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Fluorescent, internally quenched, peptides for exploring the pH-dependent substrate specificity of cathepsin B

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Abstract: Cathepsin B is a cysteine protease that in tumor tissues is localized in both acidic lysosomes and extracellular spaces. It can catalyze the cleavage of peptide bonds by two mechanisms: endoproteolytic attack with a pH optimum around 7.4, and attack from the *C*-terminus with a pH optimum at 4.5–5.5. In this work, seven fluorescent, internally quenched, decapeptides have been synthesized using the prototypical cathepsin B selective substrate Z-Phe-Arg-AMC as a lead, and used to identify the structural factors determining the susceptibility of peptides to hydrolysis at acidic and neutral pH values. Each peptide differs from the others in one amino acid (residue 6) and contains a highly fluorescent Nma group linked to the α-amino function of the *N*-terminal Orn residue and a Dnp group linked to the side chain of the Lys^8 residue acting as a quencher. Proteolytic cleavage was monitored by measuring the increase of fluorescence at 440 nm upon excitation at 340 nm, and the cleavage sites were determined by HPLC followed by ESI-MS analysis. Peptides containing Ala or Phe at position 6 are good substrates for the enzyme at both pH 5.0 and 7.4. By contrast, those containing Glu, Asp, Lys or Val are not cleaved at all by cathepsin B at pH 7.4, and are poorly hydrolyzed at pH 5.0. These findings provide new information for the rational design of cathepsin B-activated peptide-containing anticancer drugs. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: anticancer prodrugs; cathepsin B; fluorescence; internally quenched peptides

INTRODUCTION

Cathepsin B is a cysteine protease of the papain superfamily [1] that, in normal cells, is localized mainly in perinuclear acidic lysosomes and participates in the turnover of both cellular proteins and peptides taken up from the extracellular environment, as well as in prohormone processing [2]. However, this protease is also involved in a number of disease conditions such as cancer, rheumatoid arthritis and acute pancreatitis (for a review see Ref. 3). In malignant tumors and premalignant lesions, the expression of cathepsin B at mRNA, protein and activity levels is dramatically increased and the enzyme, normally present in acidic lysosomal compartments, becomes associated with the external face of the plasma membrane and is secreted by exocytosis into the pericellular space [4,5]. Plasma membrane-bound and secreted cathepsin B activities are thought to play a pivotal role in tumor cell invasion and metastasis both directly, by degrading extracellular matrix proteins, and indirectly, by activating downstream proteases, such as the urokinase-type plasminogen activator [6], in a proteolytic cascade that results in the degradation of extracellular matrices [7,8]. The relatively high level of extracellular proteolysis in tumor tissues suggests that a strategy aimed at improving the selectivity of conventional cytotoxic drugs may be the use of nontoxic peptide prodrugs that can selectively release the active drug only upon proteolytic cleavage triggered by pericellular (plasma membrane-bound and/or secreted) tumor-associated proteases. Peptide-doxorubicin conjugates activated by plasmin [9] and albumin-doxorubicin conjugates selectively cleaved by matrix metalloproteases [10–12] are examples of tumor-targeting prodrugs that are under active investigation. However, though anticancer prodrugs designed to be internalized by tumor cells and activated by lysosomal cathepsin B have been prepared [13,14], to the best of our knowledge, prodrugs designed to be specifically activated by the pericellular enzyme are not yet available.

