processing and presentation.

Table 2. CatE and CatD activity in subcellular fractions (pmol Mca liberated / min / 20 μg of total protein) of different cell lines. *Activities were determined by linear regression analysis taking at least five measurement points. Values depicted are means ± SD (DCs, n = 2; HaCaT and WT100, n = 3, where n is the number of individual experiments performed).*

Cell line	Activity	Cytosolic fraction pmol Mca liberated/min	Lysosomal fraction pmol Mca liberated /min	Endosomal fraction pmol Mca liberated/min
HaCaT	TAPA[a]	13.4 ± 2.15	106.3 ± 6.94	82.0 ± 11.33
	Cat E	13.4 ± 2.15	25.3 ± 14.01	65.4 ± 11.22
	Cat D	n.d[b]	80.9 ± 12	16.6 ± 8.40
WT100	TAPA[a]	0.49 ± 0.19	20.9 ± 1.99	13.7 ± 0.69
	Cat E	0.49 ± 0.19	9.5 ± 0.54	11.8 ± 1.23
	Cat D	n.d[b]	11.3 ± 2.54	1.9 ± 0.77
DCs	TAPA[a]	0.76 ± 0.24	210.7 ± 18.40	232.8 ± 49.65
	Cat E	0.76 ± 0.24	23.2 ± 20.54	225.7 ± 50.38
	Cat D	n.d[b]	187.5 ± 2.19	7.1 ± 0.72

[a] TAPA; total aspartic proteinase activity [b] Not detectable

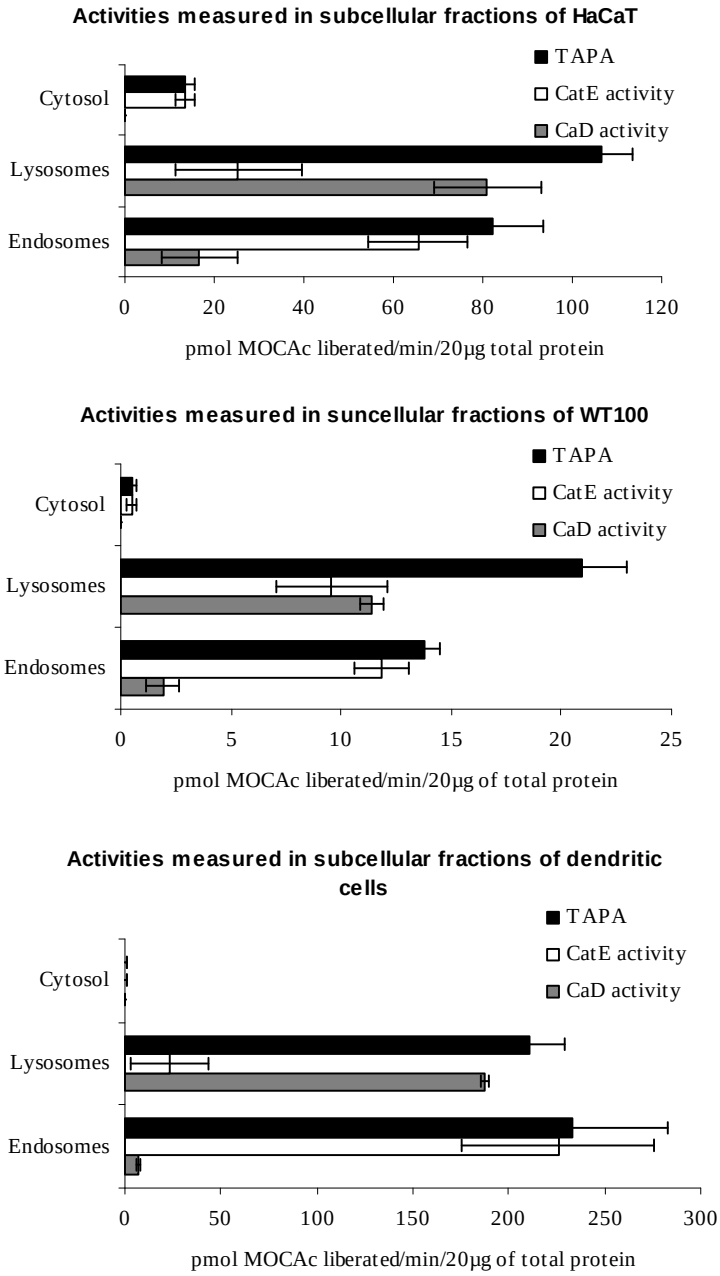

Figure 7. Distribution of TAPA (total aspartic protease activity), CatE and CatD activity in subcellular fractions of (A) HaCaT. (B) WT100. (C) DCs*. Equal amounts of total protein (20 µg) were used for the determination of CatE and CatD activities, determined by linear regression analysis using a minimum of five measurement points. Values depicted are means ± SD (DCs, n = 2; HaCaT and WT100, n = 3, where n is the number of individual experiments).*

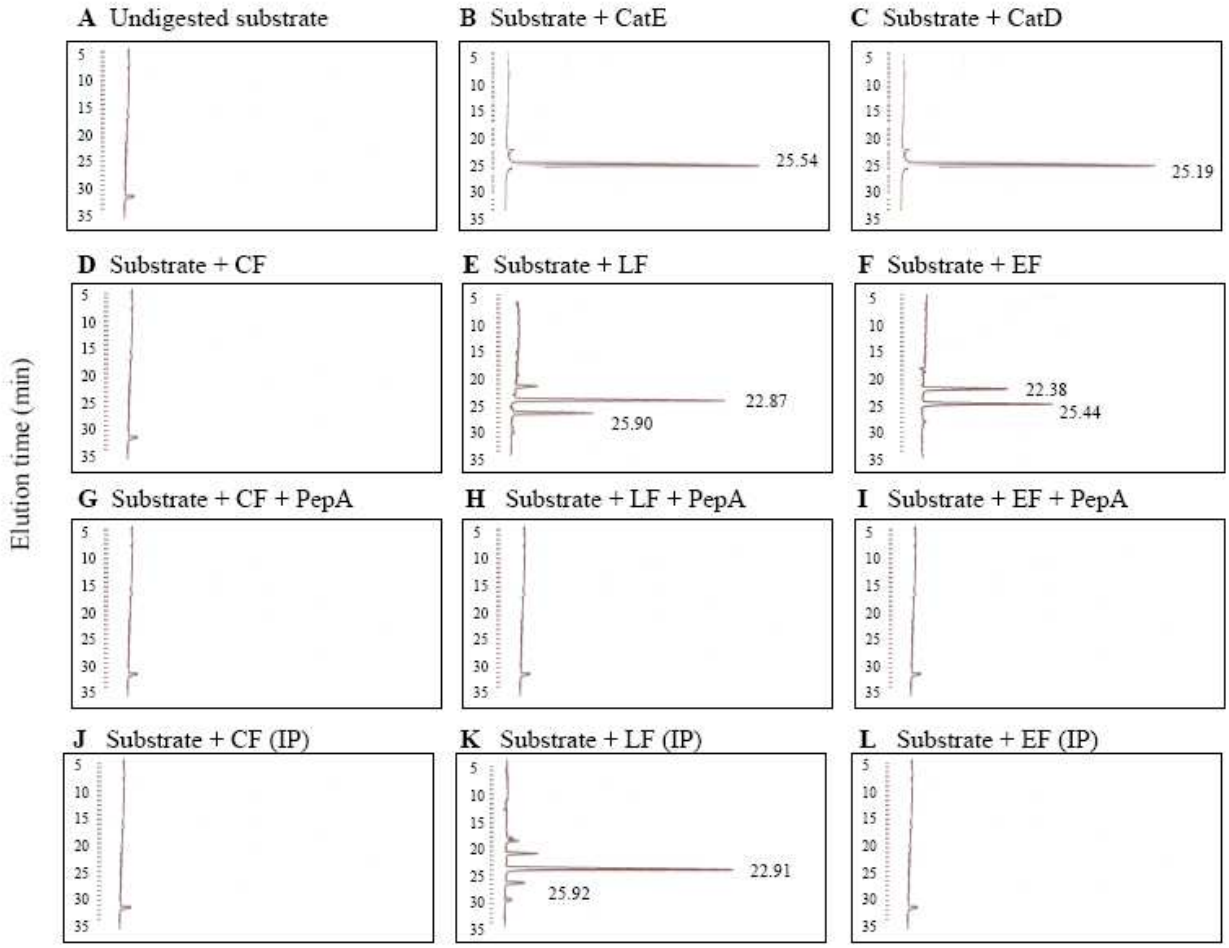

Fig 8. RP-HPLC profiles of peptide fragments obtained after digestion of the substrate with CatE, CatD or subcellular fractions of HaCaT. *Fluorogenic peptide substrate* Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 *(10μM) was incubated at 37°C in digestion buffer (50 mM sodium acetate buffer, pH 4.0) containing CatE (10 ng), CatD (10 ng) or subcellular fraction (20μg)* ***(A)*** *Undigested fluorogenic substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2.* ***(B)*** *Substrate digested with CatE* ***(C)*** *CatD* ***(D)*** *CF (Cytosolic fraction)* ***(E)*** *LF (Lysosomal fraction)* ***(F)*** *EF (Endosomal fraction)* ***(G)*** *CF after PepA treatment* ***(H)*** *LF after PepA treatment* ***(I)*** *EF after PepA treatment* ***(J)*** *CF after IP of CatE* ***(K)*** *LF after IP of CatE* ***(L)*** *EF after IP of CatE.*

Table 3. Identified peptides after digestion of fluorogenic peptide substrate by CatE, CatD and subcellular fractions of HaCaT as determined by MALDI-MS.

Retention times allude to those in figure 8.

Sample	**Digestion products**	**Retention time (min)**	**Expected mass $[M+H]^+$**	**$[M+H]^+$**	**ΔDa**
CatE	Mca-GLPILF-OH	25.54	890.80	890.77	0.03
CatD	Mca-GLPILF-OH	25.19	890.80	891.76	0.96
Lysosomal fraction	Mca-GLPILF-OH	25.90	890.80	890.90	0.1
	Mca-GLPIL-OH	22.87	743.70	743.70	0.0
Endosomal fraction	Mca-GLPILF-OH	25.44	890.80	890.80	0.0
	Mca-GLPIL-OH	22.38	743.70	743.80	0.1

3

3 A novel cell penetrating aspartic protease inhibitor blocks processing and presentation of tetanus toxoid more efficiently than pepstatin A

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3.1 Abstract

Selective inhibition of enzymes involved in antigen processing such as cathepsin E and cathepsin D is a valuable tool for investigating the roles of these enzymes in the processing pathway. However, the aspartic protease inhibitors, including the highly potent pepstatin A (PepA), are inefficiently transported across the cell membrane and thus have limited access to antigen processing compartments. Previously described mannose-pepstatin conjugates were efficiently taken up by the cells *via* receptor mediated uptake. However, cells without mannose receptors are unable to take up these conjugates efficiently. The aim of the present study was to synthesize new cell permeable aspartic protease inhibitors by conjugating pepstatin A with well-known cell penetrating peptides (CPPs). To achieve this, the most frequently used CPPs namely pAntp(43-58) (penetratin), Tat(49-60), and the 9-mer of L-arginine (R9), were synthesized followed by coupling pepstatin A to the peptides. The enzyme inhibition properties of these bioconjugates and their cellular uptake into MCF7 (human breast cancer cell line), Boleths (EBV-transformed B cell line) and dendritic cells (DC) was studied. We found that the bioconjugate PepA-penetratin (PepA-P) was the most efficient cell-permeable aspartic protease

inhibitor in comparison to PepA. Additionally, we found that PepA-P efficiently inhibited the tetanus toxoid C-fragment processing in peripheral blood mononuclear cells (PBMC), primary DC and in primary B cells. Therefore, PepA-P can be used in studying the role of intracellular aspartic proteases in the MHC class II antigen processing pathway. Moreover, inhibition of tetanus toxoid C-fragment processing by PepA-P clearly implicates the role of aspartic proteinases in antigen processing.

3.2 Introduction

Cathepsin E (CatE) and cathepsin D (CatD) are the major intracellular aspartic proteases in the endolysosomal pathway. The involvement of these proteases in cellular processes such as antigen processing and presentation is mainly demonstrated by using specific aspartic protease inhibitors [12, 13, 56, 105]. However, the aspartic protease inhibitors, including the highly potent pepstatin A (isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid) [163, 164], a peptide originally isolated from the filtrates of cultures of *Streptomyces* [165], are inefficiently transported across the cell membrane [75].

In a recent study, mannose-pepstatin conjugates were used as cell-permeable aspartic protease inhibitors, and these inhibitors blocked ovalbumin processing in dendritic cells [12, 75]. These conjugates were reported to show higher solubility in water compared to pepstatin A (PepA) and were efficiently internalized by the cells *via* receptor mediated uptake. However, the cells, which do not carry the mannose receptors such as B cells, are not able to take up these conjugates efficiently [12, 75]. Therefore, the application of these inhibitors is limited to cells bearing mannose receptors.

A valuable approach for intracellular delivery of macromolecules involves their conjugation to the cell-penetrating peptides (CPPs) [166-169]. These peptides can be internalized by most cell types and also have the capability of co-transporting the conjugated biomolecules both *in vivo* [170] and *in vitro* [171]. CPPs have been employed in the intracellular delivery of a wide range of biomolecules such as antigenic peptides [172], antisense oligonucleotides [173], full length

proteins [174, 175] or nano particles [176] and liposomes [177]. The most widely used CPPs are peptides from HIV-1 Tat [178, 179], antennapedia protein of Drosophila (penetratin) [180] and synthetic peptides such as oligo-arginine [181].

The present study illustrates the synthesis of new cell permeable inhibitors of aspartic proteases that are not cell specific. The most frequently used CPPs namely, pAntp(43-58) (penetratin), Tat(49-60) and the 9-mer of L-arginine (R9), were synthesized on a trityl resin followed by coupling pepstatin A as a complete molecule to the N-terminal amino group of the peptides with an amide bond. The enzyme inhibition properties of these bioconjugates and their cellular uptake into MCF7 (human breast cancer cell line), Boleths (EBV-transformed B cell line) and dendritic cells was examined. We found that the bioconjugate PepA-penetratin (PepA-P) is the most effective cell-permeable aspartic protease inhibitor in comparison to PepA. Moreover, it was observed that PepA-P efficiently inhibited the tetanus toxoid processing in peripheral blood mononuclear cells (PBMC), primary dendritic cells (DC) and in primary B cells. Therefore, PepA-P can be a useful tool for studies aimed at understanding the role of intracellular aspartic proteases in the MHC class II antigen processing pathway in different antigen presenting cells.

3.3 Experimental procedures

3.3.1 Enzymes and chemicals

Cathepsin D was purchased from Calbiochem (Darmstadt, Germany) and stored as a 300 U/ml stock solution in 0.1 M citrate buffer, pH 4.5, at -20 °C. Cathepsin E was purchased from R&D systems (Wiesbaden, Germany) and stored as, 0.1 mg/ml stock solution in 25 mM MES, 0.15 M NaCl, pH 6.5, and 50 % glycerol, at -20 °C. Pepstatin A was purchased from Calbiochem. The substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 [19] was obtained from Bachem (Weil am Rhein, Germany).

3.3.2 Synthesis of pepstatin-CPP conjugates

Peptide sequences are: RQIKIWFQNRRMKWKK (pAntp $_{(43-58)}$, Penetratin), RKKRRQ-RRRPPQ ($Tat_{(49-60)}$), RRRRRRRRR (R9). Peptides were synthesized using standard Fmoc/tBu chemistry [182] and synthesis was performed on the multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany) on a 0.025-mmol scale using a 6-fold molar excess of Fmoc amino acids (MultiSynTech, Witten, Germany) on TCP-resin (PepChem, Reutlingen, Germany). All reagents and solvents for peptide synthesis were purchased from Merck KGaA (Darmstadt, Germany). *In situ* activation was performed using TBTU (6 eq) and HOBt (1 eq) followed by the addition of N-methylmorpholine (12 eq) in N,N-dimethylformamide. After completion of the automated synthesis, the resin-bound peptides were Fmoc-deprotected using 20 % (v/v) piperidine in N,N-dimethylformamide twice for 15 min and washed subsequently with N,N-dimethylformamide, isopropyl alcohol, and diethyl ether.

