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Biotinylated fluorescent peptide substrates for the sensitive and specific determination of cathepsin D activity

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Abstract: Cathepsin D (CatD) is a member of the mammalian aspartic protease family and is involved in cellular protein degradation and in several pathological processes. A sensitive and specific assay for the determination of CatD activity in biological samples was developed. The peptide amide substrates Amca-EDKPILF↓FRLGK(biotin)-$CONH_2$ (I), Amca-EEKPIC(Acm)F↓FRLGK(biotin)-$CONH_2$ (II) and Amca-EEKPISF↓FRLGK(biotin)-$CONH_2$ (III) contain a CatD cleavage site (F↓F) flanked by a *N*-terminal Amca-fluorophore (7-amino-4-methylcoumarin-3-acetic acid) and a *C*-terminal biotin moiety. Substrates II and III proved to be specific substrates containing only one cleavage site for CatD. After cleavage of the Phe-Phe bond by CatD all biotin conjugated peptides were removed with streptavidin-coated magnetic beads. The remaining fluorescent peptides in solution represent the amount of digested substrate. The versatility of this CatD digest and pull down assay was demonstrated by measuring the activity of CatD in different subcellular fractions of human EBV-transformed B cells and human monocytes. The described method based on the designed CatD substrates represents a valuable tool for routine assays. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cathepsin D; fluorescent substrate; biotinylated substrate; streptavidin-coated magnetic beads

INTRODUCTION

CatD (E.C. 3.4.23.5) is a member of the aspartic protease family and widely distributed among all mammalian cells [1–4]. It is involved in the degradation of intracellular and extracellular proteins. Moreover, CatD is thought to play a dominant role in the MHC class II restricted processing of proteins and presentation of peptides to T helper cells. Apart from the processing of proteinogenic antigens in lysosomal compartments such as the proteolysis of the B chain of insulin [9], CatD seems to be involved in the processing of the MHC II-associated invariant chain [8] which is necessary for generating functional MHC class II molecules for the binding of antigenic peptides [5–7].

It has also been suggested that CatD plays a role in the processing of the β-amyloid precursor protein important for Alzheimer's disease [10–12]. Overexpression of CatD in breast cancer cells is associated with an increased risk of metastasis [13], caused by the CatD induced cancer cell proliferation [14].

In cell biological investigations, for the discovery of specific inhibitors or for clinical diagnostics it is necessary to have a fast and reproducible assay for the parallel testing of the proteolytic activity of CatD. Previously, several methods have been described for measuring the activity of aspartic proteases [15–20]. A very common method using natural protein substrates such as bovine hemoglobin is rather time consuming and lacks of specificity [15]. The generated trichloroacetic acid soluble cleavage products of hemoglobin are measured at 280 nm or the tyrosine and tryptophan content is determined using the phenol reagent of Folin and Ciocalteau [16]. A faster method uses substrates containing a chromophore, such as a nitrophenylalanine residue, at position P1. Product formation can be measured by monitoring the increase of absorbance at 300–310 nm by UV spectroscopy [17]. Recently, fluorogenic substrates containing fluorophore and quencher groups such as o-aminobenzoic acid and p-nitroanilide [18] or 5-[(2-aminomethyl)amino]naphthalene-1-sulfonic acid) (EDANS) and 4′-dimethylaminoazobenzene-4-carboxylic acid (DABCYL) [19, 22] within the same molecule have been developed. Upon cleavage of the peptide the fluorescence signal is no longer quenched and its increase can be measured continuously [23].

This paper describes the synthesis of simultaneously fluorophor and biotin labelled decapeptide amide substrates and their application to a novel heterogeneous bead assay for the determination of CatD

Abbreviations: As recommended in *J. Peptide Sci.* 2003; **9**: 1–8, with the following additions: Amca, 7-amino-4-methylcoumarin-3-acetic acid; ACN, acetonitrile; CatD, cathepsin D; CCE, crude cell extract; DABCYL, 4′-dimethylaminoazobenzene-4-carboxylic acid; DG, digestion product; E, endosomal fraction; EDANS, 5-(2-aminoethyl) aminonaphthalene-1-sulfonic acid; L, lysosomal fraction; TBST, Tris-buffered saline with Tween.

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activity. This method is applied to biological samples to analyse CatD activity in endosomal and lysosomal compartments as well as in cell lysates of human EBV-transformed B cells and human monocytes.

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals

CatD (bovine spleen and human liver) was purchased from Sigma (Taufkirchen, Germany) and stored as a 32.3 μM stock solution in 0.1 M citric acid, pH 4.5 at −20 °C prior to use. Pepstatin A, Zwittergent 3–12 and Rink amide resin were purchased from Calbiochem-Novabiochem (Schwalbach, Germany). All other chemicals and solvents were from Merck (Darmstadt, Germany). Fmoc amino acids were purchased from MultiSynTech (Witten, Germany).

