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Research paper

# Cathepsin D activity and selectivity in the acidic conditions of a tumor microenvironment: Utilization in the development of a novel Cathepsin D substrate for simultaneous cancer diagnosis and therapy

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ABSTRACT

Pro-Cathepsin D (pCD) is an aspartyl endopeptidase which is over expressed in many cancers. This over expression generally led to its secretion into the extracellular culture medium of cancer cells. Moreover, pCD can auto activate and cleave its substrates at an acidic pH compatible with that found in tumor microenvironments (TME). Thus, exploiting these two pathological characteristics of TME offers the opportunity to develop new protease-activated vector on the basis of their specific substrate structures. The aim of this study was to validate new pCD substrates in the extracellular pH conditions of TME. As a first step, we investigated the effect of pH on the catalytic activity and selectivity of mature Cathepsin D (CD). It was found that the increase in the pH of the media led to a decrease in the reaction rate. However, the specificity of mature CD was not affected by a variation in pH. In the second step, the effect of the substrate structure was studied. We demonstrated that the substrate structure had a significant effect on the catalytic activity of CD. In fact, some modifications in peptide structure induced a change in the catalytic behavior that involved a substrate activation phenomenon. We suggest that this activation may be related to the amphiphilic nature of the modified peptide that may induce an interfacial activation mechanism. Finally, pCD, which is the major form found in the extracellular culture medium of cancer cells, was used. We demonstrated that the proform of CD cleave the modified peptide **5** at pH 6.5 with the same cleavage selectivity obtained with the mature form of the protease. These data provide a better understanding of CD behavior in tumor microenvironment conditions and this knowledge can be used to develop more specific tools for diagnosis and drug delivery.



## 1. Introduction

The tumor microenvironment (TME) consists of cells, soluble factors, signaling molecules, extracellular matrix proteins, and mechanical cues that surround and feed a tumor cell [1]. TME can promote malignant transformation, and support tumor growth and invasion [2]. TME conditions are considerably different from those of normal tissues because most tumors develop a pathophysiological microenvironment characterized by low oxygen levels (hypoxia) [3], low glucose concentration [4] and micro-acidic conditions [5]. In addition, a variety of hydrolytic enzymes are over expressed by tumor cells, activated during the different stages of tumor progression and often hypersecreted, forming the *liquid milieu* of the TME [6]. Based on the understanding of chemical and functional particularities of the TME, new specific molecular targets have been identified that enhance the effective development of new diagnostic tools [7] that are more selective for cancer cells, leading to personalized treatment. For example, one chemical characteristic that is common to the majority of solid tumors is their acidic microenvironment, with an extracellular pH ($pH_e$) ranging from 5.3 to 7.2 [8]. Whatever the mechanism for acidification, the result is a pericellular environment that may favor the

*Abbreviations:* pCD, pro-Cathepsin D; TME, tumor microenvironment; CD, Cathepsin D; ppCD, pre-pro-Cathepsin D; MCA, 7-methoxycoumarin-4-acetyl; DNP, dinitrophenyl; SA, sodium acetate; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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activity of proteases such as cysteine or aspartic cathepsins that have slightly acidic pH optima. In addition, an acidic $pH_e$ induces a redistribution of lysosomes to the tumor cell surface and secretion of their proteases [9].

Most of the research in our laboratory has focused on one lysosomal enzyme, aspartic CD (CD, EC 3.4.23.5), as an entry point to innovative cancer treatments [10,11]. CD is a lysosomal aspartyl endopeptidase present in all cells and tissues and associated with tumor progression and serve as autocrine growth factor for several cancer cell types. CD may stimulate cancer growth via its enzymatic activity by digesting various chemokines and may therefore attenuate the anti-tumoral immune response [12,13]. Pre-pro-Cathepsin D (ppCD) is synthesized in the rough endoplasmic reticulum. After the signal peptide is cleaved, the 52 kDa pro-Cathepsin D (pCD) is targeted to intracellular vesicular structures like lysosomes, endosomes or phagosomes. In a tumor, pCD escapes normal targeting mechanisms and can be hypersecreted into the extracellular space. At slightly acid pH, secreted pCD undergoes partial maturation and becomes active [14,15]. Therefore, an increase in pCD level is observed in several human neoplastic tissues like breast [16], thyroid [17] and prostate sarcoma [18].

The catalytic site of CD consists of two aspartic acid residues Asp33 and Asp231, located in the triad sequences of Asp33–Thr34–Gly35 and Asp231–Thr232–Gly23 [19]. The reaction mechanism that has been generally accepted for aspartic protease involves amide bond hydrolysis through an active site water molecule [20]. In this mechanism, the hydrolysis of the peptide bonds catalyzed by CD occurs in two stages. In the first stage, two simultaneous proton transfer between the water molecule and the carboxyl ion Asp33 and between the carboxyl group of Asp231 and the oxygen atom of the carboxyl group in the substrate lead to the formation of the enzyme–substrate complex (E–S), in which the components are bound by ionic and hydrogen bonds. In the next stage, a mechanism of double transfer leads to the decomposition of the indirect product, in which a proton of the hydroxyl group is transferred onto the Asp33, whereas the Asp231 is transported onto the nitrogen atom, resulting in the hydrolysis of the peptide bond in the substrate. Concerning substrate selectivity, CD preferentially cleaves peptide bonds found within the polypeptide chain formed by hydrophobic amino acid residues like aromatic trp, tyr and phe or long-chain aliphatic amino acids such as leu and ile [21]. Moreover, peptide sequences containing less than five amino acids are resistant to CD hydrolysis [22].

