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Hydrolysis of Amino-Acid Esters by Pig-Pancreatic Kallikrein

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Initial velocities of the hydrolysis of α-*N*-acylated esters of 14 different amino acids catalyzed by pig pancreatic kallikrein were determined. Control experiments with kallikrein partially inactivated by controlled treatment with diisopropylphosphorofluoridate demonstrated the enzymic homogeneity of the preparation and the ability of kallikrein to hydrolyse (at decreasing rates) esters of basic, aromatic and neutral aliphatic amino acids, though being predominantly specific for L-arginine esters. The controversial literature on the substrate specificity of the enzyme has its origin not in the molecular heterogeneity of kallikrein, but in a contamination with other enzymes. Kinetic constants for the kallikrein-catalyzed hydrolysis of several of the esters have been determined and compared to those of trypsin and clostripain. The preference of kallikrein for an arginine over a lysine ester is still higher than with the latter enzyme. In the α-*N*-benzoylated as well as in the α-*N*-tosylated series, esters of L-arginine with different alcohol moieties exhibit not only widely different K_m, but also k_{cat} values. The latter parallel the rates of the hydroxyl-ion-catalyzed hydrolyses of the α-*N*-benzoyl-L-arginine esters which have also been determined.

Syntheses of α-*N*-benzoyl-L-lysine methyl ester hydrochloride and of α-*N*-benzoyl-L-ornithine methyl ester hydrochloride are described. A value for the rate constant of the hydroxyl-ion-catalyzed lactamization of the ornithine ester has been obtained.

Pig pancreatic kallikrein liberates a kinin peptide from a protein precursor in serum. It has long been known that the enzyme also exhibits a potent esterolytic activity against α-*N*-acylated arginine esters [1—3]. As to the specificity of kallikrein for esters of other amino acids, there are conflicting reports in the literature. (The enzyme discussed throughout this paper is that from pig pancreas.) Whereas some workers [4—6] observed only a slow hydrolysis or none at all, *e.g.* of the typical chymotrypsin substrate, *N*-acetyl-L-tyrosine ethyl ester, others reported a high activity of kallikrein with this compound [7—9]. The amino acid specificity of kallikrein is of interest not only for the characterization of the enzyme, but also for the elucidation of the process of kinin liberation. An investigation of the hydrolysis of α-*N*-acylated amino acid esters by highly purified kallikrein was therefore undertaken. As an additional precaution, the substrate specificity of the enzyme has been checked after partial inactivation of the kallikrein preparation by controlled treatment with diisopropylphosphorofluoridate. The results of this study, which are presented below, demonstrate a predominant specificity of kallikrein for esters of arginine. In this respect kallikrein, which has been shown to belong to the group of pancreatic hydrolases with serine at the active site [10] and most probably located in an Asp-Ser-Gly sequence [11], resembles far more the bacterial sulfhydryl proteinase clostripain than the pancreatic serine proteinase trypsin. For a more extensive comparison of the enzymic properties of kallikrein with that of other enzymes, the kinetics of the hydrolysis of several of the amino acid esters have been investigated. Furthermore, the hydrolysis of two series of α-*N*-acylated arginine esters of different alcohols has been studied in an effort to throw some light on the mechanism of the kallikrein-catalyzed hydrolysis of specific ester substrates. Preliminary data on problems dealt with in the present work have been communicated on several occasions [12, 13].

Dedicated to Professor Dr Dr h. c. H. Kraut on the occasion of his 80th birthday.

Abbreviations. Diisopropylphosphorofluoroidate, DFP; kallikrein partially inactivated by controlled treatment with DFP, DFP-kallikrein.

Enzymes. Kallikrein (pig pancreatic) (EC 3.4.4.21); trypsin (EC 3.4.4.4); clostripain or clostridiopeptidase B (EC 3.4.4.20); thrombin (EC 3.4.4.13); chymotrypsin (EC 3.4.4.5); chymotrypsin B (porcine) (EC 3.4.4.6); chymotrypsin C (porcine) (EC 3.4.4.–); sialidase or neuraminidase (EC 3.2.1.18).

MATERIALS AND METHODS

Substrates

The α-*N*-acylated ester hydrochlorides of glycine, L-valine, L-leucine, L-methionine, L-tryptophan, L-histidine, and α-*N*-tosyl-L-ornithine methyl ester hydro-

chloride were obtained from Cyclo Chemical Corporation (Los Angeles); the derivatives of DL-alanine, D-arginine, and phenylalanine, as well as α-*N*-benzoyl-L-arginine methyl ester, from Mann Research Laboratories (New York); α-*N*-benzoyl-L-arginine ethyl ester from E. Merck AG (Darmstadt); α-*N*-benzoyl-L-tyrosine ethyl ester and α-*N*-benzoyl-L-citrulline methyl ester, as well as dimethyl sulfoxide, from Serva Feinbiochemica (Heidelberg). Diisopropylphosphorofluoridate (DFP) was a gift from Farbenfabriken Bayer.