Synthesis of Fmoc-Lys(biotin)-Rink Amide Resin

Fmoc-Lys-OH (368.4 mg, 1.0 mmol) was dissolved in 8 ml DMF and reacted with biotin-OSu (341.4 mg, 1.0 mmol) and NMM (111 μl, 1.0 mmol) for 6 h. The reaction mixture was extracted with chloroform, washed twice with 4 M $KHSO_4$, water and finally evaporated to dryness. The obtained Fmoc-Lys(biotin)-OH derivative was coupled to Rink amide resin using the TBTU/HOBt coupling method. After this step the remaining free amino groups were blocked by acetylation with acetic anhydride/DIPEA/DMF (1 : 1 : 8, v/v/v) twice for 30 min. The degree of coupling was estimated to be 0.35 mmol/g.

Synthesis of Substrates

The peptide substrates were synthesized by solid-phase synthesis using the standard Fmoc/tBu protocol [24] on the multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany) on a 0.025 mmol scale using a six-fold molar excess of Fmoc amino acids. Side chain protecting groups were: tBu for Glu, Asp and Ser, Boc for Lys, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg and Cys was Acm-protected.

In situ activation was performed using TBTU (6 eq) and HOBt (1 eq) followed by the addition of NMM (12 eq). After the completion of the coupling the resin bound peptides were Fmoc-deprotected using piperidine/DMF (2 : 3, v/v twice for 15 min) and washed subsequently with DMF, isopropyl alcohol and diethyl ether.

On-resin Labelling of Substrates with Amca

The fluorophore Amca 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 [25]. Subsequently the resin was washed with DMF, isopropyl alcohol, diethyl ether and then dried.

Cleavage of Peptides and Purification

A cocktail of TFA/thioanisol/phenol/ethanedithiol (95 : 3 : 3 : 2, v/v/w/v) was used for 2 h to release the peptide from the resin and to remove the side chain protecting groups. After addition of cold diethyl ether, the precipitated peptides were washed three times with diethyl ether. Finally peptides were dissolved in tBuOH/H_2O (4 : 1, v/v) and lyophilized. The crude peptides were purified by preparative RP-HPLC (Nucleosil 100, 150 × 10 mm, C18 column, 7 μm particle diameter, Wicom, Heppenheim, Germany) with the following solvent system: (A) 0.055% (v/v) TFA in H_2O and (B) 0.047% (v/v) TFA in ACN/H_2O (4 : 1, v/v) on a Merck/Hitachi L-6200 delivery system (Darmstadt, Germany). The column was eluted at a flow rate of 2 ml/min with a linear gradient from 0% B to 80% B within 40 min. Detection was carried out at 214 nm (L-4000A UV Detector, Merck, Darmstadt, Germany). The identity of the peptide amides was confirmed using ESI-MS. Peptide purities of the purified fractions were analysed by analytical RP-HPLC (Nucleosil 100, 125 × 2 mm, C8 column, 5 μm particle diameter, Wicom, Heppenheim, Germany) using a 5% to 80% gradient of solvent B for 40 min with detection at 214 nm. All peptides were dissolved in DMSO at a concentration of 1 mM and stored at −20 °C until usage.

Analysis of the Digestion Products using RP-HPLC and MALDI-MS

The *in vitro* generated digestion products were separated using analytical RP-HPLC (Nucleosil 100, 125 × 2 mm, C8 column, 5 μm particle diameter, Wicom, Heppenheim, Germany) with detection at 214 nm. 0.5 μl of each fraction was then mixed with 0.5 μl of 2,5-dihydroxyacetophenone matrix (20 mg of 2,5-dihydroxyacetophenone, 5 mg of ammonium citrate in 1 ml of isopropyl alcohol/H_2O (4 : 1, (v/v)) and applied on a gold target for MALDI-MS using a MALDI time-of-flight system (G2025A, Hewlett-Packard, Waldbronn, Germany). Signals were generated by adding up 50–100 laser shots in the single shot mode.

Determination of Kinetic Characteristics of the Substrates

To measure the initial rates of substrate proteolysis and to determine the kinetic parameters of the substrates, digestion was monitored using RP-HPLC. Hydrolysis of substrates was performed in 48 μl of 50 mM Gly/HCl buffer (pH 3.5) containing 1 μl of a 0.32 μM CatD solution. CatD dilutions were prepared in 0.1 M citric acid pH 5.0 containing 0.05% (v/v) Triton X-100. The reaction was started by adding 1 μl of the appropriate substrate solution so that the final substrate concentration was between 5 and 80 μM. The reaction was performed at 37 °C.

Every 2 min 2 μl of the reaction mixture was removed and added to 38 μl of stop solution (H_2O/ACN/TFA/Zwittergent 3–12, (94 : 5 : 1 : 0.2, v/v/v/w)). The obtained solutions were applied to an analytical C8 column (Nucleosil 100, 125 × 2 mm, C8 column, 5 μm particle diameter, Wicom, Heppenheim, Germany) and eluted with a 0% to 80% gradient of solvent B for 40 min. The HPLC column eluent was analysed measuring fluorescence emission at 450 nm following excitation at 350 nm using a fluorescence HPLC monitor RF-535 (Shimadzu, Duisburg, Germany). The peak of fluorescently

labelled digestion product and the peak of undigested substrate were integrated and the amount of converted substrate in μM was determined.

