Substrate Activation of Porcine Pancreatic Kallikrein by N^{α} Derivatives of Arginine 4-Nitroanilides†

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Received September 16, 1986; Revised Manuscript Received April 6, 1987

ABSTRACT: Hydrolysis of several N^{α}-substituted L-arginine 4-nitroanilides with porcine pancreatic kallikrein was studied under different conditions of pH, temperature, and salt concentration. At high substrate concentrations a deviation from Michaelis–Menten kinetics was observed with a significant increase in the hydrolysis rates of almost all substrates. Kinetic data were analyzed on the assumption that porcine pancreatic kallikrein presents an additional binding site with lower affinity for the substrate. Binding to this auxiliary site gives rise to a modulated enzyme species which can hydrolyze an additional molecule of the substrate through a second catalytic pathway. The values of both Michaelis–Menten and catalytic rate constants were higher for the modulated species than for the free enzyme, suggesting a mechanism of enzyme activation by substrate. Kinetic data indicated similar substrate requirements for binding at the primary and auxiliary sites of the enzyme. Tris(hydroxymethyl)aminomethane hydrochloride and NaCl were shown to alter the kinetic parameters of the hydrolysis of N^{α}-acetyl-L-Phe-L-Arg 4-nitroanilide by porcine pancreatic kallikrein but not the enzyme activation pattern (ratio of the catalytic constants for the activated and the free enzyme forms). Similar observations were made when the hydrolysis of D-Val-L-Leu-L-Arg 4-nitroanilide was studied under different pH and temperature conditions.

Porcine pancreatic kallikrein (Pop-kallikrein)[1] is a true tissue or glandular kallikrein (EC 3.4.21.35) and a trypsin-like serine proteinase that exhibits practically no activity on proteins except for kininogen, its natural substrate. It has been well demonstrated that Pop-kallikrein, unlike trypsin (EC 3.4.21.4), requires other substrate structural features such as hydrophobic groups at P_2 [for nomenclature, see Schechter and Berger (1961)] to hydrolyze efficiently synthetic substrates (Fiedler & Leysath, 1979; Levison & Tomalin, 1982; Powers et al., 1984). Results obtained by high-resolution X-ray diffraction suggest that the conformations of the active site residues of Pop-kallikrein and trypsin are very similar. Therefore, the differences observed in the catalytic properties of these enzymes should result from peculiar structural features of their external loops and subsites (Bode et al., 1983; Chen & Bode, 1983).

Bovine trypsin is the only serine proteinase that has been consistently reported as exhibiting anomalously high hydrolysis rates, deviating from Michaelis–Menten kinetics, at high concentrations of some substrates (Trowbridge et al., 1963; Nakata & Ishii, 1970, 1972; Magalhães-Rocha et al., 1980; Tsunematsu et al., 1983). This property, called substrate activation, appears not to be associated with the nature of the bond cleaved by the enzyme since it was observed with esters as well as with amides. For Pop-kallikrein, there is a single report in the literature describing substrate activation of this enzyme by Bz-Arg-OEt and Tos-Arg-OMe (Fiedler & Werle, 1968).

In the present work, the hydrolysis of several N^{α}-substituted arginine 4-nitroanilides by Pop-kallikrein was studied under different conditions of pH, temperature, and salt concentration. The results show that this enzyme is strongly activated by these substrates and that this activation, unlike that described for trypsin, is closely related to the substrate structural requirements for efficient catalysis by Pop-kallikrein.

Experimental Procedures

Enzymes and Substrates. Three samples of Pop-β-kallikrein were used. Two of them containing 1374 and 1168 KU/mg (samples I and II) were kindly supplied by Prof. G. Schmidt-Kastner from Bayer AG, D-5600 Wuppertal. The third sample (III), a neuraminidase-treated Pop-kallikrein, was a kind gift of Dr. W. Müller-Sterl from the Clinic Chemistry and the Clinic Biochemistry Department, University of Munich, FRG. SDS–PAGE of these enzyme samples (20 μg) was performed in linear 7.5–15% gradient polyacrylamide gel slabs, followed by staining with Coomassie Brilliant Blue (Laemmli, 1970). Two electrophoretic bands corresponding to enzyme forms A and B were identified for samples I and II. For sample III, only one electrophorectic band, corresponding to the slower band (form B) of Pop-kallikrein, was observed. The molar concentration of the enzyme solutions in 10 mM ammonium acetate, pH 7.0, was determined by active site titration with NPGB (Fiedler et al., 1972) for samples I and II of Pop-kallikrein and by absorbance at 280 nm for sample III of the same enzyme (ϵ_{280} = 50 600 M^{-1} cm^{-1}; Fiedler et al., 1975). D-Pro-Phe-Arg-Nan and D-Val-Leu-Arg-Nan were purchased from Kabi Diagnostica. The other substrates were