Pepstatin A (25.7 mg, 0.0375 mmol) was dissolved in 1ml DMF and coupled manually to each resin bound peptide according to the aforementioned TBTU/NMM/HOBt procedure. Coupling time was 12 hrs at room temperature. The resin was washed intensively with DMF, isopropyl alcohol and finally with diethyl ether. To release the peptides from the resin and to remove the side chain protecting groups, the following solution was used: 92% (v/v) trifluoroacetic acid containing 3% (v/v) thioanisol, 3% (w/v) phenol, and 2% (v/v) ethanedithiol. The crude products were precipitated and washed twice in diethyl ether, dried, and dissolved in 80 % (v/v) tert-butanol in water followed by lyophilisation. Crude compounds were purified using preparative

reversed-phase high performance liquid chromatography (RP-HPLC) and the identity of the conjugates was confirmed using MALDI-MS. Purity of each PepA-CPP conjugate was determined *via* analytical RP-HPLC and proved to be above 95 %.

Only the peptides (penetratin, $Tat_{(49-60)}$, and R9) without PepA were also used in some experiments. These peptides were released from the resin directly as described above.

3.3.3 Proteinase assay for determination of IC_{50} values for PepA and PepA-CPP conjugates against CatE and CatD

Aspartic proteinase activity was determined fluorometrically by hydrolysis of the substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2. CatE (10 ng) or CatD (10 ng) were pre-incubated with various concentrations of PepA or PepA-CPP conjugates (at a series of twofold dilutions) in 50 mM sodium acetate buffer, pH 4.0. The reaction was started by addition of 1nmol substrate (stock solution: 1 mM in DMSO, 1 µl of stock solution was dissolved in 10 µl of the reaction buffer separately and then added to the reaction mixture). Progress of fluorescent product formation was recorded using a fluorescence reader (Tecan Spectra Fluor, Crailsheim, Germany) on kinetic mode at 37°C (λ_{ex} =340, λ_{em} = 405). Activities were determined by linear regression analysis using a minimum of 15 measurement points. Inhibitory activity of PepA and its conjugates is expressed as IC_{50}, which represents the concentration of the compound that inhibits 50 % of the maximal (uninhibited) enzyme activity.

3.3.4 Cell culture

PBMC (peripheral blood mononuclear cells) were isolated by Ficoll/Paque (PAA Laboratories Pasching Austria) density gradient centrifugation of heparinized blood obtained from buffy coats. Isolated PBMC were plated (1x 10^8 cells/8 ml/ flask) into 75 cm^2 Cellstar tissue culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) in RPMI 1640 (Gibco Life Technologies), supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS, Gibco), penicillin (final concentration: 100 U/ml, Gibco) and streptomycin (final concentration: 0.1 mg/ml, Gibco). After 1.5 hour of incubation at 37°C, non-adherent cells

were removed and adherent cells were cultured in complete culture medium supplemented with GM-CSF (Leukomax; Sandoz, Basel, Switzerland) and IL-4 (R&D systems) for six days as described previously [159]. This resulted in a cell population consisting of approximately 80% DCs (data not shown) as determined by flow cytometry (BD FACSCalibur, Heidelberg, Germany).

Boleths (EBV-transformed human B cell line) and MCF7 (human breast cancer cell line) were cultured in RPMI 1640 medium (Gibco Life Technologies, Paisly, United Kingdom) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS, Gibco), penicillin (final concentration: 100 U/ml, Gibco) and streptomycin (final concentration: 0.1 mg/ml, Gibco) at 37 °C in tissue culture flasks (Nunc, Wiesbaden, Germany).

3.3.5 Cellular uptake of PepA and PepA-CPP conjugates

PepA and PepA-CPP conjugates were dissolved in minimal amount of methanol and diluted with medium to final working concentrations. Cells (MCF7, Boleths and dendritic cells) were exposed to 1, 3, 5 and 10 µM of each conjugate for 120 min. At the end of the incubation time the loading medium was removed and cells were washed with PBS. Non-internalized, surface-bound compounds were eliminated by trypsinization for 10 min as recommended by previous studies [166, 183, 184]. Cells were lysed in NP40 lysis buffer (1% NP-40, 50 mM sodium acetate, pH 4.0).

Aspartic proteinase activity in cell extracts was determined fluorometrically by hydrolysis of the substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2, as described in proteinase assay (3.1.3). Each experiment was conducted three times. Results are expressed as % relative residual activity which represents the percent of the total activity in untreated cells (control).

3.3.6 Cell viability assay

MCF7, Boleths and dendritic cells were exposed to increasing concentrations of PepA and PepA-CPP conjugates as described above. The loading medium was removed and cells were washed with PBS. Non-internalized, surface-bound compounds were eliminated by

trypsinization for 10 min. Cells were washed with PBS and apoptosis was determined by flowcytometery (BD FACSCalibur) using 7-amino actinomycin D (7-AAD) (Sigma-Aldrich).

3.3.7 T cell proliferation assay

PBMC from healthy donors were pulsed with the corresponding tetanus toxoid C-fragment (10 µg/ml) for four days. Then the cells were harvested and stained with CD3-PerCP, CD45RA-PE, CD25-APC and CD134-FITC (BD Biosciences, Heidelberg, Germany). The co-expression of CD25 and CD134 of memory T cells (CD3+ and CD45RA-) was analysed by flowcytometry [185]. Additionally, the supernatants were collected and the cytokine production (IFN-γ and IL-2) was measured using ELISA assays, following the protocols described by the company's manual (R&D systems, Minneapolis, MN, USA).

3.4 Results

3.4.1 Synthesis of pepstatin derivatives

PepA-cell penetrating peptide (PepA-CPP) conjugates, namely, PepA-Penetratin (PepA-P), PepA-Tat(49-60), and PepA-R9, were synthesized by solid-phase synthesis on TCP-resin using standard Fmoc/tBu chemistry [182]. In general, C-terminal modified pepstatin derivatives do not alter their inhibitory activity [186]; therefore, cell penetrating peptides were synthesized on a trityl resin and then pepstatin A was coupled as a whole molecule on the N-terminal amino group of the peptide with an amide bond. The completed conjugates were cleaved from the resin by the TFA cleavage mixture. Crude compounds were purified using preparative reversed-phase high performance liquid chromatography (RP-HPLC) and identity of the conjugates was confirmed using MALDI-MS. Purity of each PepA-CPP conjugate was determined *via* analytical RP-HPLC and proved to be higher than 95 %.

The compounds were obtained without any difficulty and their characteristics are summarized in **Table 1**. PepA-Tat(49-60) and PepA-R9 were readily soluble in methanol and DMSO as PepA, whereas PepA-P was comparatively less soluble.

Table 1: Characteristics of PepA-CPP conjugates.

Pepstatin A-CPP conjugates	Conjugated CPP	Sequence of conjugated CPP	$[M+H]^+$ calculated	$[M+H]^+$ measured
PepA-P	$pAntp_{(43-58)}$; Penetratin	RQIKIWFQNRRMKWKK	2914.69	2914.34
PepA-$Tat_{(49-60)}$	HIV-$1Tat_{(49-60)}$	RKKRRQRRRPPQ	2329.91	2330.39
PepA-R9	Oligo-arginine ;R9	RRRRRRRRR	2091.62	2092.28

3.4.2 Inhibitory effect of pepstatin A-CPP conjugates on the enzymatic activity of CatE and CatD

To evaluate the usefulness of PepA-CPP conjugates as cell penetrating aspartic proteinase inhibitors it was important to determine whether or not the inhibitory effect of these conjugates was altered in comparison to PepA. Therefore, the inhibitory activity (IC_{50}) of PepA and the PepA-CPP conjugates (PepA-P, PepA-Tat(49-60) and PepA-R9) against CatE and CatD was determined using FRET-based proteinase assay.

In accordance to previous reports [163, 164], PepA inhibited CatE and CatD with IC_{50} values at subnanomolar concentrations (**Table 2**). PepA-CPP conjugates also potently inhibited CatE and CatD (**Table 2**). Hence the attachment of the cell-penetrating peptides did not result in any significant reduction in the inhibitory effects of PepA

Table 2. Inhibitory effect (IC_{50}) of PepA and PepA-conjugates against CatE and CatD. *Inhibitory activities were determined using FRET-based proteinase assay and expressed as IC_{50} (nM) means ± SD (N = 2, where N is the number of experiments performed)*

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Inhibitor	Cathepsin E	Cathepsin D
PepA	0.255 ± 0.12	0.21 ± 0.13
PepA-P	0.92 ± 0.13	0.92 ± 0.21
PepA-$Tat_{(49-60)}$	0.52 ± 0.14	0.76 ± 0.21
PepA-R9	0.47 ± 0.11	0.72 ± 0.11

3.4.3 Comparative evaluation of cellular uptake of pepstatin A-CPP conjugates

To compare the cellular uptake of PepA or PepA-CPP conjugates, human dendritic cells, human EBV-transformed B-cell line (Boleths) and human breast cell cancer cell line (MCF7) were treated with various concentrations of these compounds. These different cell types were incubated with increasing concentration of PepA and PepA-CPP conjugates, for 120 min as described in experimental procedures. Following the incubation period, cells were washed extensively and then trypsinized for 10 min in order to remove non-internalized surface-bound PepA and PepA-CCP conjugates as suggested by previous studies [166, 183, 184]. The cells were lysed and lysates were subjected to FRET-based proteinase assay in order to measure the reduction in aspartic protease activity. Relative residual activity was calculated for each sample as a percentage of aspartic proteinase activity in cells without the treatment of inhibitors.

As illustrated in **Figures 1A-C** and **Table 3,** a dose-dependent inhibition of aspartic proteinase activity was observed in MCF7 cell line after treatment with PepA and the PepA-CPP conjugates. PepA-P is the most effective cell penetrating inhibitor amongst all the inhibitors tested. At end concentration of 10 µM, PepA-P resulted in only 3.9 ± 0.3% relative residual activity, while at the same concentration PepA, PepA-Tat(49-60) and PepA-R9 resulted in 45.4 ± 5.5%, 29.8 ± 3.2% and 77.0 ± 5.2 % relative residual activities, respectively. The 10 µM concentration of PepA-P was the most effective concentration in inhibiting aspartic proteinases in MCF7 cell line. At higher concentrations of PepA-P, inhibition of aspartic proteases was lower, most likely due to the low solubility of PepA-P in aqueous solution (data not shown).

As demonstrated in **Figures 2A-C** and **Table 3**, the EBV-transformed B cell line Boleths exhibited similar dose-dependent inhibition of aspartic proteinases activity. Also, for this cell line, PepA-P was the most potent inhibitor: 10 µM concentration of PepA-P resulted in only 16.2 ± 1.5 % relative residual activity, whereas the same concentration of PepA and PepA-Tat(49-60) resulted in 88.0 ± 8.1% and 59.2 ± 5.4% relative residual activities, respectively. Of note, 10 µM concentration of PepA-R9 showed no reduction in aspartic protease activity in Boleths **(Fig. 2C, Table 3).**

Dendritic cells also showed similar results; 10 µM concentration of PepA-P (**Figures. 3A-C, Table 3**) resulted in only 14.4 ± 0.5% relative residual activity, whereas, the same concentration of PepA, PepA-Tat(49-60) and PepA-R9 resulted in 59.8 ± 1.0%, 45.4 ± 3.1 and 53.3 ± 2.1 relative residual activities, respectively.

Similar concentrations of the peptides (penetratin, Tat(49-60) and R9) showed no effect on aspartic proteinase activity in all the three cell types tested (**Figures. 1-3, Table 3**). Thus, we found that PepA-P is the most potent cell-permeable aspartic protease inhibitor in comparison to PepA and the other PepA-CPP conjugates tested in the present study.

3.4.4 Cell viability assay

Cell viability assay was performed to determine whether or not the observed reduction in aspartic protease activity in different cell types after treatment with different PepA-CPP conjugates was due to any cytotoxic effect of the conjugates. Cell viability was determined flowcytometrically by 7-AAD staining. The results of two independent experiments revealed that PepA, PepA-Tat(49-60), PepA-P and PepA-R9 were not toxic to human EBV-transformed B-cell line (Boleths) and human breast cell cancer cell line (MCF7) up to the highest concentration (10 µM) investigated in the present study. For dendritic cells PepA-P showed a mild cytotoxicity (around 27%) only at a concentration of 10 µM.

3.4.5 PepA-P reduces tetanus toxoid C-fragment (TTC) specific T cell activation

Having established that PepA-P is the most effective cell penetrating aspartic protease inhibitor in comparison to PepA or other PepA-CPP conjugates tested, the ability of the inhibitor to affect antigen-processing was investigated.

The effect of both PepA and PepA-P was tested in a T cell proliferation assay using primary human cells and the antigen tetanus toxoid C-fragment (TTC). Human peripheral mononuclear cells (PBMC) were isolated from healthy donors followed by the isolation of primary B cells (CD19+), dendritic cells (CD1c+) and untouched CD4+ T cells. Antigen presenting cells (APCs), i.e. PBMC, B cells and dendritic cells, were co-cultured with CD4+ T cells in the presence of the antigen TTC, with or without PepA-P or PepA. After four days in culture,

activated T cells were analyzed by flowcytometric analysis. Activated memory T cells were gated for their expression of CD45RA-, CD3+ and CD25+ and the activation marker CD134 as described previously [185]. T cell activation markers CD25 and CD134 were decreased to a large extent when PBMC were treated with PepA-P. In contrast, PepA treatment showed no difference in the co-expression of CD25 and CD134 in comparison to the experiment without inhibitor (**Figure. 4A**). Similar results were obtained when isolated B cells and dendritic cells were treated with PepA-P (**Figures. 4B-C**). The peptide penetratin alone had no effect on T cell activation.

To further confirm the decreased T cell activation we investigated the cytokine profile in the respective culture supernatants from PBMC. We found that IFN-γ and IL-2 were decreased when PBMC were treated with PepA-P compared to treatment with PepA or penetratin (Fig. 4A). Our data clearly show that the cell permeable aspartic protease inhibitor PepA-P, but not PepA, interfered with antigen processing of intact primary APCs resulting in inhibition of TTC-specific memory T cell activation.

***Table 3**: % Relative residual activity in cell extracts from different cell lines after treatment with PepA-CPP conjugates. Activities were determined by FRET-based proteinase assay followed by linear regression analysis taking at least five measurement points. Relative residual activity was calculated as percentage of total activity in untreated cells. Values depicted are means ± SD; n = 3, where n is the number of individual experiments performe*d.

% Relative residual activity in MCF7 cell line after treatment with different concentrations of PepA-CPP conjugates				
Inhibitor	**1 µM**	**3 µM**	**5 µM**	**10 µM**
PepA	112.8 ± 13.5	110.5 ± 14.4	82.3 ± 10.2	45.4 ± 5.5
Penetratin	116.5 ± 9.1	117.2 ± 16.0	119.3 ± 10.6	101.0 ± 13.8
PepA-P	80.5 ± 3.7	29.2 ± 1.7	14.6 ± 2.2	3.9 ± 0.3
$Tat_{(49-60)}$	89.9 ± 4.1	90.0 ± 0.65	101.1 ± 4.1	88.6 ± 4.9
PepA-$Tat_{(49-60)}$	88.6 ± 4.9	77.0 ± 3.1	73.7 ± 6.7	29.8 ± 3.2
R9	101.4 ± 3.5	97.6 ± 2.9	96.0 ± 4.2	103.4 ± 2.5
PepA-R9	102.4 ± 3.1	89.0 ± 4.6	79.3 ± 12.2	77.0 ± 5.2
% Relative residual activity in Boleths cell line after treatment with different concentrations of PepA-CPP conjugates				
Inhibitor	**1 µM**	**3 µM**	**5 µM**	**10 µM**
PepA	104.2 ± 8.2	96.5 ± 10.7	92.9 ± 7.6	88.0 ± 8.1
Penetratin	98.3 ± 7.0	102.4 ± 8.5	95.6 ± 7.4	103.0 ± 6.0
PepA-P	62.8 ± 3.3	57.8 ± 4.3	34.1 ± 3.8	16.2 ± 1.5
Tat(49-60)	99.4 ± 9.0	87.7 ± 6.9	92.5 ± 3.5	97.3 ± 7.3
PepA-Tat(49-60)	91.3 ± 3.9	95.9 ± 0.9	73.0 ± 3.8	59.2 ± 5.4
R9	90.7 ± 1.9	94.5 ± 8.2	87.4 ± 6.7	100.5 ± 3.2
PepA-R9	116.5 ± 4.0	114.2 ± 3.6	111.6 ± 1.8	114.6 ± 6.6
% Relative residual activity in Dendritic cells after treatment with different concentrations of PepA-CPP conjugates				
Inhibitor	**1 µM**	**3 µM**	**5 µM**	**10 µM**
PepA	82.5 ± 0.7	79.6 ± 3.4	59.3 ± 2.7	59.4 ± 1.0
Penetratin	94.3 ± 1.1	97.2 ± 3.3	100.0 ± 2.9	100.8 ± 3.9
PepA-P	51.9 ± 4.7	34.8 ± 2.0	15.9 ± 1.0	14.4 ± 0.5
Tat(49-60)	108.7 ± 6.0	112.7 ± 2.9	70.7 ± 7.1	87.4 ± 16.2
PepA-Tat(49-60)	65.0 ± 4.6	66.3 ± 4.0	48.7 ± 1.3	45.4 ± 3.1
R9	103.6 ± 0.2	87.2 ± 3.8	101.4 ± 2.7	100.0 ± 2.1
PepA-R9	72.9 ± 2.9	72.4 ± 3.0	70.0 ± 1.1	53.3 ± 2.1