Then the initial velocity was calculated from the linear part of the cleavage curve which proved to be between 1 and 10 min. The K_m (μM) and V_{max} ($\mu M \times s^{-1}$) values were determined using the Lineweaver-Burk method. The k_{cat} values (s^{-1}) were calculated from the equation

$$k_{cat} = \frac{V_{max}}{[CatD]_{total}} \quad (1)$$

where $[CatD]_{total}$ is the total enzyme concentration in μM and V_{max} is the maximal velocity in $\mu M \times s^{-1}$.

Digest and Pull Down Assay

The cleavage of the peptide amide substrates was performed in 48 μl 50 mM Gly/HCl buffer (pH 3.5) containing protease inhibitors (2 mM phenylmethanesulfonyl fluoride and 20 μM leupeptin for the inhibition of serine proteases, 10 mM EDTA for the inhibition of metalloproteases and 10 mM iodacetamide for the inhibition of cysteine proteases).

For the digest and pull down assay 1 μl of a 0.5 mM substrate solution in 50% (v/v) DMSO was used. The appropriate sample volume of the cell fractions containing 0.5 μg total protein determined according to Bradford [26] was then added. The calibration curve was determined using the appropriate sample volume of CatD.

After 10 min at 37 °C, 90 μl of stop solution was added to 10 μl of the reaction mixture. The fluorescence of a 50 μl aliquot of the solution was then measured using the microplate reader Spectra Fluor (Nunc, Wiesbaden, Germany) at an emission wavelength of 465 nm with excitation at 360 nm, obtaining F_1 in fluorescence units (FU). This solution was then added to 5 μl of streptavidin-coated magnetic beads (MagPrep® Streptavidin Beads by Merck, 1 μm, 1.4 g/l, 10^{12} particles/g). Magnetic beads were rinsed twice for 2 min with 50 mM Gly/HCl buffer (pH 3.5) before application. It was determined that 2 μl of the magnetic beads suspension captured up to 0.1 nmol biotinylated peptide. The mixture was stirred and kept at RT for at least 10 min but not longer than 30 min. Longer incubation led to an increase of unspecific binding. The solution should be homogeneous for complete binding of the biotinylated molecules to the streptavidin-coated magnetic beads. After 10 min the magnetic beads were separated using a permanent magnet (after 1 min the supernatant should be clear). The fluorescence of 50 μl of the obtained supernatant was measured according to F_2. The concentration of generated digestion product (DP, μM) in μmol/l per min is calculated using the equation

$$\frac{[DP]}{t} = [S_0] - \frac{F_1 - F_2}{F_1} \times [S_0] - [DP_0] \quad (2)$$

where $[S_0]$ is the initial substrate concentration (μM) and t is the digestion time in min. $[DP_0]$ is the value of biotinylated peptides not bound to the magnetic beads (μM) and was determined in parallel to each test series.

Cell Culture, Subcellular Fractionation and Western Blot Analysis

The EBV-transformed human B cell line Boleth (IMGT/HLA sequence database number: HC10329) and the human monocyte cell line U937 (ATTC number: CRL-2367) were grown at 37 °C in tissue culture flasks (Nunc, Wiesbaden, Germany) with RPMI 1640 medium (containing 10% (v/v) fetal calf serum; 2 mM L-glutamine; 25 mM Hepes; Invitrogen-Gibco, Karlsruhe, Germany) supplemented with 80 μg/ml gentamycin (Merck, Darmstadt, Germany) in a humidified atmosphere (5% CO_2/air). 10^8 cells were harvested (1000 rpm for 10 min, Heraeus-Christ Varifuge K) and subcellular fractionation was performed as previously described by Schröter *et al.* [27]. The obtained fractions were crude cell extract (CCE), endosomal fraction (E) and lysosomal fraction (L).

The subcellular fractions were separated by SDS-polyacrylamide gel electrophoresis (15 μg protein/lane) on a 15% separating gel and transferred to a PVDF-membrane (Amersham Biosciences, Freiburg, Germany) in a Novex mini-trans-blot-apparatus (Invitrogen, Karlsruhe, Germany). The membranes were blocked for 16 h at 4 °C in Tris-buffered saline with Tween 20 (TBST, 0.15 M NaCl, 10 mM Trizma® Base, 0.05% Tween 20 (v/v), pH 8.0) containing 10% (v/v) Roti® Block (Roth, Karlsruhe, Germany). Rabbit anti-human CatD (Calbiochem, Schwalbach, Germany) was diluted 1 : 5000 in TBST containing 10% (v/v) Roti® Block and incubated for 2 h. Subsequently, filters were washed three times in TBST and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno Research, West Grove, USA) diluted 1 : 10 000 in TBST containing 10% (v/v) Roti® Block. After washing the filters three times with TBST, western blots were developed according to the ECL protocol by Amersham Biosciences (Freiburg, Germany).

