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A New Fluorogenic Substrate for Plasmin

By Patricia A. PIERZCHALA, Conrad P. DORN and Morris ZIMMERMAN
Merck, Sharp and Dohme Research Laboratory, P.O. Box 2000, *Rahway, NJ* 07065, *U.S.A.*

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A new fluorogenic peptide substrate for plasmin, 7-(*N*-succinoylalanylphenylalanyllysylamido)-4-methylcoumarin trifluoroacetate salt, was prepared that can be used in a simple and direct assay. The results obtained by the assay method are linear over a wide range of enzyme concentrations and sensitive enough to detect as little as 10^{-5} CTA units of plasmin. By making use of the inhibitor Trasylol and the differences in kinetic constants, plasmin can be specifically assayed even in the presence of the plasminogen activator thrombin, as well as in culture fluids from HeLa cells.

Plasmin is a trypsin-like enzyme whose role appears to be the dissolution of blood clots. It is present in the blood as the zymogen, plasminogen, which is incorporated between the filaments when the fibrin matrix is formed. Plasminogen is converted into plasmin by the cleavage of a single Arg–Val bond through the action of a proteolytic enzyme such as plasminogen activator (Stroud, 1974; Robbins & Summaria, 1976). Plasmin breaks down the fibrin matrix by splitting Arg–X or Lys–X bonds.

There presently exist several different methods for assaying plasmin, including the use of protein substrates, e.g. fibrin or casein (Astrup & Mullertz, 1952; Johnson *et al.*, 1969), peptide esters (Sherry *et al.*, 1966; Bell *et al.*, 1974), peptide amides, e.g. nitroanilides (Mattler & Bang, 1977), and peptide 4-methoxy-2-naphthylamides (Clavin *et al.*, 1977). The standard fibrin plate method, although being highly sensitive, requires long incubation times and, as with the methods involving the use of other protein substrates, does not allow kinetic studies to be carried out conveniently. Peptide esters are generally not as specific for individual proteinases as are amides (Feinstein *et al.*, 1973; Mattler & Bang, 1977). Measurement of the rate of hydrolysis of 4-methoxy-2-naphthylamides is either indirect via coupling to a dye or direct by a fluorescent assay with lessened sensitivity. There exists a need for a direct plasmin assay method that is rapid and extremely sensitive to allow detailed kinetic studies.

Workers in this laboratory have previously reported (Zimmerman *et al.*, 1978) that the synthetic fluorogenic peptide Cbz-Gly-Gly-Arg(F_3Ac)-AMC is a highly sensitive substrate for plasminogen activator. Utilizing the same fluorescent leaving group, 7-amino-4-methylcoumarin, we have prepared a plasmin substrate, Suc-Ala-Phe-Lys(F_3Ac)-AMC, that can be used to assay extremely low concentrations of plasmin. We report here the use of this substrate in detecting plasmin impurities in samples of urokinase and thrombin as well as the detection of small amounts of plasmin in culture fluids from HeLa cells.

Abbreviations used: Suc, succinoyl; F_3Ac, trifluoroacetyl; Cbz, benzyloxycarbonyl; Boc, t-butoxycarbonyl; AMC, 7-amino-4-methylcoumarin; CTA units, Committee on Thrombolytic Agents of the NIH units, based on the digestion of a standard batch of α-casein (Johnson *et al.*, 1969); ID_{50}, 50%-inhibitory dose, concentration that inhibits 50% of the enzyme's activity under assay conditions described in the text; Tes, 2-{[-2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethane sulphonic acid.

Materials and Methods

Materials

Tes was obtained from Pierce, Rockford, IL, U.S.A. All the amino acids used were obtained from Chemalog, South Plainfield, NJ, U.S.A. The 7-amino-4-methylcoumarin was prepared as described by Zimmerman *et al.* (1976), with the following modification to remove trace fluorescent contaminants. 7-Amino-4-methylcoumarin was converted into the crystalline tosylate salt by suspending it in hot ethanol, adding toluene-*p*-sulphonic acid and allowing crystals to form by slow cooling. The salt was then converted into the free amine by suspending it in 0.5M-sodium acetate, washing with water and drying either *in vacuo* or by azeotropic distillation with toluene. The 7-(*N*-Cbz-glycylglycylargininamido)-4-methylcoumarin trifluoroacetate was prepared as described by Zimmerman *et al.* (1978). The 7-Boc-alanylphenylalanyl-ε-Cbz-lysylamido-4-methylcoumarin was prepared by a similar procedure.