The hydrochlorides of α-*N*-benzoyl-L-arginine benzyl (m.p. 90—95 °C decomp.; lit. [14] m.p. 75—78 °C), *n*-propyl (m.p. 147.5 °C; lit. [15] m.p. 146 °C), isopropyl (m.p. 178—179 °C; lit. [14] m.p. 173.5—174.5 °C), and cyclohexyl (m.p. 192—194.5 °C; lit. [14] m.p. 182—193 °C; [16] m.p. 200—210 °C; $C_{19}H_{29}O_3N_4Cl$ requires C, 57.50; H, 7.36; N, 14.12%; found[1]: C, 57.69; H, 7.36; N, 13.76%) esters and of α-*N*-tosyl-L-arginine benzyl (m.p. 70—110 °C), ethyl (m.p. 113—117 °C; lit. [17] m.p. 113—115 °C), *n*-propyl (m.p. 125—126 °C; lit. [16] m.p. 127—128 °C), and cyclohexyl (m.p. 70—120 °C; $[\alpha]_D^{26} = +11.6 \pm 0.2°$ ($c = 4$ in methanol); lit. [17] m.p. 165—167 °C; $[\alpha]_D^{17} = +11.7 \pm 0.3°$; $C_{19}H_{31}O_4N_4SCl$ requires C, 51.05; H, 6.99; N, 12.53%; found: C, 51.14; H, 7.05; N, 12.12%) esters were synthesized according to published procedures with minor modifications.

α-*N*-Benzoyl-ε-*N*-benzyloxycarbonyl-L-lysine methyl ester was prepared from 1.0 g (3 mmol) ε-*N*-benzyloxycarbonyl-L-lysine methyl ester hydrochloride (Mann) by acylation in 5 ml water with 0.46 g (3.3 mmol) benzoyl chloride in the presence of 1.2 g (12 mmol) $KHCO_3$. The oil was reprecipitated from benzene-light petroleum. Yield 1.1 g, 91%. When recrystallized from ethyl acetate—light petroleum, it solidified perfectly only after several weeks and showed an m.p. of 73—73.5 °C. Elmore *et al.* [18] quote m.p. 74—75 °C and Izumiya *et al.* [19] 74—76 °C. $[\alpha]_D^{17} = -10.0°$ ($c = 2$ in ethanol) [19]; found: $[\alpha]_D^{29} = -12.5 \pm 0.8°$.

α-*N*-Benzoyl-L-lysine methyl ester hydrochloride was obtained by hydrogenation of the above material in methanolic HCl with a palladium on charcoal catalyst. After precipitation from methanol—ether, the oil solidified in several days. The resulting, rather hygroscopic, powder was used as such. In accordance with previous observations [18, 19] the material could not be crystallized. It exhibited a melting range from about 70—120 °C. $C_{14}H_{21}O_3N_2Cl$ requires C, 55.88; H, 7.04; N, 9.32%. Found: C, 55.11; H, 7.17; N, 9.02%. $[\alpha]_D^{26} = -16.9 \pm 1.0°$ ($c = 2$ in water).

α-*N*-Benzoyl-δ-*N*-benzyloxycarbonyl-L-ornithine was prepared by acylation of δ-*N*-benzyloxycarbonyl-L-ornithine (Mann) in 1 N NaOH by means of benzoyl chloride in ether. The oil obtained by acidification of the aqueous phase solidified on storage in an ice box overnight. Yield 76%; m.p. 148—149 °C after recrystallization from ethyl acetate—light petroleum. Calculated for $C_{20}H_{22}O_5N_2$: C, 64.83; H, 5.99; N 7.57%. Found: C, 64.69; H, 5.89; N, 7.30%. $[\alpha]_D^{25} = +4.2 \pm 0.6°$ ($c = 2$ in ethanol).

α-*N*-Benzoyl-δ-*N*-benzyloxycarbonyl-L-ornithine methyl ester was prepared by esterification of 0.80 g (2.15 mmol) α-*N*-benzoyl-δ-*N*-benzyloxycarbonyl-L-ornithine as described for the corresponding α-*N*-tosylated compound [20]. Recrystallization from benzene—light petroleum yielded 0.60 g (73%) of the ester. M.p. 107—108 °C, did not change after recrystallization. Izumiya *et al.* [19] quote m.p. 105 °C and $[\alpha]_D^{17} = -10.0°$ ($c = 2$ in ethanol). Found: $[\alpha]_D^{29} = -8.2 \pm 1.8°$.

α-*N*-Benzoyl-L-ornithine methyl ester hydrochloride was obtained by hydrogenation of the above material. It was recrystallized twice from methanol—light petroleum. Yield 81%; m.p. 147—148 °C; $[\alpha]_D^{26} = -25.7 \pm 1.0°$. Izumiya *et al.* [19] found m.p. 142—143 °C and $[\alpha]_D^{10} = -27.5°$ ($c = 2$ in water). $C_{13}H_{19}O_3N_2Cl$ requires C, 54.43; H, 6.68; N, 9.77%. Found: C, 54.21; H, 6.46; N, 9.71%.

