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ORIGINAL ARTICLE

Peptide substrate for caspase-3 with 2-aminoacridone as reporting group

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Abstract Synthesis and properties of a new fluorescent/fluorogenic substrate Ac-DEVD-AMAC for caspase-3 are reported. The substrate is obtained by conventional Fmoc-based solid phase peptide synthesis and its properties are investigated with regard to fluorescence, sensitivity, applicability and kinetic constants. A non-traditional approach to assay the proteases activity using 2-amino-acridone labeled peptides is proposed. This approach utilizes the decrease of fluorescence intensity of a sample as a measure for the enzyme activity.

Keywords 2-Aminoacridone · Fluorogenic substrate · Fluorescent substrate · Caspase-3 · Apoptosis

Introduction

Fluorogenic peptide substrates have been widely used for various proteases assays. These substrates represent peptides with a fluorogenic moiety inserted at a position appropriate both for the corresponding protease specificity and reporter group sensitivity. The traditionally used peptide substrates are either non-fluorescent or of low fluorescence intensity, whereas one of the enzyme reaction products is a highly fluorescing compound. Thus, the time course of the enzyme-catalyzed reaction can be followed by tracing the increase of fluorescence due to the increasing amounts of the leaving group (Packard et al. 1997).

Caspases (cystein-dependent aspartate-specific proteases) are key regulatory enzymes in a number of cell processes such as apoptosis and inflammation (Bleackley and Heibein 2001; Fuentes-Prior and Salvesen 2004). Peptide substrates containing appropriately situated fluorophores have been widely used for the determination of caspase activity in cell culture based assays (Garcia-Calvo et al. 1999; Gurtu et al. 1997; Thornberry et al. 1997). Coumarin and rhodamine dyes are the most frequently used reporter groups (Cai et al. 2001; Gray and Sullivan 1989; Gurtu et al. 1997; Liu et al. 1999; Smith et al. 1980; Wang et al. 2005).

Many reports describe highly fluorescent acridine and acridone derivatives as effective fluorescent markers for labeling (Fan et al. 1998; Smith et al. 2004; You et al. 1999).These compounds display specific photophysical properties i.e., high fluorescent quantum yield and high molar absorption coefficients. Several studies have been reported utilizing 2-aminoacridone (AMAC) for derivatization of simple sugars and a number of linear and branched polysaccharides (Okafo et al. 1996). Substrates for trypsin and chymotrypsin with 2-aminoacridone as fluorophore have been proposed (Baustert et al. 1988). According to this survey, the enzyme cleavage of AMAC from the fluorogenic substrate is accompanied with a red shift of nearly 100 nm in the emission spectra. Those substrates possess excellent kinetic properties allowing fast assays to be performed. Substrates for L-leucine amino-peptidase and cystine aminopeptidase with AMAC as fluorophore have also been reported (Moser 1989). The last two substrates are commercially available. It has been mentioned that cleavage of the reporting group of these

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substrates is complained with decrease of fluoroescence of the analyzed sample.

In this paper, we present a non-traditional approach for caspase assay referred to utilization of decrease of fluorescence of sample as a measure of enzyme activity. This is illustrated here by the peptide substrate of caspase-3 (Ac-DEVD-AMAC) possessing 2-aminoacridone as a reporter group attached to the specific for the enzyme tetrapeptide. Synthesis, properties and application of this substrate are described.

Materials and methods

Materials

Caspase-3 (apopain, EC 3.4.22.56), 2-aminoacridone, diisopropylethylamine (DIEA), *N*-methylpyrolidone (NMP), gradient grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Germany). The Fmoc amino acids, 2-ClTrt resin, *O*-Benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU) and *N*-hydroxybenzotriazole (HOBt) were obtained from NovaBiochem (Switzerland). All other reagents were of the highest purity available.

Methods

All measurements of fluorescence spectra and caspase-3 assays were performed in 20 mM HEPES, pH 7.4 with 2 mM EDTA, 0.1% CHAPS and 5 mM DTT. The experiments were carried out on Varioscan®(ThermoElectron, USA) multifunctional reader using 96-well plates (black Microfluor® 1, ThermoElectron, USA). ^{1}H NMR spectra were recorded on Bruker Avance II+ 600.

