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Substrate Specificities of Tissue Kallikrein and T-Kininogenase: Their Possible Role in Kininogen Processing†

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ABSTRACT: The present studies demonstrate the importance of subsite interactions in determining the cleavage specificities of kallikrein gene family proteinases. The effect of substrate amino acid residues in positions P_3–P'_3 on the catalytic efficiency of tissue kallikreins (rat, pig, and horse) and T-kininogenase was studied using peptidyl-pNA and intramolecularly quenched fluorogenic peptides as substrates. Kinetic analyses show the different effects of D-amino acid residues at P_3, Pro at P'_2, and Arg at either P'_1 or P'_3 on the hydrolysis of substrates by tissue kallikreins from rat and from horse or pig. T-Kininogenase was shown to differ from tissue kallikrein in its interactions at subsites S_2, S'_1, and S'_2. As a result of these differences, Abz-FRSR-EDDnp with Arg at P'_2 is a good substrate for tissue kallikreins from horse, pig, and rat but not for T-kininogenase. Abz-FRRP-EDDnp and Abz-FRAPR-EDDnp with Pro at P'_2 (rat high molecular weight kininogen sequence) are susceptible to rat tissue kallikrein but not to tissue kallikreins from horse and pig. Arg at P'_3 increased the susceptibility of the Arg–Ala bond to rat tissue kallikrein. These data explain the release of bradykinin by rat tissue kallikrein and of kallidin by tissue kallikreins from other animal species. Abz-FRLV-EDDnp and Abz-FRLVR-EDDnp (T-kininogen sequence) are good substrates for T-kininogenase but not for tissue kallikrein. Arg at the leaving group (at either P'_1, P'_2, or P'_3) lowers the K_m values of T-kininogenase while Val at P'_2 increases its k_{cat} values. The results indicate that the enzyme subsites S'_1, S'_2, and S'_3 are important determinants for the substrate specificity of tissue kallikreins and T-kininogenase. The findings are also in agreement with the known species specificity of tissue kallikreins and the resistance of rat T-kininogen to tissue kallikreins.

Tissue kallikreins are a group of serine proteinases encoded by a multigene family which consists of very closely related members (Mason et al., 1983). Recently, a proteinase with T-kininogenase activity, isolated from rat submandibular gland, was also characterized as a member of this group (Xiong et al., 1990). Understanding the mechanism of limited proteolysis of kininogens by kallikreins and related proteinases is of interest for many reasons. These enzymes may play a regulatory role in physiological processes since the kininogen substrates, as well as the released kinin peptide products, display very important physiological functions (Schachter, 1980). Studies on kininogen processing may also provide useful information about other kallikrein gene family members such as tonin (Lazure et al., 1981) and enzymes involved in the

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processing of nerve growth factor precursors (Thomas et al., 1981; Bothwell et al., 1979) and prohormones (Taylor et al., 1970).

A wide distribution of kallikrein among different animal tissues has been described (Fiedler, 1979). The identity of rat tissue kallikrein from different tissues has been demonstrated by both specific monoclonal antibody (Woodley et al., 1985) and amino acid sequences (Swift et al., 1982). A sequence identity of 61% has been found for rat and pig pancreatic kallikrein (Tschesche et al., 1979). Rat tissue kallikrein shows a kininogen cleavage specificity which leads to bradykinin liberation (Kato et al., 1985), while tissue kallikreins from other animal species release the decapeptide lysylbradykinin (Fiedler, 1979). Differences in the susceptibilities of rat kininogen to tissue kallikreins from rat and from other animal species (Kato et al., 1985) also indicate enzymatic properties of rat tissue kallikrein distinct from those of other species. T-Kininogen is cleaved by T-kininogenase to release T-kinin (Barlas et al., 1987), whereas it is resistant to tissue kallikreins (Okamoto & Greenbaum, 1986). These results indicate significant differences in the substrate specificities of kallikrein-like enzymes.