To the mixed anhydride prepared at −30°C from

18mmol of Boc-ε-Cbz-lysine, 20mmol of *N*-methylmorpholine and 20mmol of isobutyl chloroformate in 100ml of dimethylformamide was added 20mmol of 7-amino-4-methylcoumarin. The reaction mixture was allowed to warm to room temperature and stirred overnight. The solvent was removed *in vacuo* and the residue dissolved in 100ml of methylene dichloride. After two extractions with 50ml of 2.5M-HCl, the organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Chromatography of the residue on 600g of silica gel and elution with ether/hexane (1:1, v/v) followed by ether gave 2.3g (23.8% yield) of 7-(Boc-ε-Cbz-lysylamido)-4-methylcoumarin (m.p. 139–142°C, decomp.); elemental analysis: found: C, 64.58; H, 6.59; N, 7.80; calc. for $C_{29}H_{35}N_3O_7$ (mol.wt. 537.59): C, 64.79; H, 6.56; N, 7.82%.

Selective removal of the *N*-Boc protecting group was accomplished by dissolving 4.2mmol of the above material in 20ml of trifluoroacetic acid at 0°C, allowing the reaction mixture to warm to room temperature, followed by removal of the solvent *in vacuo* to give the trifluoroacetate salt of 7-(ε-Cbz-lysylamido)-4-methylcoumarin. This crude salt and 5mmol of *N*-methylmorpholine were added to the mixed anhydride prepared from 4mmol of Boc-Phe, 4.4mmol of *N*-methylmorpholine and 4.4mmol of isobutyl chloroformate in 50ml of dimethylformamide at −30°C. The reaction mixture was stirred cold for 5min, then allowed to warm to room temperature and stirred overnight. The solvent was removed *in vacuo* and the residue was extracted between 200ml of ethyl acetate and 100ml of 0.25M-HCl. The organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Chromatography of the residue on 500g of silica gel and elution with ethyl acetate/methylene dichloride (1:1, v/v) gave 2.77g (96.3%) of 7-(Boc-phenylalanyl-ε-Cbz-lysylamido)-4-methylcoumarin (m.p. 175–177°C, decomp.); elemental analysis: found: C, 66.28; H, 6.51; N, 7.94; calc. for $C_{38}H_{44}N_4O_8$ (mol.wt. 684.76); C, 66.65; H, 6.48; N, 8.18%.

Deblocking of 3.3mmol of the above dipeptide amide in 20ml of trifluoroacetic acid under the conditions described above gave the trifluoroacetate salt of 7-(phenylalanyl-ε-Cbz-lysylamido)-4-methylcoumarin, which was used in the following step without purification.

To the mixed anhydride prepared from 3.5mmol of Boc-Ala-OH, 4.0mmol of *N*-methylmorpholine and 4.0mmol of isobutyl chloroformate in 50ml of dimethylformamide at −30°C was added the above salt in 25ml of dimethylformamide containing 4.0mmol of *N*-methylmorpholine. The reaction mixture was stirred cold for 10min, then allowed to warm to room temperature and stirred overnight. The solvent was removed *in vacuo* and the residue was taken up between 100ml of ethyl acetate and 50ml of 1M-HCl. The aqueous layer was separated and extracted with 50ml of ethyl acetate. The combined ethyl acetate layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The resulting solid was slurried with ether/hexane (1:1, v/v) and filtered to give 2.36g (94.7%) of 7-(Boc-alanylphenylalanyl-ε-Cbz-lysylamido)-4-methylcoumarin (m.p. 168–170°C, decomp.); elemental analysis: found: C, 65.11; H, 6.65; N, 9.36; calc. for $C_{41}H_{49}N_5O_9$ (mol.wt. 755.84): C, 65.15; H, 6.53; N, 9.27%.

The t-butoxycarbonyl protecting group was removed from the tripeptide amide as described above to yield the trifluoroacetate salt of Ala-Phe-ε-Cbz-Lys-AMC. To prepare the succinoyl derivative, 0.26mmol of the above material was dissolved in dimethylformamide, and then 0.26mmol of *N*-methylmorpholine and 0.26mmol of succinic anhydride were added. The reaction was stirred overnight. The solvent was then removed *in vacuo* and the residue was washed with water to yield Suc-Ala-Phe-ε-Cbz-Lys-AMC. The ε-benzyloxycarbonyl protecting group on the lysine was removed by refluxing in hot trifluoroacetic acid for 20min followed by removal of the solvent *in vacuo*.

