Synthetic Inhibitors and Ester Substrates of Pancreatic Kallikrein

Kallikreins which have been found in a variety of human and animal glandular organs, secretions and body fluids share a number of properties with trypsin. Both types of enzymes are able to release kinins from alpha globulins and to split arginyl bonds, and both are similarly inhibited by inhibitors extracted from bovine tissues and by amidino and guanidino compounds[1–5]. There exist, however, also certain definite differences between kallikreins and trypsin. In contrast to trypsin, pancreatic kallikrein, for example, effects little or no hydrolysis of casein and its activities are not blocked by either soybean trypsin inhibitor or TLCK (1-chloro-3-tosylamido-7-amino-2-heptanone)[6–8]. Furthermore, it has been reported that hydrolysis of synthetic substrates by kallikrein is not restricted to arginyl bonds, but that tyrosine and methionine esters can also serve as substrates[9].

In the study reported here, hog pancreatic kallikrein has been further characterized by its strong affinity for several small-molecular inhibitors; and 2 different preparations of pancreatic kallikrein have been investigated for their activity against a large number of ester substrates to ascertain which relationship, if any, might exist between the ability to hydrolyze arginyl linkages and the activity against bonds involving other amino acids.

Kallikrein was obtained from Farbenfabriken Bayer AG, Leverkusen, Germany. All inhibitors and substrates were purchased from commercial sources and were of purest grade. Esterolytic activity was assayed by a modified Hestrin method[10]. Hydrolysis of N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (BANA) was measured according to ERLANGER et al.[11]. Inhibition constants (K_i) were determined graphically by plotting 1/v against inhibitor concentration at 2 different substrate concentrations[12].

Table I shows the inhibitory effect of several groups of compounds on the hydrolysis of N^{α}-benzoyl-L-

Table I. Effect of inhibitors on the esterase activity of pancreatic kallikrein[a]

Inhibitor	Concentration of inhibitor (M)	
	5×10^{-3}	10^{-3}
	Inhibition (%)	
4,4′-Diamidinodiphenylamine	–	70
p-Amidinophenylpyruvic acid	–	54
p-Aminobenzamidine	62	29
Benzamidine	32	16
1,4-Diamidinobenzene	19	2
p-Hydroxybenzamidine	0	0
3-Indolepyruvic acid	27	5
Tryptamine	14	0
3-Indoleacetic acid	10	0
3-Indolelactic acid	4	0
α-Ketoisocaproic acid	15	0
p-Aminophenylpyruvic acid	–	0

[a] The reaction mixtures consisted of 1.6 ml of 0.1 M *tris*-HCl buffer (pH 8.1) containing 0.5 units kallikrein and 0.01 M N^{α}-benzoyl-L-arginine ethyl ester. Incubation was carried out for 30 min at 37 °C.

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Table II. Hydrolysis of amino acid esters by 2 different preparations of hog pancreatic kallikrein[a]

Amino acid ester	Concentration (M)	Kallikrein preparation[b]		
		A	B	Quotient, A/B
		Esterase activity, μmoles/min		
N^{α}-Benzoyl-L-arginine ethylester	0.016	100.00	100.00	1.0
N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester	0.016	7.69	13.37	0.6
N-Acetyl-L-tyrosine ethyl ester	0.015	1333.99	32.83	40.6
L-Tyrosine ethyl ester	0.025	57.11	2.08	27.5
N-Acetyl-L-tryptophan ethyl ester[c]	0.015	158.71	3.50	45.4
N-Acetyl-DL-phenylanine-β-naphthyl ester[d]	0.014	240.13	4.82	49.8
L-Phenylalanine ethyl ester	0.025	24.96	0.87	28.7
N-Benzoyl-L-leucine ethyl ester[e]	0.0038	319.19	7.05	45.3
L-Leucine ethyl ester	0.025	49.56	1.52	32.6
N-Benzoyl-DL-methionine methyl ester[f]	0.01	7.38	0.15	49.2

[a] The reaction mixtures consisted of 1.6 ml of 0.1 M *tris*-HCL buffer (pH 8.1) containing kallikrein and the given concentrations of substrate. The amounts of kallikrein were chosen differently for each substrate and ranged from 0.025–200 units per assay. The reactions were allowed to proceed for 30 min at 37 °C. [b] All values refer to amounts of the 2 enzyme preparations which were equal in their activity against BAEE, i.e., hydrolyzed 100 μmoles BAEE/min. [c] 7.5% Dimethylsulfoxide (DMSO) in assay mixtures. [d] 13.5% DMSO. [e] 15% DMSO. [f] 25% Ethanol. There was no hydrolysis of the following substrates: L-Tryptophan ethyl ester, DL-norleucine ethyl ester, L-isoleucine ethyl ester, L-cysteine ethyl ester, glycine ethyl ester, N-benzoyl-glycine methyl ester, DL-serine ethyl ester and L-glutamic acid diethyl ester.

