Highly sensitive and selective fluorescence assays for rapid screening of endothelin-converting enzyme inhibitors

Nathalie LUCIANI, Hugues DE ROCQUIGNY, Serge Turcaud, Anthony ROMIEU and Bernard P. ROQUES[1]

Département de Pharmacochimie Moléculaire et Structurale, U266 INSERM, UMR 8600 CNRS, 4 Avenue de l'Observatoire, 75270 Paris Cedex 06, France

The highly potent vasoconstrictor peptide endothelin (ET) is generated from an inactive precursor, big endothelin (bET), by endothelin-converting enzyme (ECE). ECE is a phosphoramidon-sensitive zinc metallopeptidase, which is closely related to neprilysin (neutral endopeptidase). It is possible that compounds which inhibit the formation of ET may be used as new drugs for the treatment of cardiovascular diseases. Such an approach requires a fast, simple and selective assay to measure ECE activity, allowing rapid screening of inhibitors. We describe here two new ECE substrates based on the concept of 'intramolecularly quenched fluorescence' which may fulfill this aim. One, S_1 [Pya^{21}-Nop^{22}-bET-1(19–35)], is the (19–35) fragment of the natural peptide big-ET-1(1–38), which is modified by introducing the fluorescent amino acid, pyrenylalanine (Pya), in position 21 and a quencher, *p*-nitrophenylalanine (Nop), in position 22. The second substrate (S_2) is a small peptide, Ac-Ser-Gly-Pya-Lys-Ala-Phe-Ala-Nop-Gly-Lys-NH_2, from a biased substrate peptide library. The recombinant, hECE-1c, cleaved both Pya^{21}-Nop^{22}-bET-1(19–35) and the natural substrate selectively between residues 21 and 22, whereas cleavage occurred between alanine and phenylalanine in the small peptide. In both cases, this generated intense fluorescence emission. The synthesis and kinetic parameters of these substrates are described. These assays, which can be used directly on tissue homogenates, are the most sensitive and selective described to date for ECE, and are easily automated for a high-throughput screening of inhibitors.

Key words: cardiovascular disease, high-throughput screening, quenched fluorescence, peptide synthesis, zinc metallopeptidase.

INTRODUCTION

Endothelin (ET)-1 is a potent vasoconstrictor consisting of 21 amino acids and two disulphide bridges [1]. This cyclic peptide belongs to a family of three peptides. ET-2 and ET-3 differ from ET-1 by two and six amino acids respectively, and are encoded by different genes [2]. Endogenous production of endothelins in humans contributes to the maintenance of vascular tone [3]; however, overexpression is implicated in the development and progression of several cardiovascular diseases [4], including strokes [5], chronic heart failure [6] and pulmonary hypertension [7].

ET-1 is generated by the proteolytic cleavage of an inactive precursor, big endothelin-1 [bET-1(1–38)], between tryptophan-21 and valine-22, achieved by endothelin-converting enzyme (ECE)-1. ECE-1 is a homodimer, its subunits have a mass of approx. 120 kDa and are linked by a disulphide bridge [8]. ECE-1 is a zinc metalloprotease which contains the zinc-binding motif, HExxH, and belongs to the M13 subfamily of membrane-bound endopeptidases, as does the closely related neutral endopeptidase, neprilysin (EC 3.4.24.11) [9].

The cloning of ECE-1 cDNAs has been performed from various species, such as endothelial cells from rats [10], cattle [11] and humans [12,13]. Four isoforms, ECE-1a, ECE-1b [14,15], ECE-1c [16] and ECE-1d [17], have been successively characterized. They differ only in their N-terminal regions and are derived from the same gene through the use of alternative promoters [15]. The four isoforms have similar kinetic rate constants for the cleavage of the substrate bET-1, and the mRNA of ECE-1c corresponds to the predominant isoform [15].

Because of the important role of ET in hypertension and cardiovascular diseases, an interesting therapeutic approach would be to develop potent and selective inhibitors of ECE to prevent the inactive precursor, bET-1, from maturing into ET-1. The absence of a simple, fast and efficient assay has been a major difficulty in this field. ECE-1 has long been considered to be highly specific to its natural substrate, bET-1. Indeed, bET-1 was used in enzymic assays, such as HPLC, to identify the cleavage products of ECE-1 and RIAs to detect the metabolite, ET-1 (1–21) [18]. However, a relatively short fragment of bET-1, [Phe^{22}]big-ET-1 (18–34), is cleaved by ECE with a greater efficiency that the natural substrate [19]. Similarly, various small peptides, such as angiotensin I, bradykinin, neurotensin, substance P and oxidized insulin B chain, are hydrolysed by ECE-1. This may allow more rapid and simple enzymic assays to be developed [20,21]. In the present study, we describe two assays based on the concept of intramolecularly quenched fluorescence. The pyrenylalanine residue (Pya) [22] was used as the highly fluorescent moiety and the *p*-nitrophenylalanine (Nop) moiety as the quencher moiety. The first substrate, S_1 (Figure 1), was derived from a fragment of b-ET, Pya^{21}-Nop^{22}-bET-1(19–35), and the second substrate, S_2 (Figure 1), was a 10-amino-acid peptide, Ac-S-G-Pya-K-A-F-A-Nop-G-K-NH_2, derived from a biased substrate library. Both assays were highly sensitive and selective, which allowed a very rapid and simple quantification of ECE activity in various preparations and a rapid method for screening the potential ECE inhibitors.