In this work, we describe the effects of secondary interactions on synthetic substrate hydrolysis by rat tissue kallikrein and T-kininogenase. These results were compared to those previously described for tissue kallikreins from other animal species (Fiedler, 1987; Prado et al., 1986; Araujo-Viel et al., 1988; Chagas et al., 1991). Our aim is to gain information about the role of substrate–subsite interactions in directing cleavage of the natural substrates of these proteinases and to develop specific synthetic substrates for these enzymes which could also be useful for the characterization of tissue kallikreins and kallikren-like proteinases. For these studies we used peptidyl-*p*-nitroanilides and intramolecularly quenched fluorogenic peptide substrates recently developed (Chagas et al., 1991).

Experimental Procedures

Enzymes. Previously described procedures were used to obtain homogeneous preparations of kallikrein and T-kininogenase from rat submandibular gland with some modifications (Xiong et al., 1990) and of horse urinary kallikrein (Giusti et al., 1988). A neuraminidase-treated and electrophoretically homogeneous preparation of porcine pancreatic kallikrein (form B) was kindly provided by Dr. W. Müller-Esterl, University of Mainz, Mainz, Germany; bovine trypsin, a gift from Dr. M. Mares Guia, Department of Biochemistry, UFMG, Belo Horizonte, Brazil, was prepared from a commercial enzyme preparation according to Dias and Rogana (1986). The molar concentration of the enzyme solutions was determined by active site titration with 4-nitrophenyl 4-guanidinobenzoate (Sampaio et al., 1984).

Synthetic Substrates. D-Val-Leu-Arg-pNA and D-Val-Leu-Lys-Arg-pNA[1] were purchased from AB Kabi Peptide Research (Mölndal, Sweden). Previously described procedures were used for the synthesis, purification, and analysis of other peptidyl-pNA substrates and of the intramolecularly quenched fluorogenic peptide substrates (Juliano & Juliano, 1985; Chagas et al., 1991).

Kininogen Purification. Purified human low molecular weight (LMW) kininogen was kindly provided by Dr. Y. Hojima, and bovine LMW kininogen was a gift from Dr. H. Kato of the Protein Research Institute, University of Osaka, Osaka, Japan. Low molecular weight and high molecular weight (HMW) kininogens were isolated from rat normal serum or dog plasma, and T-kininogen was purified from inflammed rat serum according to the previously described procedures (Johnson et al., 1987; Chao et al., 1989). Serum or plasma (30 mL) was made to contain 10 mM benzamidine, 40 μg/mL polybrene, 2 mM EDTA, 0.2 mM PMSF, 50 μg/mL SBTI, and 2 M NaCl. The serum was applied to a Cm-papain Affi-Gel 10 column (1.5 × 20 cm) equilibrated with a buffer containing 50 mM sodium phosphate, pH 7.5, 2 M NaCl, 1 mM benzamidine, 40 μg/mL polybrene, 1 mM EDTA, 0.2 mM PMSF, and 0.02% NaN_3. The column was washed with the equilibration buffer followed by 50 mM sodium phosphate, pH 7.5, 2 mM EDTA, 0.2 mM PMSF, and 0.02% NaN_3. Kininogens were eluted with 50 mM sodium phosphate, pH 11.5, 2 mM EDTA, and 0.02% NaN_3 in a single peak. The low molecular weight and high molecular weight kininogens were dialyzed against 10 mM sodium acetate, pH 5.5, and loaded onto a FPLC Mono Q HR 5/5 column preequilibrated with 10 mM sodium acetate, pH 5.5. The column was eluted with a linear gradient: (1) 0–0.25 M NaCl in 10 mM sodium acetate buffer, pH 5.5, in 30 min; (2) 0.25–0.50 M NaCl in 10 mM sodium acetate buffer, pH 5.5, in 5 min at 1 mL/min. LMW kininogen and HMW kininogen were eluted at 0.12 M NaCl and 0.35 M NaCl, respectively.

Kininogenase Assay. Kinin-releasing activities of tissue kallikrein and T-kininogenase were measured by incubating the enzyme (50 ng) with rat HMW kininogen, rat T-kininogen, dog LMW kininogen, human LMW kininogen, or bovine LMW kininogen (3 μg) in 0.1 M sodium phosphate, pH 8.5, in a total volume of 500 μL at 37 °C for 30 min. The reactions were stopped by boiling for 20 min. Released kinin was assayed by a kinin RIA (Shimamoto et al., 1979).