Synthesis of substrate for caspase-3

Synthesis of Fmoc-Asp-AMAC

To a solution of Fmoc-Asp (OBu^t)-OH (103 mg, 0.25 mmol) in 2 ml of NMP were added consecutively DIEA (85 ml, 0.5 mmol), TBTU (80 mg, 0.25 mmol) and 2-aminoacridone (52 mg, 0.25 mmol). After stirring for 2 h at room temperature the reaction mixture was dropped to 5% aqueous $NaHCO_3$. The resulting green-yellow precipitate was filtered, washed with water and dried over KOH under vacuum. The obtained 138 mg of Fmoc-Asp(OBu^t)-AMAC were added to 2 ml of 95% TFA and the cleavage of the protecting group was carried out for 1 h at room temperature. Then, the solution was poured dropwise into 30 ml cool dry diethyl ether. The precipitate was filtered, washed with diethyl ether and vaccum dried. Total yield is 120 mg (95%).^{1}H NMR (600 MHz, DMSO-d_6 δ ppm 12.51–12.32 (m, 1H), 11.75 (s, 1H), 10.29 (s, 1H), 8.54 (d, J = 1.98 Hz, 1H), 8.23 (d, J = 7.92 Hz, 1H), 7.96 (dd, J = 8.96, 2.17 Hz, 1H), 7.90 (d, J = 7.50 Hz, 2H), 7.85 (d, J = 7.84 Hz, 1H), 7.75 (d, J = 7.44 Hz, 2H), 7.72 (t, J = 7.64 Hz, 1H), 7.54 (d, J = 2.25 Hz, 1H), 7.52 (d, J = 3.11 Hz, 1H), 7.42 (dt, J = 7.33, 4.32 Hz, 2H), 7.34 (dd, J = 12.38, 7.18 Hz, 2H), 7.24 (t, J = 7.50 Hz, 1H), 4.56 (dd, J = 13.81, 8.11 Hz, 1H), 4.30 (dd, J = 12.64, 6.68 Hz, 2H), 4.25 (t, J = 6.88 Hz, 1H), 2.79 (dd, J = 16.54, 5.39 Hz, 1H), 2.64 (dd, J = 16.54, 8.74 Hz, 1H). MS: M^+ (m/z) 548.36.

Peptide synthesis

The loading of Fmoc-Asp-AMAC to 2-ClTrt resin was carried out as follow: a solution of Fmoc-Asp-AMAC (110 mg, 0.2 mmol) in 10 ml of DCM/DMF mixture (9/1, v/v) was added to a pre-swelled in DCM resin (0.5 g, loading 1.3 mmol/g) and then DIEA (68 ml, 0.4 mmol) was added. The reaction was carried out on rotor shaker for 4 h at room temperature. Then, the resin was filtered, washed consecutively with DCM and DMF and incubated for 30 min with 5 ml mixture of MeOH/DCM/DIEA (17/2/1, v/v/v). Finally, the resin was washed with DMF and MeOH and vaccum dried over KOH. The loading was estimated by ninhydrine test (Kaiser et al. 1970). Synthesis of the substrate was carried out by a manual approach. Fmoc deprotections were performed with 20% pyridine in DMF containing 0.1 M HOBt for 10 min. Coupling reactions were performed using three equivalents of the respective Fmoc-amino acids by TBTU/HOBt/DIEA (1/1/2 with respect to amino acid) and single 45 min coupling time. After final Fmoc deprotection, the resin was treated with acetic anhydride/pyridine (3 equivalents each) in DMF for 20 min. Then, the resin was washed consecutively with DMF, DCM and MeOH and vaccum dried. Peptide cleavage from the resin and deprotection of the aminoacids side chains were carried out with TFA/H_2O/TIS solution (90:5:5, v/v/v) for 2 h at room temperature. The resin was washed with TFA and the filtrate dropped to cool diethyl ether. The crude substrate was collected by centrifugation, dissolved in 10 mM ammonium acetate and lyophilized.