It is well established that cathepsin B has both exo- and endopeptidase activities. At the acidic pH values of the lysosomes (4.5–5.5), cathepsin B acts primarily as an exopeptidase owing to its active site being obstructed by an amino acid insertion termed the 'occluding loop' [15]. This loop, corresponding to the sequence Ile^{105}-Pro^{126}, is located toward the prime region of the active site, and makes the access of the substrates to the S'_1–S'_2 sites of the catalytic pocket of the enzyme difficult (Figure 1). The exopeptidase activity of cathepsin B is thought to be the result

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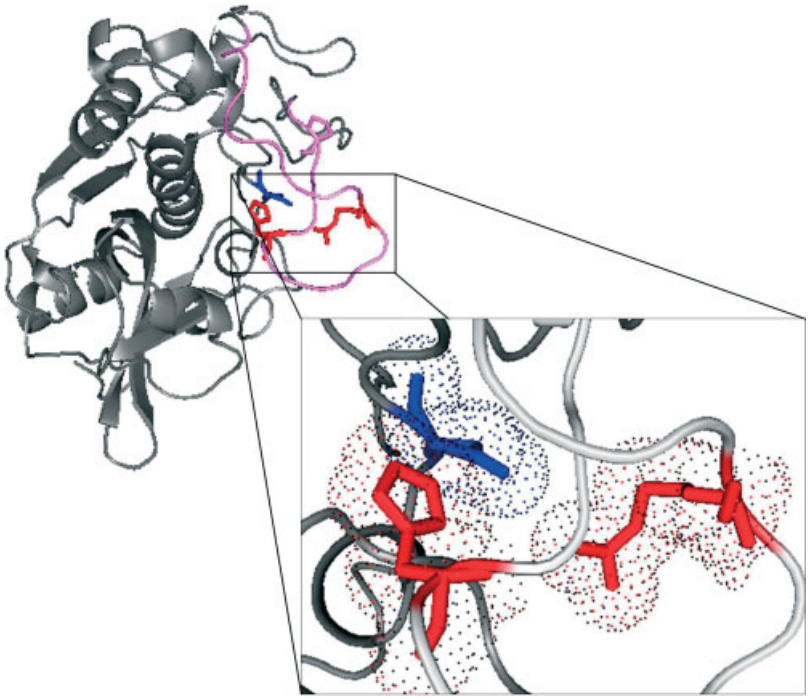

Figure 1 Conformation of cathepsin B (1HUC file from PDB). The main chain of the occluding loop is in magenta. The side chains forming salt bridges are in red (His^{110} and Arg^{116}) and in blue (Asp^{22}).

of ionic interactions between the negatively charged, *C*-terminal, carboxylate group of the substrates and the positively charged side chains of His^{110} and His^{111} residues. Additionally, His^{110} and Arg^{116} form salt bridges with Asp^{22}, which is located near the active center of the enzyme [1,16]. At higher pH values, such as those of the pericellular microenvironments of solid tumors (the average pH is typically 6.9–7.0) [17], the occluding loop is displaced from the active site cleft and the enzyme shows endopeptidase activity. This activity has a pH optimum around 7.4 [18] and deprotonation of His^{110} seems to be critical for its appearance [19].

Internal fluorescence quenching and fluorescence resonance energy transfer-based assays are becoming the most promising tools for investigating the substrate specificity of enzymes [20,21]. The basis for such an assay is the introduction in the peptide substrate of a fluorescent label (the donor) and a second label (the acceptor) that absorbs light at the same wavelength at which the donor fluoresces on opposing sides of the scissile peptide bond that is recognized by the target enzyme. The acceptor may then radiationless decay back to its ground state or it may itself fluoresce at some characteristic wavelength. We refer to the first process as quenching because the net effect is a loss of fluorescence intensity. The second situation is described as 'resonance energy transfer' because here excitation at the absorption maximum of one molecule leads to fluorescence by the other molecule (Ref. 22 for a more comprehensive treatment of fluorescence quenching and resonance energy transfer). When the peptide is intact, the donor and acceptor groups remain at a relatively fixed distance, able to energetically interact intramolecularly. Once hydrolyzed by the enzyme, however, the two fragments of the original peptide will diffuse away from each other, thus eliminating the possibility of any interaction between the donor and acceptor. The observed effect of this hydrolysis will be an increase in the fluorescence from the donor group, and, in the case of energy transfer, a concomitant decrease in the fluorescence of the acceptor group with excitation under the absorption maximum of the donor. In particular, fluorescence quenching can occur during normal intramolecular contacts, or more versatile by resonance energy transfer. In this second approach, the excited state of the fluorophore can be deactivated via dipole–dipole interactions over a separation of up to 50 Å [20].

