J. Biochem. **82**, 1495–1498 (1977)

New Fluorogenic Substrates for α-Thrombin, Factor Xa, Kallikreins, and Urokinase[1]

Takashi MORITA,* Hisao KATO,* Sadaaki IWANAGA,*
Katsumi TAKADA,** Terutoshi KIMURA,**
and Shumpei SAKAKIBARA**

*Institute for Protein Research, Osaka University, Suita, Osaka 565, and
**Peptide Institute, Protein Research Foundation, Minoh, Osaka 562

Received for publication, September 5, 1977

Twenty peptide-4-methylcoumarin amides (MCA) were newly synthesized and tested as possible substrates for α-thrombin, factor Xa, kallikreins, urokinase, and plasmin. These fluorogenic peptides contained arginine-MCA as the carboxyl-terminus. Release of 7-amino-4-methylcoumarin was determined fluorometrically. Of these peptides, the following were found to be specific substrates for individual enzymes: Boc-Val-Pro-Arg-MCA for α-thrombin, Boc-Ile-Glu-Gly-Arg-MCA, and Boc-Ser-Gly-Arg-MCA for factor Xa, Z-Phe-Arg-MCA for plasma kallikrein, Pro-Phe-Arg-MCA for pancreatic and urinary kallikreins, and glutaryl-Gly-Arg-MCA for urokinase. Moreover, these peptide-MCA substrates were resistant to plasmin.

Recently, several peptidyl-nitroanilides, which suit a specificity requirement of proteinases, have been introduced as specific substrates for blood coagulation factors (*1*, *2*), kallikreins (*3*), and urokinase (*4*). These substrates are now commercially available and various attempts to use them for specific assays of prothrombin (*5*), prekallikrein (*6*), factor Xa (*7*), antiplasmin (*8*), and antithrombin III (*9*) are being made. Peptide amides of 7-amino-4-methylcoumarin, originally developed for the sensitive assay of chymotrypsin (*10*), have also proven useful for the assay of proteases including elastase (*11*), an aminopeptidase (*12*), and X-prolyl dipeptidyl-aminopeptidase (*13*). This assay method seems more sensitive than the chromogenic assay, because the reaction product, 7-amino-4-methylcoumarin, is highly fluorescent (*10*). To extend further the availability of fluorogenic substrates, we have newly synthesized twenty peptide-MCAs and tested for their possible use as specific substrates for assay of proteolytic enzymes with limited specificity. The results indicate that the peptide-MCA substrate, which fits preferentially the specificity requirements of α-thrombin, factor Xa, urokinase, and kallikreins, is very useful for specific enzyme assay.

Peptide-MCA substrates were synthesized by standard chemical procedures, using L-amino acids. Purity of all intermediates and products was con-

[1] This study was supported in part by a grant (948205) from the Scientific Research Fund of the Ministry of Education, Science and Culture of Japan.

Abbreviations: MCA, 7-amino-4-methylcoumarin amide; Bzl, benzyl; Boc, *tert*-butoxycarbonyl; DMSO, dimethylsulfoxide.

firmed by thin layer chromatography, elemental analysis, and amino acid analysis. The experimental details of the peptide synthesis will be reported elsewhere. Highly purified α-thrombin (2,000–2,500 NIH units per mg) (*14*), factor Xa (*15*), and plasma kallikrein (*16*) from bovine sources were prepared according to the methods previously described. Bovine plasminogen, which was purified by the method of Deutsch and Mertz (*17*), was activated by human urokinase (a product of Mochida Pharmaceutical Co., Ltd., Tokyo). Pancreatic and urinary kallikreins from hog sources were highly purified materials and the former was prepared in our laboratory according to the method of Han *et al.* (*18*). The latter was a generous gift from Prof. H. Tschesche, Lehrstuhl für Org. Chemie und Biochemie der Technischen Universität München. Highly purified human urokinase (*19*), which has a molecular weight of 34,000, was a kind gift from Dr. N. Ogawa, Mochida Pharmaceutical Co.

Enzyme assays were conducted at 37°C using 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl and 10 mM $CaCl_2$, in a total volume of 2.5 ml. Substrate was first dissolved in DMSO and the solution was diluted to give a final concentration of 0.1 mM, using the buffer described above. The reaction was started by the addition of 10 μl of enzyme and the fluorescence of 7-amino-4-methylcoumarin produced was monitored using a Hitachi fluorescence spectrophotometer, model MPF-2A,

TABLE I. Hydrolysis[a] of peptide-MCAs by proteinases with limited specificity.

