Chem. Pharm. Bull. 30(5)1771—1775(1982)

Action of Various Kallikreins and Related Enzymes on Synthetic Arginine and Lysine Derivatives as Substrates[1)]

Yoshifumi Matsuda,*,[a,2a)] Kyosuke Miyazaki,[a] Yukio Fujimoto,[b] Yoshio Hojima,[a,2b)] and Hiroshi Moriya[a]

Faculty of Pharmaceutical Sciences, Science University of Tokyo,[a] Ichigaya Funagawara-cho, Shinjuku-ku, Tokyo 162, Japan and Hokkaido Institute of Pharmaceutical Sciences,[b] Katsuraoka-cho, Otaru-shi, Hokkaido 047-02, Japan

(Received September 28, 1981)

The substrate specificities of some glandular and plasma kallikreins, bovine trypsin and plasmin, and boar acrosin were investigated using five tripeptidyl-*p*-nitroanilides and ten arginine and lysine ester derivatives as substrates. Of the substrates examined, *N*-α-benzoyl-L-arginine methyl and ethyl esters(Bz–Arg–Me and Bz–Arg–Et) and *N*-α-acetyl-L-arginine methyl ester(Ac–Arg–Me) were the most effective for the pancreatic and intestinal kallikreins, while *N*-α-tosyl-L-arginine methyl ester(Tos–Arg–Me) and Bz–Arg–Me were effectives as substrates for the human salivary and guinea-pig coagulating gland kallikreins, respectively. Ac–Arg–Me and *N*-α-acetyl-glycyl-L-lysine methyl ester-(Ac–Gly–Lys–Me) were also good substrates for the boar acrosin.

All of the tripeptidyl-*p*-nitroanilide substrates examined were hydrolyzed relatively ineffectively by glandular kallikreins and boar acrosin. However, D-valyl-L-leucyl-L-arginine-*p*-nitroanilide(Val–Leu–Arg–*p*NA) and D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide(Pro–Phe–Arg–*p*NA) were found to be moderately useful substrates for various kallikreins and related enzymes.

Keywords——kallikrein; trypsin-like enzyme; substrate specificity; arginine and lysine derivative substrates; esterolytic activity; amidolytic activity

Kallikrein (EC 3.4.21.8) is known to hydrolyze *N*-substituted arginine and lysine esters,[3)] in the same way as trypsin (EC 3.4.21.4) and other trypsin-like enzymes. Two types of kallikreins are known, glandular kallikrein, which liberates lysyl-bradykinin (kallidin) from plasma kininogens, and plasma kallikrein that liberates bradykinin from plasma kininogens. Furthermore, some properties of glandular kallikreins (molecular weight, isoelectric point, behavior with inhibitors *etc.*) are different from those of plasma kallikrein. Differences of hydrolytic properties between the types of kallikreins have also been reported.[4)] However, the differences of hydrolytic actions by kallikreins present in various animal species and organs were not well characterized.

In the present paper we report on the substrate specificity of the esterolytic and amidolytic actions of some glandular kallikreins from various sources, plasma kallikreins and related enzymes such as trypsin, plasmin and acrosin.

Materials and Methods

Enzymes——Highly purified glandular (or tissue) kallikreins from human urine, dog urine, dog kidney, hog pancreas (types A and B), dog pancreas, rat pancreas, rat intestine, human salivary gland, cat submaxillary gland and guinea-pig coagulating gland were prepared in our laboratory.[5)] Human and rat plasma kallikreins were partially purified from the citrated plasma by acetone fractionation and diethylaminoethyl (DEAE)-cellulose and CM-Sephadex C-50 chromatographies.

Bovine pancreatic trypsin was purified by affinity chromatography using the method of Kasai and Ishii[6)] from the crude commercial preparation. Highly purified boar acrosin was kindly supplied by Prof. Dr. H. Fritz,[7)] University of Munich (München), West Germany. Bovine plasmin was kindly supplied by Green Cross Drug Mfg. Co., Osaka, Japan.

