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A MET-ENKEPHALIN-CONTAINING-PEPTIDE, BAM 22P, AS A NOVEL SUBSTRATE FOR GLANDULAR KALLIKREINS

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SUMMARY : Homogeneous preparations of two well-characterized glandular kallikreins have been examined for their ability to hydrolyze BAM 22P, a methionine-enkephalin-containing-peptide found in the adrenal medulla. Both enzymes cleaved preferentially the Arg^{6}-Arg^{7} bond in this substrate. The specificity constant (kcat/Km) for this cleavage was 86 mM^{-1} sec^{-1} for horse urinary kallikrein and 566 mM^{-1} sec^{-1} for porcine pancreatic kallikrein. These results demonstrate a previously undescribed specificity for glandular kallikreins and suggest a possible role for these widely distributed enzymes in prohormone processing.

Glandular (or tissue) kallikreins (EC 3.4.21.35) have a narrow and peculiar specificity. They cleave an Arg-Ser and a Met-Lys bond in bovine kininogen (for a recent review, see ref. 1). Synthetic peptide substrates with the sequence of bovine kininogen surrounding the cleavage sites (1,2,3,4) have been used for studies on the substrate specificity of glandular kallikreins. As the Met-enkephalin derivative BAM 22P (5) contains methionyl- and arginyl-peptide bonds, we decided to investigate whether it would be a substrate for

Abbreviations : BAM 22P, peptide found in the adrenal medulla (Tyr-Gly-Gly-Phe-Met^{5}-Arg-Arg-Val-Gly-Arg^{10}-Pro-Glu-Trp-Trp-Met^{15}-Asp-Tyr-Gln-Lys-Arg^{20}-Tyr-Gly) ; BK, bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) ; HMW, high molecular weight ; HoU-kallikrein, horse urinary kallikrein ; PoP-kallikrein, porcine pancreatic kallikrein ; HPLC, high performance liquid chromatography ; ME, Methionine-enkephalin (Tyr-Gly-Gly-Phe-Met).

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kallikreins from horse urine and porcine pancreas. The present study provides new data on the specificity of glandular kallikreins and considers the possible involvement of these enzymes in enkephalin biosynthesis.

MATERIALS AND METHODS

Peptides : BAM 22P (5) and standard peptides ME, ME-Arg^{6} and ME-Arg^{6}-Arg^{7} were synthesized by solid phase method and found to be pure by HPLC and aminoacid analyses.

Enzymes :HoU-kallikrein (2.87 U/mg, substrate D-Val-Leu-Arg-p-nitroanilide) was prepared and characterized as previously reported (6). Homogeneous PoP-kallikrein (5.5 U/mg, D-Val-Leu-Arg-p-nitroanilide) was kindly supplied by Bayer AG, Leverkussen, Bayer Werk FRG. The molar concentration of the HoU-kallikrein solution was determined by active site titration with p-nitrophenyl-p-guanidinobenzoate (7) ; for PoP-kallikrein, the molar concentration was calculated from the reported value of its molar absorbancy coefficient at 280 nm (8). Trypsin (EC 3.4.21.4) was obtained from Millipore Co. N.J., U.S.A. TLCK and soybean trypsin inhibitor were purchased from Sigma Co. U.S.A.

High Performance Liquid Chromatography : For the quantitative separation of ME and its derivatives by reverse phase HPLC a 25 x 0.4 cm ultrasphere octyl column (Altex) was used. Elution at 0.5 ml/min., with a linear gradient from 21 % ethanol in 0.4 M triethylamine-formic acid buffer pH 3.0 to 40 % ethanol in the same buffer (0.5 M) was monitored at 280 nm.

Kinetic constant determinations : The initial rates of hydrolysis of BAM 22P by kallikreins (30°C, pH 8.75) were determined by measuring the liberated ME-Arg^{6} by HPLC. Isocratic elution with 21 % ethanol in 0.4 M triethylamine-formic acid pH 3.0 at a flow rate of 0.5 ml/min. was used. The substrate concentrations were in the ranges of 1.41-11.28 uM for pancreatic kallikrein and 2.76-42.3 uM for urinary kallikrein. Km and V were calculated by a weighted least squares program according to Wilkinson (9).