HPLC Analysis of the Synthetic Substrates and Their Enzymatic Hydrolysis Products. The peptide solutions (80–150 μM) in 20 mM Tris-HCl, pH 8.0 or 9.0, were incubated with different proteinases at 30 °C. Samples (20 μL) of the substrate and enzyme controls, and of the enzymatic digests, were removed for analysis until 100% of substrate hydrolysis was reached. The HPLC conditions were as follows: 0.1 M NaH_2PO_4 (pH 4.2) as solvent A; acetonitrile–H_2O (9:1) as solvent B; 10–80% gradient of solvent B in 15 min; flow rate, 1 mL/min; Novapak C-18 column, 3.2 × 150 mm; detection by ultraviolet at 214 nm and by fluorescence with λ_{em} = 420 nm and λ_{ex} = 320 nm.

Enzyme Assays. Hydrolysis of the peptidyl-pNA at 30 °C in 0.05 M Tris-HCl, pH 9.0, and 1 mM EDTA was measured as described previously (Oliveira et al., 1987) by photometric determination of the 4-nitroaniline released after the addition of the enzyme solution to a 2-mL reaction buffer. The hydrolysis was carried out for 5–10 min, and the initial hydrolysis rates were calculated from the slope of time-dependent absorbance curves extrapolated to zero time. Hydrolysis of the fluorogenic peptide substrates at 30 °C in 0.02 M Tris-HCl, pH 9.0, and 1 mM EDTA was monitored by measuring the fluorescence at λ_{em} = 420 nm and λ_{ex} = 320 nm in a Perkin-Elmer spectrofluorometer, Model LC5 (Chagas et al., 1991). The 1-cm path-length cuvette containing 2 mL of the substrate solution was placed in the thermostated cell compartment for 5–10 min before the enzyme solution (10–50 μL) was added, and the increase in fluorescence with time was continuously recorded for 5 min. The slope was converted into moles of substrate hydrolyzed per minute from the fluorescence

[1] Abbreviations: Abz, *o*-aminobenzoyl; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; pNA, *p*-nitroanilide; SBTI, soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; RIA, radioimmunoassay; Cm-papain, (carboxylmethyl)papain; em, emission; ex, excitation; LMW, low molecular weight; HMW, high molecular weight.

Table I: Kinetic Parameters (±SD) for the Hydrolysis of Peptidyl-pNA by Rat Tissue Kallikrein and T-Kininogenase[a]

	substrates							
	P_3	P_2	P_1	[S] (μM)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	R^b
				Rat Tissue Kallikrein				
I	Ac	G	R	24–214	100 ± 1	1 ± 0	10	1
II	Ac	S	R	68–348	222 ± 0	20 ± 2	90	9
III	Ac	P	R	60–400	133 ± 5	32 ± 1	241	24
IV	Ac	A	R	18–186	60 ± 4	27 ± 1	450	45
V	Ac	L	R	4–225	40 ± 4	77 ± 8	1925	192
VI	Ac	F	R	15–528	33 ± 5	82 ± 8	2485	249
VII	D-P	F	R	7–43	24 ± 6	75 ± 8	3125	313
VIII	D-F	L	R	7–110	38 ± 5	69 ± 7	1815	181
IX	D-V	L	R	9–320	68 ± 10	90 ± 12	1323	132
X	D-V	L	K	13–396	295 ± 17	46 ± 4	156	16
				T-Kininogenase				
I	Ac	G	R	33–198	121 ± 17	11 ± 1	91	1
II	Ac	S	R	56–560	128 ± 31	90 ± 9	708	8
III	Ac	P	R	35–700	216 ± 10	200 ± 10	925	4
IV	Ac	A	R	18–186	99 ± 18	124 ± 11	1252	14
V	Ac	L	R	5–70	24 ± 2	90 ± 5	3750	41
VI	Ac	F	R	35–700	89–9	252 ± 20	2831	31
VII	D-P	F	R	4–100	43 ± 6	151 ± 11	3500	39
VIII	D-F	L	R	4–340	22 ± 3	98 ± 9	4450	49
IX	D-V	L	R	5–111	19 ± 2	76 ± 5	4000	44
X	D-V	L	K	13–200	154 ± 7	90 ± 2	584	6

[a] Hydrolysis conditions are at 30 °C in 50 mM Tris-HCl/0.1 mM EDTA, pH 9.0. [b] Relative specificity constant: ratios of the k_{cat}/K_m values by the value for Ac-GR-pNA.