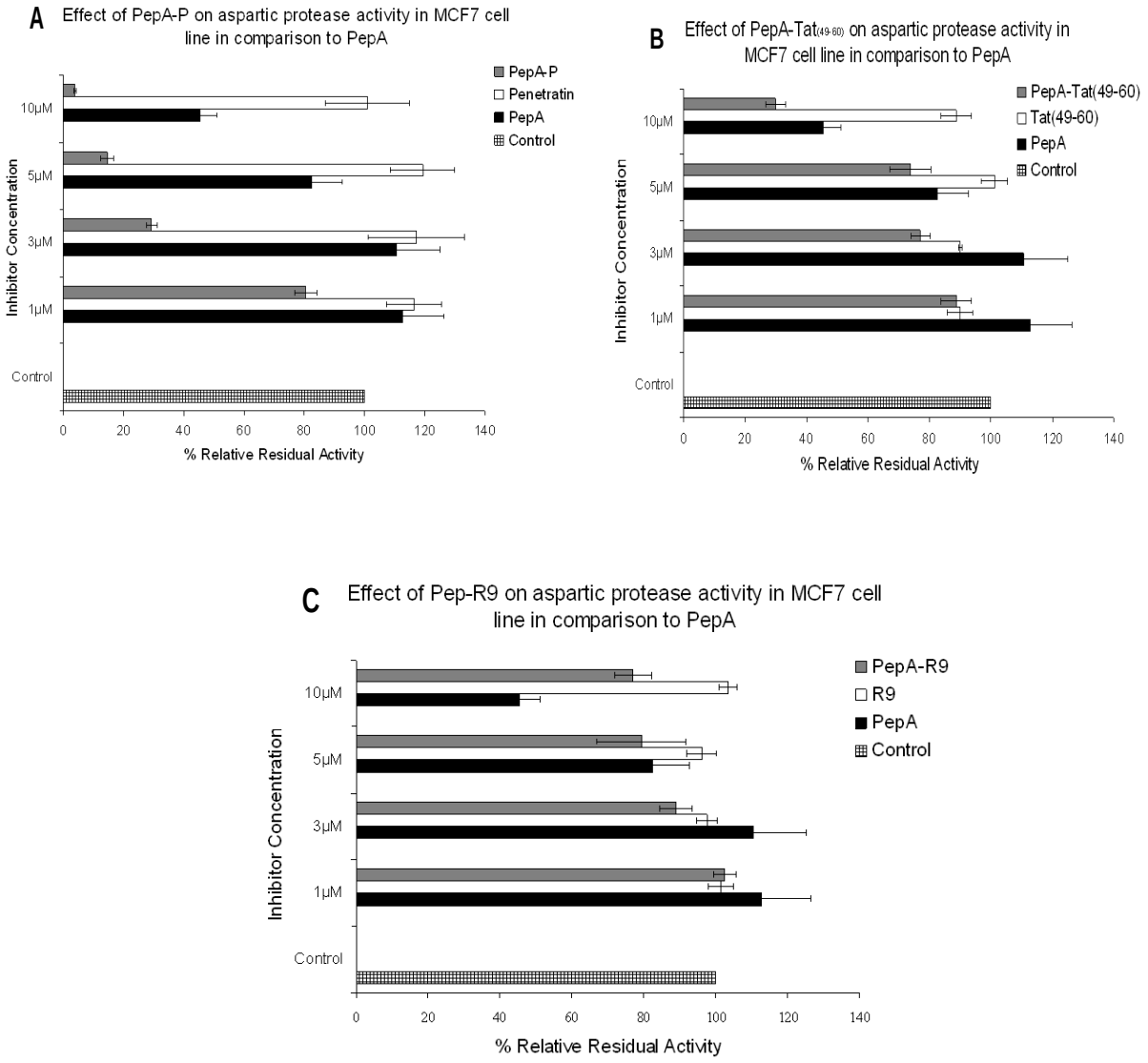

Figure 1. Dose-dependent inhibition of aspartic proteinase activity in MCF7 cell line. *MCF7 cells were incubated with increasing concentrations of PepA(1-10 µM), different PepA-CPP conjugates or CPPs for 120 mins. By trypsinization non-internalized cell surface bound conjugates and peptides were removed. Reduction in aspartic proteinase activity in the cell extracts from all the samples was determined using FRET-based proteinase assay. Effect of (A) PepA-P (B) PepA- Tat$_{(49-60)}$ (C) PepA-R9 in comparison with PepA is represented as relative residual activity, calculated as a percentage of aspartic proteinase activity in cells without the treatment of inhibitors (control). The most effective cell penetrating inhibitor was PepA-P for MCF7 cell line.*

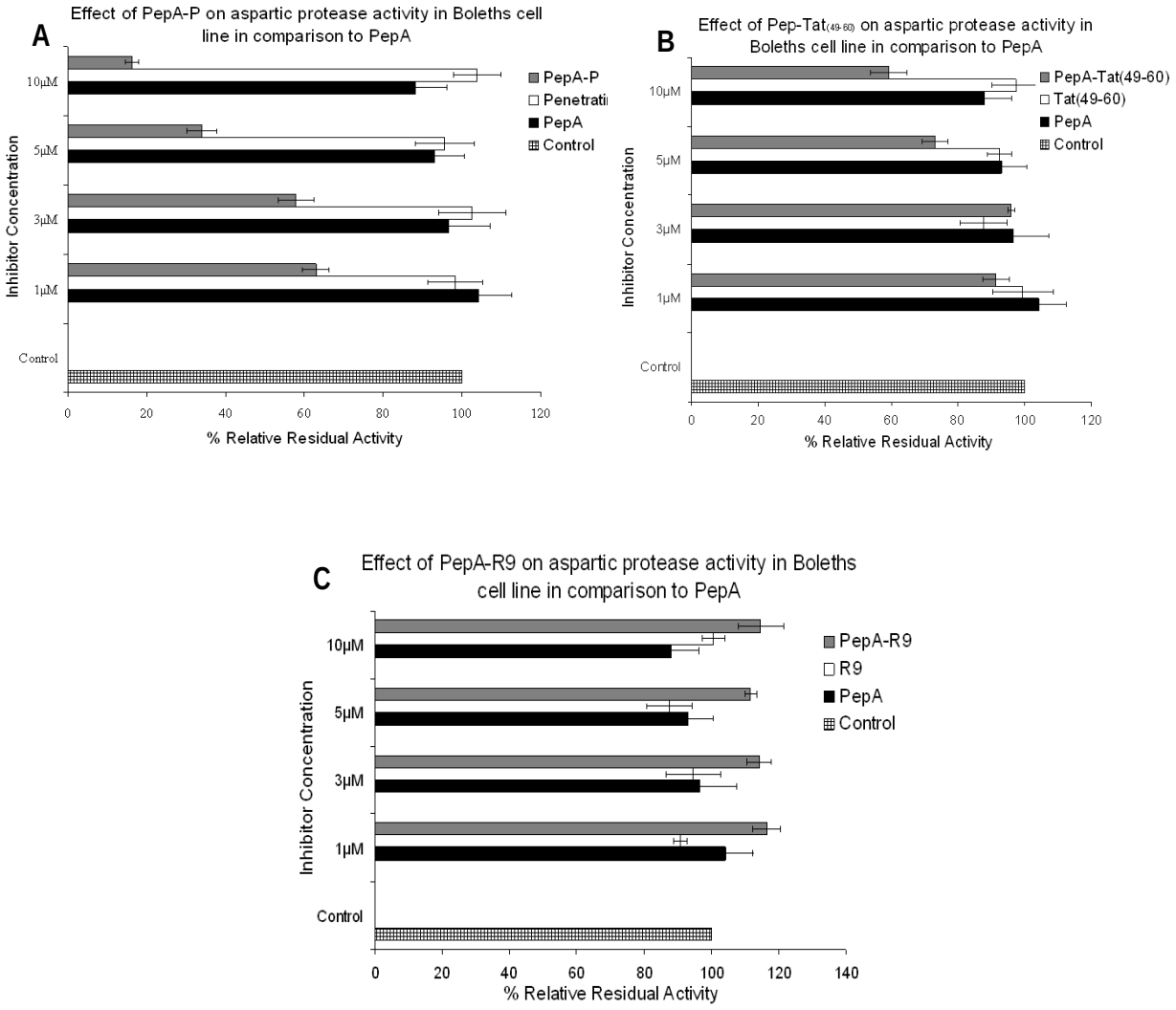

Figure 2. Dose-dependent inhibition of aspartic proteinase activity in Boleths cell line *Effect of (A) PepA-P (B) PepA- Tat$_{(49-60)}$ (C) PepA-R9 in comparison with PepA on aspartic proteinase activity in Boleths cell line. Aspartic proteinase activity in the cell extracts from all the samples was determined using FRET-based proteinase assay. For experimental conditions see legend to figure 1.*

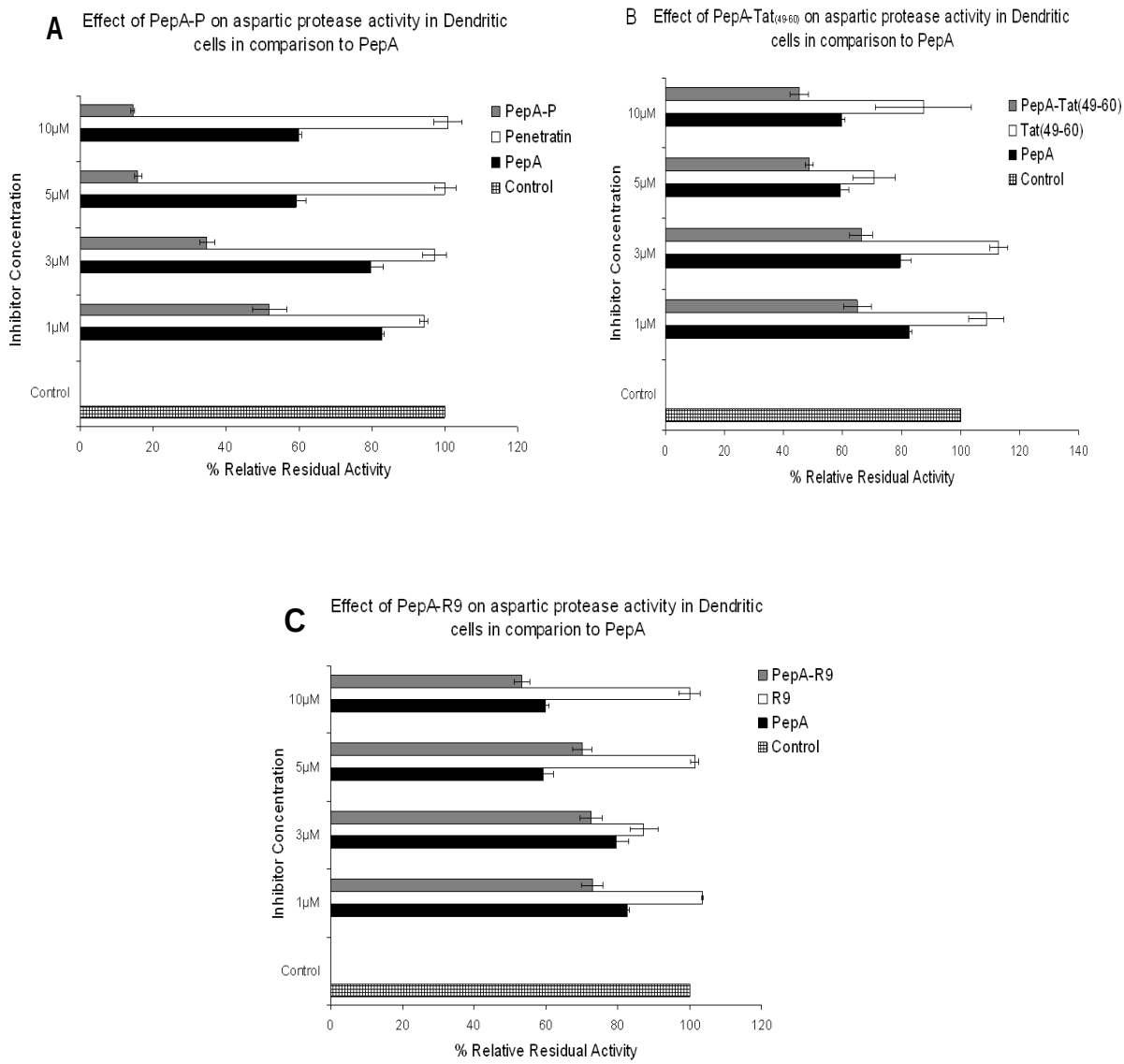

Figure 3. Dose-dependent inhibition of aspartic proteinase activity in dendritic cells. *Effect of (A) PepA-P (B) PepA- Tat$_{(49-60)}$ (C) PepA-R9 in comparison with PepA on aspartic proteinase activity in dendritic cells. Aspartic proteinase activity in the cell extracts from all the samples was determined using FRET-based proteinase assay. For experimental conditions see legend to figure 1.*

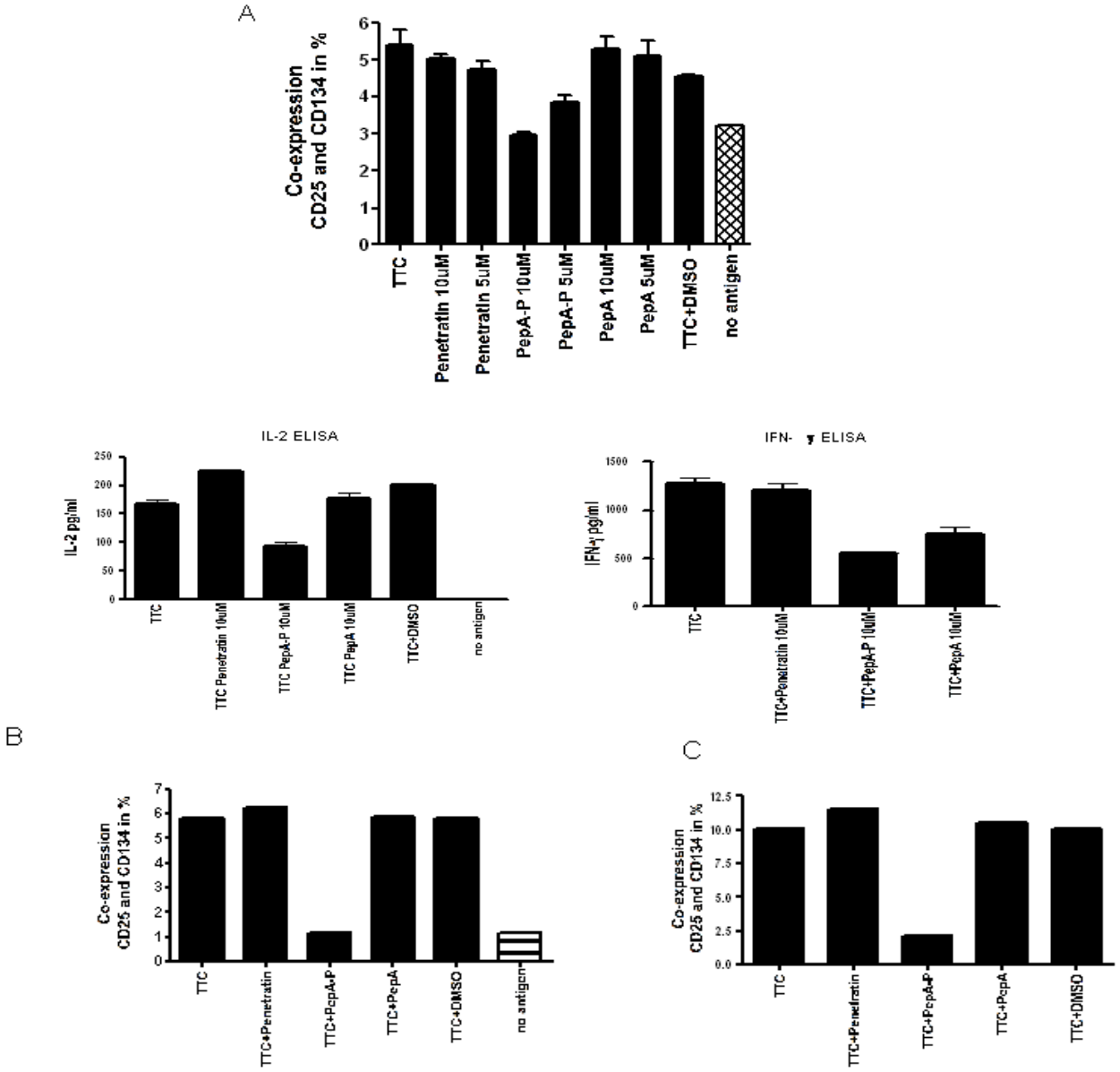

Figure 4. *T cell activation was decreased when incubated with PepA-P. (A) PBMC, (B) magnetic beads isolated B cells (CD19+) or (C) mDC (CD1c+) were co-cultured with autologous T cells in the presence or absence of penetratin, PepA-P and PepA. Tetanus toxoid C-fragment (TTC, 5 μg/ml) was then added and cultured for five days. Activation of memory T cells was determined by analyzing the co-expression of CD25 and CD134 flowcytometrically. IL-2 and IFN-γ production were identified in the respective supernatant by ELISA. Data are representative of three independent experiment*

3.5 Discussion

Our objective was to synthesize cell-penetrating inhibitors of aspartic proteases that are not only specific for any particular type of cells. To achieve this the most frequently used CPPs, namely $pAntp_{(43-58)}$ (penetratin), $Tat_{(49-60)}$ and the 9-mer of L-arginine (R9) were synthesized followed by coupling pepstatin A to the peptides. We report that the bioconjugate PepA-penetratin (PepA-P) is the most effective cell-penetrating aspartic protease inhibitor in comparison to PepA and other PepA-CPP conjugates evaluated in the present study.