RESULTS AND DISCUSSION

Pure glandular kallikrein preparations from horse urine and porcine pancreas were able to hydrolyse BAM 22P (fig. 1). Identical elution profiles were obtained in the presence of EDTA (10 mM) or on preincubation of enzymes with either TLCK or soybean trypsin inhibitor, showing that these homogeneous kallikrein preparations were free of interfering peptidases, trypsin or trypsin-like proteinases. Fig. 1 (top panel) shows that ME-Arg^{6} was the only ME-containing product formed by PoP-kallikrein. Upon hydrolysis by HoU-kallikrein (middle panel) or by trypsin (bottom panel), ME-Arg^{6} was still the main product. However, HoU-kallikrein liberated small amounts of ME and on tryptic digestion ME-Arg^{6}-Arg^{7} was also formed.

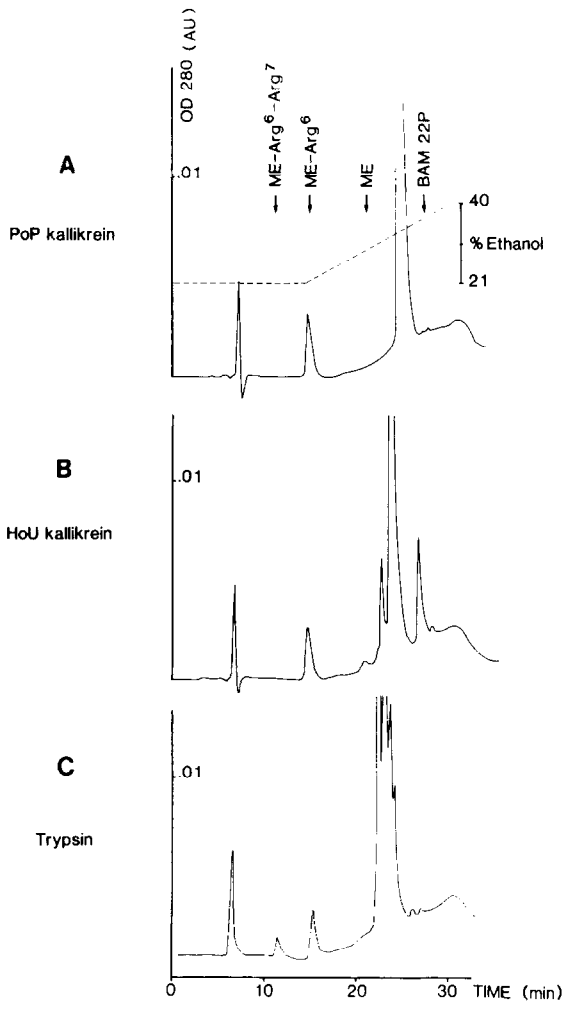

Fig. 1 : HPLC analyses of products from enzymatic cleavage of BAM 22P. BAM 22P (3.52 nmol) was incubated with : a) 40 ng of PoP-kallikrein (top panel) ; b) 37 ng of HoU-kallikrein (middle panel) ; c) 17 ng of trypsin (bottom panel). The incubation mixtures (40 ul in 0.05 M Tris HCl buffer, pH 8.75 for the kallikreins and pH 8.5 for trypsin), were mixed with 85 ul of 0.4 M triethylamine formate buffer pH 3.0 containing 20 % ethanol. Samples (100 ul, 2.8 nmol of BAM 22P) were subjected to HPLC as described in Methods. Elution position of standard peptides are indicated by arrows.

Tryptophan-containing fragments from hydrolysis of BAM 22P by the three proteinases were eluted before BAM 22P. ME-containing peptides and their recoveries obtained on complete hydrolysis of BAM 22P by the two kallikreins and trypsin are shown in Table 1. It is clear that the ratios of ME-Arg^6/ME produced on hydrolysis by HoU-kallikrein depended on the conditions used ; however, even when the maximum amount

TABLE 1: PEPTIDES OBTAINED FROM COMPLETE HYDROLYSIS OF BAM 22P WITH EITHER GLANDULAR KALLIKREIN OR WITH TRYPSIN

	[S] (μM)	[E]:[S]	PEPTIDES (MOLE/MOLE OF SUBSTRATE) ME	ME-Arg^6	ME-Arg^6-Arg^7	Bonds Cleaved
HoU-kallikrein						
a)	44	1:1300	0	1.02	0	Arg-Arg
b)	100	1:676	0.14	0.76	0	Arg-Arg Met-Arg
c)	587	1:1300	0.13	0.80	0	Arg-Arg Met-Arg
PoP-kallikrein						
	88	1:1600	0	1.01	0	Arg-Arg
Trypsin						
	88	1:5000	0	0.72	0.24	Arg-Arg Arg-Val

