Sensitive fluorometric determination of plasminogen activator in cell lysates and supernatants

A. Obrénovitch, C. Maintier, T. Maillet, R. Mayer, C. Kieda and M. Monsigny*

Centre de Biophysique Moléculaire, Centre National de la Recherche Scientifique, 1, Rue Haute, 45045 Orleans Cedex, France

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A fluorogenic substrate for plasmin, CBZ-Gly–Pro–Arg-AEC, has been synthesized and used to develop a new sensitive photometric and fluorometric assay of plasminogen activator activity. The fluorescence intensity of free AEC at 460 nm is about 3 orders of magnitude higher than that of acyl-AEC. The release of AEC from the peptidyl derivative was monitored fluorometrically after extraction of free AEC in ethylacetate. Under such conditions, the K_m was 0.16 mM. This method was used to monitor the activity of plasminogen activator synthetized by fibroblastic cells (BHK 21 C 13) either released in the supernatants or cell-associated.

Synthetic fluorogenic substrate *3-Amino-9-ethylcarbazole* *Plasminogen activator* *Serum substitute* *BHK 21 C 13 cell*

1. INTRODUCTION

Plasminogen activators are found in tissue extracts [1], in transformed cell lines [2], in primary tumors and in secondary tumors or metastases [3,4] (see also recent reviews [5–7]). Plasminogen activators and other proteases are thought to be involved in many basic physiological phenomena such as angiogenesis, cell division, tumor invasion, cell spreading and metastatic colony development.

For plasminogen activator assay several procedures have been described, amongst them: the determination of soluble peptides released from various labeled proteins such as azocasein [8] or ^{125}I-fibrin [9] in the presence of plasminogen; the direct measurement of fluorescent probes released from a fluorogenic substrate such as CBZ-Gly–Gly–Arg-AMC [10] and the colorimetric or fluorimetric measurement of an aromatic aniline released from chromogenic or fluorogenic substrates such as D-Val–L-Leu–L-Lys-pNA [11] or CBZ-Gly–Pro–Arg-MβNA [12], respectively, in the presence of plasminogen.

Here, we describe a general method allowing determination of plasminogen activator activity in cell supernatants and in cell lysates, on the basis of a spectrofluorimetric assay [13]. This method involves the use of a fluorogenic substrate CBZ-Gly–Pro–Arg-AEC, the synthesis of which is described, the extraction of the released AEC into ethylacetate, the set up of a supplemented culture medium free of or poor in plasminogen activators and plasmin inhibitors. This method allows determination of the activity of plasminogen activator associated with/or secreted by BHK 21 C13 fibroblastic cells.

* To whom correspondence should be addressed

Abbreviations: AEC, 3-amino-9-ethylcarbazole; CBZ, benzyloxycarbonyl; *p*NA, *p*-nitroaniline; AMC, 7-amino-4-methylcoumarine; MβNA, 4-methoxy-2-naphthylamide; Boc, *t*-butyloxycarbonyl; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; DMF, *N*-dimethylformamide; GMEM, Glasgow modified Eagle medium

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2. MATERIALS AND METHODS

2.1. *Materials*

N-α-Boc-L-arginine and the dipeptide *N*-α-CBZ-glycyl–L-prolyl–*N*-hydroxysuccinimide ester were purchased from Bächem (Bubendorf); 1-hydroxybenzotriazole monohydrate, 3-amino-9-ethylcarbazole (AEC) and quinine sulfate dihydrate were obtained from Aldrich Europe (Beerse). AEC was recrystallized from methanol/hexane.

N,N'-dicyclohexylcarbodiimide was from Fluka (Buchs) and *p*-toluene sulfonic acid monohydrate from Merck (Darmstadt). Dimethylformamide was distilled over *N*-α-CBZ-glycyl–*p*-nitrophenyl ester before use. For thin-layer chromatography (TLC) precoated silica gel plates from Merck were used. Plasmin from porcine blood was from Sigma (St Louis MO); purified human plasminogen from 'Centre de Transfusion Sanguine' (Orsay). Fetal bovine serum was obtained from Flow Labs. Sera substitutes: Nu-serum (Collab. Res., Waltman MA), Ultroser G and Ultroser M (Réactifs IBF, Pointet Girard, Villeneuve La Garenne).

2.2. *Peptide synthesis*

N-α-Boc-arginine (1 g) was dissolved in DMF containing 1 equiv. *p*-toluene sulfonic acid and condensed with 1 equiv. AEC under nitrogen using the classical *N,N'*-dicyclohexylcarbodiimide method in the presence of 1-hydroxybenzotriazole. *N*-α-Boc-arginyl-AEC, *p*-toluene sulfonate salt, was purified by preparative chromatography on a silica gel column equilibrated with a chloroform/methanol/acetic acid mixture (80:20:4, by vol.); R_F = 0.35, yield 60%, m.p. 240°C.

