STRIKING SUSCEPTIBILITY OF A KININ-YIELDING PENTADECA-PEPTIDE TO TRYPTIC HYDROLYSIS*

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SUMMARY: Hydrolysis of Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe--Arg-Ser-Val-Gln-Val-Ser by trypsin (EC 3.4.21.4) yields lysyl-bradykinin by rupture of the Arg-Ser bond. The k_{cat}/K_m value found for this hydrolysis was 1.4×10^{10} $M^{-1} \times sec^{-1}$, which is 10^{-5}-fold higher than that obtained for the hydrolysis of bradykinyl-Ser-Val-Gln-Val-Ser. This effect was abolished by acetylation of the lysine amino groups of the pentadecapeptide. Contrarywise, the esterolytic activity of trypsin on bradykinin methyl ester was the same as in lysyl-bradykinin methyl ester. The high susceptibility of Lys-bradykinyl-Ser-Val-Gln-Val-Ser to trypsin catalysis is striking because: a) it constitutes the first example that an amino acid residue distant from the bond split may enhance trypsin catalysis; b) this pentadeca-peptide is the best synthetic substrate so far described for trypsin and c) the value of k_{cat}/K_m for its hydrolysis is unusually high for proteases.

INTRODUCTION: In most of the kinetic studies on trypsin catalysis, N-substituted arginine or lysine esters and amides have been used as substrates. Few kinetic data have been reported for the hydrolysis of peptide bonds by trypsin. Yamamoto & Isumiya (1) studied the susceptibility to trypsin of the homologous peptides $(Gly)_2$-Lys-$(Gly)_n$, where n = 1 to 4, and Nishino, Mitsuyasu and Isumiya (2) studied the hydrolysis of synthetic insulin B chain fragments, with four and five aminoacid residues.

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We have recently (3) reported on the kinetics of the tryptic hydrolysis of two bradykinin containing peptides, BK-R and BK-OMe[1]. This paper presents the kinetic constants for the tryptic hydrolysis of Lys-BK-R and Lys-BK-OMe, and it discusses the effect of the N-terminal lysine residue on the hydrolysis of these peptides.

MATERIALS AND METHODS: Peptides were synthesized by the solid phase method (4, 5). After completion of the last coupling reactions for the synthesis of BK-R and of BK-OMe, parts of the resins were used respectively, for the synthesis of Lys-BK-R and Lys-BK-OMe. The cleavages and deprotections of the esters and of the tetra and pentadecapeptides from the solid support were made as described before (3). The peptides were purified by ion exchange chromatography until the previously described (6) purity criteria were met. The properties of BK-R and BK-OMe have been described before (3). Table I gives the properties of Lys-BK-R and Lys-BK-OMe. The relatively low peptide contents, which were identical as determined by three different methods, are due to the peptides being in the tetra-acetate form and to their water and salt contents. Ac-Lys-(Ac)-BK-R was prepared by acetylation of Lys-BK-R by the method of Vithayathil and Richards (7) until free amino groups were not detectable by fluorescamine. The concentrations of Ac-Lys(Ac)-BK-R solutions were determined by amino group analysis with TNBS[1], after complete hydrolysis with trypsin.

Enzymes - Twice crystallized, dialyzed, salt free bovine trypsin from Worthington Biochemical Corp., Freehold, N.J., was used. We verified that it was free of chymotrypsin, carboxypeptidase B and aminopeptidase activities since prolongued incubations of BK-H and Lys-BK-H with high concentrations of the trypsin did not lead to any change in their biological activity. A stock solution (1 mg/ml) of the enzyme was made in 1 x 10^{-3} M HCl and kept at 4-5°C for not more than a week. After determining the active enzyme concentration with NPGB[1] according to Chase and Shaw (8), diluted solutions were prepared in cold 10^{-3} M HCl immediately before use and kept in silicone-coated glass tubes in an ice bath. The horse urinary kallikrein (EC 3.4.21.8) preparation used was the same one described in a previous paper (3).

Other reagents - TNBS was prepared as described by Golumbic, Fruton and Bergmann (9) and purified according to Fields (10); Tos-Arg-OMe was purchased from Sigma Chemical Co., USA; fluorescamine was a generous gift from Dr. S. Udenfriend, Roche Institute, USA; NPGB was kindly supplied by Dr. E. Shaw, Brookhaven National Laboratory, USA.

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[1] Abbreviations: BK = bradykinyl:(Arg-Pro-Pro-Gly-Phe-Ser-Pro--Phe-Arg-); R = -Ser-Val-Gln-Val-Ser; TNBS: trinitrobenzenesulfonic acid; NPGB: p-nitrophenyl-p'-guanidino benzoate.