† This research was supported by Financiadora de Estudos e Projetos (FINEP), Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

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[1] Abbreviations: Pop-kallikrein, porcine pancreatic β-kallikrein; Nan, 4-nitroanilide; Bz-Arg-OEt, N^{α}-benzoylarginine ethyl ester; Tos-Arg-OMe, N^{α}-tosylarginine methyl ester; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Ac, acetyl; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The classical L indication for L isomers of amino acids is omitted; only D isomers are indicated.

0006-2960/87/0426-5032$01.50/0

Table I: Kinetic Parameters for Hydrolysis of 4-Nitroanilide Substrates by Pop-kallikrein[a]

substrate	k_2 (s^{-1})	k_{a2} (s^{-1})	k_{a2}/k_2	K_1 (μM)	K_{s1} (μM)	R^b
D-Val-Leu-Arg-Nan[c,f]	7.7 ± 0.1	46 ± 1	6.0 ± 0.2	7.5 ± 0.5	129 ± 5	3.2 ± 0.5
D-Pro-Phe-Arg-Nan[c,f]	4.3 ± 0.1	14.6 ± 0.2	3.4 ± 0.1	1.9 ± 0.1	8.6 ± 0.4	15.6 ± 0.7
Pro-Phe-Arg-Nan[c,e,g]	4.0 ± 0.1	10.6 ± 0.3	2.6 ± 0.1	50 ± 4	90 ± 10	11.4 ± 0.8
Ac-Pro-Phe-Arg-Nan[c,e,g]	2.6 ± 0.1	5.5 ± 0.3	2.1 ± 0.1	20 ± 3	80 ± 9	24 ± 3
Bz-Pro-Phe-Arg-Nan[c,e,f]	0.72 ± 0.03	2.77 ± 0.03	3.9 ± 0.2	7.5 ± 0.7	13 ± 1	7.3 ± 0.6
Ac-Phe-Arg-Nan[c,e,h]	4.7 ± 0.2	10.2 ± 0.1	2.1 ± 0.1	34 ± 3	100 ± 10	4.1 ± 0.3
Ac-Phe-Arg-Nan[d,e,h]	3.5 ± 0.3	9.1 ± 0.1	2.6 ± 0.2	24 ± 4	19 ± 2	3.5 ± 0.4
Bz-Phe-Arg-Nan[c,e,g]	0.27 ± 0.02	0.68 ± 0.02	2.6 ± 0.2	6.7 ± 0.7	10 ± 1	7.0 ± 0.8
Ac-Gly-Arg-Nan[c,g]	0.057 ± 0.006			90 ± 9		
Bz-Arg-Nan[c,e,g]	0.130 ± 0.003			150 ± 10		

[a] pH 9.0 (0.05 M Tris-HCl and 1 mM EDTA) and 37 °C; the number of runs was at least 14 for all substrates. [b] $R = K_{a1}/K_1 = K_{s2}/K_{s1}$. [c] NPGB-titrated enzyme sample I; E_0 = 1–9 nM. [d] Enzyme sample III; E_0 = 1–7 nM. [e] 10% Me_2SO (v/v). [f] S range = 3–400 μM. [g] S range = 5–1500 μM. [h] S range = 5–2500 μM.

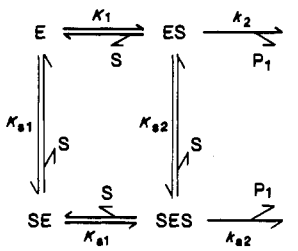
synthesized as described by Juliano and Juliano (1985).

