Vol. 97, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 17, 1980 Pages 334-339

HIGHLY SENSITIVE ASSAYS FOR PROTEINASES USING IMMOBILIZED LUMINOGENIC SUBSTRATES

Bruce R. Branchini, Francesco G. Salituro,
Jeffrey D. Hermes, and Nancy J. Post

Department of Chemistry
University of Wisconsin-Parkside
Kenosha, Wisconsin 53141

Received October 7,1980

SUMMARY

Novel assays for proteinases with immobilized substrates Affi-gel 10-Ala-Ala-Ala-Phe-Isoluminol and Affi-gel 10-Ala-Phe-Pro-Arg-Isoluminol have been devised. The substrates are luminogenic and provide extremely high sensitivity for α -chymotrypsin, trypsin, and human thrombin. Observed detection limits for the enzymes are 0.1, 0.01, and 0.5 ng respectively in 0.5 mL enzyme-substrate mixtures. A comparison of soluble and immobilized luminogenic, chromogenic, and fluorogenic substrates is presented.

The prominent involvement of proteinases in the control and maintenance of the healthy physiological state has prompted extensive investigation (1) of these enzymes. The use of synthetic peptide substrates has been essential to the progress of this research (1). We have been involved (2) in the development of sensitive peptide substrates suitable for detecting proteinase activity in body fluids and **in vitro**. Our focus has been on the use of soluble substrates designed to release chemiluminescent leaving groups (luminophores) when hydrolyzed by proteinases.

From our initial work, we concluded (2) that background light emission of unreacted substrates was a serious limitation to the potentially greater sensitivity of luminescence over absorbance- or fluorescence-based methods. We report here the use of immobilized luminogenic substrates to reduce background light emission. The matrix-bound substrates are used in chemiluminescence-based assays to detect very low levels of α -chymotrypsin, trypsin, and human thrombin.

Abbreviations: IL, isoluminol; pNA, p-nitroaniline; S-2160, Benzyl-Phe-Val-Arg-pNA; Z, benzyloxycarbonyl; Boc, **tert**-butyloxycarbonyl; TFA, trifluoroacetate; Suc, succinyl; AMC, 7-amino-4-methylcoumarin; ATMC, 7-amino-4-trifluoromethylcourmarin; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

0006-291X/80/210334-06$01.00/0

MATERIALS AND METHODS

Enzymes and reagents. Human thrombin, bovine α-chymotrypsin, N-**trans**-cinnamoylimidazole, bovine hematin chloride, and p-nitrophenyl-**p**-guanidinobenzoate-HCl were purchased from Sigma Chemical Company. Bovine trypsin was obtained from Aldrich Chemical Company, Affi-gel 10 from Bio-Rad laboratories, and 33% HBr-acetic acid from Tridom Chemical Company. Z-Phe-Pro-Arg-IL-HCl, H-Phe-Pro-Arg-IL-2HCl, and S-2160 were gifts of Kabi AB, Stockholm. The activity of chymotrypsin and trypsin samples ,was determined by titration with N-**trans**-cinnamoylimidazole (3) and **p**-nitrophenyl-**p**-guanidinobenzoate (4), respectively. Human thrombin activity was determined by S-2160 assay (5) to be 1200 NIH units/mg. Sterile charcoal-filtered distilled water was used throughout.

Immobilized substrates. H-Ala-Ala-Phe-IL-HBr. Boc-Ala-Ala-Phe-IL (30 mg, 53 μmol) (2) was stirred with 33% HBr-acetic acid (0.25 mL) for 0.5 h. Ether was added to precipitate a white solid which was collected, washed well with ether, and dried **in vacuo** to yield product (25 mg, 46 μ mol, 87%).

AG10-Ala-OH. Affi-gel 10 (25 mL settled gel) was coupled to alanine (14.3 g, 0.16 mol) in 0.1 M potassium phosphate buffer, pH 7.1 according to the suppliers instructions (6). After washing, the gel was stored in 0.02% sodium azide.