In the framework of a research program aimed at the development of anticancer prodrugs activated by tumor-associated proteases, we designed and synthesized a series of potential cathepsin B substrates containing the Arg–Arg sequence, a specific hallmark of this enzyme [23].

In particular, we synthesized a series of internally quenched, fluorescent, decapeptides derived from the sequence Nma-Orn-Ala-Gly-Arg-Arg-Xaa-Ala-Lys(Dnp)-D-Ala-Ala-OH [Nma, 2-(*N*-methylamino)benzoyl; Orn, ornithine; Dnp, 2,4-dinitrophenyl] (Figure 2), with the aim of exploring the influence of the amino acid (Xaa) bound to the carboxyl group of the Arg^5 residue on the rate of peptide hydrolysis by cathepsin B at

Table 1 Analytical data of internally quenched fluorescent peptide substrates

Peptide			M. W.		$t_{r\ (min)}$[a]
			Calculated	Determined	
1	Ala^6	$C_{54}H_{84}N_{21}O_{16}$	1283.64	1284.41	18
2	Lys^6	$C_{57}H_{91}N_{22}O_{16}$	1341.51	1342.67	24
3	Phe^6	$C_{60}H_{88}N_{21}O_{16}$	1360.51	1361.62	31
4	Glu^6	$C_{56}H_{86}N_{21}O_{18}$	1342.45	1343.60	26
5	Val^6	$C_{56}H_{88}N_{21}O_{16}$	1311.68	1312.69	27
6	β-Ala^6	$C_{54}H_{84}N_{21}O_{16}$	1284.41	1285.57	25
7	Asn^6	$C_{55}H_{85}N_{22}O_{17}$	1327.44	1326.65	25
8	Nma-Orn-Ala-Gly-OH	$C_{18}H_{26}N_5O_5$	392.19	393.10	15
9	H-Lys(Dnp)-D-Ala-Ala-OH	$C_{18}H_{26}N_6O_8$	454.18	455.38	7[b]

[a] Elution conditions are: eluants: A-0.05% TFA in water, B-0.05% TFA in 9 : 1 acetonitrile-water; column: Jupiter C_{18} column (10 μ, 250 × 4.6 mm); flow-rate 1 ml/min; isocratic elution to 20% B for 3 min and then linear gradient from 20 to 50% B in 40 min.
[b] Isocratic elution to 10% B for 3 min and then linear gradient from 10 to 40% B in 40 min.

acidic and neutral pH values. Preliminary studies have shown the importance of the distance between the fluorophore (Nma) and the quencher (Dnp). Indeed, the previously synthesized octapeptide Nma-Orn-Gly-Arg-Arg-Ala-Lys(Dnp)-D-Ala-Ala-OH is not hydrolyzed by cathepsin B (data not shown), suggesting a negative effect exerted by the proximity of the two aromatic groups. We introduced in the *C*-terminal region one residue of the D-steric series (D-Ala) to preclude the exopeptidase activity of cathepsin B.