The studies aimed at understanding the role of aspartic proteases in antigen processing are difficult to pursue mainly due to two problems. The first problem is the lack of a specific substrate for both, CatE and CatD. The two enzymes have similar enzymatic properties and it is difficult to distinguish between their activities. To address this problem we have recently described a new approach to differentiate between the activities of cathepsin E and cathepsin D in organelles relevant to antigen processing [71].

The other major problem is the absence of an inhibitor which clearly differentiates between CatE and CatD. A specific inhibitor for CatE, the *Ascaris* pepsin inhibitor, which was reported to have no activity against cathepsin D, has been described [26]. However, this inhibitor is not readily available in sufficient quantities for functional studies because of difficulties in purification. Furthermore, the recombinant inhibitor may have a slightly different inhibitory profile [74].

In order to compensate for the absence of a specific inhibitor for CatE or CatD, in recent studies on the role of cathepsin E and cathepsin D in antigen processing, the tendency has been to use a combination of genetic deficiency (CatD-deficient mice) and different aspartic protease inhibitors, mainly pepstatin A. In the cells derived from CatD-deficient mice, the aspartic protease inhibitors will selectively block CatE, as cathepsin E and cathepsin D are the only known pepstatin A-sensitive aspartic proteases that are expressed on protein level in cells of the immune system. However, the aspartic protease inhibitors, including the most widely used and highly potent pepstatin A (PepA), are inefficiently transported across the cell membrane. To overcome this problem Chain *et al.* [12] have recently described mannose derivatives of pepstatin that showed higher solubility in water as compared to pepstatin and were efficiently

taken up by the cells *via* receptor-mediated uptake. However, cells without mannose receptors can not take up these conjugates efficiently.

Over the past decade, several cell penetrating peptides have been identified and employed in a number of studies for the delivery of conjugated macromolecules [166, 167, 169, 187, 188]. In this study we report the synthesis of pepstatin A-cell penetrating peptide (PepA-CPP) conjugates. The most frequently used CPPs, namely $Tat_{(49-60)}$, $pAntp_{(43-58)}$ (penetratin) and the 9-mer of L-arginine (R9) were synthesized and then pepstatin A was coupled as a whole molecule on the N-terminal amino group of the peptides with an amide bond. Our results indicate that the attachment of the cell penetrating peptides does not result in any significant reduction in the inhibitory effects of PepA. We have demonstrated that among PepA and the three conjugates (PepA-penetratin (Pep-P), PepA-$Tat_{(49-60)}$ and PepA-R9) synthesized and tested, PepA-P was the most efficient cell permeable aspartic protease inhibitor and inhibited the aspartic protease activity in intact human dendritic cells, human EBV-transformed B-cell line (Boleths) and human breast cell cancer cell line (MCF7). Furthermore, PepA-P reduced tetanus toxoid C-fragment processing in PMBC (peripheral mononuclear blood cells), DCs (dendritic cells) and B cells. These results suggest that PepA-P possesses an inhibitory effect against aspartic proteases not only *in vitro*, but also after internalization into intact cells.

Additionally, inhibition of tetanus toxoid C-fragment processing by PepA-P clearly implicates the role of aspartic proteinases in antigen processing which confirms the previous studies reporting the processing of TTC by cathepsin E and cathepsin D [15, 189], that are the major intracellular aspartic proteinases.

Thus, in conclusion, pepstatin A- penetratin (PepA-P) conjugate can serve as an important new tool to investigate the role of aspartic proteinases in antigen processing and presentation in different antigen presenting cells.

4

4 Recombinant cathepsin E has no proteolytic activity at neutral pH

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4.1 Abstract

Cathepsin E (CatE) is a major intracellular aspartic protease, reported to be involved in cellular protein degradation and several pathological processes. Distinct cleavage specificities of CatE at neutral and acidic pH have been reported previously in studies using CatE purified from human gastric mucosa. Here, in contrast, we have analysed the proteolytic activity of recombinant CatE at acidic and neutral pH using two separate approaches, RP-HPLC and FRET-based proteinase assays. Our data clearly indicate that recombinant CatE does not possess any proteolytic activity at all at neutral pH and was unable to cleave the peptides glucagon, neurotensin and dynorphin A that were previously reported to be cleaved by CatE at neutral pH. Even in the presence of ATP, which is known to stabilize CatE, no proteolytic activity was observed. These discrepant results might be due to some contaminating factor present in the enzyme preparations used in previous studies or may reflect differences between recombinant CatE and the native enzyme

4.2 Introduction

Cathepsin E (CatE, EC 3.4.23.34) is a non-lysosomal aspartic protease with a limited distribution in certain cell types, including gastric epithelial cells [33], macrophages [29], lymphocytes [29], microglia [34] and dendritic cells [12]. It is localized in different cellular compartments. In a number of cells it appears to be present in vesicular structures associated with the endoplasmic reticulum and Golgi apparatus [30, 34, 37]. It is also reported to be localized in endosomal structures [34] and the plasma membrane [36]. It is the active form of CatE that shows endosomal localization [190]. CatE plays an important role in MHC class II antigen processing pathway in B cells [13, 35], microglia [56] and dendritic cells [12]. CatE is also expressed in pancreatic ductal adenocarcinoma [77], and its presence in pancreatic juice is reported to be a diagnostic marker for this cancer [93]. Increased levels of CatE in neurons and glial cells of aged rats are suggested to be related to neuronal degeneration and reactivation of glial cells during the normal aging process of the brain [94]. CatE-deficient mice develop atopic dermatitis-like skin lesions [43] and are more susceptible to bacterial infection associated with decreased expression of multiple cell surface Toll-like receptors [44]. According to a most recent study [45], CatE deficiency induces a novel form of lysosomal storage disorder characterized by the accumulation of lysosomal membrane sialoglycoproteins and the elevation of lysosomal pH in macrophages. CatE is normally most active at acidic pH, which corresponds to active endosomal form, preferring substrates with bulky hydrophobic amino acids at P1 and P1′ positions [155]. However, according to some reports, CatE also retains activity at neutral pH [23, 24]. In reference [24], proteolytic activity and cleavage specificity towards the B chain of oxidized insulin was examined. It was reported that the cleavage specificity changed significantly with more specific cleavage at pH 7.4 and above, as compared to pH 5.5 and 3.0. At acidic pH, several peptide bonds, especially Phe-X, Tyr-X and Leu-X were cleaved, whereas at pH 7.4 the Glu13-Ala14 bond was selectively cleaved. In a more recent study [23], preferential cleavage of Arg-X and Glu-X bonds at pH 7.4 was reported, with the Arg-Arg bond the preferred cleavage site.

This reported unique specificity of CatE at pH 7.4, together with the fact that it is localized in different cellular compartments, suggested the possibility that it could be involved in processing or degradation of certain proteins and peptides at or near neutral pH *in vivo*. However, in all the studies reporting proteolytic activity of CatE at pH 7.4, the enzyme was isolated either from human gastric mucosa [22-24] or human red blood cells [22]. In the present study, we determined the proteolytic activity of recombinant CatE in order to avoid any possible contamination of the isolated enzyme with other factors. Peptide substrates such as glucagon, neurotensin and dynorphin A were used because these were previously reported to be hydrolysed by CatE at neutral pH [23]. The experiments were carried out with or without ATP, which is reported to stabilize CatE [22]. Our data clearly indicate that at neutral pH, recombinant human CatE shows no cleavage activity and is not stabilized by ATP.

4.3 Experimental procedures

4.3.1 Materials

Cathepsin E was purchased from R&D systems (Wiesbaden, Germany) and stored as 0.1 mg/ml stock solution in 25 mM MES, 0.15 M NaCl, pH 6.5, and 50 % glycerol, at -20 °C. ATP was purchased from Sigma (Taufkirchen, Germany) prepared and used according to the instructions of the supplier.

4.3.2 Solid-phase peptide synthesis

The peptides HSQGTFTSDYSKYLDSRRAQDFVQWLMNT (Glucagon), ELYENKPRRP-YIL (Neurotensin), and YGGFLRRIRPKLKWDNQ (Dynorphin A) were synthesized using standard Fmoc/tBu chemistry [191], performed on the multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany) on a 0.025-mmol scale using a 6-fold molar excess of Fmoc amino acids (MultiSynTech, Witten, Germany) on TCP-resin (PepChem, Reutlingen, Germany). All other reagents and solvents for peptide synthesis were purchased from Merck KGaA (Darmstadt, Germany). *In situ* activation was performed using TBTU (6 eq) and HOBt (1 eq), followed by the addition of N-methylmorpholine (12 eq) in N,N-dimethylformamide. After completion of the automated synthesis, the resin-bound peptides were Fmoc-deprotected using 20% (v/v) piperidine in N,N-dimethylformamide twice for 15 min and washed subsequently with N,N-dimethylformamide, isopropyl alcohol, and diethyl ether. To release the peptides from the resin and to remove the side chain protecting groups, the following solution was used: 92% (v/v) trifluoroacetic acid containing 3% (v/v) thioanisol, 3% (w/v) phenol, and 2% (v/v) ethanedithiol. The peptides were precipitated and washed twice in diethyl ether, dried, and dissolved in 80% (v/v) tert-butanol in water followed by lyophilization. Crude peptides were purified using preparative reversed-phase high performance liquid chromatography (RP-HPLC) and their identity was confirmed using MALDI-MS. Peptide purities were determined via analytical RP-HPLC and proved to be above 95%.

A FRET-based peptide substrate derived from Glucagon sequence [Mca-KYLDSRRAQD-FVQWL-K(Dnp)-NH_2] was also synthesized. Peptide: KYLDSRRAQDFVQWL-K(Dnp)-NH_2

was synthesized as described above. After Fmoc deprotection using piperidine, on-resin labelling of the peptides with Mca ((7-methoxycoumarin-4-yl)acetyl) was performed. The fluorophore Mca was coupled in a three-fold excess directly to the α-amino group of the side chain-protected resin-bound peptide in DMF using the TBTU/HOBt activation method for 3 h in the dark [192]. Subsequently the peptides were released from the resin and processed as described above.

4.3.3 Digestion of peptides and analysis of digestion products using RP-HPLC

Hydrolysis of each peptide substrate by cathepsin E and analysis of the resulting peptides were performed as follows. Neurotensin (5µg), glucagon (5µg) and dynorphin A (5µg) were digested in absence or presence of ATP (6.25mM) at 37°C for 18 hours with cathepsin E (10 or 50 ng) in 50 mM sodium phosphate buffer, pH 7.4, or 50 mM sodium acetate buffer, pH 4.0. The digestion experiments were performed simultaneously at pH 4.0 and pH 7.4, CatE was taken from the same stock aliquot and activated at pH 4.0 prior to digestion at different pH values as recommended by the suppliers (R&D systems). The reaction was terminated by addition of 25 µl stop solution (95% (v/v) ACN, 1% (v/v) trifluoroacetic acid in water.

Peptide fragments were separated *via* analytical RP-HPLC using a C18 column (150 × 2 mm, Reprosil 100, Dr. Maisch GmbH, Tuebingen, Germany) with the following solvent system: (A) 0.055% (v/v) trifluoroacetic acid in H_2O, and (B) 0.05% (v/v) trifluoroacetic acid in ACN/H_2O (4:1, (v/v)). The column was eluted with a 20–80% gradient of solvent B for 40 min. UV detection was carried out at 214 nm (UV detector SPD-10AV, Shimadzu, Duisburg, Germany). Manually collected fractions were subsequently analyzed by MALDI-MS.

4.3.4 MALDI-MS

0.5 µl of each RP-HPLC fraction was mixed with 0.5 µl DHB-matrix (10 mg/ml (w/v) 2,5-dihydroxybenzoic acid in 60 % (v/v) ethanol containing 0.1 % (v/v) TFA) and applied on a gold target for MALDI-MS using a MALDI time-of-flight system (Reflex IV, serial number: 26159.00007, Bruker Daltonics, Bremen, Germany). Signals were generated by accumulating 120 – 210 laser shots. Raw data were analyzed using the software Flex Analysis 2.4 (Bruker Daltonics).

4.3.5 FRET-based assay for the determination of cathepsin E cleavage activity

Catalytic activity of CatE was determined fluorometrically by hydrolysis of the substrate Mca-KYLDSRRAQDFVQWL-K(Dnp)-NH_2. CatE (10 ng), was added to 80µl digestion buffer (50 mM sodium acetate buffer, pH 4.0, or 50 mM sodium phosphate buffer, pH 7.4) and the reaction was started by addition of 1 nmol substrate (stock solution 1mM in DMSO, 1 µl of substrate solution was dissolved in 10µl of the buffer separately and then added to the reaction mixture). Progress of fluorescent product formation was recorded using a fluorescence reader (Tecan Spectra Fluor, Crailsheim, Germany) on kinetic mode at 37°C (λ_{ex} =340, λ_{em} = 405). As a positive control for the Arg cleavage site, 0.1 µg trypsin was added to 80µl digestion buffer (50 mM NH_4HCO_3**,** pH 8.0) and the reaction was started by addition of 1µl substrate solution as describe above.

4.4 Results and Discussion

To investigate the proteolytic potential of recombinant CatE at neutral pH, the peptide substrates glucagon, neurotensin and dynorphin A, were tested. These peptides are previously reported to be cleaved at pH 7.4 [23]. **Figure 1** shows the expected cleavage sites in these peptides at neutral pH.

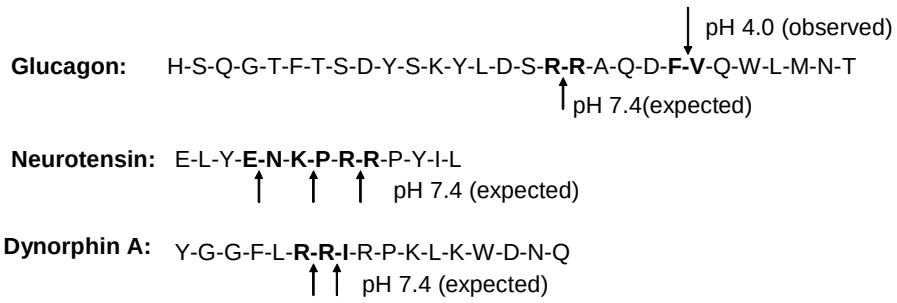


Figure 1.Sequence of different peptides used in the study and potential cathepsin E cleavage sites. *All the potential CatE cleavage sites at pH 7.4 and the observed cleavage site at pH 4.0 (glucagon) are printed in bold.*

Glucagon was digested *in vitro* with CatE and the peptide fragments thus generated were separated by RP-HPLC (**Figure 2B-E**) and identified by MALDI-MS (**Table 1**). **Figure 2A** shows the chromatogram of the undigested peptide substrate (glucagon) as a negative control. **Figure 2B** depicts the results of digestion of the glucagon with CatE at pH 4.0 in the presence of ATP at a concentration of 6.25mM, approximating that within the cells, and reported to stabilize CatE [22]. As expected, at acidic pH glucagon was cleaved between Phe-Val residues (**Figure 2B, Table 1**) as identified by MALDI-MS. Digestion of glucagon with CatE at pH 4.0 without ATP (**Figure 2C**) yielded a similar RP-HPLC-profile as in the presence of ATP. In contrast, when digested at pH 7.4 no proteolytic activity was observed with or without ATP (**Figure 2D-E, Table 1**).