RESULTS

Synthesis of Biotinylated and Fluorescent CatD Substrates

The substrate I was designed based on the published CatD peptide substrate KPILF↓FRL. Within this substrate the Phe-Phe bond is predominantly cleaved by CatD as previously described by Scarborough *et al.* [21]. This substrate was modified for our digest and pull down assay system by including the two charged amino acids glutamic acid and aspartic acid (Table 1) at the *N*-terminus in order to increase solubility. At the *C*-terminus a glycine residue was introduced as a spacer and a lysine residue for the conjugation of the biotin moiety via its ε-amino-group. This was achieved by using a Rink amide resin carrying an ε-biotinylated lysine residue (Figure 1). The peptide substrate then was synthesized by elongation via SPPS on the lysine's α-amino-group. After SPPS the substrate was then *N*-terminally labelled with the pH-insensitive fluorophore Amca due to the fact that the assay will be performed in an acidic buffer system because CatD has a pH optimum at 3.5–4.0 [20]. After deprotection and cleavage from the resin the peptide amides were purified by preparative RP-HPLC and checked for their correct masses using ESI-MS. Peptide purities were determined via analytical RP-HPLC and proved to be higher than 97%.

Table 1 Proteolytic fragments obtained after digesting substrate I, II and III with CatD as determined with MALDI-MS

	Substrate and digestion product	Expected mass $[M+H]^+$	$[M+H]^+$	ΔDa
I	Amca-EDKPILFFRLGK (biotin)-$CONH_2$	1904.3	1904.3	0.0
	Amca-EDKPILF-OH	1077.2	1077.4	0.2
	NH_2-FRLGK (biotin)-$CONH_2$	846.1	845.5	0.6
	Amca-EDKPIL-OH	930.1	930.0	0.1
	NH_2-FFRLGK (biotin)-$CONH_2$	993.3	993.3	0.0
	Amca-EDKPI-OH	816.9	817.1	0.2
	NH_2-LFFRLGK (biotin)-$CONH_2$	1106.4	n.f.[a]	—
II	Amca-EEKPIC (Acm) FFRLGK (biotin)-$CONH_2$	1979.4	1981.3	1.9
	Amca-EEKPIC (Acm) F-OH	1152.3	1151.2	1.1
	NH_2-FRLGK (biotin)-$CONH_2$	846.1	845.2	0.9
III	Amca-EEKPISFFRLGK (biotin)-$CONH_2$	1892.3	1890.9	1.4
	Amca-EEKPISF-OH	1065.2	1063.7	1.5
	NH_2-FRLGK (biotin)-$CONH_2$	846.1	845.0	1.1

[a] n.f., not found.

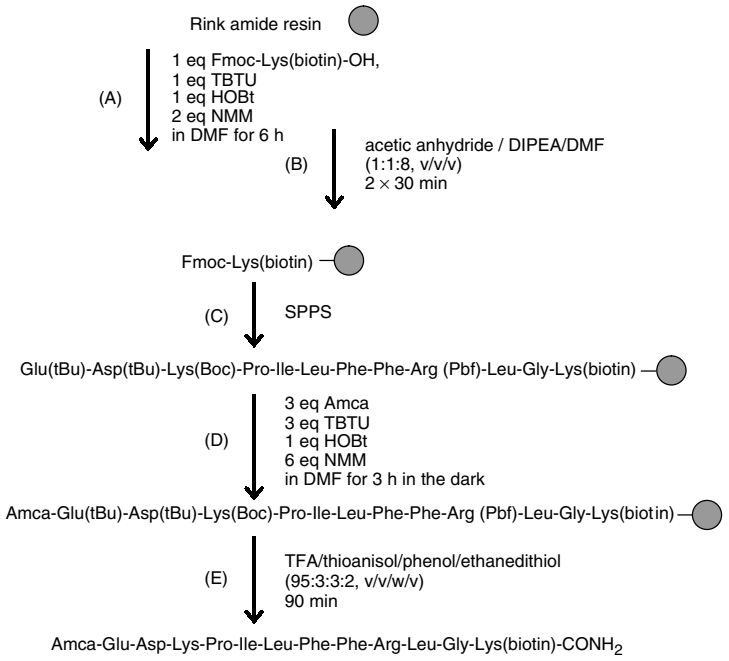

Figure 1 Synthesis of the biotinylated and fluorescent peptide amide Amca-EDKPILFFRLGK(biotin)-$CONH_2$. (A) Coupling of Fmoc-Lys(biotin)-OH to Rink amide resine. (B) Capping of the remaining amino-groups by acetylation. (C) Solid-phase peptide synthesis. (D) Introduction of the fluorophore Amca at the *N*-terminus. (E) Cleavage of the peptide from the resin and side chain deprotection.

In vitro Digestion and Optimization of the Peptide Substrate

Substrate I was digested *in vitro* with CatD and the generated peptide fragments were separated using RP-HPLC (Figure 2, panel A) and identified by MALDI-MS (Table 1). Substrate I did not possess just one single cleavage site for CatD and was cleaved between I↓L, L↓F and F↓F (Table 1, entry I). However, the development of a specific CatD assay preferably requires a substrate possessing one single cleavage site. Therefore the hydrophobic amino acid residue leucine in P2 position was replaced with the Acm-protected cysteine derivative Cys(Acm) (substrate II) or the hydrophilic amino acid serine (substrate III), respectively. The peptide amide substrates II and III were synthesized as described in the experimental section and peptide purities were determined using analytical RP-HPLC and proved to

be higher than 97%. Substrates II and III were also digested with CatD and the generated peptide fragments were separated by RP-HPLC and identified by MALDI-MS. Both substrates were only cleaved between the two phenylalanine residues yielding two peptide fragments (Table 1, entries II and III). No further digestion products were observed. These results suggest that CatD preferably cleaves peptide substrates between uncharged and hydrophobic amino acids according to previously published data [21]. Replacement of the uncharged hydrophobic amino acid leucine at P2 position renders substrates II and III more specific making them attractive tools for the development of a specific CatD assay.