	P_3	P_2	P_1	P'_1	P'_2	P'_3							P_3	P_2	P_1	P'_1	P'_2	P'_3
Bovine Kng	S	L	M	K	R	P	P	G	F	S			P	F	R	S	V	Q
Rat Kng	V	I	R	R	P	P	G	F	S				P	F	R	A	P	R
T-Kng	D	M	M	I	S	R	P	P	G	F	S		P	F	R	L	V	R

FIGURE 1: Flanking regions of the bradykinin sequence in kininogens (Kng). The bradykinin sequence is underlined. The data for bovine LMW kininogen, rat HMW or LMW kininogen, and T-kininogen were taken from Kitamura et al. (1983), Kato et al. (1985), and Furito-Kato et al. (1985).

curves for standard peptide solutions before and after total enzymatic hydrolysis. As HPLC analysis of the different fluorogenic substrates did not show any contaminant, the concentration of their solutions could be determined by the fluorometric assay of the peptide Abz-Phe-Arg-OH formed in their total tryptic hydrolysis (overnight with 0.1 μM trypsin). The standard solution of Abz-Phe-Arg-OH was prepared by alkaline hydrolysis of Abz-Phe-Arg-pNA (3 h in 1 N NaOH); its concentration was calculated from the amount of 4-nitroaniline liberated by trypsin by measuring the absorbance at 405 nm.

The standard hydrolysis conditions were strictly maintained for different substrates. Low buffer concentrations were used since rat tissue kallikrein and T-kininogenase activities, similar to the activities of tissue kallikreins from pig and horse, can be impaired by high concentrations of cations (Oliveira et al., 1987; Araujo-Viel et al., 1988); EDTA was added to avoid proteinase inhibition by heavy metals. The kinetic parameters were calculated according to Wilkinson (1961).

Results and Discussion

Hydrolysis of Peptidyl-pNA by Rat Tissue Kallikrein and T-Kininogenase: Interactions at Subsites S_1, S_2, and S_3. Hydrolysis of peptidyl-pNA by rat tissue kallikrein and T-kininogenase followed Michaelis–Menten kinetics under a wide range of substrate concentrations (Table I). Enzyme activation by the peptidyl-pNA substrates, as described for porcine pancreatic kallikrein (Oliveira et al., 1987), was not observed.

The preference of both proteinases for Arg over Lys in position P_1 was demonstrated by the kinetic parameters for D-Val-Leu-Arg-pNA and D-Val-Leu-Lys-pNA (substrates IX and X in Table I). In the hydrolysis of the peptidyl-pNA, improvements in catalytic efficiences of rat tissue kallikrein and T-kininogenase were observed for substrates having hydrophobic residues at P_2 [nomenclature of Schechter and Berg (1967)]. The relative specificity constant for these substrates (R, as defined in Table I) indicates that Phe is the most favorable P_2 residue for tissue kallikrein while T-kininogenase has a preference for Leu. These parameters also demonstrate that the effects of the hydrophobic P_2 residues are more profound for tissue kallikrein than for T-kininogenase. This pronounced secondary specificity was first described for porcine pancreatic kallikrein by Fiedler and Leysath (1979). Using peptidyl-pNA substrates, we observed the same specificity for horse tissue kallikrein (Araujo-Viel et al., 1988). This specificity has also been described for other members of the rat and mouse kallikrein gene families (Elmoujahed et al., 1990; Blaber et al., 1989).