AG10-Ala-Ala-Ala-Phe-IL and AG10-Ala-Phe-Pro-Arg-IL. The immobilized substrates were prepared by coupling Affi-gel 10-Ala-OH with H-Ala-Ala-Phe-IL-HBr 30 μ mol/ml gel) and H-Phe-Pro-Arg-IL-2HCl (18 μ mol/mL gel) by the EDAC method described for affinity gels (7). Ethylglycinate was used to block underivatized sites and gels were washed extensively with water, N,N-dimethylformamide: H_2O (1:1 v/v), and 0.05 M sodium phosphate buffer, pH 7.6. The gels were stored in 0.02% azide and washed with water and phosphate buffer before use.

Light measurements. Isoluminol production was determined by chemiluminescence measurements as described previously (2), except that assays were performed in 0.08 M Na_2CO_3, pH 11.2. Assays were carried out in triplicate with an error (standard deviation/mean) of ∿ 5%.

Enzyme assays. Assays with soluble substrates Z-Ala-Ala-Phe-IL and Z-Phe-Pro-Arg-IL were carried out as described previously (2), except that trypsin and thrombin were incubated with 0.2 mM Z-Phe-Pro-Arg-IL in 0.05 M Tris buffer, pH 7.7:H_2O:1mM HCl (7:2:1 v/v).

Assays with immobilized substrates were initiated by adding an aliquot of enzyme solution (50 μ L) to 0.45 mL of a gently stirred suspension of AG10-Ala-Ala-Ala-Phe-IL (0.017 μ mol) or AG10-Ala-Phe-Pro-Arg-IL (0.2 μ mol) in 0.05 M sodium phosphate buffer, pH 7.6 at 22±1° C. After 5 min the assay tubes (10x75 mm) were centrifuged for 15 s and a 25 μ L aliquot of supernatant was withdrawn for analysis by the light assay method described (2). Control experiments were performed by stirring immobilized substrates in the pH 7.6 phosphate buffer. Any observed background light emission was subtracted from assays containing proteinases.

Exhaustive hydrolysis of immobilized substrates. In separate experiments, 0.1 mL of AG10-Ala-Ala-Ala-Phe-IL and 0.05 mL of AG10-Ala-Phe-Pro-Arg-IL were suspended in 0.05 M sodium phosphate buffer, pH 7.6 (total volume = 0.5 mL) and gently stirred for 4 h with 0.5 μ M chymotrypsin and trypsin respectively. Luminescence assays showed that enzymic hydrolysis had produced 34 μ M and 0.37 mM isoluminol solutions, equivalent to .17 μ mol/mL gel and 3.7 μ mol/mL gel for AG10-Ala-Ala-Ala-Phe-IL and AG10-Ala-Phe-Pro-Arg-IL respectively.

Kinetic constants. K_m, k_{cat}, and V_{max} were determined for Z-Phe-Pro-Arg-IL by linear regression analysis of plots of S/V_0 versus S (8). Trypsin (7 nM) or thrombin (34

nM) was incubated with varying substrate concentrations (1-200 μ M) for one min. Aliquots (25 μ L) were withdrawn and assayed as described (2) to determine initial rate of isoluminol production.

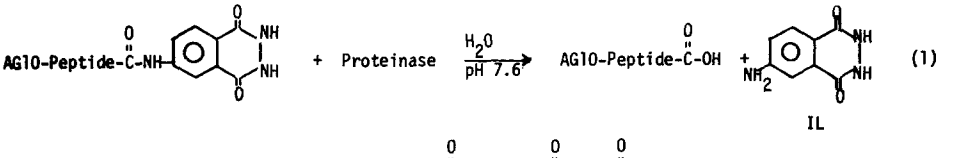
For immobilized substrates, k_{cat} values were estimated from mean determinations of isoluminol production in 5 min at 22±1° C. Values were obtained with proteinase concentrations shown in Figures 1 and 2 and effective substrate concentrations of 34μ M and 0.37 mM for AG10-Ala-Ala-Ala-Phe-IL and AG10-Ala-Phe-Pro-Arg-IL respectively.