Kallikrein

Kallikrein was obtained by chromatography, twice on hydroxyapatite-cellulose according to the method of Fritz *et al.* [21], from partially purified kallikrein preparations from porcine pancreatic autolysates generously supplied by Farbenfabriken Bayer. After gel filtration on Sephadex G-50, the enzyme preparation was lyophilized. Specific activities were determined with 10 mM α-*N*-benzoyl-L-arginine ethyl ester as substrate as described previously [11]. A single batch of enzyme (195 U/mg protein) containing about equal activities of the kallikreins A and B was used for the specificity studies compiled in Table 1 and for the preparation of the DFP-kallikrein used in the same experiments. For the isolation of kallikreins A and B, the mixture was separated by chromatography on DEAE-cellulose according to the directions of Schmidt-Kastner [22] as described by us [23]. Kallikrein B was treated with sialidase [21] and recovered by gel filtration on Sephadex G-50 and lyophilization with specific activities of 220 to 230 U/mg protein. The kallikrein A preparation used (215 U/mg protein) had been rechromatographed once on DEAE-cellulose as before.

DFP-Kallikrein

The mixture of kallikreins A and B (3 mg/ml) was incubated at 25 °C in 0.1 M sodium phosphate buffer pH 7 containing 1 mM ethylene diamine tetraacetate and 1 mM DFP. The loss of esterase activity on

[1] All analyses were performed by Mikroanalytisches Laboratorium Ilse Beetz, Kronach.

α-*N*-benzoyl-L-arginine ethyl ester was followed as described previously [10]. When about 50% inhibition had been obtained, the reaction mixture was dialyzed for 20 h at 5 °C against distilled water (3 × 5 l) and lyophilized. The preparation of DFP-kallikrein was stored like the other kallikrein preparations in dry form at −20 °C.

Hydrolysis of Esters by Kallikrein

The hydrolysis of ester substrates by kallikrein was followed with a Radiometer autotitrator TTT 1c with SBR 2c and ABU 1b (0.25 ml burette) by titration with 5 mM NaOH in 0.1 M NaCl in a volume of 2 or 20 ml. The jacketed reaction vessel was thermostated at 25.0 °C and flushed with nitrogen. The reaction medium contained 0.1 M NaCl and 0.1 mM thioglycolic acid in order to counteract the possible inhibition of kallikrein by heavy metal ions [24]. In some cases specified in the text it was necessary to add the substrate in dimethyl sulfoxide solution owing to its limited water solubility and thus to include 5 or 10% (v/v) dimethyl sulfoxide in the reaction medium. The reaction was started by the addition of kallikrein solution, 0.1 to 10 mg/ml, in 0.1 M NaCl. Such solutions were stable for at least several days. In the experiments presented in Table 1, the amount of enzyme protein was determined from the absorbance at 280 nm, using the value of $A_{280}^{1\%} = 20.5$ [25] obtained for kallikreins A and B also for DFP-kallikrein.

For the evaluation of kinetic constants, initial velocities were determined in duplicate at each substrate concentration, chosen according to the theoretical considerations of Cleland [26] whenever possible. Thus each experiment furnished from 10 to about 25 values. The data was first plotted in Lineweaver-Burk and Eadie diagrams in order to detect possible deviations from Michaelis-Menten kinetics (which had been observed previously in kallikrein-catalyzed hydrolyses of several esters at high concentrations [24]), to exclude any points grossly in error, and to obtain initial estimates of K_m and V. Refined values of these constants were then calculated by fitting the data to the hyperbolic form of the Michaelis-Menten equation by an iterative procedure [27]. The determinations of the kinetic constants were repeated 3 to 5 times on different occasions and usually with different batches of enzyme. The results are reported as the mean and its standard deviation, which generally was larger than the error calculated from a single experiment by the iterative procedure.

Values of k_{cat} were calculated from the content of enzymatically active kallikrein of the enzyme solutions used, determined with α-*N*-benzoyl-L-arginine ethyl ester as substrate, a specific activity of 300 ± 10 U/mg protein of pure kallikreins A or B, and a molecular weight of 24000 ± 860 for the protein moiety of the enzyme [28].

Hydroxyl-Ion-Catalyzed Ester Hydrolyses

The hydrolysis of the series of hydrochlorides of α-*N*-benzoyl-L-arginine esters by OH^- was also followed with the autotitrator, the sensitivity of which was increased by means of an mV-compensator PHA 802, at 25.0 °C and pH 10.0 in 0.1 N NaCl. Ester concentrations were from 0.5 to 5 mM, and either 5 or 40 mM NaOH was used for the titrations. No measurable alkali consumption of the ester solutions was observed at pH 8.0. This was taken to indicate that other pathways of ester hydrolysis besides the hydroxyl-ion-catalyzed one could be neglected at pH 10.0. The velocity v of alkali uptake at pH 10.0, corrected for the blank without ester, was therefore used for the calculation of the pseudo-monomolecular velocity constant k_{OH} of hydroxyl-ion-catalyzed ester hydrolysis at pH 10, which is defined by $v = k_{OH} \cdot [\text{ester}]$. The results were expressed as the mean and its standard deviation from 5 to 15 independent determinations.