Hydrolysis Rates. The standard conditions for the hydrolysis of nitroanilide substrates by Pop-kallikrein were pH 9.0 (0.05 M Tris-HCl containing 1 mM EDTA and optionally 10% Me_2SO v/v) and 37 °C. The enzymatic hydrolysis was monitored by measuring the absorbance at 405 nm of 4-nitroaniline (ϵ_{405} = 8900 M^{-1} cm^{-1}), in a Beckman Acta V spectrophotometer equipped with thermostated cell blocks. Two cuvettes with 1-cm path length, containing 2 mL of the same substrate solution, were left in the thermostated cell compartment until temperature equilibrium of the substrate solutions was attained (5–10 min). During this time interval no change in the differential absorbance, set initially at zero, was observed. The enzyme solution (10–50 μL) was then added, and within at least 20 s the time–absorbance data were collected, either manually at time intervals of 10 s or by continuous recording (chart speed of about 6 cm/min and expansion to 0.1 abosrbance unit), during 5–10 min. Substrate concentration was determined from the amount of 4-nitroaniline formed on hydrolysis by excess trypsin. Initial hydrolysis rates by Pop-kallikrein were calculated from the slope of time–absorbance curves extrapolated to zero time. The final enzyme concentration in the different assays was varied from 1 to 9 nM so that no more than 20–30% of the total hydrolysis was reached within at least 2 min of reaction. For substrate concentrations lower than 10 μM (A_{405} total increment <0.09), the full time–absorbance curve was recorded including the region where the substrate was depleted. At least two enzyme concentrations were used to check out the reliability of the measurements, especially for substrate concentrations below 5 μM. Absorbance variations as low as 0.002 could be reliably detected, under optimal equipment measuring conditions (absorbance scale up to 0.05).

Data Analysis. Kinetic parameters were calculated according to Wilkinson (1961) for hydrolyses following Michaelis-Menten kinetics. Data showing a systematic upward deviation from linearity on the Eadie–Hofstee plot, at higher substrate concentrations, were analyzed by an iterative regression method (Marquardt, 1963) to calculate the parameters relative to the reaction pathways of Scheme I.

Scheme I is similar to that formulated by Trowbridge et al. (1963) to interpret substrate activation of trypsin. The formalism adopted is that molecules of S interact with one putative auxiliary site of enzyme species E and ES to produce SE and SES, respectively. For trypsin, this assumption is supported by enzymatic and binding studies that suggest an auxiliary site with the ability to fit neutral rather than positively charged substrates (Sanborn & Hein, 1968; Sanborn & Bryan, 1968). For Pop-kallikrein, no direct evidence has yet been obtained, but an auxiliary site has been proposed to explain the substrate activation of this enzyme by Tos-Arg-OMe (Fiedler & Werle, 1968; Mares-Guia & Diniz, 1970).

Scheme I



Differently from Trowbridge et al. (1963), we assume that the complex SE of Scheme I is a modulated form of Pop-kallikrein. As a consequence, the substrate would be an effector in binding to the putative auxiliary site (equilibrium constants K_{s1} and K_{s2}), to modulate catalysis occurring at the level of the enzyme primary site. Therefore, two interchangeable enzyme forms E and SE might interact with the same substrate, promoting hydrolysis according to rate constants k_2 and k_{a2}, respectively, and Michaelis–Menten constants K_1 and K_{a1}, respectively. Under steady-state conditions

$$vP_1/E_0 = \frac{k_2K_{s2}S + k_{a2}S^2}{K_{a1}K_{s1} + (K_{a1} + K_{s2})S + S^2} \quad (1)$$

where vP_1 is the initial rate of leaving group (P_1) release and E_0 is the total concentration of enzyme. All constants in Scheme I may be determined by adjusting experimental data (vP_1/E_0 vs. S) to the iterative nonlinear regression to calculate the five parameters of eq 1 with the assumption that, in the steady state, $E_0 \ll S$ and $K_{s1}K_{a1} = K_1K_{s2}$. This procedure differs from that used by Nakata and Ishii (1972), who determined four parameters of Scheme I, the two catalytic rate constants and two overall equilibrium constants, for the binary and ternary enzyme–substrate complexes.