RESULTS

The accessible bound substrate content was estimated by exhaustive enzymic hydrolysis to be 0.17 μ mol and 3.7 μ mol per mL of settled gel for AG10-Ala-Ala-Ala-Phe-IL and AG10-Ala-Phe-Pro-Arg-IL respectively. Proteinase assays were carried out with the matrix-bound substrates in two successive steps (Chart 1). First, the enzyme was incubated with AG10-Ala-Ala-Ala-Phe-IL (34 μM) or AG10-Ala-Phe-Pro-Arg-IL (0.37 mM) in pH 7.6 phosphate buffer (Equation 1). Enzymic reactions were stopped by brief centrifugation and addition of aliquots of supernatant to 0.08 M Na_2CO_3, pH 11.2. In the second step, isoluminol production was determined by monitoring light emission resulting from addition of hydrogen peroxide and hematin (Equation 2) to alkaline sample mixtures. Blank assays without enzyme produced light emission from the oxidant (and possibly substrate detached from the matrix) equivalent to 0.2 nM isoluminol.

The k_{cat} values for the soluble and immobilized luminogenic substrates with α-chymotrypsin, trypsin, and human thrombin are presented in Table 1.

CHART 1



AG10 = Agarose-O-CH_2CNH$(CH_2)_2$NHC$(CH_2)_2$C-

AG10-Ala-Ala-Ala-Phe-IL, Peptide = -Ala-Ala-Ala-Phe-

AG10-Ala-Phe-Pro-Arg-IL, Peptide = -Ala-Phe-Pro-Arg-

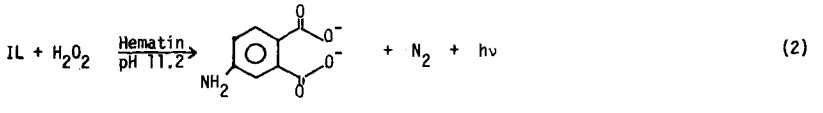


TABLE 1

COMPARISON OF SEVERAL SUBSTRATES FOR PROTEINASES

Substrate	Enzyme	k_{cat} (s^{-1})	Detection Limit (ng)
AG10-Ala-Ala-Ala-Phe-IL[a]	Chymotrypsin	0.22	0.1
Z-Ala-Ala-Phe-IL[b]	Chymotrypsin	0.43	27
TFA·Ala-Ala-Phe-AMC[c]	Chymotrypsin	0.83	25
Suc-Ala-Ala-Pro-Phe-pNA[d,e]	Chymotrypsin	45.0	2
AG10-Ala-Phe-Pro-Arg-IL[a]	Trypsin	1.5	0.01
AG10-Ala-Phe-Pro-Arg-IL[a]	Thrombin	0.05	0.5
Z-Phe-Pro-Arg-IL[a]	Trypsin	31.7	0.08
Z-Phe-Pro-Arg-IL[a]	Thrombin	4.3	1.3
Z-Gly-Gly-Arg-ATMC[a,f]	Trypsin	135.8	0.04
H-D-Phe-Pip-Arg-pNA[e,g]	Thrombin	113.6	1.1

[a]Detection limit observed experimentally. [b]Data from reference (2). [c]Data from reference (9). [d]Data from reference (10). [e]Detection limit calculated from $\Delta A_{410nm} = 0.01$ ($\varepsilon = 8800$, $b=1$, assay volume = 1 mL) in 5 min at the k_{cat} specified. [f]Data from reference (11). [g]Data from reference (12).

The relationship between α-chymotrypsin activity measured with AG10-Ala-Ala-Ala-Phe-IL and enzyme concentration was linear over the range 8.3 to 330 pM; these data are shown in Figure 1. With AG10-Ala-Phe-Pro-Arg-IL linear relationships between proteinase activity and enzyme concentration were observed for trypsin (0.86 pM to 10 nM) and human thrombin (0.03 to 3 nM); these data are shown in Figure 2. Observed detection limits for proteinases with soluble and immobilized luminogenic substrates are given in Table 1.

DISCUSSION

Enhanced sensitivity of peptide substrates for proteinases can be realized by increasing the detectability and/or rate of production of the leaving group. We have concentrated on developing assays with luminophores to improve upon the detection sensitivity possible for absorbance- and fluorescence-based methods. Detection limits for prototypic luminophores such as isoluminol (13) and luminol (13) are 30 and 1 pM; whereas those of the popular chromophore p-nitroaniline (14) and the recently described fluorophore 7-amino-4-trifluoromethylcoumarin (11), are approximately 1 μM and 20 nM respectively.