This study is a part of a project that aims to design a new intelligent CD cleavable vector for tracking and imaging the TME of breast cancer based on the response to unique pathological patterns of TMEs (micro-acidic extracellular pH associated with hypersecreted pCD). The ability to evaluate specific enzyme activity *in vivo* would thus have considerable clinical applications like the improvement of early detection of diseases and targeted drug delivery in localized areas or tissues. In the last few decades, some research teams have demonstrated the feasibility of such an approach by targeting proteases such as Cathepsin K or CD [23,24]. In this hostile environment, pCD is found in pH conditions that are far from their known optimal i.e. 3.5. We therefore started by investigating the effect of pH on the catalytic activity of the mature form of CD (34 + 14 kDa) using a commercial fluorogenic substrate. The effect of pH on CD specificity was also studied using a model synthetic substrate. As the objective is the synthesis of a CD-sensitive substrate, the effect of substrate structure on both catalytic activity and specificity of the protease was investigated using peptides selected according to the specification mentioned below. Finally, the results were validated using the pCD form that is considered to be the major form which is over expressed in the extracellular microenvironment of breast cancer.

## 2. Materials and methods

### 2.1. Materials

All reagents, unless specified otherwise, were purchased from Sigma Aldrich® (St Quentin Falavier, France).

A mature form of bovine CD (34 + 14 kDa) was also provided by Sigma Aldrich® and had an activity of 5.0 units/mg of protein. One unit, as defined by the supplier, will produce an increase in $A_{280}$ of 1.0 per min per mL at pH 3.0 and at 37 °C, measured as TCA-soluble products using hemoglobin as a substrate (1 cm light path).

Recombinant pCD (52 kDa) was purchased from R&D systems® (USA) with a purity higher than 95%.

CD activity was measured using fluorogenic peptide **1** (R[K-DNP]LRFFLIPK[G-MCA]) supplied by Sigma Aldrich® (MCA: 7-Methoxycoumarin-4-Acetyl and DNP: dinitrophenyl). Peptides **3** (LLVVFF) was prepared by standard solid phase peptide synthesis with a Fmoc strategy using a Rink amide resin (0.8–1 mmol/g loading) on a 0.1 mmol scale. The synthesis was realized using an automated microwave peptide synthesizer CEM Liberty 1 with coupling time of 15–30 min depending on the amino acid with microwave heating at 40 °C. The others peptides (Peptides **2**, **4** and **5**, see Table 3 for structures) used in this study were synthesized by Genosphere Biotechnology® (France) with purity greater than 85%.

### 2.2. Kinetics assays of CD using the fluorogenic substrate

Hydrolysis of fluorogenic peptide **1** was performed in white 96 half-well plates (Corning® #3693) using a BMG Labtech Fluostar Omega spectrofluorometer. The fluorescence was measured at $\lambda_{em} = 390$ nm and $\lambda_{ex} = 330$ nm with an interval of 20 s. The reaction was conducted at 37 °C in a final volume of 100 μL with a concentration of 8.3 ng/μL of mature CD. Three different buffers were used to adjust the reaction pH: Sodium acetate buffer (for pH 3.7, pH 4.5 and pH 5.6), 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (for pH 5.6, pH 6 and pH 6.5) and; 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer (for pH 6.5 and pH 6.8). Substrate concentrations were varied from 5 μM to 30 μM (5 μM, 10 μM, 20 μM, 25 μM, 30 μM).

### 2.3. Kinetics assays of CD using the synthetic model peptide substrates

Peptides **2**, **3**, **4** and **5** (Table 3) were first dissolved in pure DMSO at a concentration of 4 mM. Hydrolysis of the peptides was performed in a final volume of 500 μL in the presence of 8.3 ng/μL of mature CD and the reaction was conducted at 37 °C in a Radleys® reactor. Three different buffers were used to adjust the reaction pH: Sodium acetate buffer (for pH 3.7, pH 4.5 and pH 5.6), Sodium MES Buffer (for pH 5.6, pH 6 and pH 6.5) and Sodium MOPS (for pH 6.5 and pH 6.8). Substrate concentrations were varied from 10 μM to 400 μM (10 μM, 35 μM, 70 μM, 140 μM, 200 μM, 400 μM). A zero time aliquot (50 μL) was removed from the solution prior to enzyme addition. Aliquots were taken over time (0 h, 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 24 h, 28 h, 34 h, 48 h, 72 h, 120 h) and the reaction was stopped by boiling for 5 min followed by a 5 min centrifugation at 10,000× *g*. Samples were stored at −20 °C for at most 48 h prior to their analysis by LC/MS-ESI (cf. Section 2.6).

### 2.4. Phospholipid micelle preparation

Phosphatidylcholin and cardiolipin were diluted in ethanol (1 mL) at a concentration of 0.12 mM and 0.05 mM, respectively. Ethanol was dried at 37 °C and phospholipid solutes were dissolved in water (1 mL). This aqueous solution was sonicated for