MATERIALS AND METHODS

Peptide synthesis

A PerkinElmer 433 automated peptide synthesizer, coupled to a programmable absorbance detector, ABI 785A, were used to

Abbreviations used: ET, endothelin; bET, big endothelin; (h) ECE, (human) endothelin-converting enzyme; ACE, angiotensin-converting enzyme; Pya, pyrenylalanine; Nop, *p*-nitrophenylalanine; S_1 ECE substrate, Pya^{21}-Nop^{22}-bET-1(19–35); S_2 ECE substrate, Ac-Ser-Gly-Pya-Lys-Ala-Phe-Ala-Nop-Gly-Lys-NH_2; M_1, Ac-Ile-Ile-Pya-OH; M_2, Ac-Ser-Gly-Pya-Lys-Ala-OH.

[1] To whom correspondence should be addressed (e-mail roques@pharmacie.univ-paris5.fr).

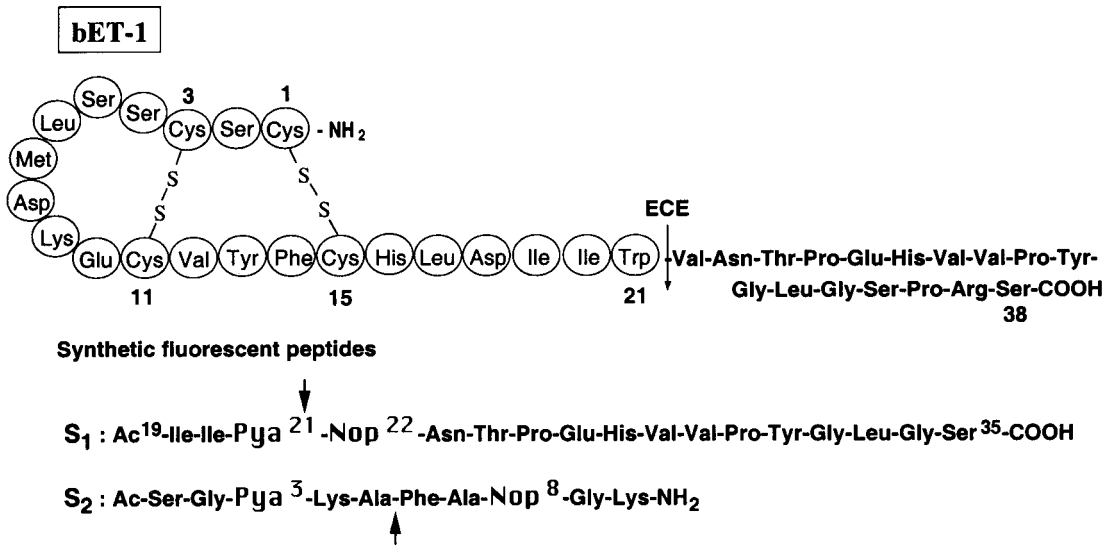


Figure 1 Primary structure of bET-1 (1–38) and substrates S_1 and S_2

ECE cleavage sites are indicated by arrows. Substrates S_1 and S_2 were both obtained by solid-phase peptide synthesis. The fluorescent chromophore, Pya, and the quencher, Nop, are in positions 21 and 22 or 3 and 8 for S_1 and S_2 respectively.

synthesize 0.1 mmol of the two ECE substrates, S_1 and S_2. Resins were 4-hydroxymethyl phenoxymethyl (1.07 mmol/g; Perkin-Elmer) for S_1 and rink amide 4-methylbenzydryl amine (0.59 mmol/g; Novabiochem) for S_2 [23]. Fluoren-9-ylmethoxy-carbonyl-protected amino acids, with the following side chain protection groups, were used: t-butyl ether, serine, threonine and tyrosine; t-butyl ester, glutamic acid; trityl, histidine and asparagine; and t-butoxycarbonyl, tryptophan. Fluoren-9-yl-methoxycarbonyl-(L)-Nop was obtained from Novabiochem (Laüfelfingen, Switzerland) and fluoren-9-ylmethoxycarbonyl-(L)-Pya was prepared as described in Soleilhac et al. [22]. Successive couplings were carried out with dicyclohexylcarbo-di-imide/hydroxybenzotriazole in *N*-methylpyrrolidone as coupling reagents. At the end of the synthesis, the peptide resin was treated for 3 h with 40 ml of trifluoroacetic acid (SDS Peypin, Peypin, France) containing water (2 ml) and triisopropylsilane (1 ml). Trifluoroacetic acid was removed under vacuum and the crude peptide was precipitated with cooled diethyl ether and centrifuged. The supernatant was then removed and the pellet vacuum dried. Purification of the crude peptide was carried out by reverse-phase HPLC (Applied Biosystems 783A) on a Vydac C_{18} column (10 mm × 250 mm, 5 μm, 300 Å) with a linear gradient of 20–70 % buffer B (0.1 % trifluoroacetic acid in water) for S_1 and 10–90 % buffer B (70 % acetonitrile and 0.09 % trifluoroacetic acid in water) for S_2 over 90 min at a flow rate of 2 ml/min. We obtained 39 mg of pure S_1 and 50 mg of pure S_2.