The *N*-α-Boc protecting group was removed by HCl/acetic acid to yield HCl, H-arginyl-AEC, *p*-toluene sulfonate, m.p. 227–229°C.

N-α-CBZ-glycyl–L-prolyl–L-arginyl-AEC, *p*-toluene sulfonate was obtained by condensation under nitrogen of *N*-α-CBZ-glycyl–L-prolyl–*N*-hydroxysuccinimide ester with HCL, H-arginyl-AEC, *p*-toluene sulfonate in DMF in the presence of 2 equiv. triethylamine.

This compound was purified by preparative chromatography using a chloroform/methanol/water mixture (90:30:5, by vol.).

2.3. *Fluorometric measurements*

Fluorescence emission or excitation spectra were recorded using a FICA 55 MK II spectrofluorimeter. The fluorimeter was daily standardized with a 0.255 μM quinine sulfate (200 ng/ml quinine sulfate dihydrate) in 0.1 N H_2SO_4; upon excitation at 345 nm, the fluorescence emission intensity at 460 nm was taken as 1 fluorescence unit.

The fluorogenic substrate (5 mg) was dissolved in *N*-dimethylformamide (0.2 ml) or in dimethylsulfoxide and diluted to the appropriate concentration with 50 mM Tris–HCl buffer (pH 8.1). Emission and excitation spectra of free and acylated AEC were monitored before and after extraction with 1 vol. distilled ethylacetate.

2.4. *Determination of the kinetic parameters of plasmin*

The kinetic constants were calculated from a double-reciprocal plot by the Lineweaver-Burk method based on initial rate determinations at 23–270 μM substrate, in the presence of 1.5 μg plasmin/ml, in 50 mM Tris–HCl buffer, pH 8.1 at 37°C. After 30 min, free AEC was extracted by 1 vol. ethylacetate and assessed spectrofluorometrically.

2.5. *Preparation of BHK cell supernatants and extracts*

Baby hamster kidney cells (BHK 21 C 13) were grown in GMEM [14] supplemented with 10% fetal bovine serum. At appropriate growth density, attached cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4) and incubated in GMEM supplemented with a serum substitute. Supernatants were collected after the required time lapses and incubated for 3 h in the presence of human plasminogen (50 μg/ml). In other experiments, cells were incubated in GMEM supplemented with a serum substitute in the presence of 50 μg human plasminogen/ml. In all cases, supernatants were assayed for plasminogen activator activity after addition of CBZ-Gly–Pro–Arg-AEC (final conc., 0.10 mM), in 0.05 M Tris–HCl buffer (pH 8.1) and incubated for 45 min at 37°C.

For the preparation of cell extracts: the cells washed twice with phosphate-buffered saline (pH 7.4) were removed from the surface by using a rubber policeman, centrifuged and lysed by incuba-

tion in 0.1 M sodium borate (pH 8.5) (1 ml for 2 × 10^6 cells), containing 0.5 g/l polyhydroxy alcanoylalkylamine (MAC 14) [15]. The solution was then acidified by adding 1.5 vol. 0.1 M glycine–HCl buffer (pH 2.7) containing 0.1% gelatin [16]. After 60 min at 37°C, the acid-treated lysate was neutralized by adding 1 M Tris–HCl buffer (pH 8.1). The lysate (500 μl) was incubated for 1 h at 37°C in the presence of plasminogen (final conc. 50 μg/ml) and finally incubated for 45 min at 37°C in the presence of CBZ-Gly–Pro–Arg-AEC (final conc. 0.1 M). Released AEC was extracted with 1 vol. ethylacetate.

3. RESULTS

3.1. *Characterization and fluorescence properties of AEC and CBZ-Gly–Pro–Arg-AEC*

CBZ-Gly–Pro–Arg-AEC synthetized by coupling the *N*-hydroxysuccinimide activated ester of CBZ-Gly–Pro with HCl, H-Arg-AEC, *p*-toluene sulfonate was obtained with a 92% yield. The same peptide was synthesized only in 40% yield by coupling CBZ-Gly–Pro–Arg-OH to AEC in the presence of 1-hydroxybenzotriazole and dicyclohexylcarbodiimide (unpublished). CBZ-Gly–Pro–Arg-AEC was homogenous in thin-layer chromatography, and its NMR, IR, UV and fluorescence spectra were in accordance to the expected ones.