Analytical (Nucleodur 100–5 C_{18} (125 × 4) column [MashereyNagel, Germany) at 1.5 ml/min] and semi-preparative purification [Partisil 10 ODS-2 (10 × 250), Whatman, Germany], RP-HPLC of the substrate were performed with binary gradient pump (model P2000, ThermoElectron, USA) equipped with a diode array detector (SPD M10Avp, Shimadzu, Germany). The solvent systems used were: A (10 mM NH_4OAc in H_2O containing 5% acetonitrile) and B (10 mM NH_4OAcin acetonitrile containing 5%H_2O). Gradient profile was from 10% B to 50% B in 25 min. ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm

11.75 (s, 1H), 10.09–9.54 (m, 1H), 8.58 (s, 1H), 8.44 (d, J = 6.96 Hz, 1H), 8.23 (d, J = 8.02 Hz, 2H), 8.18 (d, J = 7.11 Hz, 1H), 7.97 (dd, J = 8.95, 2.01 Hz, 1H), 7.91–7.89 (m, 1H), 7.71 (t, J = 7.63 Hz, 1H), 7.52 (t, J = 8.84 Hz, 2H), 7.24 (t, J = 7.52 Hz, 1H), 4.66 (dd, J = 7.12, 14.19 Hz, 1H), 4.57 (dd, J = 7.10, 14.19 Hz, 1H), 4.33–4.29 (m, 1H), 4.05 (s, 1H), 2.71–2.64 (m, 3H), 2.47–2.38 (m, 1H), 2.28–2.24 (m, 2H), 2.03–1.94 (m, 2H), 1.84 (s, 3H), 1.82–1.76 (m, 1H), 0.87 (d, J = 7.22 Hz, 3H), 0.86 (d, J = 7.22 Hz, 3H); MS: M^+ (m/z) 710.93.

Result and discussion

Synthesis of the substrate

The synthetic approach of caspase-3 substrate is presented in Fig. 1. The first step was the synthesis of corresponding *N*-acridonyl aspartamide. It was found that the reaction between Fmoc-Asp(OBu^t)-OH and AMAC was almost quantitative when TBTU was used as coupling agent. The obtained *N*-acridonyl aspartamide was tested for compatibility to Fmoc-based SPPS. Results of these studies showed that AMAC was stable for at least 10 h in 20% piperidine and more than 4 h in TFA (data not shown). After a successful loading of Fmoc-Asp-AMAC on 2-ClTrt resin, the substrate Ac-DEVD-AMAC was prepared by standard Fmoc-based SPPS protocols. After final TFA cleavage, the substrate was purified by semi-preparative RP HPLC using ammonium acetate buffers and its structure was characterized by MS and ^{1}H NMR.

This new caspase-3 substrate is well soluble in water and caspase assay buffer (clear solution was achieved even at 10 mM concentration) resulting in highly fluorescent solution.

Fluorescence properties of the substrate

We investigated the fluorescence properties of Ac-DEVD-AMAC and AMAC. Spectral scan of excitation and emission wavelengths were in accordance with previously reported data about wavelength maxima. Interesting data were obtained for the fluorescent intensity. Ac-DEVD-AMAC showed several times higher fluorescent intensity than AMAC regardless of the excitation wavelength. Moreover, AMAC is not fluorescent at 440–460 nm, where the emission maximum of Ac-DEVD-AMAC. However, in the emission maximum (530–570 nm) of AMAC the fluorescence of Ac-DEVD-AMAC was significant (Fig. 2). In addition, we found that fluorescence intensity of Ac-DEVD-AMAC was higher if excitation was performed at 290 nm instead of suggested 390 nm. The obtained data showed that peptide substrate is more suitable to be used as fluorophore than the corresponding reporting group 2-aminoacridone.