MATERIALS AND METHODS

Peptides

The fluorescent, internally quenched, peptides were synthesized by solid-phase procedures on Wang resin using an Advanced Chemtech model 348Ω peptide synthesizer and the Fmoc/HBTU [Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino) methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide] chemistry. Fmoc-Lys(Dnp)-OH was obtained by treatment of H-Lys(Dnp)-OH (Fluka, Buchs, Switzerland) with Fmoc-OSu (OSu, 1-oxysuccinimide) according to the standard procedure. Boc-Nma-OH (Boc, *tert*-butyloxycarbonyl) was synthesized from Nma (Aldrich, Milwaukee, WI) and di-*tert*-butyl dicarbonate in water/dioxane in the presence of two equivalents of $NaHCO_3$. Peptides were side-chain deprotected and removed from the resin by TFA (trifluoroacetic acid) treatment in the presence of 2.5% TIS, 2.0% anisole and 0.5% water, and then precipitated by the addition of diethyl ether. All peptides were purified by semipreparative HPLC on a Vydac C_{18} column (10 μ, 250 × 100 mm, The Separation Group, Hesperia, CA). The purity of the synthesized peptides was checked by amino acid analysis (Carlo Erba model 3A30 apparatus) and analytical HPLC using a Jupiter C_{18} column (10 μ, 250 × 4.6 mm; Phenomenex, Torrance, CA) (elution conditions and t_r values are reported in Table 1). The molecular masses of peptides were determined by ESI-MS using a Mariner API-TOF (PerSeptive Biosystem, Foster City, CA) instrument (Table 1).

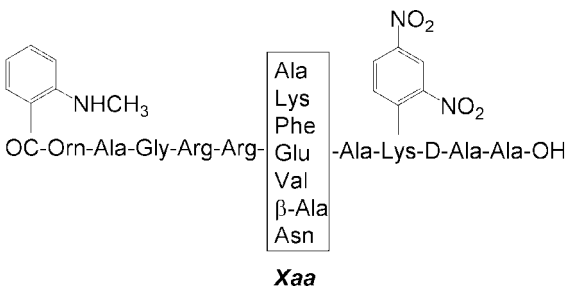

Figure 2 Primary structure of internally quenched fluorescent substrates of cathepsin B.

Enzymes and Enzyme Assays

Bovine spleen cathepsin B was purchased from Sigma-Aldrich (St Louis, MO). The concentration of enzyme water stock solution was measured spectrophotometrically using the molar extinction coefficient of 62,160 $M^{-1} \times cm^{-1}$ at 280 nm. The enzyme was preincubated in the reaction buffer for 5 min at 37 °C before addition of the peptide.

The assays were carried out in a 50 mM Tris-HCl (pH 7.4) solution containing 1 mM EDTA and 5 mM Cys, and in a 50 mM sodium acetate (pH 5.0) solution containing 1 mM EDTA and 5 mM Cys.

Concentrate aqueous stock solutions of each peptide (5 mM) were diluted with the selected buffer at substrate concentrations ranging from 0.5 to 5 μM. The final concentrations were calculated by colorimetric determination of the Dnp group (the excitation coefficient at 430 nm is 6540 $M^{-1} \times cm^{-1}$). The enzymatic hydrolysis of the internally quenched fluorescent peptides was followed at 37 °C by fluorescence measurement at 440 nm following excitation at 340 nm using a Perkin-Elmer model LS-50B spectrofluorimeter (Norwalk, CT), after addition of cathepsin B (final enzyme concentration, 91.7 nM). Fluorescence variations were converted into the amount of

hydrolyzed substrate by using standard curves obtained by the fluorescent measurements of well-defined concentrations of each substrate after complete hydrolysis by trypsin.

The kinetic parameters K_m and k_{cat} were determined from Lineweaver-Burk double reciprocal plots and from the Eisenthal-Cornish-Bowder direct plot of rates of hydrolysis *versus* initial peptide concentration [24]. The catalytic efficiency was determined as the k_{cat}/K_m ratio. The standard errors for K_m and k_{cat} determinations are less than 10%.

Determination of Substrate Cleavage Site

The cleavage points of each substrate by cathepsin B proteolysis were determined by ESI-MS of the lyophilized HPLC fractions (Jupiter C_{18} column, 250 × 4.6 mm) after incubation of substrates (160 μM) with cathepsin B (20 μM) at 37 °C for 1 h in the two previously described buffers.