MeOSuc-Ala-Phe-ε-Cbz-Lys-AMC was prepared by adding to 0.13mmol of the above trifluoroacetate salt, dissolved in dimethylformamide, 0.33mmol of *N*-methylmorpholine and 0.20mmol of 3-carbomethoxypropionyl chloride. The reaction mixture was stirred overnight. The solvent was removed *in vacuo* and the residue washed with water. The ε-benzyloxycarbonyl protecting group was removed as described above, giving MeOSuc-Ala-Phe-Lys-(F_3Ac)-AMC. The purity of these substances was determined by t.l.c. and amino acid analysis.

Highly purified urokinase, thrombin and human plasmin were obtained from Dr. Thomas Finlay, Department of Biochemistry, New York University Medical Center, New York, NY, U.S.A. A separate sample of purified urokinase was obtained from Dr. Alan Johnson, Department of Medicine, New York University Medical School, New York, NY, U.S.A. Trasylol was obtained from FBA Pharmaceuticals, New York, NY, U.S.A.

Culture fluids from HeLa cells were prepared as described by Zimmerman *et al.* (1978).

Fluorimetric assays

Enzyme assays were conducted at 24°C with the substrate in 0.05mM-Tes/NaOH buffer, pH7.5, and 20% (v/v) dimethyl sulphoxide in a final volume of 0.5ml. Dimethyl sulphoxide at concentrations up to 20% had no inhibitory or stimulatory effects on plasmin. To determine the ID_{50} of Trasylol on thrombin, 0.32mM-Ala-Phe-Lys-AMC was used with 22.2g of thrombin, and 0.12mM-Ala-Phe-Lys-AMC was used with 5×10^{-4} CTA units of plasmin.

Fluorescence of the 7-amino-4-methylcoumarin

generated was determined as described by Zimmerman *et al.* (1976). A Perkin–Elmer model 204A spectrofluorometer was standardized daily so that 10nM-7-amino-4-methylcoumarin gave 1.0 chart division (0–100 scale).

Determination of K_m

The kinetic constants were calculated from a double-reciprocal plot by the Lineweaver–Burk method, and are based on rate determinations at six different substrate concentrations. Correlation coefficients were greater than 0.99. The substrate concentrations used to determine the various K_m values generally ranged from 0.5 to 4 times the K_m value obtained.

Results

The trifluoroacetyl derivatives of Ala-Phe-Lys-AMC, Suc-Ala-Phe-Lys-AMC and MeOSuc-Ala-Phe-Lys-AMC are all excellent plasmin substrates that can be used in a simple, direct, fluorimetric assay method. The limit of detection with the succinoyl derivative is 10^{-5} CTA units of plasmin in a reaction time of 5min, and the rate of hydrolysis is linear over at least a 100-fold range of enzyme concentrations. The succinoyl and methoxysuccinoyl substrates exhibit similar kinetic constants. However, the unblocked substrate has a K_m of 0.045mM, which is 10-fold lower, and a specific activity approximately half of that for either of the blocked peptides (Table 1).

Table 1. *Kinetic constants of the hydrolysis of 7-amino-4-methylcoumarin substrates by plasmin, urokinase and thrombin*
Assays were performed as described in the text with 5×10^{-4} and 5×10^{-3} CTA units of plasmin, 0.2μg and 0.01μg of urokinase and 22.2μg and 0.222μg of thrombin with the lysine and arginine substrates respectively. The specific activities are expressed as μmol of 7-amino-4-methylcoumarin released/min per mg of protein for urokinase and thrombin and as μmol of 7-amino-4-methylcoumarin released/min per CTA unit of plasmin. The specific activities were calculated from the V values and enzyme concentrations. –, Not determined.