RESULTS

Survey of the Amino-Acid Specificity of Kallikrein in Ester Hydrolysis

The hydrolysis of α-*N*-acylated esters of 14 different amino acids by a mixture of kallikreins A and B has been studied at several substrate concentrations. A pH of 8 has been chosen, since at this pH the kallikrein-catalyzed hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester is nearly maximal [24] and spontaneous ester hydrolysis is negligible. Initial velocities were followed for several minutes and remained constant during this period in all cases.

The results of this study are compiled in Table 1, together with velocity values obtained in parallel experiments with the same batch of enzyme partially inactivated by controlled treatment with DFP. The ratio of the specific activities of the two preparations as determined with the various substrates is quoted in the last column of Table 1 and has a mean value of 0.55. As most of the velocity measurements have been done only in duplicate because of lack of enzyme, their maximum possible error is expected to amount to about ± 5%, so that values from 0.49 to 0.61 for the ratio fall within the limits of experimental precision. Larger deviations are observed only with the leucine and the alanine ester, where, as in the case of the valine ester, the experimental uncertainties might well have been somewhat higher due to the very low reaction rates. In view of the characteristic low rate of the reaction of kallikrein with DFP [10], the constancy of the ratio of the specific activities demonstrates that the kallikrein preparation used was enzymically homogeneous, though it had only two thirds the specific activity of our best preparations obtained since. It demonstrates

Table 1. *Initial velocities of the hydrolysis of α-N-acylated amino-acid esters by a kallikrein preparation and by the same preparation after partial inhibition by DFP*

The kallikrein preparation was a mixture of kallikreins A and B, 195 U/mg protein. Reactions were carried out at 25.0 °C, pH 8.0, 0.1 M NaCl, 0.1 mM thioglycolic acid, 10% (v/v) dimethyl sulfoxide

Substrate		Kallikrein at [S]=			DFP-Kallikrein at [S]=		Ratio DFP-Kallikrein / Kallikrein
Amino acid	Ester	1 mM	5 mM	25 mM	1 mM	5 mM	
		μmol × min⁻¹ × mg protein⁻¹			μmol × min⁻¹ × mg protein⁻¹		
Benzoyl-L-arginine	ethyl	182			96.2		0.53
Benzoyl-D-arginine	ethyl		<0.005				—
Benzoyl-L-arginine	methyl	191	258	258		146	0.57
Benzoyl-L-lysine	methyl	4.5	12.0	28		7.2	0.60
Benzoyl-L-ornithine	methyl	0.34	1.6		0.19		0.56
Benzoyl-L-citrulline	methyl	0.62	2.7	6.7		1.5	0.56
Benzoyl-L-histidine	methyl	0.93	4.3			2.4	0.56
Benzoyl-L-tyrosine	ethyl	1.52			0.87		0.57
Acetyl-L-tyrosine	ethyl	0.21	0.90	4.0		0.49	0.54
Acetyl-L-tryptophan	ethyl	0.20	0.58			0.34	0.59
Acetyl-L-phenylalanine	ethyl	0.28	1.10	4.8		0.63	0.57
Acetyl-DL-phenylalanine	β-naphthyl	0.39			0.22		0.56
Benzoyl-L-methionine	methyl	0.17	0.76			0.37	0.49
Benzoyl-L-leucine	methyl	0.071			0.028		0.39
Benzoyl-L-valine	methyl		0.006			0.003	0.5
Benzoyl-DL-alanine	methyl		0.026	0.087		0.018	0.69
Benzoyl-glycine	methyl		<0.005				—

further that a common active site is involved in the hydrolysis of the various substrates.

The presence of 10 or 5% (v/v) dimethyl sulfoxide had no inhibitory effect on the hydrolysis of 1 mM α-*N*-benzoyl-L-arginine ethyl ester by kallikrein (there was indeed an indication of a slight activation of the order of several per cent), but it inhibited the hydrolysis of 1 mM α-*N*-benzoyl-L-citrulline methyl ester by 75 or 45% and of 1 mM *N*-acetyl-L-tyrosine ethyl ester by 63 or 35%, respectively[2]. Though dimethyl sulfoxide thus appreciably reduces the rates of hydrolysis of the worse substrates, the observed rate differences amount to several orders of magnitude and cannot be explained by the inavoidable presence of this solvent.

Methyl esters of the aromatic amino acids were not available, and ethyl esters had to be used instead. In the α-*N*-benzoyl- and the α-*N*-tosyl-L-arginine series, ethyl esters are hydrolyzed at the least at one third the rate of the methyl esters. The nature of the α-*N*-acyl residue has a large influence on the hydrolysis of L-arginine esters by kallikrein (see below). *N*-Benzoyl-L-tyrosine ethyl ester (1 mM) is hydrolyzed about 7 times faster than the *N*-acetylated compound (Table 1). The use of α-*N*-acetylated ethyl esters will thus underestimate the substrate properties of esters of aromatic amino acids, but assuredly not enough to significantly influence this survey on kallikrein specificity.