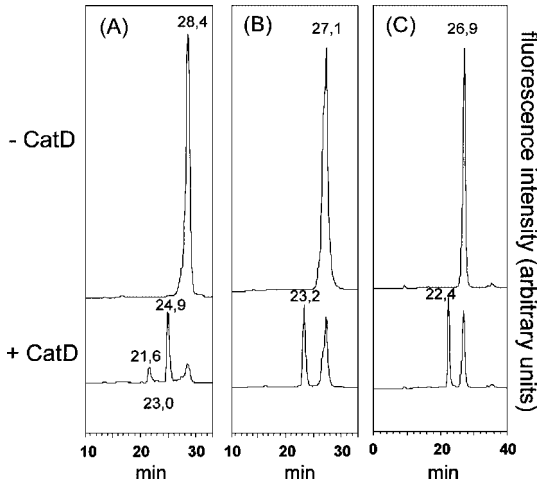

Figure 2 RP-HPLC profiles of substrates I–III after incubation with CatD at pH 3.5 in 50 mM Gly/HCl buffer with (upper line) or without (lower line) 10 μM pepstatin using fluorescence detection ($\lambda_{Ex.} = 350$ nm, $\lambda_{Em.} = 450$ nm). Apparent peaks represent the fluorescently labelled digestion products and undigested substrate. The substrates (1 μg) were incubated with 0.01 μg CatD for 10 min. (A) Digestion of substrate I with CatD leads to at least six determined cleavage products. Substrate II (B) and III (C) show only one cleavage site for CatD.

Kinetic Characteristics of the Developed CatD Substrates

Next substrates I, II and III were characterized with respect to their kinetic properties by measuring the initial velocities at various substrate concentrations using analytical RP-HPLC with fluorescence detection for the quantification of proteolytic activity. In order to obtain data that can be used for kinetic analysis, the time frame when the initial velocity was linear was determined (Figure 3). Within the first 10 min the initial velocity was linear and the obtained parameters could be used for Lineweaver-Burk analysis as described in the experimental section. The obtained K_m, k_{cat} and k_{cat}/K_m values of substrates I, II and III are listed in Table 2.

The determined kinetic parameters for the substrates were slightly higher than described for previously published substrates [19–21]. Conjugation with biotin and Amca as well as the replacement of the hydrophobic amino acid leucine in P2 position led to a small increase of the K_m values. As expected, substrate I exhibited the lowest K_m value due to the fact that it possesses several cleavage sites for CatD. Substrate III was more

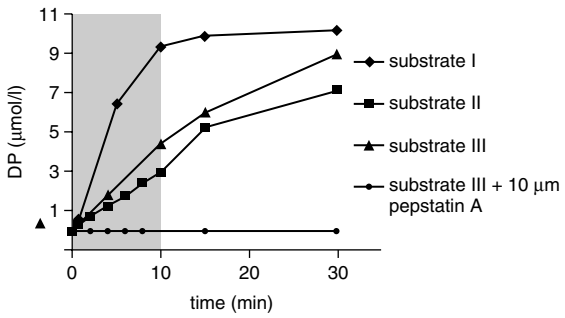

Figure 3 Time courses of the hydrolysis of 10.5 μM substrate I, II and III with 6.5 nM CatD in 50 mM Gly/HCl buffer (pH 3.5). The amount of digestion product (DP) was determined using RP-HPLC with fluorescence detection as described in the experimental section. The hatched area indicates the linear phase of the hydrolysis (0–10 min). In the presence of 10 μM pepstatin A CatD was completely inhibited.

Table 2 Kinetic parameters for the hydrolysis of the synthesized fluorescent substrates I, II and III by CatD. P1 and P1 residues are shown in bold

Substrate	Sequence	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \times s^{-1}$)	Reference
I	Amca-EDKPIL**FF**RLGK (biotin)-$CONH_2$	12.2	0.41	0.03	
II	Amca-EEKPIC (Acm) **FF**RLGK (biotin)-$CONH_2$	72.5	6.34	0.09	
III	Amca-EEKPIS**FF**RLGK (biotin)-$CONH_2$	53.0	7.28	0.14	
	Ac-EE (Edans) KPIC (Et) **FF**RLGK (Dabcyl) E-NH_2	27.5	196.0	7.10	[19]
	MOCAc-GKPIL**FF**RRLK (Dnp) γ-NH_2	3.7	57.8	15.60	[20]
	KPIR**FNph**RL-NH_2	72.0	6.0	0.08	[21]

MOCAc, (7-methoxycoumarin-4-yl) acetyl; Dnp, N-2, 4-dinitrophenyl; Nph, p-nitrophenylalanine.

specific than substrate I and had a smaller K_m value than substrate II and therefore proved to be the most suitable substrate for a rapid and specific substrate-based CatD assay. Therefore substrate III was used for further experiments.