The fluorescence spectrum of CBZ-Gly–Pro–Arg-AEC was identical to that of Boc-Val–Leu–Gly–Arg-AEC [13]. After extraction with 1 vol. of ethylacetate, 71% of CBZ-Gly–Pro–Arg-AEC (initial conc. 0.1 mM) remained in the aqueous solution. Conversely, 96% of AEC was extracted into ethylacetate, under similar conditions. In this solvent, the fluorescence spectra of free AEC and of CBZ-Gly–Pro–Arg-AEC showed excitation maxima at 370 nm and 345 nm, respectively, and emission maxima at 430 and 390 nm, respectively (fig.1). The maximum fluorescence intensity of AEC in ethylacetate is slightly lower than that of AEC in aqueous solution. So, after ethylacetate extraction and upon excitation at 370 nm, the fluorescence of the acyl-AEC derivative at 430 nm was negligible.

The amount of AEC extracted by ethylacetate from a Tris–HCl buffer solution was proportional to its initial aqueous concentration over 0.01–0.05 mM, as shown by fluorescence determination (fig.2).

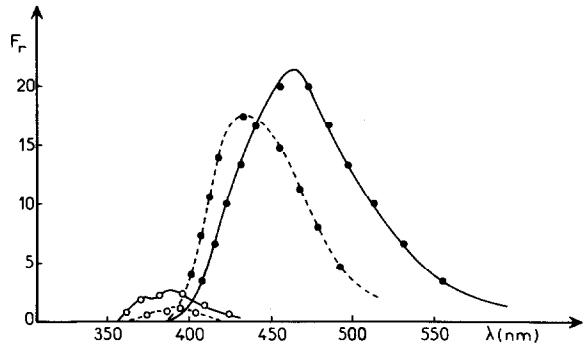

Fig.1. Fluorescence emission spectra of free AEC in Tris–HCl buffer (pH 8.1) (●——●) and in ethylacetate (o---o), of acyl-AEC in Tris–HCl buffer (pH 8.1) (o——o) and in ethylacetate (o---o). Maximum excitation wavelengths of free AEC (6.5 × 10^{-5} M): 360 nm in Tris–HCl buffer (pH 8.1); 370 nm in ethylacetate. Maximum excitation wavelengths of acyl-AEC (6.5 × 10^{-5} M); 340 nm in Tris–HCl buffer (pH 8.1); 345 nm in ethylacetate. Temperature 25°C; F_r, relative fluorescence intensity (arbitrary units).

3.2. *Plasmin activity*

Using CBZ-Gly–Pro–Arg-AEC, the K_m- and V_m-values for porcine blood plasmin were 1.6 × 10^{-4} M and 0.7 μmol.min^{-1}.mg^{-1}, respectively.

In an initial attempt to use a fluorometric method to determine proteolytic activities in a cell culture medium, it was found that medium and sera contained fluorescent and chromophoric com-

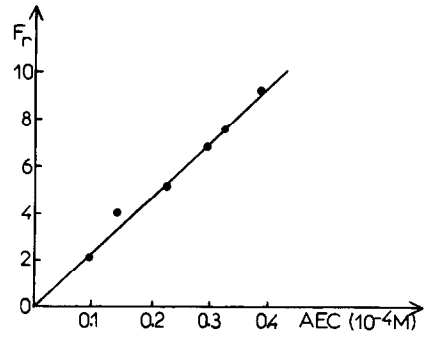

Fig.2. Relative fluorescence intensity (F_r, in arbitrary units) of AEC in ethylacetate extracts from Tris–HCl solution (pH 8.1) containing different concentrations of free AEC.

pounds which made any fluorometric method insensitive. Knowing that AEC was soluble in ethylacetate [13], media and sera were extracted with ethylacetate and it was found that no detectable amount of chromophoric or fluorescent compounds was present in the ethylacetate extract.

We had found that the fetal bovine serum and Nu-serum hydrolyzed the fluorogenic plasmin substrate and that with the Ultroser M serum no hydrolysis of the fluorogenic plasmin substrate occurred (table 1). Furthermore, after acid treatment, fetal bovine serum and Nu-serum were still unsuitable for the determination of the activity of plasminogen activator because they were not free of either proteolytic activities or plasmin inhibitors. On the contrary, Ultroser M, which contained neither proteases active on CBZ-Gly–Pro–Arg-AEC as substrate nor plasmin inhibitors, allowed further investigations.