Results and Discussion

Hydrolysis of Nitroanilide Substrates by Pop-kallikrein. The Eadie–Hofstee plot for the hydrolysis of D-Val-Leu-Arg-Nan by Pop-kallikrein (sample I) at pH 9.0 and 37 °C shows that the reaction did not follow Michaelis–Menten kinetics, displaying a biphasic pattern due to a marked upward deviation from linearity at higher substrate concentrations (Figure 1). Similar patterns were obtained with all the nitroanilide substrates assayed except Ac-Gly-Arg-Nan and Bz-Arg-Nan. The kinetic parameters of Scheme I, calculated from nonlinear regressions, are shown in Table I. The constants for the hydrolysis of Ac-Gly-Arg-Nan and Bz-Arg-Nan, which followed Michaelis-Menten kinetics in the substrate concentration range used, were included for comparison. SDS–PAGE of Pop-kallikrein samples I and II has shown

Table II: Kinetic Parameters for Hydrolysis of D-Val-Leu-Arg-Nan under Different Conditions of pH and Temperature[a]

temperature (°C)	k_2 (s^{-1})	k_{a2} (s^{-1})	k_{a2}/k_2	K_1 (μM)	K_{s1} (μM)	R^b
			pH 9.0 (0.05 M Tris-HCl)			
37	7.7 ± 0.1	46 ± 1	6.0 ± 0.2	7.5 ± 0.5	129 ± 5	13.2 ± 0.5
27	3.7 ± 0.1	25 ± 1	6.7 ± 0.4	6.1 ± 0.8	70 ± 8	11.9 ± 0.9
19	1.8 ± 0.1	8.5 ± 0.3	4.6 ± 0.2	2.0 ± 0.2	11 ± 1	61 ± 4
			pH 7.0 (0.05 M Sodium Phosphate)			
37	5.5 ± 0.1	20.4 ± 0.4	3.7 ± 0.1	17.5 ± 0.1	195 ± 8	5.0 ± 0.2
27	1.9 ± 0.1	6.2 ± 0.1	3.3 ± 0.1	5.9 ± 0.5	73 ± 8	16 ± 1
19	1.0 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	6.5 ± 0.6	15 ± 2	10.3 ± 0.8

[a] 1 mM EDTA; NPGB-titrated enzyme sample I; E_0 = 1–9 nM; S range = 3–400 μM; the number of runs was at least 15 for all series. [b] $R = K_{a1}/K_1 = K_{s2}/K_{s1}$.

Table III: Kinetic Parameters for Hydrolysis of Ac-Phe-Arg-Nan for Different NaCl Concentrations[a]

[NaCl] (M)	k_2 (s^{-1})	k_{a2} (s^{-1})	k_{a2}/k_2	K_1 (μM)	K_{s1} (μM)	R^b
0	3.8 ± 0.2	6.4 ± 0.1	1.7 ± 0.1	22 ± 3	15 ± 2	6.3 ± 0.7
0.014	3.0 ± 0.2	6.6 ± 0.1	2.2 ± 0.1	26 ± 3	19 ± 2	5.8 ± 0.7
0.055	2.8 ± 0.1	5.8 ± 0.1	2.1 ± 0.2	72 ± 8	40 ± 5	2.5 ± 0.3
0.083	3.1 ± 0.1	6.0 ± 0.1	1.9 ± 0.1	140 ± 10	52 ± 6	2.3 ± 0.2
0.138	2.5 ± 0.2	5.4 ± 0.2	2.2 ± 0.3	120 ± 10	72 ± 8	2.3 ± 0.2

[a] pH 9.0 (0.022 M Tris-HCl, 1 mM EDTA, and 10% Me_2SO v/v) and 37 °C; NPGB-titrated enzyme sample II; E_0 = 1–9 nM; S range = 5–2000 μM; the number of runs was at least 18 for all series. [b] $R = K_{a1}/K_1 = K_{s2}/K_{s1}$.

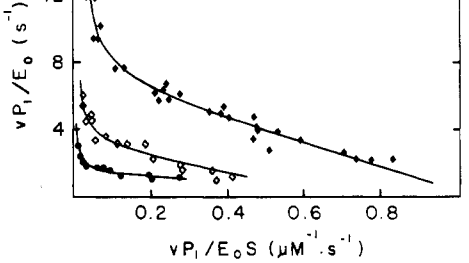


FIGURE 1: Eadie–Hofstee plots showing the effect of temperature on the hydrolysis of D-Val-Leu-Arg-Nan (S range = 3–400 μM) by Pop-kallikrein (NPGB-titrated sample I; E_0 = 1–9 nM). Reactions were carried out at pH 9.0 (0.05 M Tris-HCl and 1 mM EDTA) and at different temperatures: 37 (♦), 27 (◊), and 19 °C (●).

routinely two electrophoretic bands corresponding to the enzyme forms A and B. Therefore, the bimodal pattern shown by Pop-kallikrein (Figure 1) could also be explained by the presence of two independent enzymes in preparations I and II. However, this possibility may be ruled out since in the hydrolysis of Ac-Phe-Arg-Nan by the electrophorectically homogeneous preparation of Pop-kallikrein B (sample III), a biphasic pattern was also observed (Table I). This finding agrees with previous reports showing that Pop-kallikrein forms A and B present similar catalytic properties (Fiedler, 1976).