The hydrolysis of 5 and 25 mM *N*-acetyl-L-tyrosine ethyl ester has been studied in addition to the experiments with the mixture of kallikreins A and B also with kallikrein A and with sialidase-treated kallikrein B. Within the experimental limits of precision, the same activities relative to the hydrolysis of 1 mM α-*N*-benzoyl-L-arginine ethyl ester were obtained with all three enzyme preparations.

The results of the present work confirm previous observations on the poor substrate properties of *N*-acetyl-L-tyrosine ethyl ester for kallikrein [4—6]. The reported high activities of this enzyme with the compound mentioned [7—9] and with *N*-benzoyl-L-leucine ethyl ester [9] and *N*-benzoyl methionine methyl ester [8, 9] as well, are in contrast to our findings. The lack of specificity of kallikrein for methionine esters has been confirmed also with the corresponding DL [12] and the tosylated [6, 29] compound.

Lactamization of α-N-Benzoyl-L-Ornithine Methyl Ester

Among all the substrates used in this investigation, only the α-*N*-benzoyl-L-ornithine methyl ester showed a notable alkali uptake at pH 8.0 prior to the addition of enzyme. The initial rate of this uptake was proportional to ester concentration in the range from 0.2 to 7.5 mM, and on the basis of a 1:1 stoichiometry a first order rate constant of $(11.2 \pm 0.62) \cdot 10^{-3}$ min^{-1} ($n = 26$) was calculated for the process.

[2] A referee suggested that denaturation could possibly occur which is stabilized by the best substrates, but not by the worse substrates. A time-dependent denaturation of kallikrein appears improbable in view of the constancy of the rates of hydrolysis during several minutes. Control experiments with 10-min preincubation of the enzyme with 5 or 10% dimethyl sulfoxide prior to addition of substrate (1 mM of the arginine or the tyrosine ester) led to results practically identical with those obtained in experiments with a reversed order of addition of reagents.

Table 2. *Kinetic constants for the hydrolysis of several α-N-acylated amino-acid esters by (A) sialidase-treated kallikrein B, (B) bovine trypsin and (C) clostripain*
Conditions in (A) were pH 8.0, 25.0 °C, 0.1 M NaCl, 0.1 mM thioglycolic acid, in (B) pH 8.0, 25.0 °C and (C) pH 7.8, 25.0 °C except where stated otherwise

Enzyme	Substrate: Amino acid	Substrate: Ester	Substrate: Concentration	K_m	k_{cat}	k_{cat}/K_m	References
			mM	mM	s^{-1}	$mM^{-1}\,s^{-1}$	
(A) Kallikrein	α-*N*-Tosyl-L-arginine	methyl	0.005... 0.5	0.031 ± 0.008	2.7 ± 0.33	150	This work
	α-*N*-Benzoyl-L-arginine	ethyl	0.01 ... 1	0.104 ± 0.008	123 ± 8	1180	
	α-*N*-Benzoyl-L-arginine	methyl	0.01 ... 1	0.125 ± 0.020	154 ± 13	1230	
	α-*N*-Benzoyl-L-lysine	methyl	0.05 ... 3	2.00 ± 0.28	15.5 ± 1.4	7.75	
	α-*N*-Benzoyl-L-ornithine	methyl	0.15 ... 2	2.44 ± 0.30	1.80 ± 0.25	0.74	
	α-*N*-Benzoyl-L-citrulline	methyl	0.5 ...35[a]	12.3 ± 1.4	8.64 ± 0.81	0.70	
	α-*N*-Acetyl-L-tyrosine	ethyl	1 ...25[a]	50 ± 14	11.2 ± 2.2	0.22	
(B) Trypsin	α-*N*-Tosyl-L-arginine	methyl	—	0.0125	60	4800	[33]
	α-*N*-Benzoyl-L-arginine	ethyl	—	0.0025...0.0043	14.6 ...31.1	3400... 9600	[34–36]
	α-*N*-Benzoyl-L-arginine	methyl	—	0.001 ...0.01	15	1500...15000	[17,31]
	α-*N*-Benzoyl-L-lysine	methyl	—	0.0167	16.6	994	[18]
	α-*N*-Tosyl-L-ornithine	methyl	—	15.3; 19	3.34; 1.4	0.22; 0.74	[17,37][b]
	α-*N*-Benzoyl-L-citrulline	methyl	—	41	0.14	0.0034	[37][c]
	α-*N*-Acetyl-L-tyrosine	ethyl	—	42; 62	14.5; 57	0.35; 0.92	[35,34][d]
(C) Clostri-pain	α-*N*-Tosyl-L-arginine	methyl	—	0.022	9.8	440	[38]
	α-*N*-Benzoyl-L-arginine	ethyl	—	0.25; 0.24	91; 92	364; 382	[38]
	α-*N*-Benzoyl-L-lysine	methyl	—	3.0	22	7.3	[38]

[a] 5 % (v/v) dimethyl sulfoxide.
[b] pH 7; pH 6.75.
[c] pH 7.0.
[d] 5 % dioxane; 10 % 2-propanol.