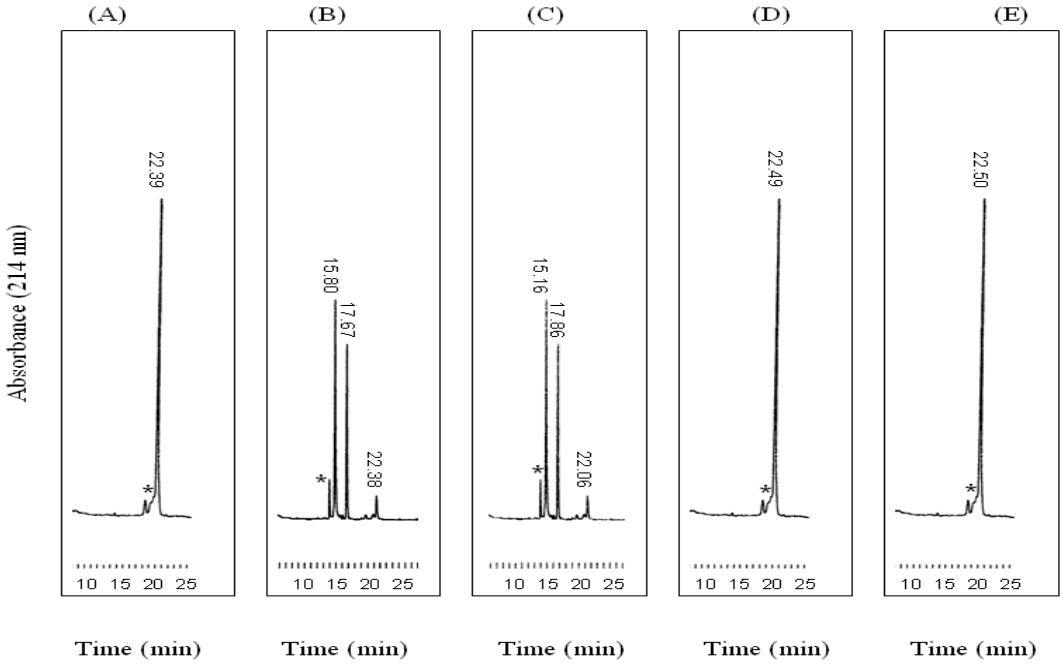


Figure 2. RP-HPLC profiles depicting digestion of synthetic glucagon peptide with CatE. *(A) Undigested peptide. (B) Peptide digested with CatE (10ng) at pH 4 (0.05 M sodium acetate buffer) in presence of ATP (6.25 mM). (C) Peptide digested with CatE (10ng) at pH 4.0 without ATP. (D) Peptide digested with CatE (50ng) at pH 7.4 (0.05M sodium phosphate buffer) in the presence of ATP (6.25 mM). (E) Peptide digested with CatE (50ng) at pH 7.4 without ATP. Digestion of glucagon with CatE at pH 4.0 with or without ATP resulted in one cleavage reaction giving two peaks. Whereas, digestion at pH 7.4 with or without ATP showed*

*no cleavage activity even when 5 times larger amount of CatE was applied. Peaks were collected and peptides were analysed using MALDI-MS (see table 1). * corresponds to peptides with oxidized methionine.*

Neurotensin, previously reported to be cleaved at Arg-Arg, Lys-Pro, and Glu-Asn **(Figure 1)** [23] by human CatE at pH 7.4 was also examined (**Figure 3**). Again no cleavage activity was observed at pH 7.4 with or without ATP (**Figure 3 B-C, Table 1**). Finally, digestion of dynorphin A with CatE at pH 7.4 also showed no proteolytic activity with or without ATP (**Table 1**).

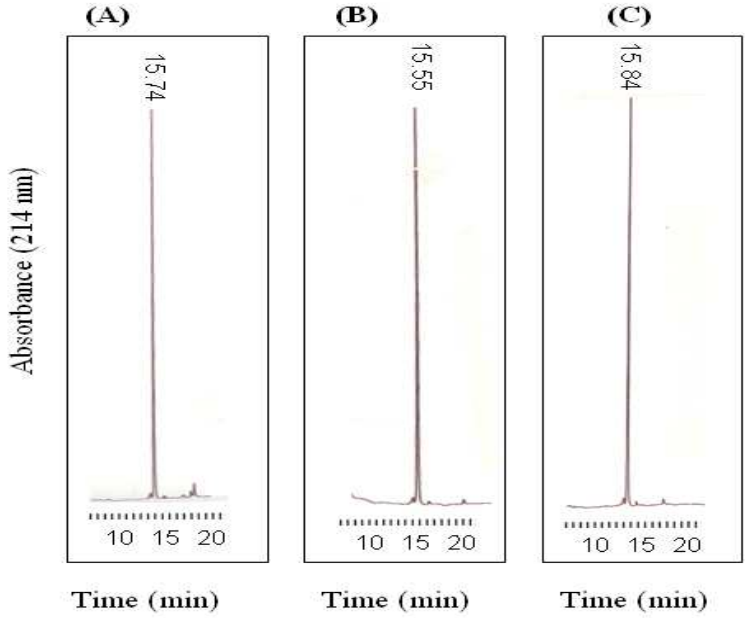


***Figure 3. RP-HPLC profiles depicting digestion of synthetic neurotensin peptide with CatE**. (A) Undigested peptide (B) Peptide digested with CatE (50ng) at pH 7.4 (0.05M sodium phosphate buffer) in the presence of ATP (6.25 mM). (C) Peptide digested with CatE (50ng) at pH 7.4 without ATP.*

In order to confirm the above mentioned results another approach, i.e FRET-based proteinase assay, was used for determining proteolytic activity at neutral pH. According to the sequence of glucagon, a FRET-based peptide substrate for CatE i.e. Mca-KYLDSRRAQDFVQWL-K(Dnp)-NH_2 [where Mca is (7-methoxycoumarin-4-yl)acetyl and Dnp is dinitrophenyl] was

synthesized. It is an intramolecularly-quenched fluorogenic peptide derivative in which the fluorescent signal of the fluorophore Mca is quenched by the chromophoric residue Dnp. After cleavage of the peptide, the quenching efficiency is decreased, resulting in an increase in fluorescence. Using this substrate, cleavage activity of CatE at pH 7.4 and 4.0 was determined. As shown in **Figure 4**, no increase in fluorescence was observed at pH 7.4 whereas at pH 4.0 there was a rapid increase. To have a positive control for the Arg cleavage site trypsin was included in the experiment as it cleaves C-terminal to Arg residues. As also shown in **Figure 4**, trypsin cleaved the substrate efficiently at pH 8.0.

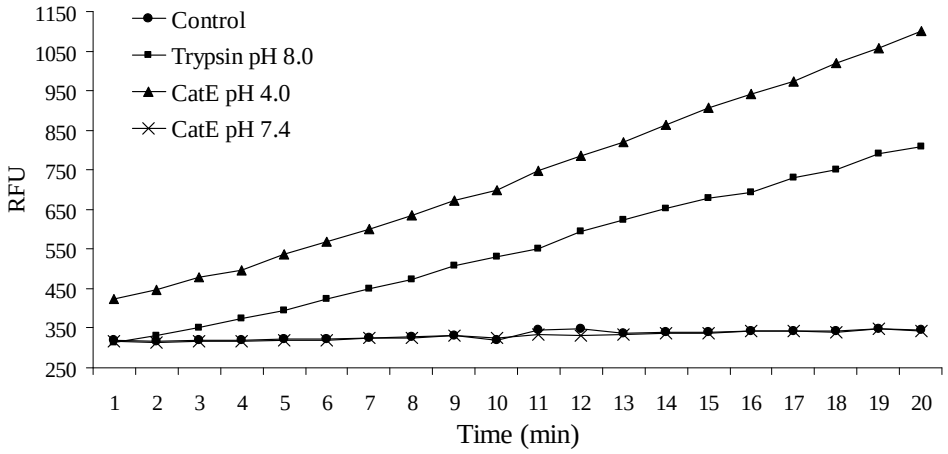


Figure 4. FRET-based assay for the determination of CatE cleavage activity at pH 4.0 and 7.4. *Hydrolysis of the fluorogenic peptide substrate based on glucagon sequence (Mca-KYLDSRRAQDFVQWL-K(Dnp)-NH_2)(1µM) by 10 ng of CatE in 50mM sodium acetate buffer (pH 4) at 37°C. or 0.05M sodium phosphate buffer (pH 7.4) at 37°C or 0.1 µg of trypsin in 50mM NH_4HCO_3 (pH 8.0). Control is the sample containing only the substrate and no enzyme.*

Our results fail to reveal any proteolytic activity of recombinant CatE at neutral pH even in the presence of ATP. Thus, the purified enzyme used in previous studies [18, 19] might have been contaminated with some other factor. Alternatively, these discrepant results may reflect conformational differences between recombinant CatE and the native enzyme. These differences may also arise from posttranslational modification of the native enzyme in vivo.

Table 1. Identified peptides after digestion of neurotensin, glucagon, and dynorphin A by CatE, as determined by MALDI-MS *(digestion experiments performed in the presence of 6.25 mM ATP).*

Substrate	pH	Peptides identified by MALDI-MS	$[M+H]^+$		Retention time
			Theoretical	Observed	
Glucagon HSQGTFTSDYSKYL-DSRRAQDFVQWLMNT	4	HSQGTFTSDYSKYLDSRRAQ-DF	2609.77	2609.39	15.8
		VQWLMNT	891.06	891.06	17.67
		Glucagon (uncleaved)	3482.82	3481.95	22.38
	7.4	No cleavage activity was observed. Only undigested glucagon recovered.	3482.82	3481.86	22.49
Neurotensin ELYENKPRRPYIL	7.4	No cleavage activity was observed. Only undigested neurotensin recovered.	1690.98	1690.88	15.55
Dynorphin A YGGFLRRIRPKLKWD-NQ	7.4	No cleavage activity was observed. Only undigested dynorphin A recovered.	2147.53	2147.53	17.60

5

5 . Substrate profiling of Cathepsin E and D

5.1 Abstract

The substrate specificities of cathepsin E and D were studied in detail. Previously described peptide substrate for CatE and CatD i.e. G-K-P-I^{P3}-L^{P2}-F^{P1}-$F^{P1´}$-$R^{P1´}$-$L^{P1´}$ (digested at Phe-Phe bond, by both CatE and CatD) was modified systematically. Positions P1, P2, P3, P1´, P2´ and P3´ of this substrate were each substituted with 20 different naturally occurring amino acid residues and norleucine. Screening of several peptides provided distinct amino acid preferences by cathepsin E and D. It was identified that within a limited range of enzyme concentration (0-5nM) and time (0-120 min) provided for hydrolysis, peptides with basic residues at position P3 were only cleaved by cathepsin E. But these peptides were also cleaved by CatD at higher concentration and after longer incubation time. Thus, we were unable to identify exclusively specific substrates for either CatE or CatD but, the detailed screening provides better understanding of the substrate specificity profiles of the two cathepsins. This study provides useful information for developing specific substrates and selective inhibitors for these two otherwise very similar proteases.

5.2 Introduction

Proteases, also referred as proteolytic enzymes or proteinases, constitute one of the largest and most important protein families. They are of great relevance to biology, medicine and biotechnology. The catalytic function of proteases is to hydrolyze amide bonds in proteins and peptides. They differ in their ability to hydrolyze various peptide bonds. The biological significance of proteases is illustrated by the fact that they comprise over 2% of the human genome [193]. An important feature of any protease is its substrate specificity that results in preferential cleavage of its specific substrate in presence of several other proteins and peptides. Therefore, specificity information gives a better understating of the physiological role of any protease and also helps in designing the specific substrates and selective inhibitors.

Cathepsins E and D are the major aspartic proteinases in the endolysosomal pathway. These enzymes have been implicated in in several physiological processes and as well as in pathalogical conditions [194, 195]. They have similar enzymatic properties, e.g. susceptibility to various proteinase inhibitors, similar substrate preferences [155] and similar pH optimum.

Previous reports have described highly selective substrates for aspartic proteinases, but none of the described substrates is exclusively specific for CatE or CatD [19, 20, 156]. It is known that cathepsin E and cathepsin D both prefers hydrophobic amino acids at P1 and P1` positions. For cathepsin E, β-branched residues, e.g. V and I are not allowed at P1 [28] while position P2´ accepts a broad range of amino acids, including charged and polar ones [28]. For CatE, a basic residue e.g. K is acceptable at position P2, which is not the case for CatD [64]. The presence of proline at P4 seems to be important for CatE [17]. For CatD, leucine is strongly favoured at P1, at P2 and P1´ the hydrophobic requirements are less strict [27]. A charged preferably basic residue is found mostly at ´positions P2´and P5; at least 1 basic residue appears to be required by CatD at either position [27].

On the basis of available information about substrate specificity, several synthetic chromogenic or fluorogenic substrates have been developed to measure CatE or CatD activity [19, 68-70]. These methods are simple and fast, but the described substrates are restricted in their selectivity. Recently, a new selective substrate for cathepsin E, based on the cleavage site

sequence of α2-macroglobulin, has been described [87]. However, this substrate was also not exclusive for CatE because it had low level of activity for CatD and pepsin as well. In order to understand the physiological role and enzymatic properties of cathepsin E and D in detail, there is an urgent need of a specific and sensitive substrate for each of these enzymes.

In this study, substrate specificity of cathepsin E and D was examined in detail. To achieve this the most commonly used substrate for CatE and CatD, G-K-P-I^{P3}-L^{P2}-F^{P1}-$F^{P1´}$-$R^{P1´}$-$L^{P1´}$[19] (digested at Phe-Phe bond, by both CatE and CatD) was modified systematically. Positions P1, P2, P3, P1´, P2´ and P3´ of this substrate were each substituted with 20 different naturally occurring amino acid residues and norleucine. Although, no exclusively specific substrates for either CatE or CatD were identified but, the detailed screening offers a better understanding of the substrate specificity profiles for the two enzymes. The presented data will facilitate in designing specific substrates or selective inhibitors of cathepsin E and D.

5.3 Experimental procedures

5.3.1 Enzymes and chemicals

Cathepsin E was purchased from R&D systems (Wiesbaden, Germany) and stored as 0.1 mg/ml stock solution in 25 mM MES, 0.15 M NaCl, pH 6.5, and 50 % glycerol, at -20 °C. CatD (bovine kidney) was purchased from Calbiochem (Darmstadt, Germany) and stored as a 0.4 mg/ml stock solution in 0.1 M citrate buffer, pH 4.5, at -20 °C.

5.3.2 Solid-phase peptide synthesis

The peptides GKPILFFRX, GKPILFFXL, GKPILFXRL, GKPILXFRL, GKPIXFFRL and GKPXLFFRL (were X represents any of the 20 naturally occurring amino acids or norleucine) were synthesized using standard Fmoc/tBu chemistry [191], performed on the multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany) on a 0.025-mmol scale using a 6-fold molar excess of Fmoc amino acids (MultiSynTech, Witten, Germany) on TCP-resin (PepChem, Reutlingen, Germany). For the position X, a mixture of five different amino acids was loaded. Thus, a total of 23 peptide series each containing 5 different peptides were synthesized. All reagents and solvents for peptide synthesis were purchased from Merck KGaA (Darmstadt, Germany). *In situ* activation was performed using TBTU (6 eq) and HOBt (1 eq) followed by the addition of N-methylmorpholine (12 eq) in N,N-dimethylformamide. After completion of the automated synthesis, the resin-bound peptides were Fmoc-deprotected using 20% (v/v) piperidine in N,N-dimethylformamide twice for 15 min and washed subsequently with N,N-dimethylformamide, isopropyl alcohol, and diethyl ether. To release the peptides from the resin and to remove the side chain protecting groups, the following solution was used: 92% (v/v) trifluoroacetic acid containing 3% (v/v) thioanisol, 3% (w/v) phenol, and 2% (v/v) ethanedithiol. The peptides were precipitated and washed twice in diethyl ether, dried, and dissolved in 80% (v/v) tert-butanol in water followed by lyophilization. Peptides were seperated using preparative reversed-phase high performance liquid chromatography (RP-HPLC) and their identity was confirmed using MALDI-MS. Peptide purities were determined *via* analytical RP-HPLC and proved to be above 95%.