Group	Substrates	α-Thrombin	Factor Xa	Kallikreins			Urokinase	Plasmin
				Plasma	Pancreatic	Urinary		
A-1	Boc-Val-Pro-Arg-MCA	60.0	0.4	ND[b]	ND	ND	ND	0.3
A-2	Z-Pro-Arg-MCA	6.8	ND	ND	ND	ND	ND	ND
B-1	Boc-Ile-Glu-Gly-Arg-MCA	0.2	2.4	ND	ND	ND	0.6	ND
B-2	Acetyl-Glu-Gly-Arg-MCA	0.1	0.7	ND	ND	ND	1.5	ND
B-3	Boc-Val-Leu-Gly-Arg-MCA	0.3	1.7	0.3	ND	ND	0.4	ND
B-4	Boc-Leu-Gly-Arg-MCA	0.8	1.7	0.1	ND	ND	0.9	ND
B-5	Z-Leu-Gly-Arg-MCA	1.1	2.2	0.5	ND	—[c]	0.8	ND
B-6	Boc-Val-Ser-Gly-Arg-MCA	0.2	0.4	0.1	ND	ND	2.5	ND
B-7	Boc-Ser-Gly-Arg-MCA	0.8	2.8	0.3	ND	ND	0.8	ND
B-8	Boc-Ser(0-Bzl)-Gly-Arg-MCA	1.3	3.0	0.3	ND	ND	0.5	ND
C-1	Pro-Gly-Arg-MCA	0.1	0.1	0.1	ND	ND	0.4	ND
C-2	Z-Pro-Gly-Arg-MCA	0.8	0.2	0.1	ND	ND	ND	ND
C-3	Glutaryl-Gly-Arg-MCA	ND	0.1	ND	ND	ND	6.2	ND
C 4	Glu-Gly-Arg-MCA	0.1	0.2	ND	ND	ND	5.5	ND
C-5	Z-Gly-Arg-MCA	0.2	0.1	ND	ND	ND	0.6	ND
C-6	Gly-Arg-MCA	ND	ND	ND	ND	ND	0.5	ND
D 1	Z-Pro-Phe-Arg-MCA	0.1	0.1	2.9	2.9	4.3	ND	0.2
D-2	Pro-Phe-Arg-MCA	0.1	0.2	3.3	6.3	9.7	ND	0.4
D-3	Z-Phe-Arg-MCA	ND	0.2	4.6	0.8	0.5	ND	0.3
D-4	Phe-Arg-MCA	ND	—[c]	0.2	0.2	0.2	ND	ND

[a] Values are expressed as μmoles hydrolyzed per min per mg protein (α-thrombin, factor Xa, urinary kallikrein, and urokinase) or absorbance unit=1.0 at 280 nm (plasma kallikrein, pancreatic kallikrein, and plasmin). [b] ND, not detectable; Boc, *tert*-butoxycarbonyl; Z, carbobenzoxy; Bzl, benzyl. [c] —, not determined.

equipped with a recorder. The measurements were carried out with excitation at 380 nm and emission at 460 nm. The instrument was standardized so that at 10 μM solution of MCA in 0.1% DMSO gave 1.0 relative fluorescence unit.

Table I shows a comparison of the rates of hydrolysis of different peptide-MCA substrates by α-thrombin, factor Xa, various kallikreins, urokinase, and plasmin. The rates are expressed as μmoles hydrolyzed per min per mg protein or absorbance unit=1.0 at 280 nm. Under the assay conditions used, the linearities of the rates of 7-amino-4-methylcoumarin released *versus* incubation time (within 10 min) and various enzyme concentrations (at least 10-fold range) were satisfactory for all the enzymes, although the data are not shown.

For α-thrombin, Boc-Val-Pro-Arg-MCA (A-1 in Table I) was specific and no detectable hydrolysis was observed with plasma and tissue kallikreins, and urokinase. Factor Xa showed activity of less than 1% of that of α-thrombin. This tripeptide-MCA was based on the information from the COOH-terminal sequence of the "activation peptide," which is liberated during the conversion of plasma factor XIII into its active transglutaminase in the presence of thrombin (*20*). The importance of the Pro-Arg sequence as P_1 and P_2 binding sites for α-thrombin has been suggested (*21*).

Factor Xa hydrolyzed all the peptide-MCAs having the COOH-terminal Gly-Arg sequence, although these substrates were essentially resistant to plasmin and tissue and plasma kallikreins. Of the substrates, Boc-Ile-Glu-Gly-Arg-MCA (B-1), Boc-Ser-Gly-Arg-MCA (B-7), and Boc-Ser(0-Bzl)-Gly-Arg-MCA (B-8) seemed to be the most specific for factor Xa. The tetrapeptide sequence of B-1 originates from the sequence located close to the cleavage site required for the activation of prothrombin by factor Xa (*22*).