RESULTS AND DISCUSSION

Substrate Specificity

In order to study the pH effect on substrate hydrolysis by cathepsin B, we synthesized a series of seven peptides derived from the lead Nma-Orn-Ala-Gly-Arg-Arg-Xaa-Ala-Lys(Dnp)-D-Ala-Ala-OH containing an Nma-Dnp fluorescent donor-acceptor pair. The quencher (Dnp) was introduced on the Lys side-chain amino group, whereas the fluorophore (Nma) was linked to the Orn *N*-terminal amino group. The Xaa position was alternatively replaced by residues showing different physico-chemical properties (polarity, charge, dimension) (Figure 2).

The UV-Vis absorption spectrum of the H-Lys(Dnp)-D-Ala-Ala-OH tripeptide (peptide **9** in Table 1) and the emission spectrum of the Nma-Orn-Ala-Gly-OH tripeptide (peptide **8**) are shown in Figure 3. The observed overlap between the absorption band of the Dnp group and the emission spectrum of the Nma group suggested that the quenching effect present in these kinds of substrates can be essentially attributed to a nonradiative, long-range resonance energy transfer mechanism rather than collisional effects.

The emission spectra of the intramolecularly quenched fluorogenic substrates **1**–**7** as well as the emission spectrum of the Nma-tripeptide **8** upon excitation at 340 nm were recorded and the quenching efficiencies, expressed by the gradient $(F_0\text{-}F)/F_0$, where F_0 and F are the fluorescence intensities of the Nma donor in the absence (peptide **8**) and in the presence of the Dnp acceptor (peptides **1**–**7**), respectively, were calculated. The results are summarized in Figure 4(A).

The quenching efficiency reflects the flexibility of the designed substrates, which can result in a shorter average distance between the Nma and Dnp groups, consequently increasing the quenching phenomenon. Only peptide **6** (**Xaa→β-Ala**) is characterized by a relatively low quenching efficiency (83%) as compared to those of the other substrates tested (mean quenching efficiency 96%). This finding suggests that the introduction of the β-Ala residue into the peptide chain increases the average distance between the donor (Nma) and the acceptor (Dnp) groups.

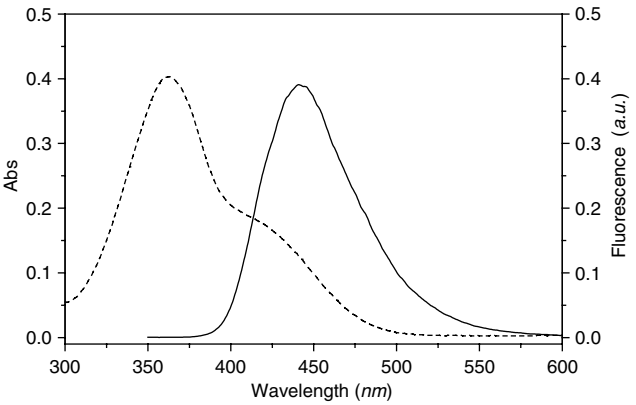

Figure 3 Overlapping of the Dnp absorption spectrum (- - - -) of reference peptide **9** and the Nma fluorescence spectrum (———) of reference peptide **8**.

The relative (F_t/F_i) ratio of the fluorescence of each peptide solution after (F_t) and before (F_i) cathepsin B hydrolysis were calculated and compared to the same value obtained by treatment with trypsin at pH 7.4 (Figure 4(B)). Only peptides **1** and **3** (**Xaa→Ala** or **Phe**, respectively) result in more than a 10-fold fluorescence increase after cathepsin B treatment at both the pH values tested. On the other hand, the data obtained at pH 5.0 show that only peptide **4** (**Xaa→Glu**), in addition to peptides **1** and **3**, has a (F_t/F_i) ratio higher than 10. By contrast, all of the peptides, with the exception of peptide **6** (**Xaa→β-Ala**), show a high value for the (F_t/F_i) ratio after treatment with trypsin. Fluorescence increase is typically related to peptide structure and in particular to peptide flexibility, as well as to the aptitude to hydrolysis of the substrate by the assayed enzyme. The results summarized in Figure 4(B) permit the screening of selective substrates for cathepsin B at different pH values, confirming the high sensitivity of these kinds of substrates toward the activity of this enzyme [20,21].