Table I shows that the catalytic constants k_{a2} are consistently higher than the corresponding constants k_2. A similar pattern was previously reported for the hydrolysis of several substrates by trypsin (Trowbridge et al., 1963; Nakata & Ishii, 1970, 1972; Magalhães-Rocha et al., 1980; Tsunematsu et al., 1983), characterizing a mechanism of substrate activation for this enzyme. For Pop-kallikrein, activation is also supposed to be due to substrate binding to an enzyme auxiliary site (Scheme I) as indicated by the values of K_{s1} and K_{s2} (equal to $K_{s1}R$ where $R = K_{a1}/K_1 = K_{s2}/K_{s1}$; Table I). As a result of this mechanism, substrate binding to the enzyme primary site is weaker for activated forms of Pop-kallikrein, as is shown by the higher values of K_{a1} (equal to K_1R) as compared to K_1 (Table I). Substrate binding to the auxiliary and the primary sites of Pop-kallikrein was always stronger for the free enzyme E than for binary complexes SE and ES as indicated by $R >$ 1 (Table I). These values vary within the same order of magnitude for all substrates that display biphasic hydrolysis. From this relative constancy, it may be assumed that the structural features important for a favorable substrate binding at the auxiliary site (thus producing enzyme activation) are equally important for substrate binding at the primary site of Pop-kallikrein. The parallelism between the variations in the values obtained for K_{s1} and K_1 strengthens this assumption (Table I).

The kinetic parameters in Table I are compatible with the well-known preference of Pop-kallikrein subsite S_2 for hydrophobic residues (Fiedler & Leysath, 1979; Levison & Tomalin, 1982; Powers et al., 1984). Moreover, the ratios k_2/K_1 and k_{a2}/K_{a1} (calculated from constants in Table I) are higher for nitroanilide tripeptides containing D-amino acid residues with a free α-amino group in position P_3 (D-Pro and D-Val) than for those with free or N^{α}-substituted Pro residues at the same position and for dipeptides. The favorable interaction of a D residue possessing a free α-amino group with subsite S_3 has recently been proposed for synthetic substrates for plasmin. For this enzyme, a negatively charged locus at the subsite S_3 is suggested in order to accommodate the free α-amino group of these substrates (Okada et al., 1986). However, for Pop-kallikrein, no indication for such a subsite locus has been obtained from X-ray studies (Chen & Bode, 1983), and therefore, no explanation for the preference of the enzyme for D-amino acid residues at P_3 may be presented at the moment. Ac-Gly-Arg-Nan and Bz-Arg-Nan are the worst Pop-kallikrein substrates of those listed in Table I, and their enzymatic hydrolyses followed a monophasic pattern. By taking an average value of the R values for other nitroanilide substrates (Table I), it may be predicted that evidence for enzyme activation by those two substrates would only be obtained if a larger number of experimental points at higher substrate concentration were determined.

Effect of pH and Temperature on the Hydrolysis of D-*Val-Leu-Arg-Nan by Pop-kallikrein.* The biphasic pattern for the hydrolysis of D-Val-Leu-Arg-Nan by Pop-kallikrein (sample I) was maintained under different temperatures, both at pH 9.0 (0.05 M Tris-HCl and 1 mM EDTA) and at pH 7.0 (0.05 M sodium phosphate and 1 mM EDTA). This behavior, illustrated in Figure 1, is similar to that reported for tryptic catalysis of Tos-Arg-OMe (Bechet & Yon, 1964).