Experimental masses of 2101.61 Da and 1269.38 Da were obtained for S_1 and S_2, compared with theoretical masses of 2100.7 Da and 1268.96 Da respectively.

The fluorescent metabolites Ac-Ile-Ile-Pya-OH (M_1) and Nop-Asn-Thr-Pro-Glu-His-Val-Val-Pro-Tyr-Gly-Leu-Gly-Ser (M_1') for S_1, and Ac-Ser-Gly-Pya-Lys-Ala-OH (M_2) and Phe-Ala-Nop-Gly-Lys-NH_2 (M_2') for S_2 were synthesized as described for S_1 and S_2. Due to its high hydrophobicity, M_1 was purified by single ether precipitation. After freeze-drying, M_1 was analysed by HPLC. This revealed one peak at $\lambda = 210$ and 343 nm. M_2 was purified by HPLC as described above for S_2. M_2 and S_2 were analysed by ^{1}H NMR spectroscopy at 270 MHz and electrospray MS (M_1, $MM_{calc} = 557.49$; $MM_H^+ = 558.38$ and M_2, $MM_{calc} = 674.52$; $MM_H^+ = 675.63$). Stock solutions of substrates and metabolites (1×10^{-3} M) were prepared in 50 % DMSO and 50 % ethanol and stored at 4 °C. Subsequent dilutions were made with 50 mM Tris/maleate (pH 6.8). M_1' and M_2' were purified by reverse-phase HPLC, as described above for S_1 and S_2, and analysed by NMR and MS.

Spectrometric measurements

The fluorometric properties of S_1, S_2, M_1 and M_2 were determined using a PerkinElmer LS50B luminescence spectrometer equipped with a thermostat-regulated cell holder. For rapid screening of inhibitors and routine enzyme assays a multiwell plate reader fluorimeter (Cytofluor® Series 4000; PerSeptive Biosystems) was used.

Crude preparation of ECE from rat lung microsomes

All procedures were performed at 4 °C [24]. Briefly, rat lungs were washed twice in PBS and homogenized in 20 ml of the homogenization buffer (50 mM Tris/maleate, pH 6.8, and 0.1 mM PMSF) and then centrifuged at 800 ***g*** for 10 min. The supernatant was further centrifuged at 100000 ***g*** for 30 min and the microsomes obtained were washed twice by resuspension in 15 ml of homogenization buffer followed by centrifugation. The homogenate was suspended in 1 ml of 50 mM Tris/maleate, pH 6.8, containing 1 % (w/v) *N*-octyl glucoside (ICN Biomedicals), stirred, and kept on ice for 1 h before centrifugation at 20000 ***g*** for 60 min. The supernatant was stored at 4 °C and used directly for enzyme assays. The protein concentration was determined by the method of Bradford, using BSA as a standard.

ECE preparation from rat brain cortex membranes

All procedures were performed at 4 °C. The brain cortex was washed twice in PBS, and membranes were prepared by homogenizing the cortex in 15 ml of homogenization buffer in a Teflon/glass homogenizer, followed by centrifugation at

100000 ***g*** for 30 min. This step was repeated, and the final membrane pellet was dissolved in 500 μl of 50 mM Tris/maleate, pH 6.8, containing 1 % (w/v) *N*-octyl glucoside for 1 h on ice, before centrifugation at 20000 ***g*** for 60 min. The supernatant was stored at 4 °C and used directly for enzyme assays. The protein concentration was determined by the method of Bradford using, BSA as a standard.