The plot of Ac-DEVD-AMAC and AMAC concentration versus fluorescence intensity were investigated. The calibration curves (in range 0.6–10 μM) for both fluorophores Ac-DEVD-AMAC (λ_{ex} 290 nm, λ_{em} 455 nm and λ_{ex} 390 nm, λ_{em} 455 nm) and AMAC (λ_{ex} 450 nm, λ_{em} 555 nm) were performed. The obtained data clearly showed that equal changes in the concentrations of the substrate and the reporting group result in quite different variations in the fluorescence intensity. It was found that a variation of 1 μM corresponded to a change of 123 ± 3 RFU for Ac-DEVD-AMAC and 0.19 ± 0.01 RFU for AMAC, respectively (Fig. 3). In other words, assays based on measuring the changes in fluorescence intensity of the substrate would be more sensitive as compared to methods based on the use of the reporting group.

These facts and compatibility of AMAC with Fmoc-based SPPS suggest that AMAC should be very practical marker for fluorescent labeling of peptides.

The observed fluorescent properties of 2-acylamino-acridone derivatives might provide a new mode for application of AMAC labeled peptide substrates in proteases assays. The protease-catalyzed hydrolysis of these substrates would be accompanied by a decrease in the fluorescence intensity and this could be used for measuring the enzyme activity.

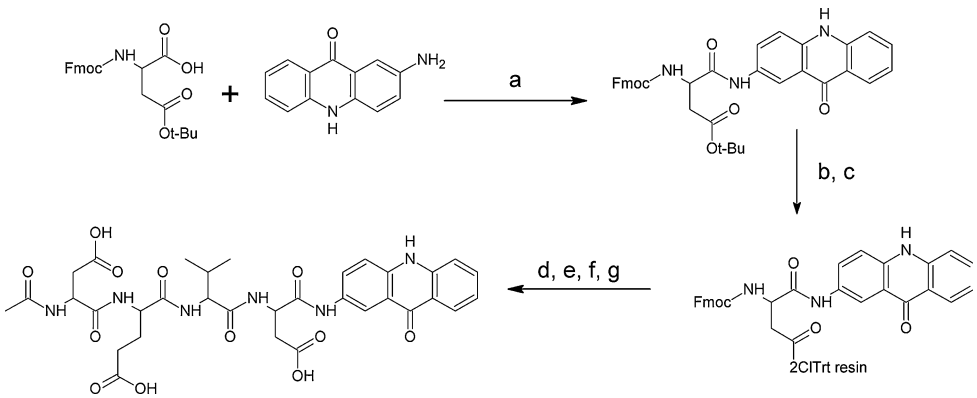

Fig. 1 Synthesis of substrate Ac-Asp-Glu-Val-Asp-AMAC. Reagents (*a*) TBTU/DIEA in NMP, 2 h; (*b*) 95% TFA, 1 h; (*c*) 2-ClTrt resin/DIEA in DCM/DMF; (*d*) 20% Pip/DMF, 10 min; (*e*) Fmoc-AA-OH/TBTU/DIEA in DMF, 45 min; (*f*)Ac_2O/Pyr in DMF, 20 min; (*g*) TFA/H_2O/TIS (90/5/5), 2 h