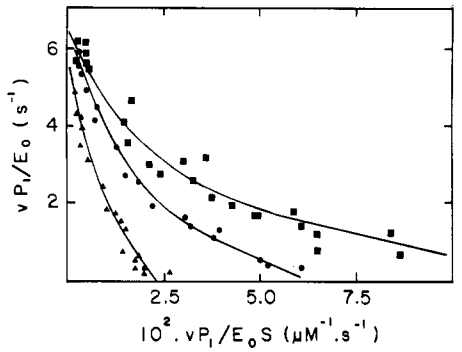


FIGURE 2: Eadie–Hofstee plots showing the effect of NaCl on the hydrolysis of Ac-Phe-Arg-Nan (S range = 5–2500 μM) by Pop-kallikrein (NPGB-titrated sample II; E_0 = 1–9 nM). The substrate was hydrolyzed at pH 9.0 (0.022 M Tris-HCl, 1 mM EDTA, and 10% Me_2SO v/v) and 37 °C, in the absence (■) or in the presence of NaCl: 0.14 (●) or 0.083 M (▲). The hydrolysis rate was measured 30 s after adding the enzyme to the substrate solution in different salt concentrations.

The kinetic parameters (Table II) show that, by decreasing the temperature at pH 9.0 and 7.0, both rate constants k_2 and k_{a2} are decreased without any significant change in the k_{a2}/k_2 ratios. Regarding the enzyme–substrate binding constants, under the same conditions, a decrease of K_1 and K_{s1} was observed, and this effect, except for pH 9.0 and 19 °C, seems to affect in a similar way the K_{a2} and K_{s2} values so that the corresponding R values are maintained (Table II).

Effect of Tris-HCl and Sodium Chloride on Hydrolysis of Ac-Phe-Arg-Nan by Pop-kallikrein. The hydrolysis of Ac-Phe-Arg-Nan by Pop-kallikrein (sample II) at 37 °C and pH 9.0 was substantially affected by the concentrations of Tris-HCl and of NaCl but with a different concentration dependence for each salt. Since, for Tris-HCl alone, those modifications were detectable only above 0.05 M, lower buffer concentrations (0.022 M) were used to study the effect of NaCl. The effect of this salt on the hydrolysis of Ac-Phe-Arg-Nan by Pop-kallikrein is shown in Figure 2. When analyzed according to Scheme I, kinetic data indicated that the constants K_1 or K_{s1}, associated with substrate binding to the primary and auxiliary sites of Pop-kallikrein, respectively, were increased as a function of NaCl concentration (Table III). Since, under the same conditions, a less pronounced increase was observed for constants K_{a1} and K_{s2}, the resulting lower values for R ratios make the biphasic pattern of the hydrolysis by Pop-kallikrein less conspicuous at higher salt concentrations. In contrast, catalytic rate constants k_{a2} and k_2 were less affected than enzyme–substrate binding constants.

Increasing concentrations of NaCl decrease the hydrolysis rates of Ac-Phe-Arg-Nan by Pop-kallikrein at the substrate concentration range used in our assays (Figure 2). No evidence is presently available to indicate that this behavior is also shown by other substrates. Previous reports about cationic effects on the hydrolysis of Bz-Arg-OEt by Pop-kallikrein (Fiedler & Werle, 1968; Takami, 1968; Worthington & Cuschieri, 1974) are not conclusive since they have been based mostly on experiments using a single substrate concentration. Nevertheless, it seems plausible that the hydrolytic activity of Pop-kallikrein should be also modulated by cations as has been observed for urinary kallikreins (Prado et al., 1963; Lieberthal et al., 1982; Chao et al., 1983; Murthy et al., 1986).

Adherence to Michaelis–Menten kinetics has been reported for the hydrolysis of several nitroanilide and methyl ester substrates by Pop-kallikrein (Powers et al., 1984; Fiedler & Leysath, 1979; Levison & Tomalin, 1982). On the basis of the evidence presented in this work, it might be suggested that substrate activation of the enzyme was not observed, at least in the case of nitroanilide substrates, because of the high cationic buffer or NaCl concentration and the narrow substrate concentration range used.

Registry No. D-Val-Leu-Arg-Nan, 64816-14-4; D-Pro-Phe-Arg-Nan, 64816-19-9; Pro-Phe-Arg-Nan, 71973-96-1; Ac-Pro-Phe-Arg-Nan, 103418-72-0; Bz-Pro-Phe-Arg-Nan, 58840-30-5; Ac-Phe-Arg-Nan, 103418-66-2; Bz-Phe-Arg-Nan, 103418-68-4; Ac-Gly-Arg-Nan, 103418-61-7; Bz-Arg-Nan, 6208-93-1; Tris-HCl, 1185-53-1; NaCl, 7647-14-5; kallikrein, 9001-01-8.

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