The peptide cleavage sites were determined by ESI-MS of the lyophilized HPLC fractions of the cathepsin B hydrolyzed peptides. The results show that the cleavage site at pH 7.4, as well as at pH 5.0, corresponds to the Xaa–Ala bond. Peptide **3** was also cleaved at the Arg–Xaa bond, even if this second cleavage site corresponds to the minor fraction of the isolated fragments.

Kinetic Analysis

Peptide hydrolyses were monitored by the increase of fluorescence at 440 nm following excitation at 340 nm of the substrate solutions after cathepsin B addition

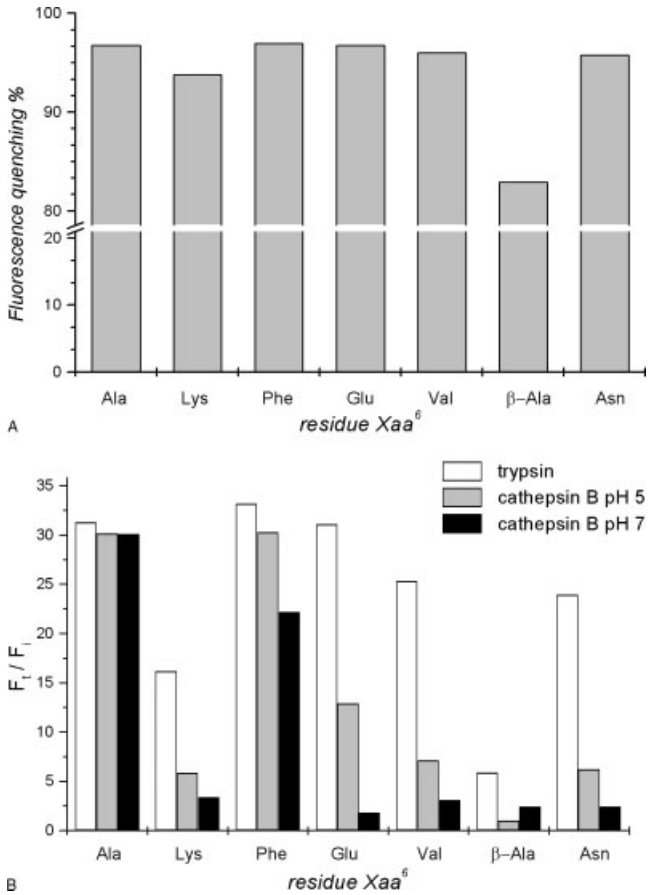

Figure 4 Fluorescence quenching percentages of the substrates (A); and (F_t/F_i) ratios after cathepsin B (pH 5.0 and 7.4) and trypsin (pH 7.4) hydrolysis (B). The amino acid residue at position Xaa^6 is indicated on the *x*-axis.

(as an example, the increase of fluorescence related to hydrolysis of peptide **1** is reported in Figure 5). Time courses resulting from hydrolysis of the internally quenched fluorescent peptides in the two different buffer solutions are reported in Figure 6, while the corresponding kinetic parameters are summarized in Table 2.

Peptides with hydrophobic residues such as Ala and Phe (peptides **1** and **3**), but not Val (peptide **5**), at the Xaa position are preferred substrates for cathepsin B at both pH values tested. By contrast, substrates with a negatively charged amino acid (Glu, peptide **4**), a polar uncharged amino acid (Asn, peptide **7**) or a positively charged amino acid (Lys, peptide **2**) at the same position are poorly hydrolyzed by cathepsin B only at pH 5.0 owing to either reduced k_{cat} or increased K_m, or both. Similarly, peptide **5** (**Xaa→Val**) is poorly hydrolyzed by the enzyme at pH 5.0, even if it shows a low K_m value. The introduction at the Xaa position of the β-Ala residue (peptide **6**) is detrimental for the enzyme activity at both pH values tested.