FRET-based peptide substrates Mca-Gly-Lys-Pro-His-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2, Mca-Gly-Lys-Pro-Lys-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 and Mca-Gly-Lys-Pro-Arg-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 were synthesized. The peptides without Mca were synthesized as described above. After Fmoc-deprotection using piperidine, on-resin labeling of the peptides with Mca ((7-methoxycoumarin-4-yl)acetyl) was performed. The fluorophor Mca was coupled at a three-fold excess directly to the α-amino group of the side chain-protected resin-bound peptide in DMF using the TBTU/HOBt activation method for 3 h in the dark [192]. Subsequently, the peptides were released from the resin and processed as described above.

5.3.3 Digestion of peptides and analysis of digestion products

Hydrolysis of each peptide substrate by cathepsin E or D and analysis of the resulting peptides were performed as follows. All the peptides were digested at 37°C for30 min with CatE (1.19 nM) or with CatD (4 nM) in 50 mM sodium acetate buffer, pH 4.0. The concentration of CatE (1.19 nM) and CatD (4 nM) for each reaction were chosen, as these amounts of enzymes were able to digest 50% of the control peptide (GKPILFFRL) in 30 min. The reactions were terminated by boiling the samples for 5 min at 98°C.

Peptide fragments were separated *via* analytical RP-HPLC using a C18 column (150 × 2 mm, Reprosil 100, Dr. Maisch GmbH, Tuebingen, Germany) with the following solvent system: (A) 0.055% (v/v) trifluoroacetic acid in H_2O, and (B) 0.05% (v/v) trifluoroacetic acid in ACN/H_2O (4:1, (v/v)). The column was eluted with a 20–80% gradient of solvent B for 40 min. UV detection was carried out at 214 nm (UV detector SPD-10AV, Shimadzu, Duisburg, Germany).

5.3.4 MALDI-MS

0.5 µl of each RP-HPLC fraction was mixed with 0.5 µl DHB-matrix (10 mg/ml (w/v) 2,5-dihydroxybenzoic acid in 60 % (v/v) ethanol containing 0.1 % (v/v) TFA) and applied on a gold target for MALDI-MS using a MALDI time-of-flight system (Reflex IV, serial number: 26159.00007, Bruker Daltonics, Bremen, Germany). Signals were generated by accumulating 120 – 210 laser shots. Raw data were analyzed using the software Flex Analysis 2.4 (Bruker Daltonics).

5.4 Results and Discussion

5.4.1 Synthesis of peptide libraries

Different peptide series were synthesized for substrate profiling of CatE and CatD. To achieve this, the previously described peptide substrate Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu [19] which is digested at Phe-Phe bond, by both CatE and CatD was manipulated. The positions P1, P2, P3, P1´ P2´ and P3´ of this peptide were each substituted with 20 different naturally occurring amino acids and norleucine. Five peptides were synthesized together in one series as described in experimental procedures. The peptides were separated by means of preparative RP-HPLC; five fractions were separately collected, lyophilized and identified by MALDI-MS **(Figure 1).** Some of the synthesized peptides were lost during the processes of synthesis, separation or purification. **Table 1** shows the sequence and characteristics of all the synthesized peptides that were recovered from different peptide series and analysed by digestion experiments. The coding system of peptides is also explained in **Figure 1**.

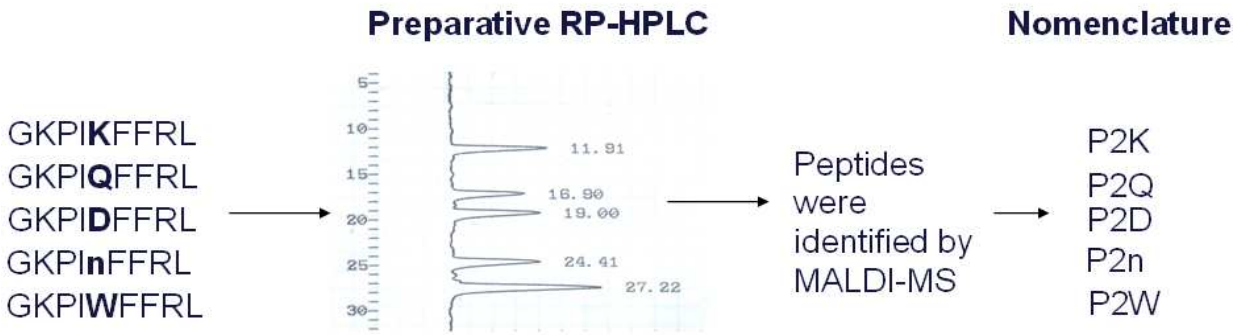


Figure 1. Schematic representation of synthesis, purification and coding of the peptides*. Five peptides were synthesized simultaneously in one series. For the altering amino acid a mixture of five amino acids was loaded on the peptide resin (the representative scheme shows altering P2 position). Peptides were separated and purified via preparative RP-HPLC and identified by MALDI-MS and named according to the variation related to the control peptide (GKPILFFRL), e.g., when position P2 was substituted with K, the peptide is named P2K.*

Table 1. Peptides analyzed in the present study.

Peptide I.D	Peptides	Expected Weight	Observed Weight	ΔDa	Retention Time (min)
P_3'					
P_3'H	GKPILFFR**H**	1114.37	1114.71	0.34	28.24
P_3'S	GKPILFFR**S**	1064.31	1064.69	0.38	29.47
P_3'V	GKPILFFR**V**	1076.36	1076.79	0.43	32.92
P_3'F	GKPILFFR**F**	1124.41	1124.81	0.40	36.83
P_3'K	GKPILFFR**K**	1105.40	1105.50	0.10	16.30
P_3'Q	GKPILFFR**Q**	1105.36	1150.53	0.17	18.14
P_3'D	GKPILFFR**D**	1092.32	1092.50	0.18	19.29
P_3'W	GKPILFFR**W**	1163.44	1163.59	0.15	32.86
P_3'R	GKPILFFR**R**	1133.42	1133.95	0.53	10.90
P_3'A	GKPILFFR**A**	1048.31	1048.87	0.56	15.59
P_3'M	GKPILFFR**M**	1108.42	1108.89	0.47	17.03
P_3'I	GKPILFFR**I**	1090.39	1090.88	0.49	17.63
P_3'N	GKPILFFR**N**	1091.33	1091.43	0.10	17.14
P_3'E	GKPILFFR**E**	1106.34	1106.60	0.26	18.06
P_3'T	GKPILFFR**T**	1078.33	1078.53	0.20	18.54
P_3'Y	GKPILFFR**Y**	1140.40	1140.48	0.08	21.23
P_3'L	GKPILFFR**L**	1090.39	1090.54	0.15	27.82
P_2'					
P_2'H	GKPILFF**H**L	1071.34	1071.42	0.08	24.14
P_2'S	GKPILFF**S**L	1021.28	1021.38	0.10	28.12
P_2'G	GKPILFF**G**L	991.25	991.37	0.12	29.08
P_2'V	GKPILFF**V**L	1033.33	1033.59	0.26	36.30
P_2'F	GKPILFF**F**L	1081.38	1081.51	0.13	44.08
P_2'K	GKPILFF**K**L	1062.37	1062.34	0.01	14.52
P_2'Q	GKPILFF**Q**L	1062.33	1062.41	0.08	20.35
P_2'D	GKPILFF**D**L	1049.29	1049.37	0.08	21.92
P_2'Nle	GKPILFF**n**L	1047.36	1047.44	0.08	30.78
P_2'W	GKPILFF**W**L	1120.41	1120.67	0.26	32.61
P_2'R	GKPILFF**R**L	1090.39	1090.56	0.17	30.60
P_2'P	GKPILFF**P**L	1031.32	1031.54	0.22	35.70
P_2'I	GKPILFF**I**L	1047.36	1047.75	0.39	42.17
P_2'N	GKPILFF**N**L	1048.30	1048.72	0.42	24.80
P_2'Y	GKPILFF**Y**L	1097.38	1097.57	0.19	30.57
P_2'L	GKPILFF**L**L	1047.36	1047.57	0.21	35.58
P_1'					
P_1'H	GKPILF**H**RL	1080.35	1080.60	0.25	16.90
P_1'G	GKPILF**G**RL	1000.26	1000.59	0.33	22.11

P_1´V	GKPILF**V**RL	1042.34	1042.59	0.25	26.92
P_1´F	GKPILF**F**RL	1090.39	1090.61	0.22	31.98
P_1´K	GKPILF**K**RL	1071.38	1071.36	0.02	17.96
P_1´Q	GKPILF**Q**RL	1071.34	1071.85	0.51	23.08
P_1´D	GKPILF**D**RL	1058.30	1058.39	0.09	24.15
P_1´W	GKPILF**W**RL	1129.42	1129.60	0.18	33.31
P_1´R	GKPILF**R**RL	1099.40	1099.40	0.00	18.85
P_1´A	GKPILF**A**RL	1014.29	1015.01	0.72	24.00
P_1´P	GKPILF**P**RL	1040.33	1040.87	0.54	24.74
P_1´M	GKPILF**M**RL	1074.41	1074.99	0.58	28.90
P_1´I	GKPILF**I**RL	1056.37	1056.88	0.51	30.24
P_1´N	GKPILF**N**RL	1057.31	1057.48	0.17	7.92
P_1´F	GKPILF**E**RL	1072.33	1072.66	0.33	9.14
P_1´Y	GKPILF**Y**RL	1106.39	1106.65	0.26	11.74
P_1´L	GKPILF**L**RL	1056.37	1056.65	0.28	15.98
P_1					
P_1H	GKPIL**H**FRL	1080.35	1080.76	0.41	8.27
P_1G	GKPIL**G**FRL	1000.26	1000.72	0.46	10.92
P_1S	GKPIL**S**FRL	1030.29	1030.64	0.35	10.99
P_1V	GKPIL**V**FRL	1042.34	1042.77	0.43	16.03
P_1F	GKPIL**F**FRL	1090.39	1090.83	0.44	19.88
P_1K	GKPIL**K**FRL	1071.38	1071.79	0.41	7.66
P_1Q	GKPIL**Q**FRL	1071.34	1071.93	0.59	10.97
P_1D	GKPIL**D**FRL	1058.30	1058.76	0.46	12.26
P_1X	GKPIL**X**FRL	1056.37	1056.88	0.51	18.26
P_1W	GKPIL**W**FRL	1129.42	1129.72	0.30	20.72
P_1R	GKPIL**R**FRL	1099.40	1099.84	0.44	8.44
P_1A	GKPIL**A**FRL	1014.29	1014.76	0.47	11.72
P_1P	GKPIL**P**FRL	1040.33	1040.80	0.47	13.44
P_1M	GKPIL**M**FRL	1074.41	1074.81	0.40	16.18
P_1I	GKPIL**I**FRL	1056.37	1056.82	0.45	17.90
P_1N	GKPIL**N**FRL	1057.31	1058.03	0.28	10.71
P_1E	GKPIL**E**FRL	1072.33	1072.77	0.44	12.43
P_1Y	GKPIL**Y**FRL	1106.39	1106.92	0.53	14.51
P_1L	GKPIL**L**FRL	1056.37	1056.89	0.52	18.72
P_2					
P_2H	GKPI**H**FFRL	1114.37	1114.70	0.33	10.67
P_2G	GKPI**G**FFRL	1034.28	1034.65	0.37	14.40
P_2S	GKPI**S**FFRL	1064.31	1064.69	0.38	14.49
P_2V	GKPI**V**FFRL	1076.36	1076.81	0.45	18.37
P_2F	GKPI**F**FFRL	1124.41	1124.84	0.43	23.09
P_2K	GKPI**K**FFRL	1105.40	1105.87	0.47	11.91

P_2Q	GKPI**Q**FFRL	1105.36	1105.86	0.50	16.90
P_2D	GKPI**D**FFRL	1092.32	1092.84	0.52	19.00
P_2Nle	GKPI**n**FFRL	1090.39	1090.91	0.52	24.41
P_2W	GKPI**W**FFRL	1163.44	1163.93	0.49	27.22
P_2R	GKPI**R**FFRL	1133.42	1133.51	0.09	9.97
P_2A	GKPI**A**FFRL	1048.31	1048.46	0.15	13.79
P_2P	GKPI**P**FFRL	1074.34	1074.48	0.14	15.03
P_2M	GKPI**M**FFRL	1108.42	1108.51	0.09	18.40
P_2I	GKPI**I**FFRL	1090.39	1090.45	0.06	19.99
P_2N	GKPI**N**FFRL	1091.33	1091.44	0.11	11.80
P_2E	GKPI**E**FFRL	1106.34	1106.54	0.20	11.89
P_2Y	GKPI**Y**FFRL	1140.40	1140.62	0.22	15.28
P_2L	GKPI**L**FFRL	1090.39	1090.63	0.24	18.56
P_3					
P_3H	GKP**H**LFFRL	1114.37	1114.48	0.11	8.83
P_3G	GKP**G**LFFRL	1034.28	1034.53	0.25	11.34
P_3V	GKP**V**LFFRL	1076.36	1076.31	0.05	15.53
P_3F	GKP**F**LFFRL	1124.41	1124.62	0.21	19.83
P_3K	GKP**K**LFFRL	1105.40	1105.56	0.16	12.78
P_3Q	GKP**Q**LFFRL	1105.36	1105.74	0.38	16.51
P_3D	GKP**D**LFFRL	1092.32	1092.66	0.34	17.26
P_3Nle	GKP**n**LFFRL	1090.39	1090.72	0.33	23.63
P_3W	GKP**W**LFFRL	1163.44	1163.69	0.25	26.36
P_3R	GKP**R**LFFRL	1133.42	1133.73	0.31	5.41
P_3A	GKP**A**LFFRL	1048.31	1048.57	0.26	7.14
P_3P	GKP**P**LFFRL	1074.34	1074.66	0.32	7.19
P_3M	GKP**M**LFFRL	1108.42	1108.51	0.09	11.62
P_3I	GKP**I**LFFRL	1090.39	1090.24	0.15	13.34
P_3N	GKP**N**LFFRL	1091.33	1091.28	0.05	4.56
P_3E	GKP**E**LFFRL	1106.34	1106.32	0.02	5.48
P_3H	GKP**Y**LFFRL	1140.40	1140.40	0.00	8.23
P_3L	GKP**L**LFFRL	1090.39	1090.41	0.02	10.72

5.4.2 Screening of the peptide libraries for the selection of preferable substrates for cathepsin E or D

In the first step of screening all peptides were digested with cathepsin E (1.19 nM) and D (4 nM) for 30min as described in materials and methods. The digestion products were separated by analytical RP-HPLC and the digestion percentage was calculated by area of digestion peaks and starting material (uncleaved substrate) **(Figure 2).**

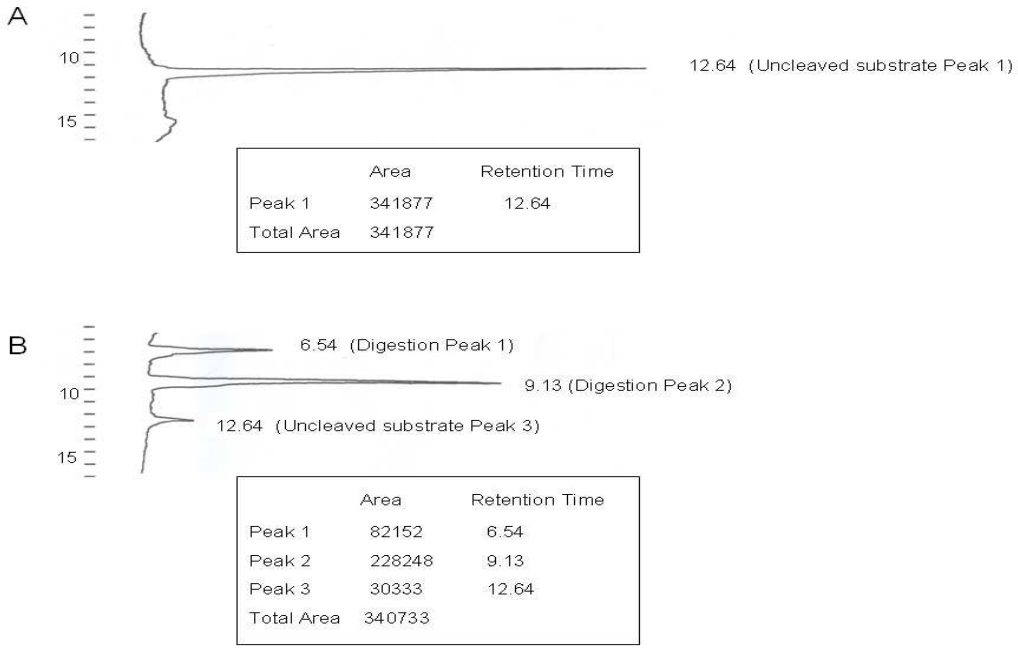


Figure 2. Calculation of digestion percentage for a peptide substrate using area of digestion peaks and uncleaved residual substrate peak on RP-HPLC chromatograms. *Chromatograms represent peptide P3H, (A) Undigested, (B) Digested with cathepsin E (1.19 nM) for 60 min. Total area for both the chromatograms is similar. Therefore, it can be used to calculate the digestion percentage. Digestion % was calculated using the following formula:*

$$\text{Digestion \%} = \frac{\text{Area of digestion peak 1} + \text{Area of digestion peak 2}}{\text{Total area}} \times 100$$

For the represented case:

$$\text{Digestion percentage} = \frac{82152 + 228248}{340733} \times 100 = 91.09\%$$

As shown in **Figures 3A and B,** both enzymes showed similar preferences for P1 positions. F, n (norleucine), M and L were the most preferable amino acids at this position. Whereas, I and V were not allowed at this position which is consistent with previously published studies [28]. For P1´, CatE accommodated a relatively broader range of residues, but like CatD, a higher preference was observed for F, M and I **(Figures 3C and D)**. Thus, no significant difference was observed for these positions with the exception of the peptide P1´P: P at P1´ was not allowed for cleavage by CatD, but CatE allowed this residue. The digestion was comparatively

low (<10%), but specific for CatE. Therefore this peptide was selected for second stage of screening.