Chemicals——*N*-α-Tosyl-L-arginine methyl ester(Tos–Arg–Me), *N*-α-benzoyl-L-arginine ethyl ester(Bz–

Arg–Et), *N*-α-acetyl-L-arginine methyl ester(Ac–Arg–Me) L-arginine methyl ester(Arg–Me), *N*-α-tosyl-L-lysine methyl ester(Tos–Lys–Me), acetyl-glycyl-L-lysine methyl ester(Ac–Gly–Lys–Me) and L-lysine methyl ester(Lys–Me) were obtained from the Peptide Institute Inc., Osaka, Japan. L-Phenylalanyl-L-arginine methyl ester(Phe–Arg–Me) and *N*-α-carbobenzoxy-L-arginine methyl ester(Z–Arg–Me) were kindly supplied by Dr. K. Dobashi, Teikoku Hormone Co., Tokyo, Japan. *N*-α-Benzoyl-L-arginine methyl ester(Bz–Arg–Me) was obtained from Serva Chemical Co., West Germany.

D-Valyl-L-leucyl-L-arginine-*p*-nitroanilide(Val–Leu–Arg–*p*NA) and D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide(Pro–Phe–Arg–*p*NA) were supplied by Kabi Chemical Co., Switzerland. *N*-α-Benzoyl-D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide(Bz–Pro–Phe–Arg–*p*NA), *N*-α-tosyl-glycyl-D-prolyl-L-arginine-*p*-nitroanilide(Tos–Gly–Pro–Arg–*p*NA) and *N*-α-tosyl-glycyl-D-prolyl-L-lysine-*p*-nitroanilide(Tos–Gly–Pro–Arg–*p*NA) were from Pentapharm Chemical Co., Sweden. All other chemicals used were of analytical reagent grade from commercial sources.

Enzyme Assay Methods——Vasodilator activity was assayed by the method of Moriya *et al.*,[8] by measuring the increase of femoral artery blood flow in the dog, and expressed in terms of the kallikrein unit (KU).

Esterolytic activities were determined by two methods. Bz-Arg-Et-hydrolyzing activity wasmeasured by the spectrophotometric method,[9] and other esterolytic activities were assayed by the colorimetric method with chromotropic acid.[10] All esterolytic activities were expressed in terms of μmol of substrate hydrolyzed per min at pH 8.0, 30°C (or 25°C for Bz-Arg-Et esterolytic activity).

Amidolytic activities were determined by the modified method of Amundsen *et al.*[11] using tripeptidyl-*p*-nitroanilides as substrates. Activities of amidolysis were expressed in terms of μ mol of substrate hydrolyzed per min at pH 8.0, 30°C.

Results and Discussion

The results of our survey of the action of the glandular kallikreins on some arginine and lysine esters are summarized in Table I. The first group of glandular kallikreins was from the urinary organs, human urine, dog's urine and kidney; the second group was from the pancreas (hog, dog and rat) and intestine (rat) and the third group was from human saliva, cat submaxillary and guinea-pig coagulating grands. The substrate concentration in the esterolytic assay was fixed at 5 mM, on the grounds that the *K*m values for usual substrates of kallikreins from various sources did not vary greatly.[12]

The ratio of the hydrolyzing ability towards Bz–Arg–Me to that towards Tos–Arg–Me of the first and third groups were calculated to be 0.6 to 1.6, but those in the second group were 3.4 to 23.3. Ac–Arg–Me, Z–Arg–Me, Bz–Arg–Et were found to be useful substrates for the second group, but were not always useful for the first and third groups. The hydrolyzing activities of glandular kallikreins towards the lysine esters were less than those towards the arginine esters, with some exceptions. The results for some arginine and lysine esters with hog pancreatic kallikrein were in good agreement with those reported by other investigators.[13] Nustad and Pierce reported that Z–Arg–Me was the most effective substrate for rat urinary kallikrein.[14] We found that Z–Arg–Me was also most exsily hydrolyzed by dog urinary kallikrein. However, Z–Arg–Me was hydrolyzed to a lesser extent by human urinary kallikrein than Tos–Arg–Me, and Phe–Arg–Me seemed to be the best substrate for the human urinary kallikrein. These differences in the substrate specificities of closely related kallikreins can be attributed to the species differences.

Substrate specificities of two plasma kallikreins (human and rat), trypsin (bovine pancreatic) and trypsin-like enzymes (bovine plasmin and boar acrosin) are shown in Table II. The arginine and lysine esters were well hydrolyzed by the bovine pancreatic trypsin, and Tos–Arg–Me was the best substrate. Z–Arg–Me and Tos–Arg–Me were hydrolyzed by the two partially purified plasma kallikreins, but the hydrolyzing ability seemed to be much lower than that of the glandular kallikreins. The lysine esters such as Tos–Lys–Me and especially Ac–Gly–Lys–Me were hydrolyzed well by bovine plasmin, and some arginine esters including Z–Arg–Me, Ac–Arg–Me and Bz–Arg–Me were also good substrates for the plasmin. The hydrolyzing abilities of bovine trypsin and plasmin were generally in good agreement with those reported by other investigators.[15] Tos–Arg–Me, Ac–Arg–Me and Ac–Gly–Lys–Me were effectively hydrolyzed by boar acrosin and the best substrate was Ac–Arg–Me.