Influence of pH on the Catalytic Activity

A clear picture of the impact of pH values on the cathepsin B activity emerges from the data shown in Figures 4 and 6. In agreement with its biological function, cathepsin B displays higher and broader proteolytic activity at pH 5.0 than at pH 7.4. Indeed, at pH 5.0 all studied peptides containing an α-amino acid at the Xaa position are hydrolyzed, though with different efficiency (Figure 7). On the contrary, only peptides **1** and **3** (**Xaa→Ala** or **Phe**, respectively) are hydrolyzed by cathepsin B at pH 7.4. The effect of pH value on the enzyme activity is more evident for peptide **1** than for peptide **3**. Indeed, the catalytic efficiency of the enzyme toward peptide **1** is twofold higher at pH 5.0 than at pH 7.4, while the enzyme efficiency toward peptide **3** is comparable with the two pH values tested.

CONCLUSIONS

In summary, all of the designed peptides, with the exception of peptide **6** (**Xaa→βAla**), are substrates for cathepsin B at pH 5.0, despite the noteworthy differences. The best substrate at this pH value is

Table 2 Kinetic constants for cathepsin B hydrolyses of the Xaa^6-peptide analogs

Peptide		pH 5.0			pH 7.4		
		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM × s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM × s^{-1})
1	Ala^6	16.2	10.3	635.8	4.4	1.44	327.2
2	Lys^6	3.3	$9.0 \cdot 10^{-3}$	2.7	—	Resistant	—
3	Phe^6	30.0	0.26	8.7	48.0	0.36	7.5
4	Glu^6	20.7	$4.0 \cdot 10^{-2}$	1.9	—	Resistant	—
5	Val^6	7.1	$1.3 \cdot 10^{-2}$	1.8	—	Resistant	—
6	β-Ala^6	—	Resistant	—	—	Resistant	—
7	Asn^6	14.3	$1.4 \cdot 10^{-2}$	0.9	—	Resistant	—

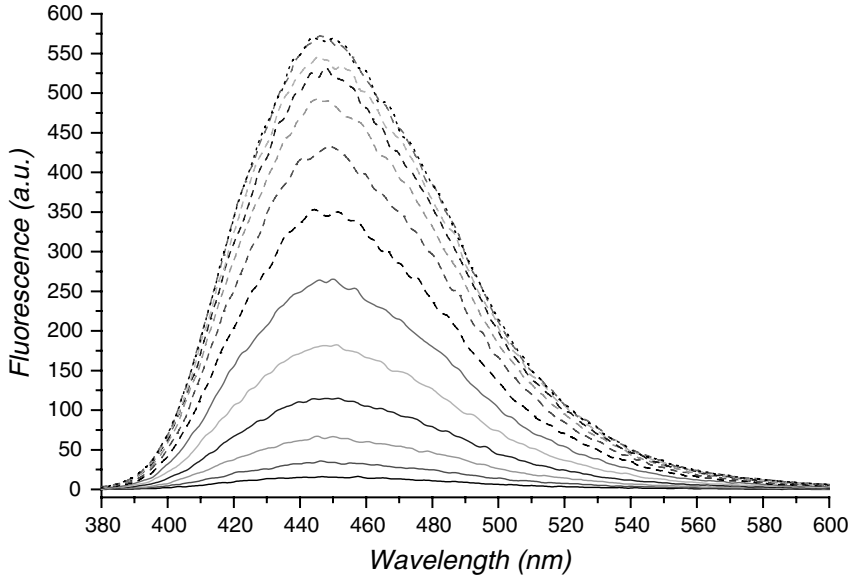

Figure 5 Fluorescence emission spectra of peptide **1** in Tris buffer, pH 7.4 (5.6 μM, λ_{ex} 340 nm) after cathepsin B (91.7 nM) addition. The spectra were recorded with a delay of 30 s.