The cause of this exceptional behaviour of the ornithine ester is most probably lactamization [19,20,30]. Analyzing the data as described in [30], using the value of 8.97 determined for the pK_a of the δ-amino group of α-*N*-tosyl ornithine methyl ester [30] also for the α-*N*-benzoylated ester, a value of 128 ± 7.1 $mM^{-1} \cdot min^{-1}$ was calculated for the second-order rate constant of the presumed hydroxyl-ion-catalyzed lactamization of the unprotonated molecular species of α-*N*-benzoyl-L-ornithine methyl ester.

For the corresponding α-*N*-tosylated DL-ornithine compound, the value of this constant is reported as 46.6 ± 9.3 $mM^{-1} \cdot min^{-1}$ [30]. The benzoylated ester is thus lactamized at (2.75 ± 0.57) times the rate of the tosylated ester. A direct comparison of the rates of alkali uptake of α-*N*-benzoyl- and α-*N*-tosyl-L-ornithine methyl ester at 2 mM concentration at pH 8 led to a velocity ratio of 4.3, in reasonable agreement with the former result. These values resemble the ratio of the rates of the hydroxyl-ion-catalyzed hydrolysis of α-*N*-benzoyl *versus* α-*N*-tosyl arginine methyl ester, 3.5 ± 1.3 [31]. The influence of the tosyl group on ornithine ester lactamization obviously goes in the same unexpected direction as its effect on arginine ester hydrolysis [32]. This result is in contrast to observations in the α,γ-diaminobutyrate series [30], where an α-*N*-tosyl group leads to lactamization more readily than does an α-*N*-benzoyl group. (The reported almost instantaneous lactamization of α-*N*-benzoyl-L-ornithine methyl ester [19] has probably occurred only under the influence of the alkaline hydroxylamine reagent used for analysis.)

Kinetics of the Kallikrein-Catalyzed Hydrolysis of Amino-Acid Esters

For a number of α-*N*-acylated amino acid esters, kinetic constants of the kallikrein-catalyzed hydrolysis have been determined and are compiled in Table 2 together with corresponding data from the literature for trypsin and clostripain. In the range of substrate concentrations used for the present study, the reactions of all the esters obeyed Michaelis-Menten kinetics, as was true also for the experiments presented in Tables 3 and 4. Deviations from earlier reported values [12,13,24] result from the larger number of experiments conducted in the meantime, from the more refined treatment of the data, and from the use of revised values for the molecular weight and the specific activity of kallikrein.

In the case of the ornithine ester, relatively large corrections for nonenzymatic alkali uptake were necessary. No inhibition of the kallikrein-catalyzed reaction of 1 mM α-*N*-benzoyl-L-ornithine methyl ester was observed in the presence of 0.5 mM of the lactam obtained by incubation of an ester solution

Table 3. *Kinetic constants for the hydroxyl-ion-catalyzed and the kallikrein-catalyzed hydrolyses of esters of α-N-acylated L-arginine*
For the hydroxyl-ion-catalyzed reactions the first-order rate constant k_{OH} was determined at pH 10.0, 25.0 °C, 0.1 M NaCl. For the kallikrein-catalyzed reactions sialidase-treated kallikrein B was used at pH 8.0, 25.0 °C, 0.1 M NaCl and 0.1 mM thioglycolic acid

Ester substrate	α-N-Benzoyl-L-arginine esters				α-N-Tosyl-L-arginine esters		
	$k_{OH} \cdot 10^3$	[S]	K_m	k_{cat}	[S]	K_m	k_{cat}
	min^{-1}	μM	μM	s^{-1}	μM	μM	s^{-1}
Benzyl	16.2 ± 2.3	10...1000	4.1 ± 0.5	226 ± 14	5...500	3.2 ± 1.3	8.5 ± 0.57
Methyl	14.2 ± 1.6	10...1000	125 ± 20	154 ± 13	5...500	31 ± 8	2.7 ± 0.33
Ethyl	5.16 ± 0.58	10...1000	104 ± 8	123 ± 8	10...200	16 ± 3	0.65 ± 0.10
n-Propyl	4.01 ± 0.25	10... 500	46 ± 3	114 ± 7	15...100	18 ± 4	1.20 ± 0.09
Isopropyl	0.81 ± 0.11	30...1000	186 ± 8	42 ± 3			
Cyclohexyl	0.58 ± 0.02	10...1000	24 ± 3	28 ± 3	15...300	31 ± 8	0.30 ± 0.04

Table 4. *Kinetic constants for the hydrolysis of α-N-benzoyl-L-arginine esters by sialidase-treated kallikrein B at pH 9.0 and 5.5*
Conditions: 25.0 °C, 0.1 M NaCl, 0.1 mM thioglycolic acid. The values were obtained from single experiments at 10 to 20 different substrate concentrations