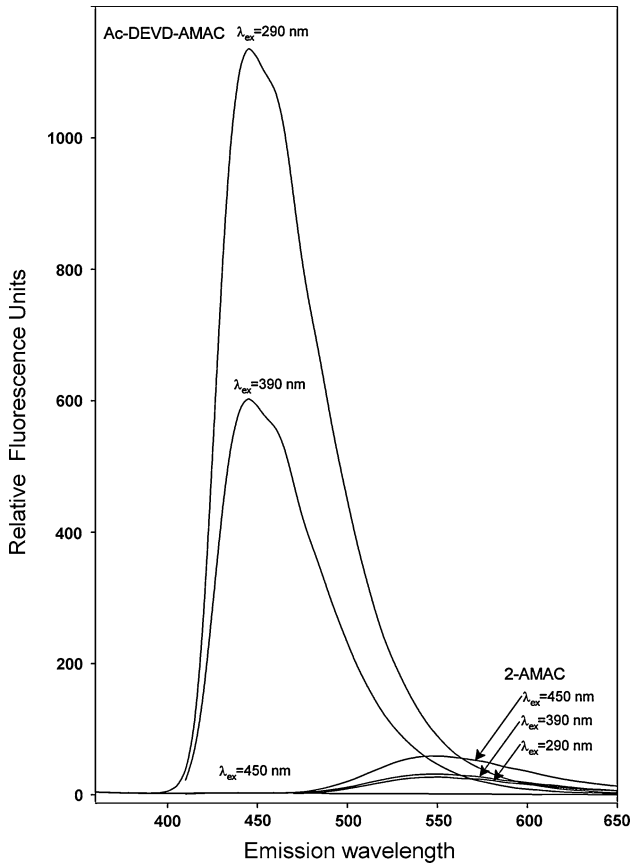

Fig. 2 The emission spectra of Ac-DEVD-AMAC and AMAC. The spectra were recorded in caspase assay buffer using equimolar concentration

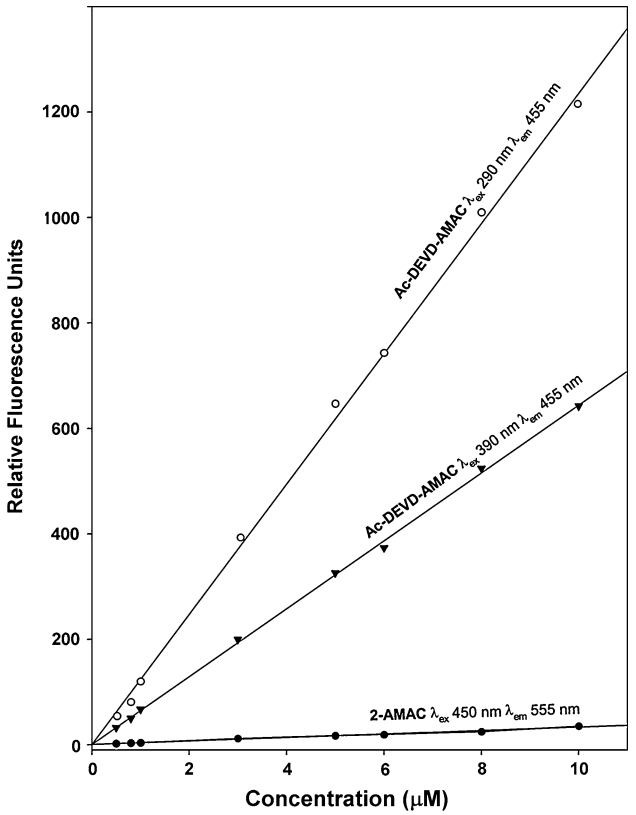

Fig. 3 Calibration curves for Ac-DEVD-AMAC and AMAC

Caspase-3 assay

The newly synthesized substrate for caspase-3 with 2-aminoacridone as a reporter group attached to the specific for this enzyme sequence Asp-Glu-Val-Asp was used as a model system for verification of the proposed assay approach. Time-course of Ac-DEVD-AMAC hydrolysis by human recombinant caspase-3 is presented on Fig. 4. The reaction was monitored simultaneously using two sets (excitation/emission wavelengths) of instrument parameters that are specific for the substrate (Ac-DEVD-AMAC) and for the reporting group (AMAC). The obtained data unambiguously showed that measuring the decrease of fluorescence of a sample using AMAC-labeled substrate represented a much more sensitive approach than the registration of the fluorophore release.

The sensitivity of the assay in respect of detection limit of caspase-3 was investigated. For this purpose, a defined concentration of the substrate was incubated with different concentrations of recombinant caspase-3.The results of these experiments are presented on Fig. 5. According to the obtained data, at least 1 ng caspase-3 could be detected in a sample (Fig. 6).