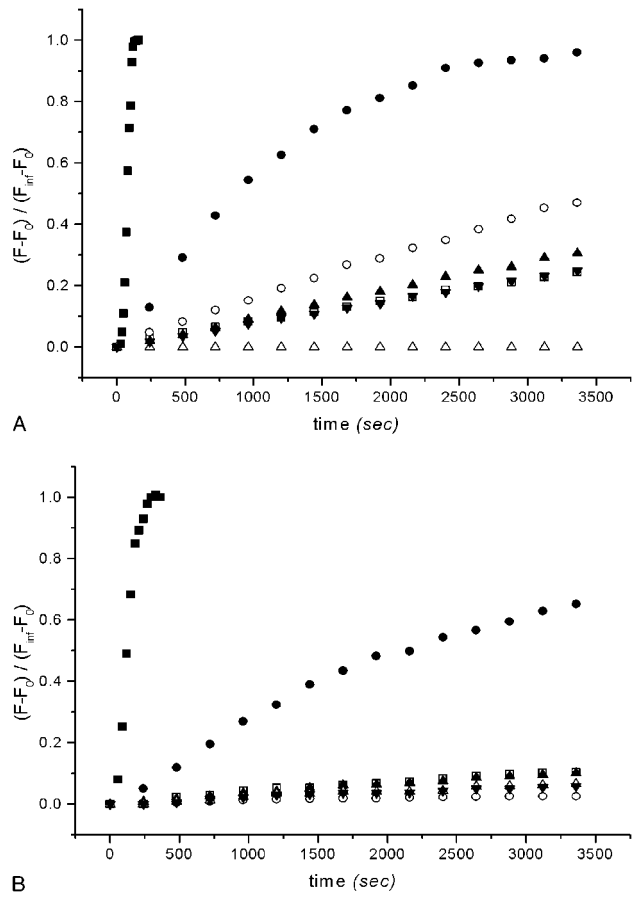

Figure 6 Time courses of internally quenched fluorescent substrate hydrolyses by cathepsin B. A: pH 5.0, B: pH 7.4 (■, Ala; □, Lys; ●; Phe; ○, Glu; ▲, Val; ▼, β-Ala; Δ, Asn).

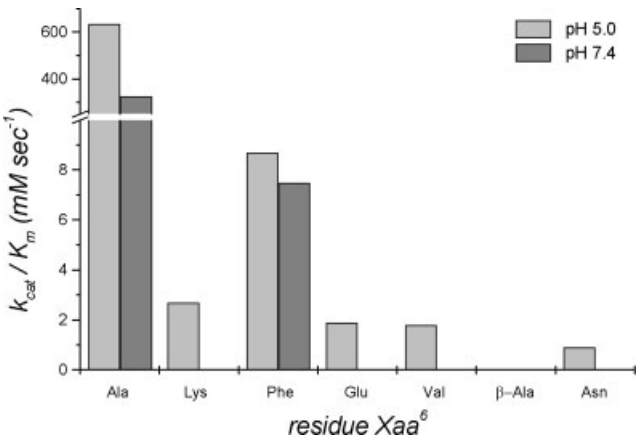

Figure 7 Specificity constants (k_{cat}/K_m) for the hydrolyses of the internally quenched fluorescent substrates by cathepsin B. The amino acid residue at position Xaa^6 is indicated on the *x*-axis.

that containing an Ala residue at the Xaa^6 position (peptide **1**). The replacement of this residue in the internally quenched substrate strongly decreases its susceptibility to enzymatic hydrolysis. In particular, the introduction of Val, Glu, Lys or Asn residue at the Xaa position confers to the peptides selectivity for hydrolysis by cathepsin B at acidic pH values, making them convenient substrates for analyzing the lysosomal activity of this enzyme. On the other hand, peptides **1** and **3** could represent useful tools to explore the proteolytic activity of the enzyme at both acidic and neutral pH values, the latter being typical of extracellular environments. Efforts are ongoing in our laboratory to synthesize a putative cathepsin B-activated anticancer prodrug by coupling peptide **1** and **3** sequences to conventional anticancer drugs.

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