	Plasmin		Urokinase		Thrombin	
	K_m (mM)	Sp. activity	K_m (mM)	Sp. activity	K_m (mM)	Sp. activity
Ala-Phe-Lys-AMC	0.045	210	0.90	100	0.80	9
Suc-Ala-Phe-Lys-AMC	0.40	370	0.80	62	–	–
MeOSuc-Ala-Phe-Lys-AMC	0.44	470	–	–	–	–
Cbz-Gly-Gly-Arg-AMC	0.45	10	0.13	1600	0.11	475

Table 2. *Detection of plasmin in the presence of urokinase, thrombin or plasminogen activator*
Assays were carried out as described in the text with 0.045mM-Ala-Phe-Lys-AMC, 0.32mM-Cbz-Gly-Gly-Arg-AMC and 0.8mM-Suc-Ala-Phe-Lys-AMC as substrates and 1.4μM-Trasylol where indicated. The urokinase and thrombin concentrations per assay were 50ng and 222ng respectively. The asterisk (*) indicates that the assay mixture contained plasmin: urokinase*, 1.25×10^{-5} CTA units of plasmin was added to the assay, and thrombin*, 6.0×10^{-5} CTA units of plasmin was added. The activities are expressed as nmol of 7-amino-4-methylcoumarin released/min per μg of protein for urokinase and thrombin and nmol of 7-amino-4-methylcoumarin released/min for urokinase*, thrombin* and HeLa cells. –, Not determined.

	Ala-Phe-Lys-AMC		Cbz-Gly-Gly-Arg-AMC		Suc-Ala-Phe-Lys-AMC	
	–Trasylol	+Trasylol	–Trasylol	+Trasylol	–Trasylol	+Trasylol
Urokinase	–	–	1013	960	–	–
Urokinase*	0.06†	0.0	–	–	–	–
Thrombin	0.0	–	387	401	–	–
Thrombin*	0.44	0.0	–	–	–	–
HeLa (0.1ml)	–	–	–	–	0.08	0.02
HeLa (0.2ml)	–	–	0.26	0.30	0.15	0.05
HeLa (0.4ml)	–	–	0.54	0.54	0.21	0.06

† Amount of plasmin added is at the detection limit for this assay.

Highly purified urokinase and thrombin also hydrolyse Ala-Phe-Lys-AMC, but at a much lower rate and with K_m values of 0.9mM and 0.8mM respectively (Table 1). The large difference between the K_m with plasmin and that with either urokinase or thrombin has enabled us to assay specifically for plasmin in the presence of urokinase or thrombin. Samples of urokinase and of thrombin, deliberately contaminated with 1.25×10^{-5} and 6.0×10^{-5} CTA units of plasmin respectively, were assayed with 0.045mM-Ala-Phe-Lys-AMC. In both cases the activity of the small amount of plasmin was able to be measured (Table 2).

With the substrate Ala-Phe-Lys-AMC, the ID_{50} of Trasylol with plasmin was found to be $<0.05\mu M$ and with thrombin $>23\mu M$. Zimmerman *et al.* (1978) have reported that Trasylol will completely inhibit plasmin with no effect on urokinase activity. With the plasminogen activator substrate Cbz-Gly-Gly-Arg-AMC they report an ID_{50} of $>12\mu M$ with urokinase. However, when urokinase was assayed with Ala-Phe-Lys-AMC 50% inhibition was observed on the addition of only $1.4\mu M$-Trasylol. A similar result was obtained with a different sample of purified urokinase. When the assay was repeated with Cbz-Gly-Gly-Arg-AMC as the substrate, no inhibition of urokinase activity was found on Trasylol addition (Table 2), thus suggesting a slight contamination of 'pure' urokinase with plasmin. When a mixture of plasmin and thrombin or plasmin and urokinase was assayed with Ala-Phe-Lys-AMC, the enzyme activity was totally inhibited by the addition of $1.4\mu M$-Trasylol (Table 2). This result supports the observation that the plasmin activity was able to be measured specifically in the presence of other similar enzymes.

When assaying plasmin activity in crude extracts, one of the blocked substrates must be used to avoid non-specific substrate hydrolysis by aminopeptidases. Culture fluids from HeLa cells were assayed with 0.8mM-Suc-Ala-Phe-Lys-AMC and 0.32mM-Cbz-Gly-Gly-Arg-AMC. As shown in Table 2, both substrates were hydrolysed. To ensure that the activity observed with the high concentration of Suc-Ala-Phe-Lys-AMC was due to plasmin rather than plasminogen activator, Trasylol was added to the assay mixture. It inhibited most of the activity (70%) with the lysine substrate, but had no effect on the hydrolysis rate with the arginine substrate (Table 2).