Substrates containing the COOH-terminal Pro-Phe-Arg sequence of bradykinin were all susceptible to hydrolysis by plasma and tissue kallikreins, although there is a significant difference in the rates of hydrolysis by the different kallikreins. These peptide-MCA substrates were slightly hydrolyzed by α-thrombin, factor Xa, and plasmin but not by urokinase. Plasma kallikrein hydrolyzed Z-Phe-Arg-MCA (D-3) more readily than Z-Pro-Phe-Arg-MCA (D-1), while the inverse was observed with tissue kallikreins. There was no specificity difference between pancreatic and urinary kallikreins towards these peptide-MCA substrates.

Urokinase similar to factor Xa showed a broad specificity towards all the peptide-MCA substrates having the COOH-terminal Gly-Arg sequence. Among them, glutaryl-Gly-Arg-MCA (C-3) was found to be the best substrate for urokinase. This substrate is useful for urokinase assay.

Plasmin hydrolyzed some of the peptide-MCA substrates shown in Table I. However, all the substrates containing COOH-terminal arginine were essentially resistant to plasmin, indicating that a specific assay for each enzyme is possible even in the presence of plasmin. More detailed studies including the kinetic parameters of hydrolysis for the enzymes used here are now under investigation.

We are indebted to Professor S. Fujii of the institute, for his support and encouragement. We also wish to express our thanks to Professor H. Tschesche and Dr. N. Ogawa, for providing the purified urinary kallikrein and urokinase, respectively. We thank Prof. L.M. Greenbaum, Dept. of Pharmacology, College of Physicians & Surgeons, Columbia University, for his kind help in preparing the English manuscript.

REFERENCES

1. Svendsen, L., Blombäck, B., Blombäck, M., & Olsson, P. (1972) *Thrombosis Research* 1, 267–278
2. Claeson, G., Aurell, L., Karlsson, G., & Fiberger, P. (1977) in *New Methods for the Analysis of Coagulation Using Chromogenic Substrates* (Witt, I., ed.) pp. 37–48, Walter de Gruyter, Berlin, New York
3. Amundsen, E. & Svendsen, L. (1977) in *New Methods for the Analysis of Coagulation Using Chromogenic Substrates* (Witt, I., ed.) pp. 211–219, Walter de Gruyter, Berlin, New York
4. Svendsen, L. (1977) in *New Methods for the Analysis of Coagulation Using Chromogenic Substrates* (Witt, I., ed.) pp. 251–259, Walter de Gruyter, Berlin, New York
5. Bergström, K. & Blombäck, M. (1974) *Thrombosis Research* 4, 719–729
6. Meier, H.L., Pierce, J.V., Colman, R.W., & Kaplan, A.P. (1977) *J. Clin. Invest.* 60, 18–31
7. Abildgaad, U., Lie, M., Teisen, A.N., & Ødegård, O.R. (1975) In Abstracts, Vth Congr. Int. Soc. Thrombosis & Haemostasis, p. 112, Paris

8. Edy, J., De Cock, F., & Collen, D. (1976) *Thrombosis Research* **8**, 513–518
9. Teisen, A.N., Lie, M., & Abildgaad, U. (1976) *Thrombosis Research* **8**, 413–416
10. Zimmerman, M., Yurewicz, E.C., & Patel, G. (1976) *Anal. Biochem.* **70**, 258–262
11. Zimmerman, M., Ashe, B., Yurewicz, E.C., & Patel, G. (1977) *Anal. Biochem.* **78**, 47–51
12. Kanaoka, Y., Takahashi, T., & Nakayama, H. (1977) *Chem. Pharm. Bull.* **25**, 362–363
13. Kato, T., Nagatsu, T., Kimura, T., & Sakakibara, S. (1977) *Seikagaku* (in Japanese) **49**, 990
14. Morita, T., Iwanaga, S., & Suzuki, T. (1974) *J. Biochem.* **76**, 1031–1048
15. Morita, T. & Iwanaga, S., *J. Biochem.* in press
16. Takahashi, H., Nagasawa, S., & Suzuki, T. (1972) *J. Biochem.* **71**, 471–483
17. Deutsch, D.G. & Mertz, E.T. (1970) *Science* **170**, 1095–1096
18. Han, Y.N., Kato, H., Iwanaga, S., & Komiya, M. (1978) *J. Biochem.* **83**, No. 1, in press
19. Ogawa, N., Yamamoto, H., Katamine, T., & Tajima, H. (1975) *Thrombosis Diathes. Haemorrh.* (Stuttg.) **34**, 194–209
20. Nakamura, S., Iwanaga, S., & Suzuki, T. (1975) *J. Biochem.* **78**, 1247–1266
21. Gráf, L., Barát, E., Borvendég, J., Hermann, I., & Patthy, A. (1976) *Eur. J. Biochem.* **64**, 333–340
22. Magnusson, S., Sottrup-Jensen, L., & Petersen, T.E. (1975) in *Prothrombin and Related Coagulation Factors* (Hemker, H.C. & Veltkamp, J.J., eds.) pp. 25–46, Leiden University Press, Leiden