Expression, solubility and characterization of recombinant human ECE-1c

Recombinant human ECE-1c (hECE-1c) was expressed in Cos-7 cells. The plasmid used contained the full-lengh cDNA encoding human ECE-1c inserted into a pcDNA3 expression vector. Cos-7 cells were grown in Dulbecco's modified Eagle's medium complemented with 10 % fetal calf serum, and 1×10^6 cells were transfected with 50 μg of plasmid by electroporation (240 V and 1000 μF; Bio-Rad electroporator). Each pool of transfected cells was incubated in a Petri dish (10 cm diameter) at 37 °C for 48 h. The cells were then washed twice and harvested by scraping in PBS. After rapid centrifugation at 2000 ***g***, the pellet was re-suspended in ice-cold 50 mM Tris/maleate, pH 6.8, and membranes were prepared by homogenizing the cells in a Teflon/glass homogenizer, followed by centrifugation at 100000 ***g*** for 30 min at 4 °C. This step was repeated, and the resultant membrane pellet was dissolved in 100 μl/Petri dish of 50 mM Tris/maleate, pH 6.8, containing 1 % (w/v) *N*-octyl glucoside for 1 h on ice, before centrifuging at 20000 ***g*** for 15 min. The supernatant was stored at 4 °C and used for Western-blot analysis and enzyme assays. In these conditions, enzyme activity was found to be stable for at least 1 month, and after freezing at −80 °C it was stable for several months. Neprilysin and angiotensin-converting enzyme (ACE, EC 3.4.15.1) were purified by standard methods [25,26].

Characterization of ECE by Western blotting and control of enzymic activity by RIA

Proteins from the different preparations (lung, brain and recombinant hECE-1c) were separated by 7.5 % SDS/PAGE and electroblotted on to nitrocellulose filters for 1 h at 4 °C and 100 V (Amersham International). The blots were equilibrated in PBS/1 % Tween containing 0.5 % (w/v) BSA and then blocked for 1 h in 5 % (w/v) BSA PBS Tween. Antiserum raised against ECE (a gift from Servier Laboratories, Croissy, France) was added for 1 h at room temperature. After three successive washes with PBS Tween horseradish peroxidase-linked anti-rabbit immunoglobulin from sheep (Amersham International) was used to detect immune complexes. Peroxidase activity was visualized with a chemiluminescence detection kit (Amersham International). Bovine ECE-1 purified at homogeneity (a gift from Servier Laboratories) was used as a control.

HECE-1c was incubated with bET-1 (Bachem Biochemi SARL, Voisins-le-Bretonneux, France) and the ET 1-21-specific ^{125}I assay kit (Amersham International) was used to measure the amount of ET formed [24].

ECE fluorometric enzyme assays with S_1 or S_2 substrates

Calibration curves

Calibration curves connecting the fluorescence increase and the substrate degradation, were established by adding increasing quantities of the fluorescent metabolite (M_1 or M_2) and decreasing quantities of substrate (S_1 or S_2) to 100 μl in 50 mM Tris/maleate buffer (pH 6.8). This volume was commonly used in robotized assays.

The fluorescence was measured at $\lambda_{ex} = 340$ nm and $\lambda_{em} = 400$ nm on a multiwell plate reader fluorimeter coupled to Cyto2® software.

Assays for ECE activity

ECE activity was studied in black 96-well microplates in a final volume of 100 μl. S_1 (20 μM final concentration) and 4 μg of recombinant hECE-1c were incubated in 50 mM Tris/maleate (pH 6.8) for 1 h at 37 °C. The reaction was stopped by cooling to 4 °C for 10 min, and the fluorescence was then measured ($\lambda_{ex} =$ 340 nm; $\lambda_{em} = 400$ nm). S_2 (10 μM final concentration) was incubated with 1 μg of recombinant hECE-1c for 30 min at 37 °C. For enzyme assays with crude membrane preparations, 32 μg of protein from rat lungs or 80 μg of protein from rat brain cortex were incubated with 20 μM S_1 at 37 °C for 1 h. Blanks consisted of all reagents except the enzyme and were treated under the same conditions as above. The enzymic reactions could also be interrupted by addition of 10 μl of acetonitrile and the fluorescence measured immediately or after 30 min. In these conditions, the fluorescence of the formed metabolites M_1 and M_2 is slightly decreased but did not modify the easy quantification of the hECE-1 activity (results not shown).

HPLC analysis of cleavage products

The reaction products formed by the hydrolysis of S_1 and S_2 were characterized by HPLC (Shimadzu L10A). A total of 50 μl of the reaction mixtures were injected on to a Kromasil C_{18} column (5 μm, 100 Å, 4.6 mm × 250 mm; Touzart et Matignon, Les Ulis, France). Elution was carried out for 30 min in a 0–100 % gradient of buffer B [buffer A, 0.05 % trifluoroacetic acid in water; buffer B, 0.038 % trifluoroacetic acid in CH_3CN/H_2O, 90:10 (v/v)] with a flow rate of 1 ml/min. The UV unit of the HPLC apparatus (Shimadzu SPD 10A) was used to detect the formed products at $\lambda = 210$ nm. The synthetic substrates and metabolites were used as references.

Determination of the kinetic constants of S_1 and S_2 for recombinant hECE-1

Recombinant hECE-1c (4 μg or 1 μg) was added to 100 μl of 5–100 μM substrate (S_1 or S_2) in 50 mM Tris/maleate (pH 6.8). The mixture was incubated at 37 °C for 1 h (or 30 min). The reaction was stopped on ice and the fluorescence was measured as above. K_m values were calculated using the ENZFITTER program (Biosoft, Paris, France).