Application of the Substrates for the Determination of CatD Activity

Figure 4 shows the principle of our novel digestion and pull down assay. After incomplete digestion of substrate III the following peptides were in solution: intact substrate and both fluorescent and biotinylated digestion products. First, the total fluorescence of the reaction solution was measured at an emission wavelength of 465 nm with excitation at 360 nm immediately after digestion. The obtained value is termed F_1 and represents the total amount of fluorescent peptides in solution, i.e. digested and undigested fluorescent substrate. In the next step all biotinylated peptides were removed using streptavidin-coated magnetic beads, leaving only fluorescent digestion products in solution. Then the remaining fluorescence was measured yielding the value F_2. Under the conditions described in the experimental section about 95% of all biotinylated molecules were bound and removed (Figure 5A) from the solution within 10 min (Figure 5B). Longer incubation times or the application of more magnetic beads did not improve the efficiency of the removal of the biotinylated peptides. The difference between the values F_1 and F_2 coincides proportionally with the amount of digested substrate. Using equation (2) shown in the experimental section, the concentration of the digestion product (DP) after the appropriate time can be calculated.

Determination of absolute CatD activity in biological samples requires a calibration curve. For this purpose substrate III was incubated with different amounts of CatD in the range 15 mU/ml to 45 mU/ml (Figure 6). The obtained values were interpolated leading to the following equation

$$CatD = \frac{[DP]}{t \times 0.1656} \tag{3}$$

where [*DP*] is the concentration of the digestion product in µmol/l, t is the digestion time in min and *CatD* is the enzyme activity in mU/ml. The constant factor 0.1656 represents the slope of the calibration curve. One unit was defined as an increase in absorbance at 280 nm of 1.0 in 30 min at pH 3.3 and at 37 °C measured as trichloroacetic acid soluble products using acid-denaturated hemoglobin as substrate (Sigma-Aldrich, Taufkirchen, Germany).

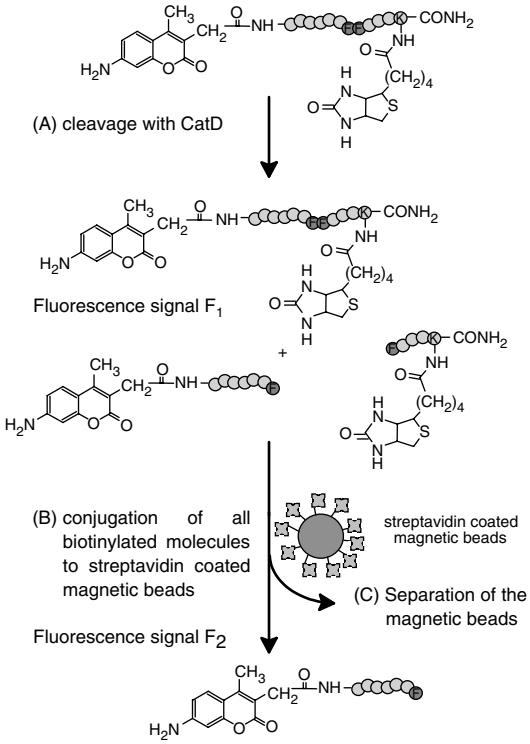

Figure 4 Principle of the digest and pull down assay: (A) Incomplete digestion of peptide substrate and fluorescence measurement (F_1). (B) Capturing of biotinylated digestion products with streptavidin-coated magnetic beads. (C) Measurement of fluorescence (F_2) after separation of magnetic beads with a permanent magnet.

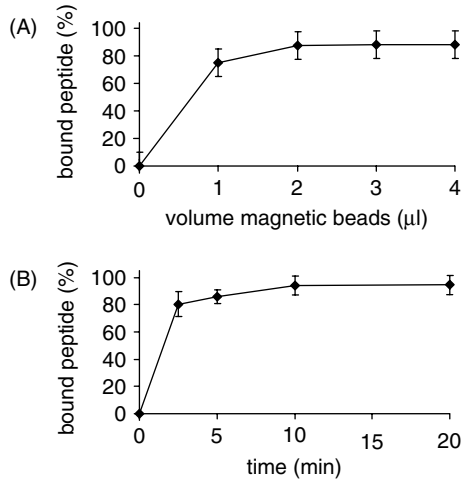

Figure 5 Characterization of the streptavidin-coated magnetic beads. A solution of 0.1 nmol substrate III in 50 µl Gly/HCl buffer (pH 3.5) was incubated with different amounts of streptavidin-coated magnetic beads (MagPrep® Streptavidin Beads by Merck, Darmstadt, Germany, 1 µm, 1.4 g/l, 10^{12} particles/g) (1–4 µl) for different time periods (3, 5, 10 and 20 min). The amount of bound peptide was then determined using analytical RP-HPLC with fluorescence detection by measuring the solution before and after bead incubation. The difference of the areas under the peaks represents the relative amount of bound peptide in percent. (A) Binding capacity of the streptavidin-coated magnetic beads. (B) Time course of the conjugation of biotinylated peptide substrate III to 2 µl of the streptavidin-coated magnetic beads.