The digestion profile for the peptides varying at P2 position was slightly different for CatE and CatD. The amino acid G and the basic residues H, K and R were not allowed for cleavage by CatD at this position, but CatE accepted these residues, though the degree of digestion was lower in comparison to the control peptide **(Figures 3E and F).** Thus, peptides P2G, P2H, P2K and P2R were selected for second stage of screening. Moreover, preference for the resides, S, V, Q, D, and W was also different between CatE and D. For these residues, CatE showed a higher degree of digestion compared to CatD that was minimal in comparison to the control peptide.

The digestion pattern for the peptides varying at P2´position was similar for both, CatE and CatD, therefore, from this series none of the peptides were further pursued **(Figures 2G and H).**

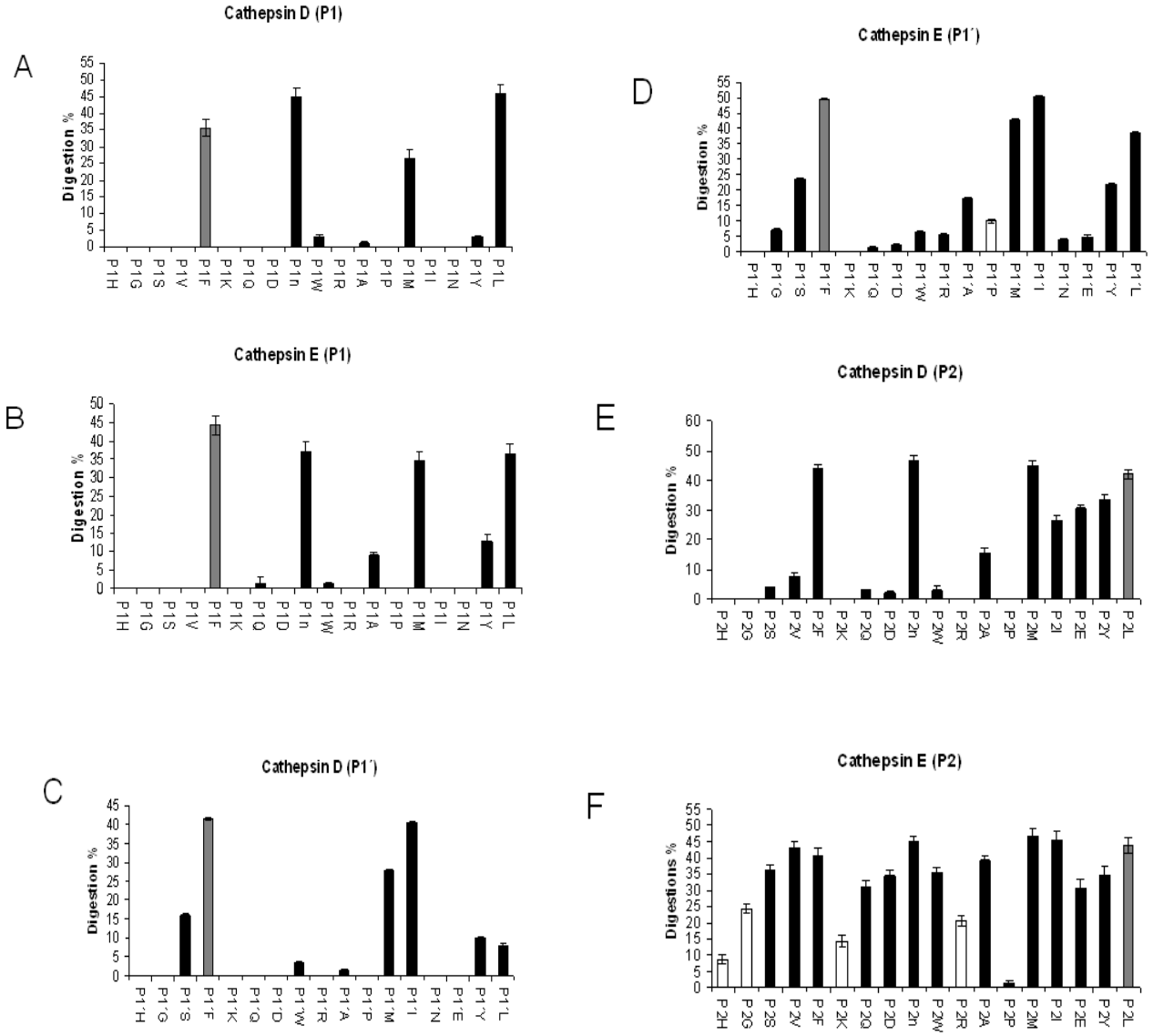
The peptides with basic residues at position P3 also showed differences in digestion pattern between CatE and CatD. The basic residues H, K, and R at this position were not allowed for cleavage by CatD, but were accommodated by CatE. Thus, the peptides P3K, P3R and P3H were selected for second stage of screening **(Figures 3I and J)**.

For the position P3´, the only differences between CatE and D were for the residue R. Again, this residue was allowed for cleavage by CatE, but not CatD. The corresponding peptide P3´R was selected for second stage of screening **(Figures 3K and L)**.

The peptides selected after first stage of screening, i.e. P2H, P2G, P2K, P2R, P3H, P3K, P3R, P1´P and P3´R were all more or less specific for CatE. Unfortunately, we were unable to find any specific substrate for CatD among the peptides analysed in the present study.

In the second stage of screening, all the selected peptides were digested by CatE under similar conditions as described above for preliminary screening. On the other hand, the digestion time for CatD was increased to 120 min in order to confirm the specificity of the selected peptide for CatE.

Figures 4A and B show the digestion profiles for the selected peptides by CatD and CatE, respectively. It was observed that even after 120 min of incubation with cathepsin D (4 nM), the peptides with basic residues at P3 position i.e. P3H, P3K and P3R were not cleaved. Moreover, the only cleavage site for CatE in these peptides was between two phenylalanine residues as determined by RP-HPLC and MALDI.MS. These substrates were further investigated as potential specific substrate for CatE.



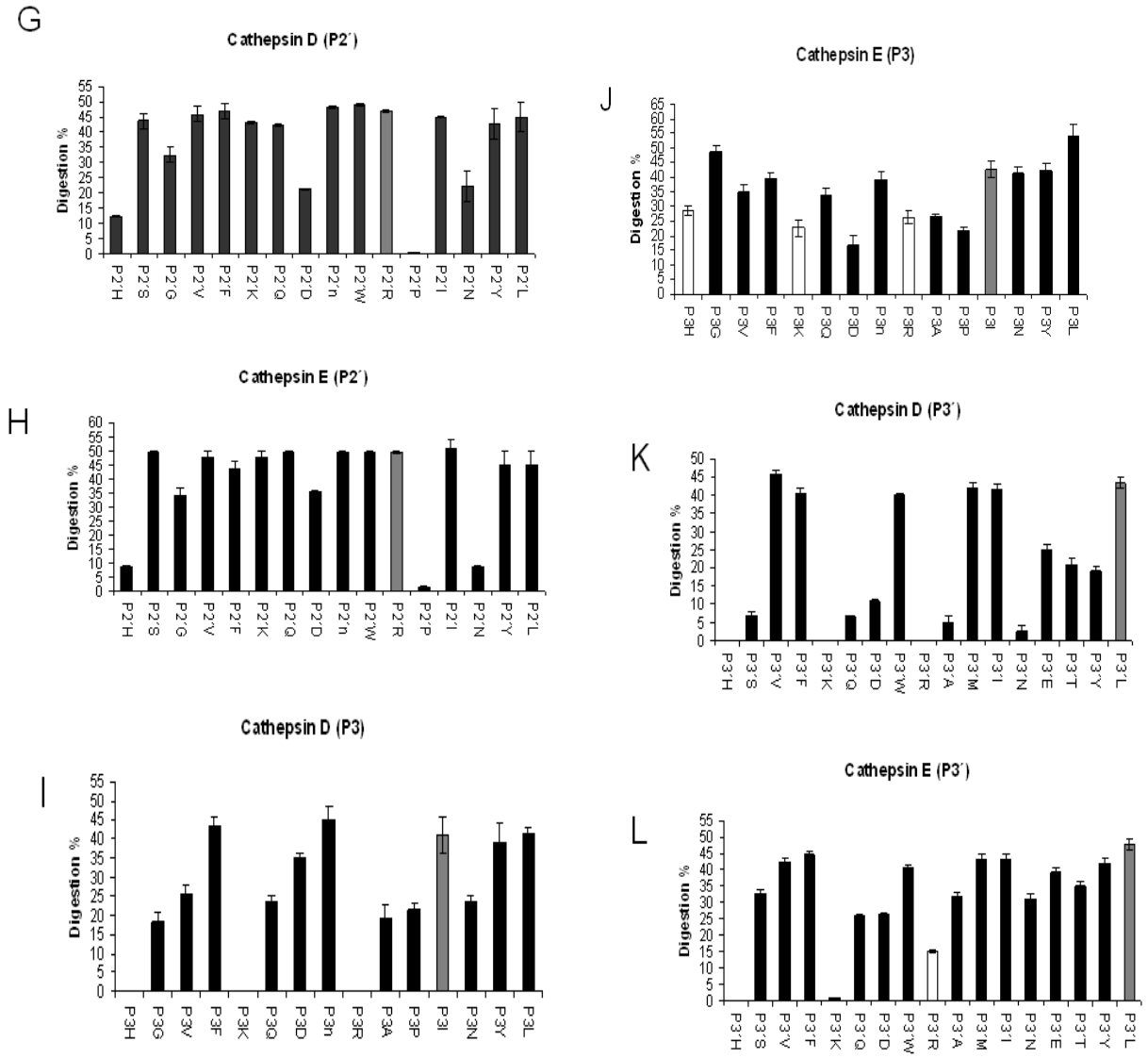


Figure 3. Preliminary screening of peptide series for determination of substrate specificity of cathepsin E and D. *All the peptides were digested with CatE (1.19 nM) or CatD (5 nM) for 30 min at 37°C in digestion buffer (50 mM sodium acetate buffer, pH 4.0). The digestion products were separated by RP-HPLC and the digestion percentage was calculated as described in* ***Figure 2****. The digestion percentage (vertical axis) is plotted for different peptides as indicated on the horizontal axis. White bars indicate the peptides which were specifically cleaved by CatE and selected for second stage of screening, whereas the grey bar in each panel indicates the control peptide. Values depicted are means ± SD (n = 2) where n is the number of individual experiments.*

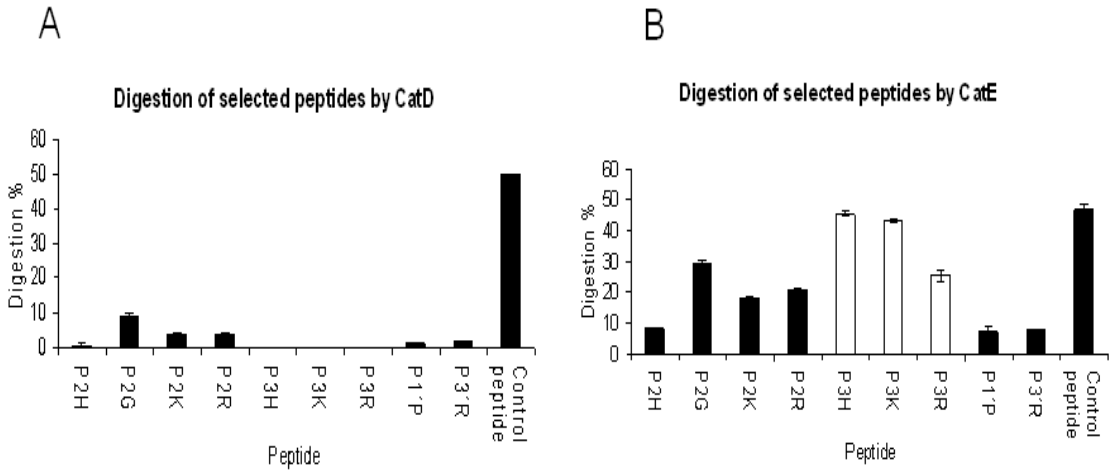


Figure 4. Digestion of the peptides selected after preliminary screening with cathepsin E and D. *The peptides that were specifically cleaved by CatE in preliminary screening were subjected to second stage of screening. All the peptides were digested with CatE (30min) or CatD (120 min) (for experimental details see legend to figure 1). Values depicted are means ± SD (n = 3). It was observed that peptides with basic amino acid residues at P3 position i.e. P3H, P3R and P3K were not cleaved by CatD even after 2 hours of incubation. These peptides were further pursued for their potential as specific CatE substrates.*

5.4.3 Effect of incubation time, enzyme concentration and pH and on hydrolysis of selected substrates by cathepsin E and D

The substrates selected to be specific for cathepsin E after screening i.e. P3H, P3K and P3R were subjected to further investigation. The effects of incubation time, enzyme concentration and pH on hydrolysis of selected substrate by cathepsin E and D were studied. As mentioned above, after 120 min of incubation with cathepsin D in second stage of screening, these substrates were not cleaved, whereas CatE hydrolyzed approximately 30-50% of these peptide substrates within 30 minutes.

The selected substrates were incubated with cathepsin E and D for different time points (60-300 min). It was observed that after 180 min of incubation with cathepsin D all the substrates were digested **(Figure 5 A)**.

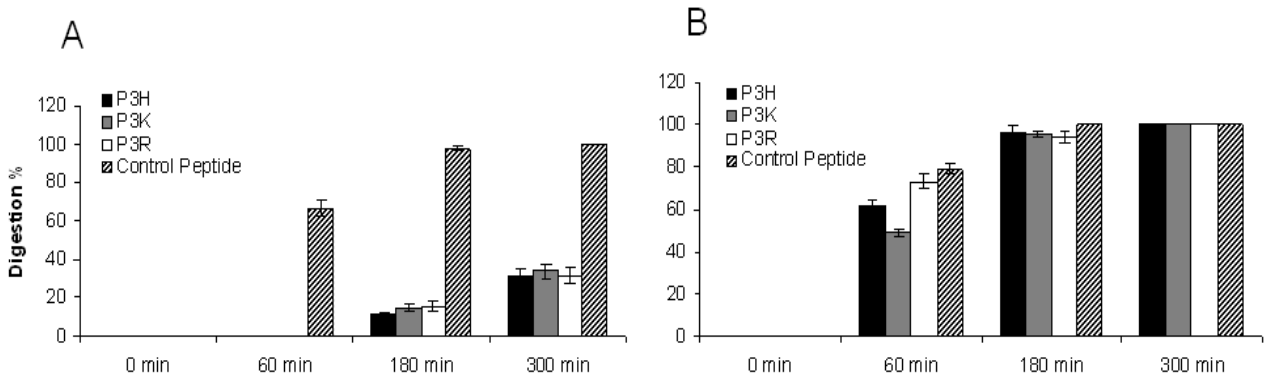


Figure 5. Digestion of the selected peptides (i.e. GKPHLFFRL, GKPKLFFRL and GKPRLFFRL) with (A) CatD (B) CatE at different time points. *Substrates P3H, P3K, P3R, and the control peptide were each incubated with CatE (1.19 nm) or CatD (4nm) at 37°C in digestion buffer (50 mM sodium acetate buffer, pH 4.0) for different time points (0-300 min). The substrates that were previously observed to be specific towards CatE* (*i.e. P3H, P3K and P3R) after 120 min of incubation were found to be digested even with CatD when incubation time was increased to 180 min.*

Moreover, the selected substrates were also incubated with increasing concentrations of CatE and CatD. It was observed that the selected substrates were cleaved also by cathepsin D above the concentration of 6nM after 30 min of incubation **(Figure 6 A).** Thus, the above described specificity of the selected substrates for CatE was limited to the lower concentration of enzymes and shorter incubation time provided for hydrolysis. This is the major drawback as in physiological conditions the concentration of cathepsin D is higher than cathepsin E. Therefore, the selected substrates might show selectivity towards cathepsin E, but are not exclusively specific for cathepsin E.