arginine ethyl ester (BAEE) by kallikrein. The list includes amidines and indole derivatives, most of which have previously been shown to inhibit trypsin[13–15], and the series is rounded off by 2 strong inhibitors of chymotrypsin, α-ketoisocaproic acid[16] and *p*-aminophenylpyruvic acid[17]. The most effective inhibitors of kallikrein were among the amidines, and the ranking of these compounds as to potency was the same as described earlier for trypsin with the only notable exception that 4,4′-diamidinodiphenylamine moved into first place ahead of *p*-amidinophenylpyruvic acid. 3-Indolepyruvic acid turned out to be a stronger inhibitor of kallikrein than tryptamine, while the converse holds true for trypsin. Benzamidine, one of the compounds investigated here, has previously been studied by MARES-GUIA and DINIZ[5] for its influence on rat urinary kallikrein and the inhibition constant was reported as $1.15 \times 10^{-4} M$. Using the same substrate as those investigators, i.e., BANA, the K_i value of benzamidine for hog pancreatic kallikrein was determined as $6 \times 10^{-4} M$, the value of *p*-aminobenzamidine as $2.4 \times 10^{-4} M$, and the value of 4,4′-diamidinodiphenylamine as $1.5 \times 10^{-5} M$. The type of inhibition was competitive for all compounds.

The results in Table II confirm the findings of ROCHA E SILVA[9] that tyrosyl and methionyl bonds are hydrolyzed by commercial kallikrein. In addition, it is evident that esters of L-tryptophan, L-phenylalanine and L-leucine were readily split, and especially so when the alpha amino group was acylated. A comparison of the 2 kallikrein preparations reveals that the material in the first column had a much higher activity against non-arginyl esters than the one in the second column. However, the ratios of corresponding non-arginyl esterase activities in the 2 preparations were remarkably constant. This observation suggests that a single enzyme might have been responsible for all the chymotrypsin-like activities. Contamination by chymotrypsin itself could be ruled out by the absence of any hydrolysis of DL-norleucine ethyl ester, a substrate which is split by chymotrypsin almost as rapidly as the kallikrein substrate L-leucine methyl ester. In view of the recent discovery of a number of different kallikreins in hog pancreas[18], it has to be considered whether one or several of these enzymes may not be endowed with the ability to split also non-arginyl bonds. The mode of purification of kallikrein might favor the presence of one form over the other in different enzyme preparations, thus accounting for the variations in the ratios between BAEE-ase activity and non-arginyl esterase activity. The suggestion that native kallikrein or a modified form may possess the ability to split non-arginyl bonds is supported by the finding that 4,4′-diamidinodiphenylamine strongly blocks not only the hydrolysis of BAEE, but of all the other esters as well, that L-tyrosine ethyl ester (TEE) inhibits competitively the hydrolysis of BANA, and that, on the other hand, BANA impedes the hydrolysis of TEE[19].

Zusammenfassung. Es wird in 4,4′-Diamidinodiphenylamin der bis jetzt stärkste niedermolekulare Hemmstoff für Kallikrein aus Schweinepankreas gefunden, mit der Fähigkeit, Tryptophan-, Phenylalanin- und Leucin-Ester zu spalten.

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Occurrence of Rhodoquinone-9 in the Muscle of *Ascaris lumbricoides var. suis*

Rhodoquinone was first isolated by GLOVER and THRELFALL[1] from *Rhodospirillum rubrum*. Further work by MOORE and FOLKERS[2] has shown the true structure of rhodoquinone, in which one methoxyl group of ubiquinone-10 is replaced by amino group. Recently POWLS and HEMMING[3] isolated rhodoquinone-9 from *Euglena gracilis*. The occurrence of rhodoquinone in nature has hitherto been confined to only these few microorganisms in contrast to the ubiquitous existence of ubiquinone in the living systems. During the survey study on ubiquinone in parasitic nematodes, the authors have obtained the evidence that the mitochondrial fraction of *Ascaris* muscle contained a rhodoquinone analogue in the place of ubiquinone[4]. This was the first evidence showing the occurrence of rhodoquinone in an animal tissue. In order to characterize rhodoquinone from *Ascaris* muscle, further examination with a large amount of *Ascaris* muscle was carried out. Now the quinone has been isolated in a crystalline form and the spectroscopic examinations revealed that it must be rhodoquinone-9 just like that from *Euglena gracilis*.

Methods and results. Adult round worms, *Ascaris lumbricoides* var. *suis*, were collected freshly from the slaughter house. Worms were cut open longitudinally and freed from intestine, eggs, etc. The muscular layer was then scraped from the cuticle, and washed several times with 0.9% NaCl solution. Muscle strips thus obtained were stored at −15 °C. Homogenized in Waring blender the frozen materials were extracted with 10 volumes of ethanol-ether (3:1, v/v) for about 15 h. The combined ethanol-ether extracts were evaporated under reduced pressure over a warm bath. When the volume of the extract was reduced to 1/10 the original volume, aqueous ethanol suspension was extracted with *n*-hexane.

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