3.3. *Activity of plasminogen activator in cell lysates*

The determination of cell-associated plasminogen activator requires lysis of the cells. Among various detergents (table 2), the polyhydroxyalkanoylaminoalkane MAC 14 [15] was found to be suitable not only to obtain effective cell lysis but also to determine the released AEC from CBZ-Gly–Pro–Arg-AEC by either plasmin or activated plasminogen by plasminogen activator. When CBZ-Gly–Pro–Arg-AEC was incubated with 10^6 BHK 21 C 13 cells lysed in the presence of 0.5% MAC 14, free AEC was released very rapidly, showing the presence of a cell associated plasmin-like activity; this plasmin-like activity was quantitatively abolished by an acid treatment (60 min, 37°C, pH 2.7) followed by

Table 1

Proteolytic activity of bovine plasmin in the presence of fetal bovine serum or of serum substitutes

Sera	Acid treatment	Proteolytic activity (F_r)	Activity of added plasmin (%)
Control	–	–	100
Fetal bovine serum	–	>5	n.d.
	+	0	0
Nu-serum	–	>5	n.d.
	+	0	4
Ultroser G	–	>5	n.d.
	+	0	0
Ultroser M	–	0	100
	+	0	100

n.d., not done; F_r, relative fluorescence intensity

Proteolytic activity was determined by measuring the fluorescence intensity of the released AEC from (0.1 mM) CBZ-Gly–Pro–Arg-AEC after 45 min incubation at 37°C. Other experimental conditions are in section 2

Table 2

Hydrolysis of CBZ-Gly–Pro–Arg-AEC by plasmin in the presence of detergent or surfactants

Detergent or surfactants	Conc. (g/l)	Hydrolysis rate (v_F)
Control	–	100
Triton X-100	5	0
Triton X-100	10	0
MAC-18	5	0
	2.5	0
MAC-16	5	4
MAC-14	5	104

v_F, change fluorescence intensity/min, arbitrary units

CBZ-Gly–Pro–Arg-AEC (0.1 mM) was incubated at 25°C in the presence of 1 μg bovine plasmin/ml and of 2.5–10 mg detergent/ml

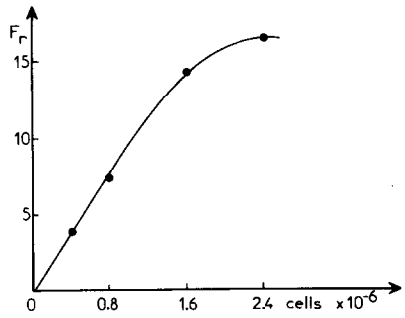

Fig.3. Plasminogen activator activity in BHK 21 C 13 cell lysates: relative fluorescence intensity (F_r) of released AEC by hydrolysis of CBZ-Gly–Pro–Arg-AEC in the presence of plasminogen plus acid-treated cell lysate. Average of two independent experiments in triplicates, deviation <10%.

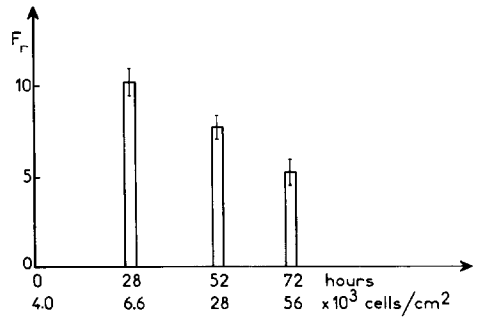

Fig.4. Decrease of plasminogen activator activity in cell lysates upon cell density increase. F_r, relative fluorescence intensity of released AEC from a 0.1 mM solution of CBZ-Gly–Pro–Arg-AEC, in the presence of 50 μg plasminogen/ml and of the lysate from 5×10^5 cells. Average of independent expt in triplicate, deviation < 10%.

neutralisation to pH 8.1. Thus, the acid-treated MAC 14 lysate was suitable to monitor the activity of plasminogen activator, in cell lysate. The fluorescence intensity of released AEC from CBZ-Gly–Pro–Arg-AEC in the presence of plasminogen and acid-treated cell lysate was concentration-dependent (fig.3) and lysates from 5×10^5 cells contained enough plasminogen activator to allow accurate measurements. The plasminogen activator activity of acid-treated cell lysates on the basis of plasminogen activator activity from a constant number of cells (5×10^5 cells) decreased when cell density increased (fig.4).