Determination of the inhibitory potencies of various compounds towards hECE-1

To determine the K_i values, 4 μg (or 1 μg) of hECE-1c/well, was pre-incubated for 10 min at 37 °C in 50 mM Tris/maleate, pH 6.8, with increasing concentrations of the inhibitor (from 1×10^{-10} M to 1×10^{-4} M, final concentration) in black 96-well microplates. Fluorescent substrates S_1 (20 μM) or S_2 (10 μM) were added to a final volume of 100 μl and incubated at 37 °C for 1 h (or 30 min). The reaction was stopped by cooling on ice, and the fluorescence measured with a multiwell plate reader fluorimeter. Samples with 0 % hydrolysis were obtained by adding the substrate to the buffer, and samples with 100 % relative activity were prepared by omitting the inhibitors. The percentage of degradation was evaluated compared with 100 % relative activity, and the IC_{50} values of tested inhibitors were determined

accordingly. The K_i values of the competitive inhibitors were determined by the Cheng–Prussof equation [27]:

$$K_i = IC_{50}/(1+[S]/K_m)$$

Due to the huge increase in fluorescence generated by the cleavage of S_1 or S_2 the substrate incubation period could be greatly reduced, thus shortening the assay.

RESULTS

Expression of human recombinant ECE-1c in Cos-7 cells

hECE-1c was transiently transfected in Cos-7 cells. pcDNA3 was transfected as a control. Western-blot analysis showed that hECE-1c and purified bovine ECE-1 have similar apparent molecular masses (results not shown). ECE-1 was not detected in cell extracts transfected with the control plasmid pcDNA3. The conversion of bET-1 by hECE-1c was verified using the RIA kit, with ^{125}I-ET-1 as competitor. The conversion was inhibited by phosphoramidon. No activity was detected in cell extracts transfected with the control plasmid.

Fluorescence properties of S_1 and M_1

The fluorescence spectra of S_1 and its metabolite, M_1, display two broad emission maxima at approx. 377 nm and 398 nm and an excitation maximum at 343 nm. This is consistent with the parameters reported for pyrenylalanine [22]. Owing to near-complete fluorescence quenching, S_1 alone gives a very small residual signal. Therefore the fluorescence intensity of S_1 and M_1 were compared at 50 μM S_1 and 2.5 μM (5% cleavage) M_1 (Figure 2a). In these conditions, M1 fluorescence emission was 550-fold higher than that of S_1.

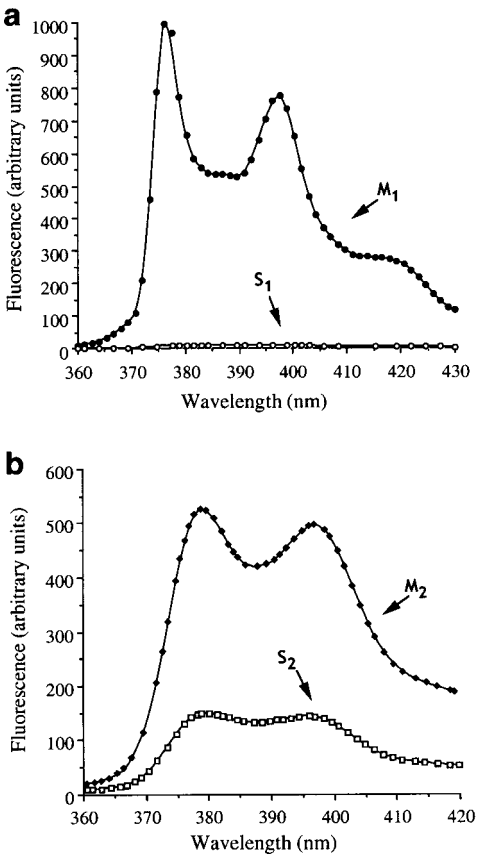


Figure 2 Fluorescence emission spectra (λ_{ex} = 343 nm) of S_1 and S_2

(**a**) S_1 (○) and M_1 (●) at 50 μM and 2.5 μM (5% cleavage) in 50 mM Tris/maleate (pH 6.8). (**b**) S_2 (□) and M_2 (■) at 1 μM and 0.05 μM (5% cleavage) in 50 mM Tris/maleate (pH 6.8).