Table I - Properties of Lys-BK-OMe and Lys-BK-R

	Aminoacid composition[a] (mol/mol peptide)								Peptide content[b] %(w/w)			Eletrophoretic mobility[c] pH 5	R_f[d]				Biological Activity[e]
Peptide	Lys	Arg	Pro	Gly	Phe	Ser	Val	Glu	I	II	III	R_{Arg}	A	B	C	D	
Lys-BK-OMe	1.03	2.10	2.84	1.02	2.09	0.93	-	-	67	70	69	0.96	0	0.03	0	-	0.06
Lys-BK-R	0.81	2.05	2.98	1.16	1.96	2.84	2.13	1.05	68	63	60	0.68	0	0.18	0.36	0.12	nil

a) Aminoacid analysis of 72 hr hydrolysate (6N HCl, 110°C) made in a Beckman 120C aminoacid analyzer.

b) Determined by I) aminoacid analysis; II) TNBS method; III) bioassay of Lys-BK-R released after complete hydrolysis by trypsin.

c) High voltage electrophoresis migration relative to Arg , developed with ninhydrin and Sakaguchi reagents.

d) Thin layer chromatography on 0.1 mm silica gel plates (Eastman "Chromagram") with solvent systems A) n-butanol-acetic acid-water (4:1:1); B) n-butanol:ethylacetate:acetic acid-water (1:1:1:1); C) n-butanol:pyridine:acetic acid:water (30:20:6:4); D) chloroform:methanol:ammonia (2:2:1). Developped with fluorescamine.

e) Assayed on the ileum preparation; Lys-BK-H activity = 1

Table II - Kinetics constants for the hydrolysis at 30°C, pH 8.1 of synthetic peptides by trypsin and horse urinary kallikrein

Enzyme	[E] (M)	Substrate	[S] range[a] (10^{-4} M)	K_m^b (10^{-3}M)	k_{cat}^b (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})	Assay
Tryp[c]	2.4×10^{-14}	Lys-BK-R	0.3-0.8	0.12±0.05	(17± 5)x10^5	1.4×10^{10}	TNBS
Tryp	1.3×10^{-13}	Lys-BK-R	0.7-2.1	0.09±0.02	(8±0.9)x10^5	0.9×10^{10}	Bioassay
Tryp	2.7×10^{-9}	Ac-Lys(Ac)-BK-R	0.7-3.5	0.10±0.005	40±0.8	4×10^5	Fluram[d]
Tryp	6.0×10^{-9}	BK-R	0.9-1.7	1.70±0.27	250±31	1.5×10^5	TNBS
Tryp	6.0×10^{-9}	BK-R	0.9-4.4	0.91±0.22	148±37	1.6×10^5	Bioassay
HoUK[c]	1.6×10^{-8}	Lys-BK-R	0.7-2.8	0.25±0.03	21±1.5	8.5×10^4	Bioassay
HoUK	1.6×10^{-8}	BK-R	0.85-1.70	0.44±0.11	14±2.7	3.2×10^4	Bioassay

a) Concentrations of Lys-BK-R and BK-R stock solutions were determined by aminoacid analysis, reaction with TNBS and from the amount of liberated kinin following complete hydrolysis with trypsin; for Ac-Lys(Ac)-BK-R, see text.

b) Average values ± S.D.

c) Tryp: trypsin; HoUK: horse urinary kallikrein.

d) Fluorescamine

Hydrolysis rates - For measuring the hydrolysis of Lys-BK-R and BK-R the following conditions were used: 200 µl of peptide solution in 0.017 M N-ethyl morpholine acetate buffer, 0.025 M $CaCl_2$, pH 8.1, were incubated at 30°C with 20 µl of trypsin or 10 µl of kallikrein solutions. Final concentrations of enzymes and substrates are given in Table II. Aliquots were removed at different times up to 20 minutes of reaction, for the bioassay of the kinin formed and for measuring the increase of amino groups. Single and double doses of the standard kinins and conveniently diluted aliquots from the incubates were assayed on the isolated guinea pig ileum preparation. The amino groups were measured by the TNBS method as described by Field (10). The hydrolysis of Ac-Lys(Ac)-BK-R was followed only by measuring liberated amino groups with the fluorescamine method (11), because Ac-Lys(Ac)-BK-OH has no detectable biological activity. Initial rates for the hydrolysis of Lys-BK-OMe and BK-OMe were determined by Schwabe's method (12) in 0.025 M $CaCl_2$, 2 x 10^{-4} M barbital indicator, pH 8.1 at 30°C, with the enzyme and substrate concentrations given in Table III. The amounts of liberated acid were calculated from the measured absorbances at 245 nm, using a calibration curve of peptide-barbital solution titrated spectrophotometrically with standard HCl. Initial rates were also determined by bioassay of the kinin formed. Hydrolysis of Tos-Arg-OMe in 0.1 M NaCl was followed with a Radiometer autotitrator (TTT1C, SBR 2c and AB12 autoburette, 0.25 ml capacity) by titration with 0.01 M NaOH at pH 8, 30°C in 3 ml final volume.