X-ray diffraction studies have shown that the Phe side chain is the most likely structure to fit properly in the wedged-shaped hydrophobic cavity formed between the Tyr-99 and Trp-215 side chains at the porcine kallikrein S_2 subsite (Chen & Bode, 1983). The hydrophobic pair Tyr-99 and Trp-215 has been identified in the sequences of tissue kallikreins from rat, human, and other animal species. The specificity of tissue kallikrein subsite S_2 residues has been considered as a determinant for liberating kinin by the limited proteolysis of kininogens (Fiedler, 1987; Elmoujahed, 1990). In favor of this notion is the presence of hydrophobic residues at the P_2 positions of the two bonds split by tissue kallikreins in kininogens from different animal species (Figure 1). In the present studies we have demonstrated that this specificity is also a

Table II: Kinetic Parameters (±SD) for the Hydrolysis of Fluorogenic Tetrapeptides Abz-Phe-Arg-X-Y-EDDnp (X = Ser or Arg; Y = Arg, Val, or Pro) by T-Kininogenase (T-Kngase) and Rat Tissue Kallikrein (Rat TK)[a]

		substances X-Y (P'_1–P'_2)				
	enzymes	Ser-Arg (XI)	Ser-Val (XII)	Ser-Pro (XIII)	Arg-Val (XIV)	Arg-Pro (XV)
[S] (μM)	T-Kngase	0.29–3.90	0.20–0.32	0.70–5.60	0.30–3.00	0.17–3.70
	rat, TK	0.06–11.40	0.85–6.85	0.70–10.40	0.04–4.00	0.02–3.70
K_m (μM)	T-Kngase	0.64 ● 0.09	10.0 ± 2.0	12.0 ± 1.0	1.30 ± 0.30	0.90 ● 0.10
	rat TK	0.24 ± 0.07	1.6 ± 0.1	5.2 ● 0.6	0.31 ● 0.10	0.62 ± 0.60
k_{cat} (s^{-1})	T-Kngase	0.33 ● 0.01	54.0 ± 0.9	0.15 ± 0.01	10.0 ± 0.9	0.02 ● 0.01
	rat TK	9.80 ± 0.40	6.3 ● 0.2	0.76 ± 0.06	6.5 ± 0.3	2.70 ± 0.20
k_{cat}/K_m[b] (s^{-1} mM^{-1})	T-Kngase	516	5400	13	7936	19
	rat TK	40833	3930	116	20960	4630
	pig TK	32182	737	122	6167	102
	horse TK	38000	1000	91	6333	170

[a] Hydrolysis conditions are at 30 °C, in 20 mM Tris-HCl/1 mM EDTA, pH 9.0. [b] The k_{cat}/K_m values for tissue kallikreins (TK) from pig pancreas and horse urine (Chagas et al., 1991) are shown for comparison.

Table III: Hydrolysis of Fluorogenic Substrates Having the Rat Kininogen Sequence at the C-Terminal Cleavage Site by T-Kininogenase (T-Kngase) and Tissue Kallikreins (TK)[a]

enzyme	no.	substrates[b]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
T-Kngase	XVI	Abz-FRAP-EDDnp	28 ± 5	0.09 ± 0.03	3
	XVII	Abz-FRAPR-EDDnp	2 ± 0	0.32 ± 0.03	152
rat TK	XVI	Abz-FRAP-EDDnp	23 ± 5	0.59 ± 0.07	26
	XVII	Abz-FRAPR-EDDnp	8 ± 1	4.05 ± 0.02	506

enzyme	no.	substrates[b]	[S] (μM)	velocity [μmol/(s·μmol of E)]
horse TK	XVII	Abz-FRAPR-EDDnp	30	0.04
pig TK	XVII	Abz-FRAPR-EDDnp	30	0.03

[a] Hydrolysis conditions are at 30 °C in 20 mM Tris-HCl/1 mM EDTA, pH 9.0. [b] Substrate concentration ranges [S]: peptide XVI, 3.0–40 μM for both enzymes; substrate XVII, 0.5–3.7 μM and 1.5–30 μM for T-kininogenase and tissue kallikrein, respectively (at higher substrate concentrations T-Kngase inhibition was observed).