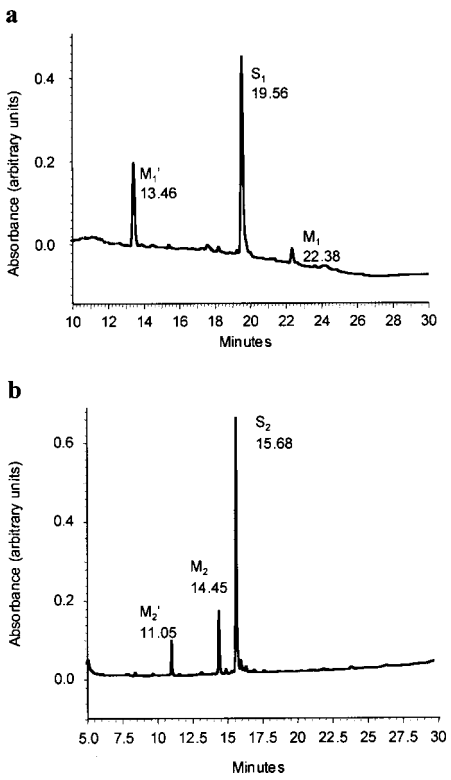


Figure 3 HPLC analysis of cleavage of synthetic substrates by hECE-1c

(**a**) Cleavage of S_1. S_1 (20 μM) was incubated with 4 μg of hECE-1c at 37 °C for 1 h. The reaction was stopped by cooling to 4 °C and 50 μl of the solution were injected and analysed by HPLC, as described in the Materials and methods section. Detection was monitored by UV at 210 nm. The position of the peaks corresponding to S_1 and the formed metabolites, M_1 and M_1', corresponded exactly to those of synthetic references. (**b**) Cleavage of S_2. S_2 (10 μM) was incubated with 1 μg of hECE-1c at 37 °C for 30 min. At the end of the reaction, 50 μl of the solution was analysed by HPLC, as described in the Materials and methods section. Eluted peptides were detected in UV at 210 nm. The position of the peaks corresponding to S_2 and the formed metabolites, M_2 and M_2', were identical to those of synthetic references. These experiments demonstrated the selective cleavage by hECE-1c of both S_1 and S_2.

Fluorescence properties of S_2 and M_2

The fluorescence spectra of S_2 and its fluorescent metabolite, M_2, display excitation maximum at 343 nm and two broad emission maxima at approx. 380 nm and 398 nm (Figure 2b). The basal fluorescence in this substrate is greater than that observed in the bET substrate, S_1, due to the presence of four residues between the fluorophore, Pya, and the quencher, Nop. To compare the fluorescence intensity of S_2 with M_2, the spectra of 1 μM and 0.05 μM solutions respectively (5% cleavage) were monitored (Figure 2b). In these conditions, the emission of the metabolite was 70-fold higher than the substrate.

Selectivity and HPLC analysis of S_1 or S_2 cleavage by hECE-1

S_1 and S_2 were incubated with hECE-1c to investigate the selectivity of the cleavage. The mixtures were analysed by HPLC (Figure 3). In each case, only two metabolites were observed (M_1

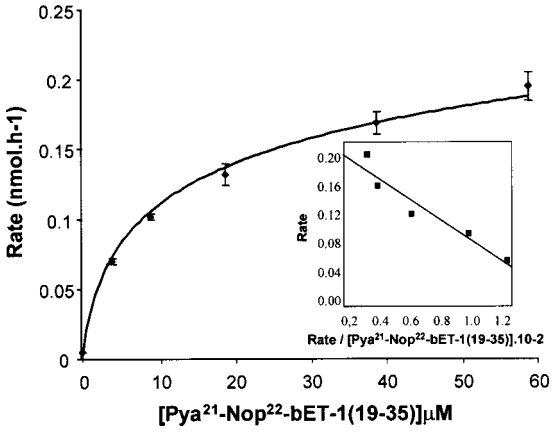


Figure 4 Rate of S_1 cleavage by hECE-1c

Different concentrations of S_1 were treated for 1 h at 37 °C in 100 μl of 50 mM Tris/maleate buffer (pH 6.8) containing hECE-1c. The K_m was estimated from Eadie–Hofstee representation (inset).

Table 1 Kinetic constants for hECE-1c with various substrates

Substrate	K_m (μM)	V_{max} (nmol/mg of protein/min)
bET-1	0.20 ± 0.03	0.27 ± 0.05
S_1	20.1 ± 0.9	2.7 ± 0.3
S_2	21.3 ± 0.9	20.5 ± 0.3

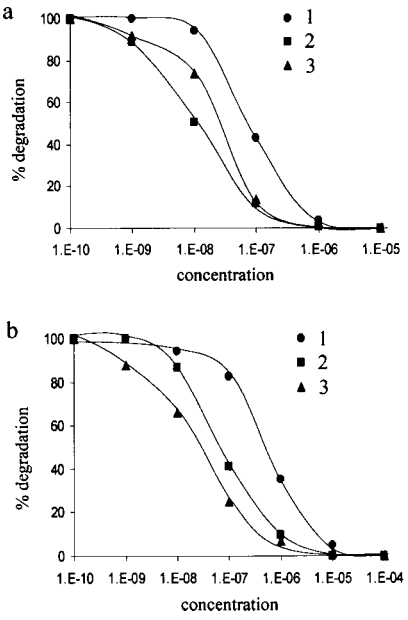


Figure 5 IC_{50} determination for compounds 1 (●), 2 (■) and 3 (▲)

(**a**) Fluorescent substrate S_1. (**b**) Fluorescent substrate Pya^3-Nop^8-(Lys-Ala-Phe-Ala).

and M_1' for S_1, and M_2 and M_2' for S_2), indicating that the two fluorescent substrates were cleaved by hECE-1 at a single peptide bond. These metabolites were identified by the use of synthetic standard references.