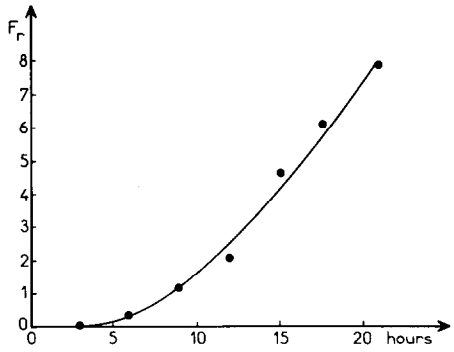

Fig.5. Activity of plasminogen activator in supernatants of BHK 21 C 13 cells. Cells were incubated in GMEM supplemented with Ultroser M in the presence of 30 μg human plasminogen/ml. After the required time lapse, plasmin was determined as in section 2. F_r, relative fluorescence intensity.

3.4. *Activity of plasminogen activator in cell supernatant*

No plasminogen activator activity was detected in cell supernatant up to 3 h after addition of plasminogen in Ultroser M-supplemented medium; then, the plasminogen activator activity increased rapidly (fig.5). In the absence of plasminogen, no plasmin-like activity was detectable even after 24 h incubation in Ultroser M-supplemented medium.

4. DISCUSSION

With the aim of setting up a sensitive assay of cell-associated proteases and of secreted proteases, we have developed new fluorogenic peptides [13]. The specific determination of a given cell protease is based on the use of oligopeptides (tri- or tetrapeptides). To determine plasminogen activator activity, we selected the sequence CBZ-Gly–Pro–Arg which was shown to be specific for thrombin [12] and plasmin [17]. Indeed, while CBZ-Gly–Pro–Arg-AEC was easily hydrolysed by plasmin, it was not hydrolyzed by 100 μg/ml of elastase, urokinase or kallikrein (not shown).

The affinity of CBZ-Gly–Pro–Arg-AEC to porcine plasmin is relatively high as shown by K_m = 0.16 mM which is 3-fold better than the K_m of MeOSu-Ala–Phe–Lys-AMC [18], and close to the K_m of Boc-Val–Leu–Lys-AMC or Boc-Gly–Lys–Lys-AMC [19]. The rate of hydrolysis of CBZ-Gly–Pro–Arg-AEC is in the same range as those of the above AMC derivatives [19]. Furthermore, the fluorescence characteristics of free AEC and acyl AEC either in aqueous buffer [13] or in ethylacetate made this type of derivative very suitable for determination of the activity of proteases. The easy extraction from an aqueous solution of free AEC by ethylacetate allows one to stop the protease activity and to avoid interferences due to fluorescent or chromophoric components present in sera, cell supernatants, or cell lysates. Proteases which use CBZ-Gly–Pro–Arg-AEC as substrate are present in sera [12], serum substitutes and cell lysates, but are absent from the serum substitute Ultroser M. Proteases hydrolysing CBZ-Gly–Pro–Arg-AEC and inhibitor present in cell lysates were deactivated by the acid treatment proposed by Loskutoff and Edginton [16] which does not seem to alter the activity of plasminogen activator. The medium supplemented with Ultroser

M did not hydrolyse CBZ-Gly–Pro–Arg-AEC; under the above conditions, no trace of free AEC was detected after 4 h incubation at 37°C. Therefore, the measured amount of free AEC after 45 min incubation at 37°C must be due to the cell proteases. The use of medium supplemented with the non-interfering serum substitute (Ultroser M) allows cells to grow for $\geqslant$72 h (fig.4).

The method described allows quantitative determination of plasminogen activator activity both in cell lysates and cell supernatants. Supernatants from 5×10^5 cells did not need to be concentrated before the monitoring step, while the direct assay described by Zimmerman et al. [10] required at least a 100-fold concentration.

The cell-associated plasminogen activator detected under the described conditions may also include the corresponding proenzyme. Indeed, plasminogen activator activity was determined by activation of plasminogen and therefore if the pro-plasminogen activator was present, it should be activated as soon as a trace of plasmin is formed [20].

We have described a sensitive fluorometric assay which is suitable for monitoring the activity of cell-associated plasminogen activator and that present in supernatants. This method is based on the use of CBZ-Gly–Pro–Arg-AEC as plasmin substrate, plasmin being produced by specific activation of added plasminogen. The released AEC is specifically extracted by ethylacetate allowing the enzymatic reaction to be stopped and avoiding various interferences coming from chromophoric and fluorescent compounds present in media, sera and cell lysates. The use of a serum substitute such as Ultroser M is required to allow cell growth and to avoid interferences due to other protease or protease inhibitors present in fetal bovine serum. Recently, using the same method, it was possible to study various aspects of plasminogen activators secreted by Lewis lung carcinoma cells from primary tumors and metastasis. These data will be published elsewhere. Using this method, it was possible to monitor the plasminogen activator activity with regard to cell density and cell growth of the fibroblastic like BHK 21 C 13 cells.

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