Ester	pH 9.0				pH 5.5			
	[S]	K_m	k_{cat}	$\frac{k_{cat}\ (pH\ 9.0)}{k_{cat}\ (pH\ 8.0)}$	[S]	K_m	k_{cat}	$\frac{k_{cat}\ (pH\ 8.0)}{k_{cat}\ (pH\ 5.5)}$
	mM	μM	s^{-1}		mM	mM	s^{-1}	
Methyl	0.1 ...1	68 ± 5	166 ± 10	1.08	0.03...3	1.65 ± 0.08	7.0 ± 0.4	22
Ethyl	0.1 ...1	82 ± 8	144 ± 8	1.17	0.03...3	1.73 ± 0.42	6.0 ± 1.0	20
Isopropyl	0.1 ...1	135 ± 11	46 ± 3	1.10				
Cyclohexyl	0.04...1	16 ± 2	32 ± 2	1.14				

for about 10 min at pH 10. The product of this incubation, the presumed lactam, did not react measurably with kallikrein at pH 8. It has not been examined whether the product of the kallikrein-catalyzed reaction of the ornithine ester was α-*N*-benzoyl-L-ornithine or possibly the lactam.

Takami [6] stated that α-*N*-benzoyl-L-ornithine methyl ester is not attacked by kallikrein. No data characterizing the ester used by him are given, and no mention is made of a nonenzymic reaction. We had obtained a sample designated α-*N*-benzoyl-L-ornithine methyl ester · H_2O from Cyclo Chemical Corporation, Los Angeles (Cat. No. 3820; lot P-2527) which was not hydrolyzed by kallikrein and showed no nonenzymic alkali uptake at pH 8 even at 5 mM concentration.

Only a few values of K_m for the kallikrein-catalyzed hydrolysis of some of the amino acid esters have been reported by other workers. Trautschold and Werle [4] determined K_m for α-*N*-benzoyl-L-arginine ethyl ester spectrophotometrically as 0.27 mM, while Chambers *et al.* [5] obtained at pH 7 a value of 0.37 mM and for the methyl ester 0.18 mM with quite a crude kallikrein preparation. In view of the different reaction conditions, the agreement may be considered fair. Some data published by Takami [6] have been obtained at 40 °C. The values of 1.1 mM for α-*N*-benzoyl-L-arginine ethyl ester and of 0.33 mM for α-*N*-tosyl-L-arginine methyl ester appear rather high. At least with the latter compound, substrate activation [24] might be expected at the substrate concentrations used by this author. The *V* for this substrate relative to *V* for α-*N*-benzoyl-L-arginine ethyl ester reported by him as 7.1% in contrast to 2.2% calculated from the results of the present work also supports this possibility, unless the influence of temperature on kallikrein-catalyzed reactions is quite exceptional. There are also differences in the kinetic constants for α-*N*-benzoyl-L-lysine methyl ester, for which Takami [6] reported a K_m of 0.93 mM and a relative *V* of 3.7%, whereas 12.6% is calculated from our results.

Kallikrein-Catalysed and Hydroxyl-Ion-Catalyzed Hydrolyses of α-N-Benzoyl- and α-N-Tosyl-L-Arginine Esters with Different Alcohols

In an effort to reveal some features of the mechanism of hydrolysis of specific amino acid ester substrates by kallikrein, we studied the hydrolysis of a number of esters of α-*N*-benzoyl- and α-*N*-tosyl-L-arginine with different alcohols (Table 3). k_{cat} values vary by a factor of 8 with the benzoylated and 28 with the tosylated compounds. That this observation is not the result of different pH-dependencies of the reactions of the various esters is borne out by the data of Table 4. The K_m values at pH 8 are also vastly different. There is no correlation between k_{cat} and K_m recognizable, except that both benzyl esters exhibit the highest k_{cat} and by far the lowest K_m.

The only other regularity evident from the data was a possible correlation of k_{cat} with the expected rates of the alkaline hydrolysis of the various esters with different alcohol moieties. Since rate constants for the hydroxyl-ion-catalyzed hydrolysis of only the methyl and ethyl esters could be found in the literature, the first-order rate constants k_{OH} of the non-enzymic hydrolysis of the α-*N*-benzoyl-L-arginine esters have been determined at pH 10.0 (Table 3). The experimental hydrolysis velocities were proportional to ester concentration. The data reported for the methyl ester, $10.0 \cdot 10^{-3}$ min^{-1} [32] and $(13.8 \pm 2.1) \cdot 10^{-3}$ min^{-1} calculated from the data of [31], and the ethyl ester, $3.41 \cdot 10^{-3}$ min^{-1} [32] and $(4.8 \pm 1.2) \cdot 10^{-3}$ min^{-1} [31], are in reasonable agreement with the results of the present study. A comparison of k_{OH} with k_{cat} of the kallikrein-catalyzed hydrolysis of the benzoyl-arginine esters reveals indeed a parallelity, but no strict proportionality of the two velocity constants. The same is true for k_{cat} of the tosyl-arginine series with the exception of the ethyl/*n*-propyl ester pair. k_{cat}/K_m values correlate much less with k_{OH}. No better correlation is obtained if one tries to calculate rate constants of acylation reactions with the assumption of deacylation being rate-limiting in the case of the most rapidly hydrolyzed benzyl esters.