Moreover, the S_1 peptide was not cleaved after 2 h incubation at 37 °C with 300 ng of purified neprilysin or 85 ng of purified ACE. The incubation of S_2 for 30 min at 37 °C with ACE led to 0 % cleavage, whereas the incubation of S_2 with neprilysin in the same conditions resulted in less than 2 % cleavage (results not shown).

Kinetic parameters of hECE-1 substrates S_1 and S_2

A concentration of 20 μM for S_1 and 10 μM for S_2 was chosen for enzyme assays and inhibitors screening. With these concentrations, the steady state activity was measured after 4 h of incubation for S_1 and after 40 min for S_2. From standard calibration curves, kinetic constants for the enzymic cleavage of S_1 and S_2 by hECE-1c were determined in conditions that led to less than 10 % of cleavage, and with substrate concentrations of between 5 and 100 μM. The Michaelis–Menten constants, K_m and V_{max}, were calculated from the Eadie–Hofstee representation (Figure 4 and Table 1). The values are the means of three experiments carried out in duplicate. The K_m values were similar for the two substrates, but the second substrate, S_2, was cleaved approx. 8-fold faster than S_1 (Table 1).

High throughput assay for inhibitor screening

Three compounds were tested as ECE inhibitors with substrates S_1 and S_2: a thiol inhibitor synthesized in our laboratory [28] (compound **1**); a thiol amino acid compound [29] (compound **2**); and a phosphonate-containing inhibitor, CGS-26303 [30] (compound **3**) (Figure 5 and Table 2). In each experiment, the enzyme was initially pre-incubated with the inhibitor for 10 min at 37 °C and subsequently incubated with S_1 or S_2 at 37 °C for 30 min. The K_i values determined for these inhibitors were, as expected, identical with both S_1 and S_2, and were in the same range as those cited in the literature (Table 2).

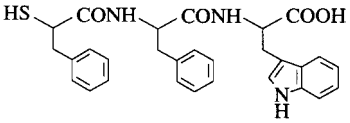
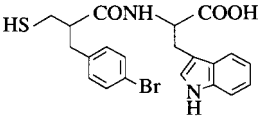
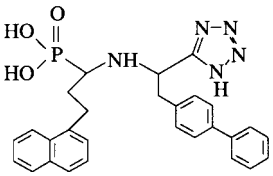
Routine experiments were carried out with a robot (Genesis RSP 100; Tecan, Mechelen, Belgium) in black 96-well microplates. Each component (buffer, inhibitor, enzyme and substrate) was diluted in a sufficiently large volume to be distributed by the robot. A final volume of 100 μl was usually used, but a final volume of 50 μl was tested and did not modify the fluorescent and kinetic parameters. Thus the putative ECE-inhibitory potencies of a large series of compounds could be rapidly estimated by use of two concentrations, 1×10^{-5} M and 1×10^{-6} M, of each product and measurement of fluorescence following ECE action.

Screening assay on crude enzymic preparations

The high sensitivity and selectivity of S_1 is illustrated by the K_i values determined for ECE inhibitors using crude membrane preparations from rat lungs or brain cortex as source of ECE. The substrate was incubated with these easily accessible enzyme preparations, as described in the Materials and methods section, for 2 h at 37 °C. The reactions were initiated by the addition of 20 μM (final concentration) of the fluorescent substrate, S_1. The rat lung preparation had an ECE activity of 0.17 nmol/mg of protein/min and the rat brain cortex homogenate had an ECE activity of 0.033 nmol/mg of protein/min. These results are consistent with reports demonstrating that the lung contains very high concentrations of ECE compared with other tissues [24]. In both cases, only one cleavage site was observed (results not shown).

The K_i values of compounds **1** and **2** with S_1 and the lung preparation were determined. K_i was 1.03×10^{-7} M for compound

Table 2 K_i **values of inhibitors for hEDE-1c**

Compound	Structure	K_i (M) S_1	S_2	Published value
1		$(1.7 \pm 0.4) \times 10^{-7}$	$(0.6 \pm 0.02) \times 10^{-7}$	
2		$(2.2 \pm 0.17) \times 10^{-8}$	$(0.8 \pm 0.02) \times 10^{-8}$	2.5×10^{-8} [29]*
3		$(1.1 \pm 0.02) \times 10^{-8}$	$(1.8 \pm 0.07) \times 10^{-8}$	$(1.7 \pm 0.39) \times 10^{-8}$ [30]*

*bET-I [18] was used as substrate.

1 and 6.8×10^{-8} M for compound **2**. These values were very close to those determined using recombinant hECE-1c (Table 2).