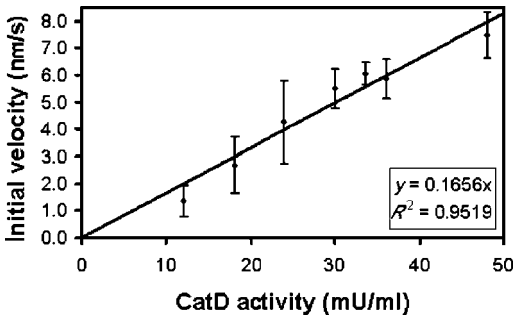

Figure 6 Calibration curve for the determination of CatD activity using the bead-assay. Varying concentrations of CatD were incubated with substrate III (10.6 μM) in 50 μl 50 mM Gly/HCl buffer (pH 3.5) at 37 °C for 10 min. Values represent the mean of at least three independent experiments.

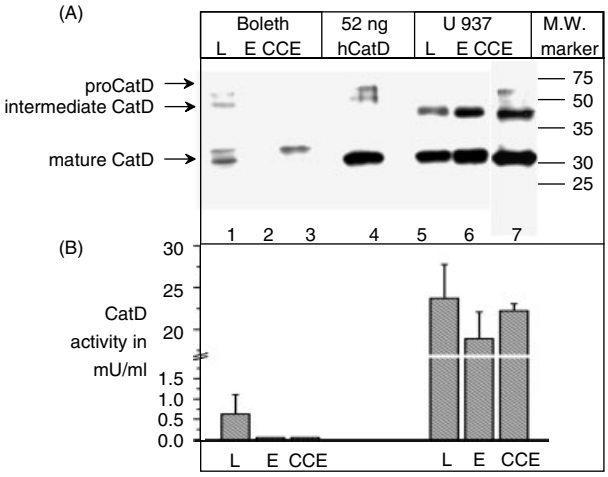

Figure 7 (A) Distribution of CatD in subcellular fractions of human EBV-transformed B cells and human monocytes (U937) detected by western blot analysis: 15 μg protein per lane. 52 ng of hCatD was used as a positive control and shows a signal at 31 kDa (mature CatD) and at 51–55 kDa (proCatD). In all fractions of U937 and in the lysosomal fraction of Boleth cells the 31 kDa-mature CatD band is visible. The intermediate single chain form of CatD migrating at 44 kDa could only be detected in U937. For determination of molecular weights the full range rainbow marker (Amersham Biosciences, Freiburg, Germany) was used. (B) CatD activity in mU/ml as determined by the bead-assay using the calibration curve shown in Figure 6 (L, lysosomal fraction; E, endosomal fraction; CCE, crude cell extract).

Determination of CatD Activity in Subcellular Compartments of Human B Cells and Monocytes

To demonstrate the applicability of the digest and pull down assay for biological samples the distribution of CatD in different subcellular fractions of the human EBV-transformed B cell line Boleth and the human monocyte cell line U937 was analysed. The two cell lines were selected because of their different CatD expression levels. The cell line U937 exhibits a very high level of CatD compared with the Boleth cell line where no CatD was detectable in cell lysates using western blot analysis [28].

In order to validate the versatility of the novel digest and pull down assay for cellular CatD activity measurements it was compared with the standard western blot analysis. Therefore the two cell lines were homogenized and fractionated as previously described [27] yielding the following subcellular fractions: crude cell extract (CCE), endosomal fraction (E) and lysosomal fraction (L). The total amount of protein in each fraction was determined using the method of Bradford [26]. 15 μg total protein of the subcellular fractions was applied to a 15% SDS-polyacrylamide gel and the proteins then were transferred to a PVDV-membrane followed by western blot analysis using a rabbit anti-human CatD antibody (Figure 7A). Lanes 1, 2 and 3 and 5, 6, 7 represent the lysosomal fraction, endosomal fraction or crude cell extract of the Boleth cell line and U937 cell line, respectively. Human CatD was used as a positive control and is shown in lane 4. The band migrating at 31 kDa represents the active mature form of CatD. Two weaker bands at 44 kDa and 51–55 kDa represent the intermediate single chain form of CatD and glycosylated proforms of CatD, respectively [29]. The control human CatD showed a major band at 31 kDa and a small amount of the intermediate single chain form and the proform of CatD due to the fact that it was isolated from human liver.

In the endosomal fraction and in crude cell extracts of the Boleth cell line no CatD could be detected. The band visible in the crude cell extract and in the lysosomal fraction of the Boleth cell line migrating at 33 kDa is not mature CatD due to the fact that it migrates above the mature CatD band. This CatD form has not been identified before. Only in the lysosomal fraction of the Boleth cell line was a small amount of mature CatD as well as the intermediate single chain CatD and the proform of CatD detected.

In contrast to this intracellular CatD distribution in the Boleth cell line, in all fractions of the U937 cell line almost the same amount of mature CatD was detected. The single chain intermediate form of CatD is predominantly found in the endosomal fraction and to a smaller extent in the lysosomal fraction and crude cell extract.