Table IV: Hydrolysis of Fluorogenic Substrates Having the T-Kininogen Sequence at the C-Terminal Cleavage Site by T-Kininogenase (T-Kngase) and Tissue Kallikreins[a]

enzyme	no.	substrates[b]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
T-Kngase	XVIII	Abz-FRLV-EDDnp	6.6 ± 1.1	2.1 ± 0.2	318
	XIX	Abz-FRLVR-EDDnp	0.8 ± 0.2	9.0 ± 2.0	11250

tissue kallikreins	no.	substrates[b]	[S] (mM)	velocity [μmol/(s·μmol of E)]
rat	XIX	Abz-FRLVR-EDDnp	20	0.34
horse	XIX	Abz-FRLVR-EDDnp	2	0.04
			20	0.40
pig	XIX	Abz-FRLVR-EDDnp	2	0.01
			20	0.02

[a] Hydrolysis conditions are at 30 °C in 20 mM Tris-HCl/1 mM EDTA, pH 9.0. [b] Substrate concentration ranges [S]: peptide XVIII, 1.3–6.3 μM; peptide XIX, 0.15–0.58 μM (at higher substrate concentrations T-Kngase inhibition was observed).

feature for rat tissue kallikrein (Table I). However, this P_2 specificity does not explain differences in kininogen cleavage by rat tissue kallikrein and kallikreins from other animal species, which lead to bradykinin and Lys-bradykinin release, respectively.

Regarding subsite S_3 specificity, it has been previously reported that D-amino acid residues, with the free amino group at P_3, increase the substrate susceptibility to tissue kallikreins (Oliveira et al., 1987; Araujo-Viel et al., 1988) and the inhibition rates of rat and human tissue kallikreins by peptidyl-Arg chloromethyl ketones (Kettner et al., 1980). In contrast, our kinetic analyses using tripeptidyl-pNA substrates indicate that, for rat tissue kallikrein and T-kininogenase, the effect of D-amino acids at P_3 is not significant (Table I).

Hydrolysis of Quenched Fluorogenic Peptides by Tissue Kallikreins and T-Kininogenase: Interactions at Subsites S'_1, S'_2, and S'_3. A single fluorescent peptide identified by HPLC as Abz-Phe-Arg was formed in the hydrolysis of all quenched fluorogenic peptides presented in Tables II–IV by tissue kallikreins and T-kininogenase. The highest specific constant (k_{cat}/K_m) for Abz-Phe-Arg-Ser-Arg-EDDnp (substrate, XI, Table II) and the lowest one for Abz-Phe-Arg-Ser-Pro-EDDnp (substrate XIII, Table II) in the hydrolysis by rat tissue kallikrein demonstrate that this proteinase, like those from other animal species, has a preference for P'_2 Arg and interacts very poorly with Pro at this position (Prado et al., 1986; Araujo-Viel et al., 1988; Chagas et al., 1991). A favorable interaction of subsite S'_1 with Arg could also be demonstrated for rat tissue kallikrein by comparison of the kinetic data for substrates having P'_1 Arg (peptides XIV and XV, Table II) and those having at this position Ser (substrates XII and XIII, Table II), Ala (substrate XVI, Table III), and Leu (substrate XVIII, Table IV). Kinetic parameters for the hydrolysis of peptides XVI and XVII (Table III), having the rat HMW kininogen sequence at the C-terminal cleavage site (-Phe-Arg-Ala-Pro-Arg-), indicate that the Arg residue in P'_3 increases the susceptibility of the Arg–Ala bond to rat tissue kallikrein. In contrast, peptide XVII (Abz-Phe-Arg-Ala-Pro-Arg-EDDnp) was resistant to both horse and porcine tissue kallikreins (Table III).