DISCUSSION

The design of substrates with intramolecular fluorescence quenching leads frequently to a very sensitive method and presents favourable disposition for automation. The fluorophore and quencher were originally chosen because the theory of non-radiative resonance energy transfer states that the emission spectrum of the fluorescent donor group at least partially overlaps with the absorption spectrum of the quenching acceptor moiety of the molecule. Nevertheless, intramolecular quenching can also be achieved in the absence of spectral overlap [31], giving a wider choice of donor–acceptor pairs. In this case, quenching is caused by collisions between the concerned residues. Thus the closer the fluorophore–quenching pair, the higher the quenching efficiency will be.

To be used routinely in an enzymic assay, an ECE substrate must fulfill several criteria: (i) easy preparation; (ii) good enzyme affinity and efficient hydrolysis to minimize the amount of enzyme used and the reaction time; (iii) selective cleavage by the enzyme at a single peptide bond between the fluorophore and the quencher; (iv) high quenching efficiency leading to a high sensitivity; and (v) a simple method to quantify the cleavage and to measure the K_i of inhibitors in an automated assay.

As previously shown, hydrophobic residues interacting with the S_1' subsite [32] of the metallopeptidase are essential for efficient interactions between a putative substrate and the ECE-1 active site [30]. Consequently, two strategies could be developed for designing efficient ECE-1 substrates. First, the 'fluorophore–quencher' pair could be introduced at the cleavage site of a substrate. Secondly, the fluorophore and quencher residues may be placed on both sides of a small peptide (three or four amino acids) designed to optimally recognize the ECE-1 active site.

These two strategies have been used in parallel, allowing their efficiency to be compared. In a continuation of our work on the fluorescence properties of the Pya–Nop pair in the development of new fluorogenic substrates of high sensitivity for peptidase activity measurements [22,33], these two residues were introduced in the substrates S_1 and S_2 described in the present paper.

The fluorophore–quencher pair was introduced into the smallest cleavable fragment of bET (19–35), in positions 21 and 22 corresponding to the natural site of hydrolysis of bET (Figure 1). Because of the close vicinity of the two chromophores in S_1, the fluorescence was very efficiently quenched (Figure 2). Accordingly, a very large increase in fluorescence was observed after incubation of hECE-1c, reflecting the separation of Pya and Nop. S_1, and its corresponding fluorescent metabolite M_1, had very similar fluorescence spectra, with broad emission maxima at 377 nm and 388 nm. However, very large differences in their fluorescence intensity led to an M_1/S_1 fluorescence ratio of approx. 550. The kinetic constants $K_m = 20.1 \pm 0.9\ \mu$M and $V_{max} = 2.7 \pm 0.33$ nmol/mg of protein/min, indicated that S_1 has a higher K_m value than the natural bET-1 substrate ($K_m = 0.2\ \mu$M and $V_{max} = 0.27$ nmol/mg per min), but its rate of cleavage is approx. 10 times higher. Moreover, the absence of cleavage of S_1 by pure related enzyme, such as ACE or neprilysin (reviewed in [9]), and the complete protection from degradation by crude lung or brain membranes by a selective ECE inhibitor (results not shown), indicated that S_1 is highly specific for ECE-1. Therefore S_1 behaves as a highly sensitive substrate which could be used with preparations of ECE that have been crudely purified.

S_2 was derived from a biased substrates library. It consists of two short N- and C-terminal domains with hydrophilic residues. These residues surround a peptide which contains the two chromophores separated by four variable amino acids which optimally recognize the active site of the targeted enzyme [33]. The hydrophilic moieties ensure that the substrates dissolve well in aqueous media. One of the most efficient variable amino acid

sequences between Pya and Nop for a rapid and selective cleavage by hECE-1 was found to be Lys-Ala-Phe-Ala.

The wavelength of the emission maxima of S_2 is similar to that of S_1, but owing to the relative position of their chromophores, the internal quenching is not as efficient as in S_1. This leads to a lower fluorescence intensity ratio between M_2 and S_2 (ratio 70) than between M_1 and S_1 (ratio 550). However, this decreased sensitivity is compensated by the kinetic parameters of S_2 and the fact that it is easier to synthesize than S_1. Although the K_m value of S_2 ($21.3 \pm 0.9\ \mu$M) is not significantly different from that of S_1 ($20.1 \pm 0.9\ \mu$M), its V_{max} (20.5 nmol/mg of protein/min) is approximately eight times higher. Consequently, with S_2 the screening of inhibitors requires less enzyme and lower incubation times (2-fold decrease) than S_1.

In conclusion, the use of pyrenylalanine as a fluorophore in substrates with internal quenching leads to very sensitive protease assays [33]. This is illustrated here for ECE with S_1 and S_2, which resulted in the simplest and most selective method reported to date for enzymic or anatomical studies on this physiologically important zinc metallopeptidase. Moreover the development of ECE inhibitors should be facilitated by using this fast and robotized enzymic assay.

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