Table I. Hydrolyzing Activities of Glandular Kallikreins towards Some Arginine and Lysine Esters as Substrates

Substrate	Kallikrein from									
	First Group			Second Group				Third Group		
	Urinary		Renal	Pancreatic			Intestinal	Salivary and Submaxillary		Coagulating gland
	Human	Dog	Dog	Hog	Dog	Rat	Rat	Human	Cat	Guinea-pig
Bz–Arg–Et	6.8	13.8	—a)	97.0	—a)	—a)	—a)	7.6	—a)	1.8
Tos–Arg–Me	14.5	11.3	0.39	12.6	1.7	20.4	13.3	24.3	15.8	3.2
Bz–Arg–Me	8.8	17.2	0.63	294	22.9	58.0	110	23.0	18.4	3.7
Ac–Arg–Me	5.7	7.0	0.36	277	17.7	80.0	224	8.6	Trace	0.4
Z–Arg–Me	10.8	37.4	—a)	32.6	21.2	41.5	—a)	17.8	—a)	—a)
Phe–Arg–Me	20.3	—a)	—a)	—a)	—a)	—a)	—a)	—a)	—a)	—a)
Arg–Me	1.9	0.5	Trace	3.4	4.1	1.3	—a)	10.3	1.6	Trace
Tos–Lys–Me	3.9	3.9	0.30	3.1	—a)	1.8	6.4	15.0	1.8	1.2
Ac–Gly–Lys–Me	2.5	6.9	0.15	24.6	1.3	14.0	25.3	10.0	Trace	0.20
Lys–Me	0.3	0.3	Trace	1.3	2.7	Trace	—a)	12.6	3.8	Trace

a)—:not measured.
Esterolytic activities were expressed in terms of μmol of substrate hydrolyzed/min/mg of enzyme at pH 8.0, 30°C, and substrate concentrations were 25 mM each. For Bz–Arg–Et hydrolyzing activity, substrate concentration was 0.5 mM and the temperature was 25°C.[9]

Table II. Hydrolysis of Some Arginine and Lysine Esters by Two Plasma Kallikreins and Trypsin-like Enzymes

Substrate	Enzyme				
	Plasma kallikrein		Trypsin-like enzyme		
	Human	Rat	Trypsin Bovine pancreas	Plasmin Bovine plasma	Acrosin Boar sperm
Tos–Arg–Me	0.094	0.158	224	1.18	9.9
Bz–Arg–Me	0.062	0.156	67	1.10	10.0
Ac–Arg–Me	0.020	0.062	78	1.32	16.4
Z–Arg–Me	0.130	0.174	—a)	1.28	—a)
Arg–Me	0.094	0.280	38	0.12	0.56
Tos–Lys–Me	0.060	0.260	124	2.20	4.3
Ac–Gly–Lys–Me	Trace	0.066	33	7.4	15.5
Lys–Me	Trace	Trace	36	0.47	0.30

a)—:not measured.
Activity was expressed in terms of μmol of substrate hydrolyzed/min/mg of enzyme at pH 8.0, 30°C and substrate concentrations were 25 mM each.

Amidolytic activities of human and dog urinary, dog renal, hog pancreatic hog pancreatic types A and B (heterogeneous forms), rat intestinal and human salivary kallikreins, and boar acrosin are summarized in Table III. The amidolytic activities towards the tripeptidyl-*p*-nitroanilides were lower than the esterolytic activities towards Tos–Arg–Me and Bz–Arg–Et (or Me) as substrates (Tables I and II). However, among these tripeptidyl-*p*-nitroanilide substrates, Val–Leu–Arg–*p*NA was the most effective substrate with one exception, and Tos–Gly–Pro–Arg–*p*NA and Tos–Gly–Pro–Lys–*p*NA were ineffective substrates. Pro–Phe–Arg–*p*NA, which had been synthesized as a substrate for plasma kallikreins, was also hydrolyzed by the rat intestinal, hog pancreatic and hog pancreatic types A and B (heterogeneous forms) kallikreins. These results also suggested that the enzymatic actions of the pancreatic