DISCUSSION

As a possible explanation of the varying results on the amino acid specificity of kallikrein [4—9] obtained with different preparations of the enzyme, the existence of several forms of kallikrein has been mentioned [9]. The two principal forms of the enzyme, kallikreins A and B, however, resemble each other closely. They can be purified to the same specific activity (on protein basis) with α-*N*-benzoyl-L-arginine ethyl ester as substrate and appear to differ only in carbohydrate content (unpublished results from this laboratory). The easy enzymatic removal of sialic acid residues which evidently are the cause of the electrophoretic heterogeneity of kallikreins A and B [21, 39] suggests their peripheral position on the enzyme molecule. Thus, the existence of major differences in the catalytic properties of the various electrophoretically distinguishable forms of kallikrein, especially with small substrate molecules, is not to be expected. Indeed, kallikreins A and B, as well as their electrophoretically discernible subfractions and the sialidase-treated enzyme, cause quantitatively the same lowering of canine blood pressure [21]. Kallikrein A and sialidase-treated kallikrein B react with DFP at an identical rate [40], and sialidase-treated kallikreins A and B behave identically in their reactions with cinnamoyl and indoleacryloyl imidazole [25] and with *p*-nitrophenyl-*p'*-guanidinobenzoate [28]. An equal specific activity in the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester, though quite a low one, has been demonstrated in the course of the present work for a mixture of kallikreins A and B as well as for kallikrein A and for sialidase-treated kallikrein B.

A much more straightforward and likely explanation for the reports of a high activity of kallikrein with esters of neutral amino acids [7—9] is contamination of the kallikrein preparations used with other esterolytic enzymes, conceivable impurities with pertinent specificities in preparations of the anionic [39] porcine kallikrein being anionic chymotrypsins B [41] or C [42]. Such a contamination of our kallikrein preparations is ruled out by the results of the experiments with the partially DFP-inhibited enzyme, so that the data presented in Tables 1 and 2 can safely be regarded as pertaining to kallikrein itself and can be compared with the substrate specificities of other proteinases.

The most striking feature distinguishing kallikrein from bovine trypsin, both members of the serine proteinase family of pancreas, is the strong preference of α-*N*-benzoyl-L-arginine methyl ester over the corresponding lysine compound. This preference expresses itself in K_m as well as in k_{cat}, and the combined effects lead to a specificity constant [43] k_{cat}/K_m 159 times higher for the arginine than for the lysine ester. This ratio is even higher than the value of 50 found for the sulfhydryl proteinase clostripain [38], whereas trypsin hardly discriminates between esters of the two amino acids and thrombin even shows a slight preference for lysine [16]. Thus it becomes understandable why kallikrein liberates Met-Lys-bradykinin from a Gly-(or Ser)-Arg-Met-Lys-bradykinin peptide, while bradykinin is the final product of the action of trypsin [44].

In their behaviour towards esters of α-*N*-acylated neutral amino acids, kallikrein and trypsin resemble each other in that these esters are hydrolyzed, but are vastly inferior substrates compared to arginine esters. Corresponding derivatives of ornithine are also not endowed with better substrate properties for both enzymes. Incidentally, α-*N*-benzoyl-L-ornithine methyl ester $\cdot H_2O$ obtained from Cyclo Chemical Corporation was not attacked by trypsin [45], a fact not surprising in view of our experience with this material. The existence of the ester in the form of the free base is most improbable, and the Cyclo product is presumably the lactam.

As to the mechanism of the kallikrein-catalyzed hydrolysis of specific ester substrates, in contrast to observations with trypsin [14, 17] the rate constants of the hydrolysis of two series of arginine esters by kallikrein have been found to depend on the nature of the alcohol moiety and to run roughly parallel to the rates of hydroxyl-ion-catalyzed hydrolyses. This observation suggests a close relationship between the mechanism of the two types of catalysis. Such a

relationship has been corroborated with more arguments in the case of chymotrypsin [46]. As in other serine proteinases, the nucleophile involved might conceivably be the hydroxyl group of the essential serine residue of kallikrein [10], assisted by a histidine residue the cooperation of which reveals itself in the pH-dependence of the kallikrein-catalyzed hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester [24].

The present results do not allow any conclusion to be drawn on the operation of an acyl enzyme mechanism in the hydrolysis of specific ester substrates by kallikrein. There exist, however, several pieces of evidence that the enzyme is able to form acyl enzymes. Kallikrein is inactivated by reaction with a single molecule of DFP. Serine phosphate can be detected after mild acid hydrolysis[10]. Cinnamoyl and indoleacryloyl imidazole allow the spectrophotometric identification of the corresponding acyl kallikreins [25]. In the reaction of kallikrein with *p*-nitrophenyl-*p'*-guanidinobenzoate, the typical biphasic liberation of nitrophenol indicative of acyl enzyme formation is observed [28]. Thus it appears reasonable to assume that an acyl enzyme mechanism is also operating in the kallikrein-catalyzed hydrolysis of aliphatic esters of amino acids, as it is in other serine proteinases [43]. If this be the case, evidently it is the acylation reaction that is rate-limiting with at least the majority of the arginine esters studied.

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