BAM 22P (S) Sequence: Tyr-Gly-Gly-Phe-Met^5-Arg-Arg-Val-Gly-Arg^{10}-
-Pro-Glu-Trp-Trp-Met^{15}-Asp-Tyr-Gln-Lys-Arg^{20}-Tyr-Gly.
The Met-enkephalin (ME) sequence is underlined;

(14 %) of ME was produced (Table 1, condition b) the predominant product was still ME-Arg^6. This demonstrates the high preference of HoU-kallikrein for the Arg^6-Arg^7 bond over the Met^5-Arg^6 bond. This observation is in accordance with the previously demonstrated (4,5) low susceptibility of the Met-Lys bond in the bradykinin derivatives Gly (Ser)-Met-Lys BK to both HoU- and PoP-kallikreins.

In BAM 22P, the hydrolysis of the Arg^6-Arg^7 bond by both kallikreins followed the Michaelis-Menten kinetics. The kinetic constants in Table 2 demonstrate that this peptide is a good substrate for both enzymes. The specificity constant (kcat/Km) for PoP-kallikrein is of the same order of magnitude as those for bovine HMW kininogen (9) and for Ac-Phe-Arg-Ser-Val-NH_2 (1). This last compound is the best peptide substrate so far described for PoP-kallikrein. BAM 22P, in spite of having a methionyl residue in position P_2 (Schechter & Berger's nomenclature, (11)), which is known to

TABLE 2: KINETIC CONSTANTS (± s.d.) FOR THE HYDROLYSIS OF BAM 22P BY KALLIKREINS AS COMPARED TO THOSE OF SOME STANDARD PEPTIDES AND KININOGENS

	Bond split	Km μMolar	kcat sec^{-1}	kcat/Km $mM^{-1}sec^{-1}$
PoP kallikrein				
BAM 22P	Arg-Arg	5.41±1.45	3.03±0.43	566
Ac-Phe-Arg-Ser-Val-NH_2 (1)	Arg-Ser	40	11.6	290
Bovine HMW kininogen[a] (10)	Met-Lys Arg-Ser	-	0.94	120
HoU kallikrein				
BAM 22P	Arg-Arg	9.9±2.06	0.86±0.43	86
Lys-BK-Ser-Val-R[b] (2)	Arg-Ser	250±3	21±1.5	84
Horse LMW kininogen[c] (3)	X-Lys Arg-X	0.77±0.15	1.20±0.07	1558

Cleavage site sequences- a)-Leu-Met-Lys-Arg-Pro- and -Pro-Phe--Arg-Ser-Val-; b)-Pro-Phe-Arg-Ser-Val- ; c)complete sequence not yet determined;

be less favorable than a phenylalanyl (2), is slightly better than Ac-Phe-Arg-Ser-Val-NH_2 as a kallikrein substrate. Regarding HoU-kallikrein, the kcat/Km values for both peptide substrates (Tables 2) are identical, but they are 18-fold lower than the specificity constant for the liberation of Lys-BK from horse kininogen, the natural substrate (6).

Fiedler (4) observed that PoP-kallikrein splits with a very low kcat/km (0.8 $mM^{-1}sec^{-1}$) the Arg-Arg bond in the peptide Ac-Phe-Arg-Arg-Pro-NH_2 ; he suggests that this resistance could be due to the inability of the enzyme to accommodate an arginyl (or an arginyl-prolyl) residue at the subsites S'_1 (and S'_2). However, as the Arg^6-Arg^7 bond in BAM22P was easily split by glandular kallikreins, the resistance of the Arg-Arg bond in Fiedler's model peptide, as well as that of the Lys-Arg bond in bovine kininogen should be attributed to the proline residue in position P'_2.

The present results add important information concerning the specificity of kallikreins. The splitting of the Arg-Arg

bond by these enzymes indicate that they may be involved in the processing of prohormones where the active hormone is flanked by a pair of basic amino acids (12,13,14). In the case of proenkephalin it is interesting that BAM 22P, whose sequence appears within proenkephalin, is a good substrate for glandular kallikreins. We are now investigating larger fragments of proenkephalin as substrates for these enzymes.

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