The two cell lines were analysed for their CatD activity using substrate III and the novel digest and pull down assay. The digest and pull down assay was performed with 0.5 μg total protein of the subcellular fractions. The digestion products were then separated by RP-HPLC and identified by MALDI-MS. Substrate III was only cleaved between the two phenylalanine residues and no further cleavage sites were observed when using the inhibitor mix described in the experimental section (data not shown). These findings proved that substrate III can be used for the specific determination of CatD in subcellular fractions of human B cells and human monocytes, respectively.

Substrate III was also incubated for 10 min with the subcellular fractions and analysed using a microplate reader at an emission wavelength of 465 nm with excitation at 360 nm obtaining F_1 and F_2, respectively. The amount of digestion product (μM) was calculated using equation (2). For the determination of the absolute CatD activity in the subcellular fractions the obtained amount of digestion product was inserted in equation (3) yielding the absolute CatD activity in mU/ml sample volume. The results are shown in Figure 7B and represent the mean of at least three different experiments.

In the endosomal fraction and in the crude cell extract of the Boleth cell line no CatD activity could be detected. Only weak CatD activity was measured in the lysosomal fraction. These results concerning the distribution of CatD in subcellular compartments agree well with those obtained by western blot analysis.

As previously described, the U937 cell line exhibits a higher CatD expression level [28] corresponding to the results obtained with the digest and pull down CatD assay. About a 40-fold higher CatD activity was found in fractions of the U937 cell line compared with the Boleth cell line. Another difference between these two cell lines is the intracellular distribution of CatD. The lysosomal fraction of U937 shows about a 20% higher CatD activity than the endosomal fraction, whereas CatD in the Boleth cell line could only be detected in the lysosomal fraction and not in the endosomal fractions. Furthermore, the crude cell extract of the U937 cell line exhibits almost the same CatD activity as the lysosomal fraction of U937. These results suggest that CatD may be only slightly enriched in lysosomal fractions and otherwise more ubiquitously distributed in the human monocyte cell line U937.

The developed digest and pull down assay proved to be suitable for the determination of CatD activity in biological samples. The measured activity using substrate III is specific for CatD under the conditions described since there was no other cleavage site observed.

DISCUSSION

This contribution describes a robust and specific method for the determination of CatD activity in biological samples. It presents the solid-phase synthesis and *in vitro* digestion of biotinylated fluorescent CatD substrates and their application to a novel digest and pull down assay. The CatD digest and pull down assay presented here is based on the CatD-mediated cleavage of peptidic substrates bearing a fluorophore at their *N*-terminus and a biotin moiety at their *C*-terminus. The biotin tag and the fluorophore are separated upon digestion with CatD. Subsequent removal of all biotin-carrying peptides from the assay solution using streptavidin-coated magnetic beads leaves only digested fluorescent substrate in solution. From this amount of fluorescent peptide the enzymatic activity of CatD can easily be determined.

The digest and pull down assay offers several advantages over other state-of-the-art protease assays. Commercially available sensitive assays (Amersham Biosciences, Freiburg, Germany) are based on the application of substrates bearing two different fluorophores [30]. The synthesis of such doubly labelled fluorescent peptides goes beyond standard SPPS [31–33]. Moreover, many fluorescent dyes used for these substrates are reasonably expensive in milligram quantities, clearly limiting their broad applicability in straightforward solid-phase synthesis which routinely requires high molar excesses. The choice of the two fluorescent dyes for these substrates needs to be evaluated carefully since the two different fluorophores can either undergo FRET or quench each other significantly [32].

Another commercially available CatD assay (Molecular Probes, EnzChek™ protease assay kit, Leiden, the Netherlands) is based on casein derivatives that are labelled with many fluorescent dyes, resulting in almost total quenching of the conjugate's fluorescence because of the high density of fluorophores within the casein protein. After cleavage of the fluorescent casein the quenching of the dyes is abolished and the net fluorescence increases significantly [34]. However, this system lacks specificity since there are many potential cleavage sites in casein for other proteases.

The presented digest and pull down assay proved to be more specific for CatD since substrate III was only cleaved between the two phenylalanine residues by reaction with the subcellular fractions. Another advantage of the digest and pull down assay over the assays using quenched peptide substrates is that a change of the fluorescence signal can only result from cleavage of the substrate and not from conformational changes of the peptide backbone, e.g. caused by binding to another protein contained in the sample.

In the digest and pull down assay described here, short incubation times were selected for a fast assay performance. In order to increase the assay's sensitivity, longer incubation times may also be applied. The described synthesis of fluorescent peptide substrates is simple and can easily be transferred to peptide substrates of other proteases and would clearly extend the applicability of this digest and pull down assay. Moreover other pH-stable fluorescent dyes, such as rhodamine derivatives, may be used for *N*-terminal labelling of the protease substrates and thus increase the sensitivity of the digest and pull down assay due to higher quantum yields.

For technical applications and high-throughput screening the digest and pull down assay can be performed in 96-well plates. All steps including enzymatic hydrolysis, removal of biotinylated peptides

using the streptavidin-coated magnetic beads and fluorescence measurements can be made in parallel and performed in the 96-well plate format. This option underlines that the digest and pull down assay offers a fast alternative to other commercially available assays.

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