Discussion

We have described a new fluorogenic peptide substrate allowing the detection of extremely low concentrations of plasmin. The assay method is rapid and direct, with no observable spontaneous hydrolysis of the substrate.

The sequence chosen was based on the chloromethyl ketone inhibition data reported by Kettner *et al.* (1977), which showed Ac-Ala-Phe-Arg-CH_2Cl to inhibit plasmin 475-fold better than it inhibits urokinase. Since plasmin prefers lysine at the bond cleaved, Ala-Phe-Lys-AMC was synthesized.

Unlike the 4-methoxy-2-naphthylamide substrate described by Clavin *et al.* (1977), Suc-Ala-Phe-Lys-AMC can be used directly to detect as little as 10^{-5} CTA units of plasmin, compared with the 5×10^{-2} CTA unit detection limit that they report. Nieuwenhuizen *et al.* (1977) have also reported a fluorogenic peptide substrate for plasmin and plasminogen activator with the use of a β-naphthylamide leaving group; however, it is a better substrate for urokinase than for plasmin. Alternately, Ala-Phe-Lys-AMC has a 20-fold lower K_m with plasmin than with either urokinase or thrombin, thus enabling plasmin contamination in either preparation to be readily measured. The sensitivity and specificity of Ala-Phe-Lys-AMC is such that trace amounts of another proteinase, presumably plasmin, were detected in supposedly pure preparations of urokinase. Confirmation of these results, achieved through the use of the macromolecular inhibitor Trasylol, which completely inhibits plasmin activity with little of no effect on urokinase or thrombin, demonstrates the utility of combining the use of this new substrate with Trasylol to check the purity of enzymes isolated from biological fluids.

The succinoyl and methoxysuccinoyl substrates both have much higher K_m values than the unblocked substrate. Possibly the carboxy group and its methyl ester interfere with binding (assuming $K_m = K_s$), and studies on whether extending the peptide chain will optimize the substrate should be undertaken.

The blocked substrate Suc-Ala-Phe-Lys-AMC was used when assaying crude extracts to avoid any possible aminopeptidase action on the substrate. Although there is only a 2-fold difference in K_m, discrimination between plasmin and urokinase was possible in the presence of Trasylol. Culture fluids from malignant cells (HeLa) were found to contain low concentrations of plasmin in addition to plasminogen activator. This method can be applied to other cell systems to assay directly and specifically for plasmin.

Suc-Ala-Phe-Lys-AMC has been shown to be a much more highly sensitive and specific plasmin substrate than any other reported. By using both Cbz-Gly-Gly-Arg-AMC and Suc-Ala-Phe-Lys-AMC it is now possible to assay the same cell fluids for both plasminogen activator and plasmin activity.

References

Astrup, T. & Mullertz, S. (1952) *Arch. Biochem. Biophys.* **40**, 346–351

Bell, P. H., Dziobkowski, C. T. & Englert, M. E. (1974) *Anal. Biochem.* **61**, 200–208
Clavin, S. A., Bobbitt, J. L., Shuman, R. T. & Smithwick, E. L., Jr. (1977) *Anal. Biochem.* **80**, 355–365
Feinstein, G., Kupfer, A. & Sokolovsky, M. (1973) *Biochem. Biophys. Res. Commun.* **50**, 1020–1026
Johnson, A. J., Kline, D. L. & Alkjaersig, N. (1969) *Thromb. Diath. Haemorrh.* **21**, 259–271
Kettner, C., Ruscica, J. & Shaw, E. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 2599
Mattler, L. E. & Bang, N. U. (1977) *Thromb. Haemostasis* **38**, 776–791
Nieuwenhuizen, W., Wijngaards, G. & Groeneveld, E. (1977) *Anal. Biochem.* **83**, 143–148
Robbins, K. C. & Summaria, L. (1976) *Methods Enzymol.* **45B**, 257–273
Sherry, S., Alkjaersig, N. & Fletcher, A. P. (1966) *Thromb. Diath. Haemorrh.* **16**, 18–31
Stroud, M. (1974) *Sci. Am.* **231**, 74–88
Zimmerman, M., Yurewicz, E. & Patel, G. (1976) *Anal. Biochem.* **70**, 258–262
Zimmerman, M., Quigley, J., Ashe, B., Dorn, C., Goldfarb, R. & Troll, W. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 750–753