Next, the kinetic constants of the new substrate were studied. Active site titration of the enzymes using Z-VAD-fluoromethylketone was performed according to the procedures, described by Stennicke and Salvesen (2000).Kinetic constant determination was performed in the caspase assay buffer using peptide concentrations ranging from 0.6 to 10 μM.The initial velocity was determined from the linear portion of the progression curves and plotted versus the initial substrate concentrations. The K_m and V_{max} values were calculated by fitting the data to the Henri-Michaelis-Menten equation by non-linear regression using software SigmaPlot (SPSS Inc., Richmond, CA). K_{cat} values were calculated using the V_{max} value, and were based on the concentration of the enzyme. All kinetic assays were performed in triplicate; the rates for each substrate concentration were also measured in triplicate. Results compared with the available data are shown in Table 1. The obtained value for K_m clearly indicates that AMAC as reporting group is very well fitted to active site of caspase-3. This lower K_m value results in significant increase of the ratio k_{cat}/K_m for the proposed substrate compared to other substrates.

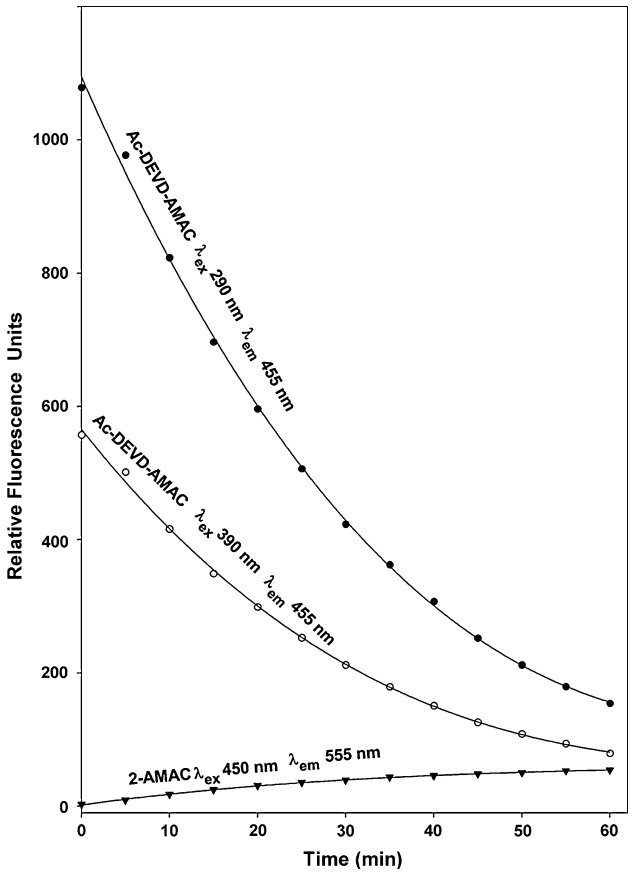

Fig. 4 The time-course of Ac-DEVD-AMAC(6 μM) hydrolysis by human recombinant caspase-3. The decrease of fluorescence of the sample due to hydrolysis of Ac-DEVD-AMAC and increase of fluorescence resulting from liberation of AMAC were measured simultaneously

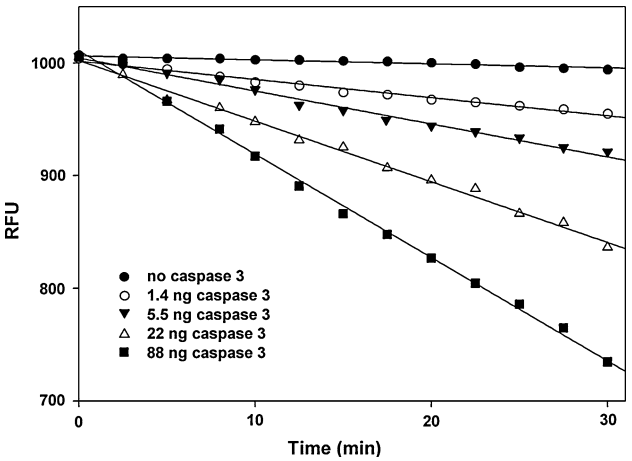

Fig. 5 The time-course of Ac-DEVD-AMAC (6 μM) hydrolysis by various concentrations of human recombinant caspase-3. The reaction was monitored for 30 min at 3 min interval