Bradykinin is released from rat HMW or LMW kininogen by the hydrolysis of arginyl bonds with Pro at P'_2 positions of both cleavage sites (Figure 1). These bonds were shown to be very resistant to tissue kallikreins from horse, human, and pig (Prado et al., 1986; Araujo-Viel et al., 1988; Chagas et al., 1991). However, as we have demonstrated in the hy-

Table V: Kinin-Releasing Activities of Rat Tissue Kallikrein and T-Kininogenase

substrate	specific activity[a]	
	tissue kallikrein	T-kininogenase
rat T-kininogen	2.6 ± 1.0	192.9 ± 25.9
rat HMW kininogen	93.8 ± 13.7	48.0 ± 12.5
dog LMW kininogen	151.7 ± 7.6	74.5 ± 14.6
bovine LMW kininogen	45.1 ± 9.2	41.6 ± 12.0
human LMW kininogen	145.4 ± 23.4	160.0 ± 39.7

[a] Specific activity is expressed as micrograms of kinin released per milligram of enzyme in 30 min at 37 °C. Values represent mean ± SD (n = 3–5).

drolysis of the oligopeptides (Table I), the arginine residue in position P'_1 at the N-terminal side and in position P'_3 at the C-terminal side of the kinin moiety in rat HMW kininogen (Figure 1) increases the susceptibility to rat tissue kallikrein but not to the tissue kallikreins from horse and pig. These data explain bradykinin liberation by rat tissue kallikrein (Kato et al., 1985) and the resistance of rat kininogen to heterologous tissue kallikreins.

In kininogens from bovine or other animal species (Figure 1), Pro is the P'_2 residue for the Lys–Arg bond at the N-terminal cleavage site which is only susceptible to rat tissue kallikrein. In contrast, the Arg–X bond at the C-terminal cleavage site is cleaved by all the true tissue kallikreins. Cleavage of the Met–Lys bond by the kallidin-liberating kallikreins may be directed by the optimal interactions of these enzymes with hydrophobic residues at P_2 and with Arg at P'_2. However, the importance of further interactions cannot be ruled out in view of the described resistance of the peptide Gly-Leu-Met-Lys-bradykinin to horse urinary and porcine pancreatic kallikrein (Araujo-Viel et al., 1981; Fiedler, 1983).

The very weak T-kinin releasing activity of rat tissue kallikrein from the T-kininogen substrate demonstrated previously (Okamoto & Greenbaum, 1986) was confirmed with the enzyme and the purified T-kininogen preparation used in the present work (Table V). In agreement with this observation, the peptide having the sequence of T-kininogen at the C-terminal cleavage site (Abz-Phe-Arg-Leu-Val-Arg-EDDnp) was shown to be a very poor substrate for rat as well as for porcine and horse tissue kallikreins (Table IV).

When peptide substrates with Ser-Pro-EDDnp, Arg-Pro-EDDnp, or Ala-Pro-EDDnp as leaving groups were used (Tables II and III, substrates XIII, XV, and XVI), T-kininogenase displayed very low activity. The results demonstrate that this proteinase, like human or porcine tissue kallikreins, does not accept Pro at P'_2. On the other hand, Abz-Phe-Arg-Ser-Arg-EDDnp (peptide XI), which was shown to be the best substrate for rat, porcine, and horse tissue kallikreins (Chagas et al., 1991), was a poor substrate for the T-kininogenase (Table II). Peptides XII and XIV (Table II) with Ser-Val-EDDnp and Arg-Val-EDDnp as leaving groups presented high susceptibility to T-kininogenase, while peptide XVIII (Table IV), having P'_2 Val but a hydrophobic residue (Leu) at P'_1, was a poor substrate. As can be seen in Tables II and III, Arg at the leaving groups (at either P'_1, P'_2, or P'_3) lowers the K_m value of T-kininogenase while Val at P'_2 increases the k_{cat} values.

An observation that still awaits further studies was the T-kininogenase inhibition by the substrates that presented higher hydrolysis rates (substrates XII, XIV, and XIX in Tables II and IV). The parameters for these hydrolyses were determined at substrate concentrations that still observe Michaelis–Menten kinetics. These data show that the best peptide substrate for T-kininogenase is Abz-Phe-Arg-Leu-Val-Arg-EDDnp (peptide XIX) mimicking the T-kininogen sequence (Figure 1).