Table III - Kinetic constants for the hydrolysis at 30°C, pH 8.1 of synthetic esters by trypsin[a]

Substrate	[S] range (10^{-5}M)	K_m[b] (10^{-6}M)	k_{cat}[b] (sec^{-1})	$10^{-6} \times k_{cat}/K_m$ (M^{-1} sec^{-1})	Assay
BK-OMe	0.7-2.7	25±0.2	115±4.6	4.6	A_{245}
BK-OMe	0.7-1.7	52±14	225±52	4.3	Bioassay
Lys-BK-OMe	0.6-2.9	100±19	194±31	1.9	Bioassay
Tos-Arg-OMe	7.5-15	41± 5	160± 4	3.9	Titration

a) The trypsin concentration was 1 x 10^{-9} M for the peptide esters and 1.25 x 10^{-9} for Tos-Arg-OMe.

b) Mean values ± S.D.

Kinetic constants - K_m and V_{max} were calculated by a weighted least-squares program described by Wilkinson (13) written in Basic for a VARIAN 620/L-100 computer.

RESULTS AND DISCUSSION: As it was expected from the well known trypsin specificity, only the Arg-Ser bonds in Lys-BK-R and Ac-Lys(Ac)-BK-R were cleaved by trypsin. The amounts of amino groups released on complete tryptic hydrolysis of both peptides were consistent with the splitting of a single peptide bond; the biological activity of Lys-BK-R hydrolysate corresponded to the theoretical amount of Lys-BK-OH (0.97 mol/mol) and the Ac-Lys(Ac)--BK-R hydrolysate had no biological activity. The hydrolysis of both peptides followed Michaelis-Menten kinetics in the range of substrate concentrations used. In spite of some discrepancies found for values of K_m and k_{cat} determined with the same peptide, by the two methods (Table II), the differences in the kinetic constants for Lys-BK-R and BK-R are of a much higher magnitude by both methods. Comparison of the parameter k_{cat}/K_m shows that Lys-BK-R is 10^5-fold more susceptible than BK-R to trypsin hydrolysis. It is worth emphasizing that no significant difference in the susceptibilities of Lys-BK-R and BK-R to kallikrein hydrolysis was observed (Table II); this enzyme, like trypsin, splits only the Arg-Ser bond of both substrates.

The similarity of the k_{cat}/K_m values for the tryptic hydrolysis of Ac-Lys(Ac)-BK-R and BK-R hydrolysis (Table II) indicates that either the ε or the α or both free amino groups of the lysine residue are important for the high susceptibility of Lys-BK-R to trypsin catalysis. The k_{cat}/K_m value for Lys-BK-R is the highest so far reported for tryptic hydrolysis and it is higher than the values reported for the best substrates for elastase (14), pepsin (15), chymotrypsin (16) and papain (17).

The extremely large difference between the susceptibilities of Lys-BK-R and BK-R to trypsin is mostly due to the striking difference in the k_{cat} values. This constitutes the first evidence that an aminoacid residue distant from the scissible bond may alter trypsin catalysis. The differences in substrate susceptibilities to trypsin described by Yamamoto et al. (1) and Nishino et al. (2) were mainly due to the effect of the free carboxyl group in the vicinity of the lysine and arginine residues.

When the esters BK-OMe and Lys-BK-OMe were used as substrates, no significant differences were found in their susceptibility to trypsin (Table III). The values of k_{cat}/K_m were of the same order of magnitude as those for Tos-Arg-OMe and other N-substituted arginine esters (18). The fact that the k_{cat} value for Lys-BK-R was 10^4-fold higher than the value for Lys-BK-OMe hydrolysis suggests possible differences in the mechanisms of hydrolysis of these substrates by trypsin. In this respect, it is interesting mentioning that Bresler, Krutyakov and Vlasov (19) concluded that a single mechanism may not explain the catalytic cleavage of esters, amides and peptides by trypsin. Kinetic studies on the hydrolysis of bradykinyl and lysyl-bradykinyl amides, in progress in our laboratory, should give useful information on this important point.

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