Table III. Amidolytic Activities of Glandular Kallikreins and Boar Acrosin

Substrate	Enzyme								
	Glandular kallikrein								Acrosin
	Urinary		Renal	Pancreatic			Intestinal	Salivary	Sperm
	Human	Dog	Dog	Hog	Hog–A[a]	Hog–B[a]	Rat	Human	Boar
Vasodilator activity	382	1250	22	—[c]	1350	1400	—[c]	880	—[c]
Tos–Arg–Me[b]	14.5	11.3	0.39	12.6	11.1	11.5	13.3	24.3	9.9
Val–Leu–Arg–*p*NA	0.59	2.6	0.027	3.09	2.36	2.86	1.68	1.84	0.62
Pro–Phe–Arg–*p*NA	0.072	0.43	0.014	2.33	1.73	1.88	2.70	0.14	0.59
Bz–Pro–Phe–Arg–*p*NA	0.007	0.029	—[c]	0.030	0.033	0.032	—[c]	—[c]	0.003
Tos–Gly–Pro–Arg–*p*NA	0.003	0.013	—[c]	0.037	0.025	0.018	—[c]	—[c]	0.002
Tos–Gly–Pro–Lys–*p*NA	0.001	0.008	—[c]	0.010	0.003	0.002	—[c]	—[c]	0.15

a) Hog pancreatic kallikrein types A and B, see the text.
b) From Tables I and II.
c)—:not measured.
Amidolytic activities were expressed in terms of μmol of substrate hydrolyzed/min/mg of enzyme and substrate concentrations were 2.0 mM each. Tos–Arg–Me hydrolyzing and vasodilator activities are given as μmol/min/mg of enzyme and kU/mg of enzyme, respectively.

and intestinal (second group), and the urinary (first group) or the submaxillary (third group) kallikreins were different. The substrate specificities of the human urinary and some glandular kallikreins towards the tripeptidyl-*p*-nitroanilide substrates were in good agreement with other reports,[11,16] except for minor differences with the hog pancreatic kallikreins.

The above results seem to justify the classification of the glandular kallikreins into three groups (urinary organs, pancreas and intestine, and others), based on their esterolytic and amidolytic activities. The glandular kallikreins can be classified into three groups on the basis of the esterolytic activities using Tos–Arg–Me, Bz–Arg–Et (or –Me) and Ac–Arg–Me as substrates, and a clear difference in the substrate specificity of the esterolytic actions between the dog urinary organ and pancreatic kalikreins was recognized in this investigation. On the other hand, a recent report suggested that the urinary kallikrein may originate in part from the pancreas and salivary grands, at least as far as the hog species is concerned.[17]

References and Notes

1) Abbreviations: KU, kallikrein unit; Tos–Arg–Me, N-α-tosyl-L-arginine methyl ester; Bz–Arg–Et, *N*-α-benzoyl-L-arginine ethyl ester; Bz–Arg–Me, *N*-α-benzoyl-L-arginine methyl ester; Ac–Arg–Me, *N*-α-acetyl-L-arginine methyl ester; Ac–Aly–Lys–Me, *N*-α-acetyl-glycyl-L-lysine methyl ester; Phe–Arg–Me, L-phenylalanyl-L-arginine methyl ester; Z–Arg–Me, *N*-α-carbobenzoxy-L-arginine methyl ester; Tos–Lys–Me, *N*-α-tosyl-L-lysine methyl ester; Arg–Me, L-arginine methyl ester; Lys–Me, L-lysine methyl ester; Val–Leu–Arg–*p*NA, D-valyl-L-leucyl-L-arginine-*p*-nitroanilide; Pro–Phe–Arg–*p*NA, D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide; Bz–Pro–Phe–Arg–*p*NA, N-α-benzoyl-D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide; Tos–Gly–Pro–Arg–*p*NA, *N*-α-tosyl-glycyl-D-prolyl-L-arginine-*p*-nitroanilide; Tos–Gly–Pro[Lys–*p*NA, *N*-α-tosyl-glycyl-D-prolyl-lysine-*p*-nitroanilide.
2) *a*) Present address: *Department of Biochemistry, Meiji College of Pharmacy, Nozawa-cho, Setagaya-ku, Tokyo 154, Japan*; *b*) *Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California, 92037, U.S.A.*
3) M.E. Webster and J.V. Pierce, *Pro. Soc. Exp. Biol. Med.*, **107**, 186 (1961); M. Schachter, *Phys. Rev.*, **49**, 510 (1969).
4) S. Iwanaga, T. Morita, H. Kato, T. Harada, N. Adachi, T. Sugo, I. Maruyama, K. Takada, and S. Sakakibara, "Adv. Exp. Med. Biol. Kinins II," 120A, ed. by S. Fujii, H. Moriya, and T. Suzuki, Plenum Press, New York, 1979, p. 83.
5) H. Moriya, Y. Matsuda, Y. Fujimoto, and C. Moriwaki, "Kininogenases," Vol. 1, ed. by G.L. Haberland, and J.W. Rohen, Springer-Verlag, Berlin, 1972, p. 37; Y. Matsuda, K. Miyazaki, Y. Fujimoto, Y. Ho-