Addition of dithiothreitol (5 mM) had no effect on the kinetic parameters for D-Val-Leu-Arg-pNA and Abz-Phe-Arg-Leu-Val-Arg-EDDnp hydrolysis by T-kininogenase. These data constitute a further indication that the increase of the kinin releasing activity in the presence of thiol reagents (Barlas et al., 1987) may be related to an effect on the natural substrate but not on the enzyme (Xiong et al., 1990). The fact that T-kinin released from T-kininogen involves the cleavage of a Met–Ile bond suggests that the increase on T-kininogenase activity by thiol reagents may be related to the reduction of possible oxidized Met residues in the natural substrate. In favor of this hypothesis is our previous demonstration that oxidation of the Met residue in the sequence Gly(or Ser)-Leu-Met-Lys-bradykinin impairs the cleavage activity of the Met–Lys bond by tissue kallikreins (Araujo-Viel et al., 1981).

T-Kininogenase was shown to release kinin peptides from kininogens of several animal species (Table V). Hydrolysis of the peptides Abz-Phe-Arg-Ser-Val-EDDnp (Table II) and Abz-Phe-Arg-Ala-Pro-Arg-EDDnp (Table III) confirms the ability of T-kininogenase to cleave the Arg bond at the kinin C-terminal side in bovine and rat kininogen (Figure 1). On the other hand, the resistance of Abz-Phe-Arg-Arg-Pro-EDDnp makes bradykinin liberation by T-kininogenase unlikely.

In contrast to peptidyl-Arg-pNA, some of the fluorogenic peptide substrates described in this and previous work (Chagas et al., 1991) may be considered up to now as specific substrates for members of the kallikrein gene family proteinases, as follows: (1) for Abz-Phe-Arg-Ser-Arg-EDDnp, its specificity for true tissue kallikreins described previously was confirmed by the high k_{cat}/K_m value for rat tissue kallikrein and the 77-fold lower value for T-kininogenase (Table II). (2) Abz-Phe-Arg-Arg-Pro-EDDnp was shown to be a much better substrate for tissue kallikrein from rat than from other animal species (Table II). (3) Abz-Phe-Arg-Leu-Val-Arg-EDDnp is a good substrate for T-kininogenase but is resistant to tissue kallikreins (Table IV). These substrates may be potentially valuable to the isolation and characterization of other kallikrein-like enzymes.

The data presented in this present work on the hydrolysis of synthetic substrates by tissue kallikreins and T-kininogenase indicate that subsites S'_1, S'_2, and S'_3 are important determinants for the substrate specificity of tissue kallikreins and related proteinases. The findings are also in agreement with the known species specificity of tissue kallikreins and the resistance of rat T-kininogen to tissue kallikreins.

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Stereochemical and Positional Specificity of the Lipase/Acyltransferase Produced by *Aeromonas hydrophila*†

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ABSTRACT: *Aeromonas* species secrete a glycerophospholipid–cholesterol acyltransferase (GCAT) which shares many properties with mammalian plasma lecithin–cholesterol acyltransferase (LCAT). We have studied the stereochemical and positional specificity of GCAT against a variety of lipid substrates using NMR spectroscopy as well as other assay methods. The results show that both the primary and secondary acyl ester bonds of L-phosphatidylcholine can be hydrolyzed but only the *sn*-2 fatty acid can be transferred to cholesterol. The enzyme has an absolute requirement for the L configuration at the *sn*-2 position of phosphatidylcholine. The secondary ester bond of D-phosphatidylcholine cannot be hydrolyzed, and this lipid is not a substrate for acyl transfer. In contrast to the phospholipases, but similar to LCAT, the enzyme does not interact stereochemically with the phosphorus of phosphatidylcholine. In fact, the phosphorus is not required for enzyme activity, as GCAT will also hydrolyze monolayers of diglyceride, although at much lower rates.

Lipases are found throughout nature. All those which have been studied have been classified as members of the family of serine esterases, distinguished by their ability to act at lipid–water interfaces. They have recently attracted a great deal of attention, partly because of the important roles they play in lipid metabolism, as well as because of the many applications they may have in industry and medicine (Harwood, 1989). The glycerophospholipid–cholesterol acyltransferase (GCAT), released by *Vibrio* species, and lecithin–cholesterol acyltransferase (LCAT), found in mammalian plasma, may be grouped with the lipases as they each have small regions corresponding to the lipase consensus sequence in which their

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