The pH dependence of the hydrolysis of the selected substrates by CatE and CatD was also examined. In concordance with previous reports [20, 196] both, CatE and CatD gave their maximal activity at pH 4. At pH 2.0, CatE showed less than 15% of its maximum activity whereas CatD was completely inactive **(Figures 7 A-B)** (the point of maximum activity was taken 100%. In each case, reaction was much slower for of CatD).

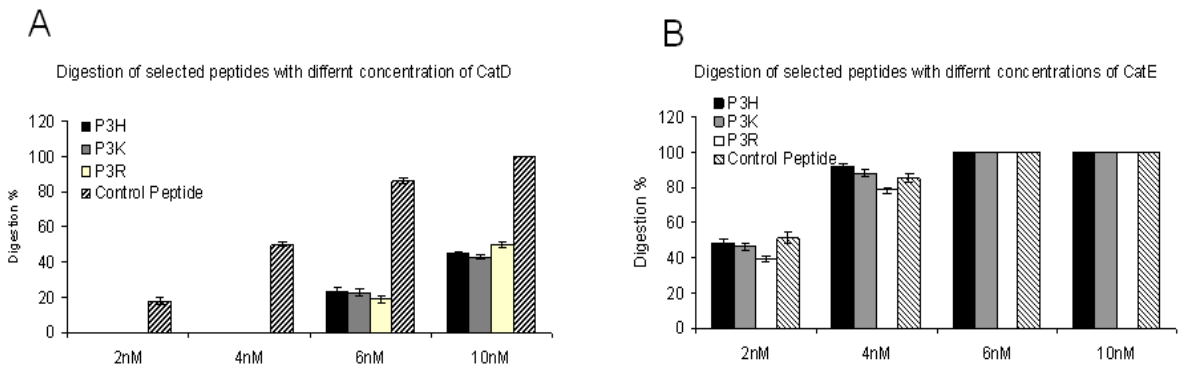


Figure 6. Digestion of selected peptides with different concentration of (A) CatD and (B) CatE. *Substrates, selected to be specific for CatE after preliminary screening (i.e. P3H, P3K andP3R), and the control peptide were each incubated at 37°C in digestion buffer (50 mM sodium acetate buffer, pH 4.0) for 30 min with increasing concentrations of CatE or CatD (2-10 nm).Each substrate was cleaved by CatD above 6 nm concentration.*

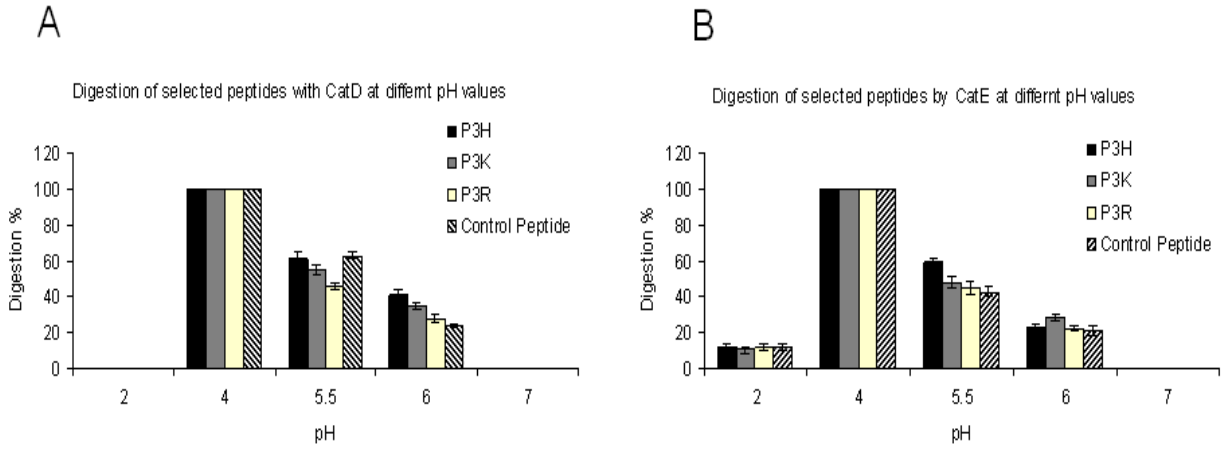


Figure 7. Digestion of the selected peptides with of (A) CatD and (B) CatE at different pH values. . *Substrates P3H, P3K, P3R, and the control peptide were each incubated with CatE (1.19 nm) or CatD (4nm) at 37°C for 30 min at different pH values. (the point of maximum activity was taken 100%. Iin each case reaction was much slower for CatD).*

5.4.4 FRET labeling of the selected substrates

The selected substrates (i.e. P3H, P3K and P3R*)* were also synthesized as FRET-based peptides [Mca-GKP**H**LFFRL-Lys(Dnp)-DArg-NH_2, Mca-GKP**K**LFFRL-Lys(Dnp)-DArg-NH_2 and Mca-GKP**R**LFFRL-Lys(Dnp)-DArg-NH_2 where Mca is (7-methoxycoumarin-4-yl)acetyl and Dnp is dinitrophenyl] to study the kinetic parameters.

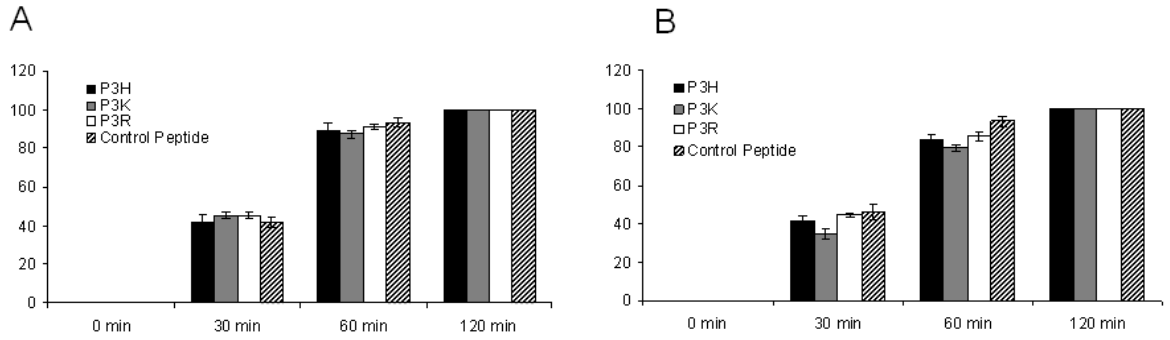


Figure 7. Digestion of the FRET-based peptides by (A) CatD and (B) CatE at different time points. *Substrates P3H, P3K, P3R, and the control peptide were each synthesized as FRET-based peptide (Mca/Dnp type). These substrates were each incubated with CatE (1 nm) or CatD (1 nm) at 37°C for different time points. All of these substrates were cleaved by both, cathepsin E and D equally.*

It was observed that the unlabelled peptides with the similar sequence that were found to be selective for CatE (i.e. GKP**H**LFFRL, GKP**K**LFFRL and GKP**R**LFFRL) when synthesized as FRET-based substrates (Mca/Dnp type) lost their specificity and were cleaved by both, cathepsin E and D equally **(Figures 7A and B)**. The reaction was completed in 2 hours with both, CatE and CatD, as observed by fluorescence (no increase in fluorescence was detected after 2 hours). The loss of specificity of these substrates for CatE can not be explained with our data, but we speculate that it is due to change of conformation of the lengthed peptides.

The investigations, presented in this chapter, on the substrate G-K-P-I^{P3}-L^{P2}-F^{P1}-$F^{P1´}$-$R^{P1´}$-L^{P1}, modified systematically at positions P1, P2, P3, P1´ P2´, and P3, allows detailed and accurate mapping of the substrate specificity profiles of cathepsin E and D. It was observed that cathepsin E prefers basic residues especially at position P3. We were unable to identify exclusively specific substrates for either CatE or CatD, but the presented data provide a better understanding

of their substrate specificity profile. These results can be used for designing of specific substrates and selective inhibitors for cathepsin E and D.

6

6 . Key findings, conclusions and future perspectives

6.1 Key findings and conclusions:

The studies based at understanding the role of cathepsin E and D are limited by two major obstacles; one is the absence of an assay or substrate that distinguishes between the two enzymes in crude biological samples. Second is the absence of a specific inhibitor. In the present thesis both of these problems have been addressed. .

Following are the major goals accomplished during the course of this PhD project.

- This thesis presents an assay combining a new monospecific CatE antibody and substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 [where Mca is (7-methoxycoumarin-4-yl)acetyl and Dnp is dinitrophenyl]. This substrate is digested by both proteinases and therefore can be used to detect the total aspartic proteinase activity (TAPA) in biological samples. After depletion of CatE by immunoprecipitation, the remaining activity is due to CatD, and the decrease of activity can be assigned to CatE. This assay distinguishes between the activities of enzymatically similar proteinases CatE and CatD and therefore can be used in studies aimed at understanding the involvement of these enzymes in antigen processing and presentation.

- Using the above described assay, the activity of CatE and CatD in cytosolic, endosomal and lysosomal fractions of B cells, dendritic cells and human keratinocytes was determined. The data clearly indicates that CatE activity is mainly located in endosomal and that of CatD in lysosomal compartments. Hence, this assay can also be used for characterization of subcellular fractions using CatE as an endosomal marker, whereas CatD is a well-known lysosomal marker.

- New cell permeable aspartic protease inhibitors were synthesized by conjugating pepstatin A with well-known cell-penetrating peptides (CPPs). To achieve this, the most frequently used CPPs, namely pAntp(43-58) (penetratin), Tat(49-60), and 9-mer of L-arginine (R9) were synthesized followed by coupling pepstatin A to the peptides. The enzyme inhibition properties of these bioconjugates and their cellular uptake into MCF7 (human breast cancer cell line), Boleths (EBV-transformed B cell line) and dendritic

cells (DC) was studied. It was found that the bioconjugate PepA-penetratin (PepA-P) was the most efficient cell-permeable aspartic protease inhibitor in comparison to PepA. Additionally, we found that PepA-P efficiently inhibited the tetanus toxoid C-fragment processing in peripheral blood mononuclear cells (PBMC), primary DC and in primary B cells. This inhibition of tetanus toxoid C-fragment processing by PepA-P clearly implicates the role of aspartic proteinases in antigen processing.

- In some of the previous studies, distinct cleavage specificities of CatE at neutral and acidic pH have been reported. Here, the proteolytic activity of recombinant CatE at acidic and neutral pH using two separate approaches, RP-HPLC and FRET-based proteinase assays, was determined. The data clearly indicated that recombinant CatE does not possess any proteolytic activity at neutral pH and was unable to cleave the peptides glucagon, neurotensin and dynorphin A that were previously reported to be cleaved by CatE at neutral pH. These discrepant results might be due to some contaminating factor present in the enzyme preparations used in previous studies or may reflect differences between recombinant CatE and the native enzyme.
- The substrate profiling of cathepsin E and D was performed in detail. It was found that presence of basic residues i.e. R, K and H especially at position P3 is preferred by cathepsin E. Unfortunately we were unable to identify exclusive substrate for CatE or D. However, the detailed specificity profiling of cathepsin E and D led to the identification of preferable residues at different positions that can help in designing of specific substrates or selective inhibitors for cathepsin E or D.

6.2 Future perspectives

The findings of the present work may facilitate further studies regarding cathepsin E and D or other proteases that exhibit similar enzymatic characteristics. E.g., the assay that was developed to distinctly measure the activity of CatE and CatD in biological samples is flexible and can be used for discriminating activity of other proteinases with similar enzymatic properties.

The present study has also described PepA-penetratin (PepA-P) conjugate that can serve as an important new tool to investigate the role of aspartic proteinases in antigen processing and presentation in different antigen presenting cells.

Moreover, the current thesis provides the detailed specificity information for cathepsin E and D that can give clues about biological functions of the two proteases and may facilitate the designing of specific substrates and selective inhibitors.

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Publications and poster presentations

Original Publications:

1. **Nousheen Zaidi,** Timo Herrmann, Daniel Baechle, Sabine Schleicher, Jeannette Gogel, Christoph Driessen, Wolfgang Voelter and Hubert Kalbacher. A new approach for distinguishing cathepsin E and D activity in antigen-processing organelles, *FEBS Journal 2007 Jun;274(12):3138-49.*
2. **Nousheen Zaidi,** Timo Herrmann, Wolfgang Voelter and Hubert Kalbacher. Recombinant cathepsin E has no proteolytic activity at neutral pH. *Biochem Biophys Res Commun. 2007 Aug 17;360(1):51-5. Epub 2007 Jun.*
3. **Nousheen Zaidi,** Timo Burster, Vinod Sommandas, Timo Herrmann, Bernhard O. Boehm, Christoph Driessen, Wolfgang Voelter, Hubert Kalbacher. A novel cell penetrating aspartic protease inhibitor blocks processing of tetanus toxoid more efficiently than pepstatin A. *Biochem. Biophys. Res. Commun. (2007), Dec 14;364(2):243-9. Epub 2007 Oct 4.*
4. **Nousheen Zaidi,** Hubert Kalbacher. Cathepsin E: A mini review. *Biochem. Biophys. Res. Commun. (2008*) Mar 14;367(3):517-22. Epub 2008 Jan 4.
5. **Nousheen Zaidi,** Hubert Kalbacher. Cathepsin D: A mini review. *(Manuscript Submitted).*
6. **Nousheen Zaidi,** Nicolas Lützner, Wolfgang Voelter and Hubert Kalbacher. Substrate profiling of cathepsin E and D. *(Manuscript Submitted).*
7. Rizwana Waraich**, Nousheen Zaidi**, K. Moeschel, A. Beck, Wolfgang Voelter, Hubert Kalbacher1, and R.Lehmann.Development and precise characterization of phospho-site-specific antibody for novel Ser357 of IRS-1: elimination of cross reactivity with adjacent Ser358 *(Manuscript Submitted).*

Abstracts/ Poster presentation:

During the period of this dissertation the work was presented in the following conferences:

1. **Nousheen Zaidi,** Timo Herrmann, Daniel Baechle, Sabine Schleicher, Jeannette Gogel, Christoph Driessen, Wolfgang Voelter and Hubert Kalbacher. A new approach for distinguishing cathepsin E and D activity in antigen-processing organelles. . *Poster presentation at Annual Meeting of the German Society for Immunology, 5^{th}-8^{th} September 2007 Heidelberg, Germany.*
2. **Nousheen Zaidi**, Timo Burster, Vinod Sommandas, Timo Herrmann, Bernhard O. Boehm, Wolfgang Voelter, Hubert Kalbacher. Synthesis of novel cell penetrating aspartic proteases inhibitors as the targeted inhibitors of antigen processing. *Poster presentation at Annual Meeting of the German Society for Immunology, 5^{th}-8^{th} September 2007 Heidelberg, Germany.*

3. Timo Herrmann, **Nousheen Zaidi**, Hubert Kalbacher New strategies for the analysis of processed antigens in dendritic cells. . *Poster presentation at Annual Meeting of the German Society for Immunology, 5th-8th September 2007 Heidelberg, Germany.*
4. Herrmann T, **Zaidi N**, Brandenburg J, Kalbacher H. Proteomics of processing relevant organelles in antigen presenting cells. *Poster presentation at 3rd Summer School in Proteomic Basics. "Exploring the Diversity of Proteins", 13th-19th August 2006 Kloster Neustift (Brixen/Bressanone, South Tyrol, Italy).*

Liste der akademischen Lehrer

W.Voelter	PhD research work
H. Kalbacher	PhD research work
F.Abbas	Research work
A.Saddiqui	Biochemistry
S.A.Qureshi	Biochemsitry/Molecular biology
Z.Hasan	Signal transduction
R.Hussain	Molecular immunology
S.Hussain	Cell biology
P.M.Frossard	Molecular biology
S.A.Saeed	Physiology
N.Ahmad	Molecular genetics
O.Y.Khan	Advance evolution
S.R.Farooqi	Human genetics
A.Wahidi	Plant genetics
M.Maqsood	Biochemsitry

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Title: Entwicklung und Anwendung Neuer Verfahren zur Untersuchung der Beteiligung von Cathepsin E und D am MHC II-Stoffwechsel.