jima, C. Moriwaki, and H. Moriya, *J. Biochem.* (Tokyo), **80**, 671 (1976); H. Moriya and Y. Matsuda, "Kininogenases," Vol. 4, ed. by G.L. Haberland, J.W. Rohen, and T. Suzuki, Springer-Verlag, Berlin, 1977, p. 29; C. Moriwaki, K. Miyazaki, Y. Matsuda, H. Moriya, Y. Fujimoto, and H. Ueki, *J. Biochem.* (Tokyo), **80**, 1277 (1976); H. Moriya, Y. Fukuoka, Y. Hojima, and C. Moriwaki, *Chem. Pharm. Bull.*, **26**, 3178 (1978); Y. Hojima, M. Yamashita, N. Ochi, C. Moriwaki, and H. Moriya, *J. Biochem.* (Tokyo), **81**, 599 (1977); Y. Hojima, C. Moriwaki, and H. Moriya, *Chem. Pharm. Bull.*, **23**, 1128 (1975); C. Moriwaki, H. Fujimori, and Y. Toyono, "Kininogenases," Vol. 4, ed. by G.L. Haberland, J.W. Rohen, and T. Suzuki, Springer-Verlag, Berlin, 1977, p. 283; Y. Fujimoto, H. Moriya, and C. Moriwaki, *J. Biochem.* (Tokyo), **74**, 239 (1973); Y. Fukuoka, Y. Hojima, S. Miyaura, and C. Moriwaki, *ibid.*, **85**, 549 (1978); C. Moriwaki, N. Watanuki, Y. Fujimoto, and H. Moriya, *Chem. Pharm. Bull.*, **22**, 628 (1974).
6) K. Kasai and S. Ishii, *J. Biochem.* (Tokyo), **78**, 653 (1975).
7) S. Palm and H. Fritz, "Kininogenases," Vol. 2, ed. by G.L. Haberland, J.W. Rohen, C. Schirren, and P. Huber, Springer-Verlag, Berlin, 1974, p. 17.
8) H. Moriya, K. Yomazaki and H. Fukushima, *J. Biochem.* (Tokyo), **58**, 201 (1965).
9) G.W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta*, **16**, 570 (1955).
10) C. Moriwaki, Y. Hojima, N. Inoue, and H. Moriya, *Yakugaku Zasshi*, **91**, 413 (1970).
11) E. Amundsen, J. Puter, P. Firberger, M. Knos, M. Larsbraten, and G. Claseson, "Adv. Exp. Med. Biol., Kinin II," 120A ed. by S. Fujii, H Moriya, and T. Suzuki, Plenum Press, New York, 1979, p. 83.
12) F. Fiedler, "Handb. Exp. Pharmacol., XXV," ed. by E.G. Erdös, Springer-Verlag, Berlin, 1979, p. 103.
13) T. Takami, *J. Biochem.* (Tokyo), **66**, 651 (1969); F. Fiedler, G. Leysath, and E. Werle, *Eur. J. Biochem.*, **36**, 152 (1973).
14) K. Nustad and J.V. Pierce, *Biochemistry*, **13**, 2312 (1974).
15) W. Troll, S. Sherry, and J. Wachman, *J. Biol. Chem.*, **208**, 85 (1954); S. Sherry, M. Alkjaersig, and A.P. Fletcher, *Am. J. Physiol.*, **209**, 577 (1965).
16) T. Morita, H. Kato, S. Iwanaga, K. Takada, T. Kimura, and S. Sakakibara, *J. Biochem.* (Tokyo), **82**, 1495 (1977).
17) F. Fiedler and W. Gerhard, *Z. Physiol. Chem.*, **361**, 1661 (1980).