Enzyme assays with soluble luminogenic substrates are approximately as effective as the chromogenic and fluorogenic peptide analogs (Table 1). The sensitivity of lumino-

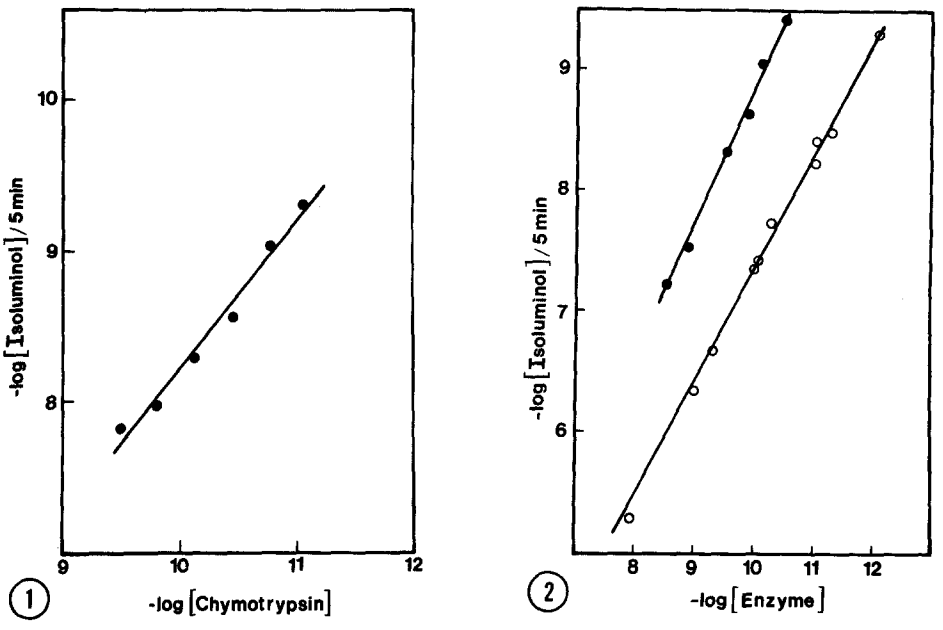


Figure 1. Relationship between isoluminol production and chymotrypsin concentration. Enzyme solutions, 8.3 to 330 pM, were assayed as described in Materials and Methods with AG10-Ala-Ala-Ala-Phe-IL (34 μ M) in 0.05 M sodium phosphate buffer, pH 7.6 (total volume = 0.5 mL) at 22±1° C.

Figure 2. Relationship between isoluminol production and trypsin or human thrombin concentration. Solutions of trypsin (O), 0.86 pM to 10 nM, or human thrombin (●), 0.03 to 3 nM, were assayed as described in Materials and Methods with AG10-Ala-Phe-Pro-Arg-IL (0.37 mM) in 0.05 M sodium phosphate buffer, pH 7.6 (total volume = 0.5 mL) at 22±1° C.

genic assays is decreased, however, by the intrinsic light emission of unreacted substrate. For example, the detection limit of isoluminol is raised to 0.7 μ M in assays with Z-Ala-Ala-Phe-IL (2). Attaching luminogenic substrates to an insoluble agarose matrix obviates this problem. Enzymic hydrolysis of immobilized substrates (Equation 1) releases the luminophore into a solution essentially free of unreacted substrate making it possible to achieve the reported pM detection limit for isoluminol (13). A comparison of the various types of substrates for α-chymotrypsin, trypsin, and human thrombin shows that the immobilized luminogenic substrates provide superior sensitivity for each proteinase examined (Table 1).

We are presently investigating the cause for the decrease in k_{cat} that occurs when the luminogenic trypsin/thrombin substrate is immobilized. Similar decreases with immobilized fluorogenic substrates have been reported (15). Work is in progress to

further extend the usefulness of immobilized luminogenic substrates to new classes of proteinases and direct clinical applications.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial support of Kabi AB and helpful discussions with M. T. Marron and G. Claeson. We thank L. Aurell, S. Arielly, and R. Simonsson for their support and the generous gifts of H-Phe-Pro-Arg-IL-2HCl, Z-Phe-Pro-Arg-IL-HCl, and S-2160.

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