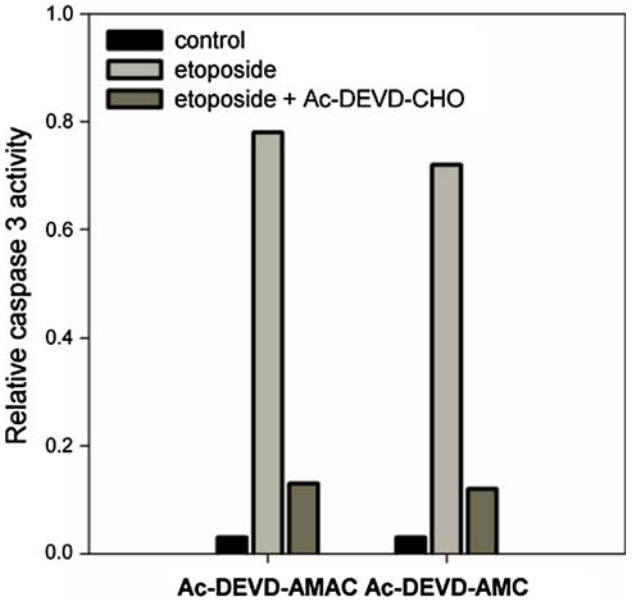

Fig. 6 Caspase-3 assay of etoposide induced apoptosis in A431 cells

Finally, the new substrate was used to assay caspase-3 activity in cell culture lysates. It was assayed after etoposide induced apoptosis (Mizukamia et al. 1999) in A431 cell line. The applicability of the new substrate for this kind of assays was demonstrated by performing parallel experiments under identical conditions with the commercial assay kit (Sigma-Aldrich, Germany). Untreated cells and caspase-3 inhibitor Ac-DEVD-CHO were used as controls. In the case of AMAC substrate, some changes in the manufacturer's protocol were introduced: (1) the substrate was dissolved in the corresponding assay buffer; (2) calibration curve for substrate dilutions was drawn; (3) the decrease of the fluorescence intensity (λ_{ex} 290 nm, λ_{em} 455 nm) was monitored. The results obtained with the two substrates were comparable. Furthermore, using Ac-DEVD-AMAC as substrate the initial velocity of the enzyme reaction was found to be higher than the rate with Ac-DEVD-AMC.

In conclusion, here we demonstrate a new approach for the assay of a protease activity using 2-aminoacridone labeled peptides. The proposed method utilizes the decrease of fluorescence intensity of the sample as a measure of enzyme activity. This approach was successfully applied for the assessment of caspase-3 activity in cell lysates.

The described approach for protease assay has some advantages over the conventional one. There is no need of background correction, because the hydrolysis products in these assay conditions are not fluorescent and the sample background is already taken into account. The enzyme activity could be monitored immediately after the addition of the analyzed sample without the need of fluorophore accumulation in order to record the signal.

Table 1 Kinetic constants of Ac-DEVD-AMAC in comparison with known data

Substrate	K_M μM	k_{cat} s^{-1}	10^{-6} k_{cat}/K_M s^{-1} M^{-1}	References
Ac-DEVD-AMAC	4.68	9.95	2.13	Determined in this study.
Ac-DEVD-AMC[a]	10	14	1.4	Garcia-Calvo et al. 1999
Ac-DEVD-AMCA[b]	13.6	5.86	0.43	Lien et al. 2004
Ac-DEVD-AFC[c]	16.8	nd	nd	Sun et al. 1997
Z-DEVD-aminoluciferin	7.66	nd	nd	O'Brien et al. 2005

[a] AMC-7-amino-4-methylcoumarin

[b] AMCA-7-amino-4-methyl-3-coumarinylacetamide

[c] AFC-7-amino-4-trifluoromethylcoumarin

nd not described

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