

Characteristics and Kinetics of Kallikrein Enzyme from Arabian Camel Pancreas

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Abstract. The characteristics and kinetics of kallikrein enzyme from camel was investigated in this study. The enzyme activity was inhibited by urea, phenylmethyl sulfonyl fluoride (PMSF), leupeptin, cations and aprotinin. The enzyme activity was optimum at pH 10.0 and was stimulated by different concentrations of deoxycholate. The enzyme was shown to be heat labile enzyme. Its activity was not affected by heating of the enzyme at 45°C for 30 min. However, this kallikrein retained 75% of its activity by its heating at 50°C for 30 min. Latter temperatures above 50°C caused partial degradation of the enzyme reaching 78.5% at 70°C. The rate of thermal inactivation of the enzyme at 50°C, 60°C, 70°C showed first order kinetics with constant rates of about 8.98×10^{-3} , 3.22×10^{-2} , 9.60×10^{-2} / min. respectively. The Michaelis – Menten constant K_m and V_{max} were showed to be 40.0 $\mu\text{mole/min}$ and 8.53 $\mu\text{mole/min}$. respectively.

Introduction

The tissue kallikrein enzyme was identified by the use of immunochemical and histochemical techniques (Weigershausen et al., 1468). The enzyme appears to be predominately in liver, kidney, pancreas as localized in epithelial cell membranes at a site though to be involved in transcellular electrolyte transport (Carretero and Scicli, 1992). The dietary and hormonal influences are known to change the amount of kallikrein in many body organs (Al-Hamidi et al., 1992). Kallikreins are acidic glycoproteins that exist in two forms; an active enzyme and inactive zymogens called prokallikrein (Margolius, 1976). As Kallikreins release kinin from tissue protein or the so-called kininogen, they referred as kininogenases (Pisano, 1978).

The cellular actions of kinins are modified by their ability to stimulate the release of many second-generation mediators, for example, platelet-activating factor. Kinins also stimulate the secretion of renin from the kidney, release vasopressin from the neurohypophysis and secretion of catecholamine from the adrenal medulla (Carretero and Scicli, 1992). In addition, the kallikrein therapy lowered the blood pressure significantly and normalized the reduced urinary kallikrein excretion in patients with essential hypertension (Overlack et al., 1980).

In a previous study (Al-Hamidi, 2005), Kallikrein enzyme from camel was extracted and purified. This study suggested three step purification procedures for extraction and purification of Kallikreins from camel. To complete our work in this above mentioned subject, the present study was undertaken to study the characteristics of kallikrein enzyme from camel in Saudi Arabia.

Materials and Methods

Chemicals and buffers:

All chemicals used were provided from Sigma Chemical Company (Ltd, Poole, Dorset, UR). These chemicals are analytical grade reagents. SBTI; PMSF; EDTA; SPA, LBTI were designated and abbreviated to denote on soyabean trypsin inhibitor; phenylmethyl sulfonyl fluoride; ethylenediaminetetraacetic acid; specific activity; Lima bean trypsin inhibitor respectively. All experimental procedures were carried out at 4°C unless otherwise stated.

Extraction of kallikrein and kininogen preparation:

Kallikrein enzyme was extracted from camel pancreas by the procedures of Al-Hamidi, (2005). Crude kininogen was isolated from camel blood as described by (Al-Hamidi et al., 1992). Crude

Kininogen was partially purified from the diluted plasma by ion-exchange chromatography on sephadex A-50 resin as described previously by the above mentioned author.

Activation of prokallikrein:

0.2 ml enzyme samples were incubated with 0.2 ml of trypsin for 30 min. at 25°C to produce an appropriate ratio of enzyme to trypsin depending on the enzyme purity. The reaction was terminated by addition of 0.1 ml of SBTI to produce a ratio of 1:25 trypsin to SBTI and further incubated for another 30 min. (Al-Hamidi, 2005).

Kallikrein purification and kininogenase assay:

Kallikrein enzyme was purified from camel pancreas by the procedures adopted by Al Hamidi (2005). 0.1 ml of the purified and activated enzyme were added to 0.3 ml samples of partially purified sheep kininogen at 25°C for 10 min. Then reaction was stopped by boiling in water both for another 10 min. The reaction mixture was then cooled and the liberated kinin was measured as described by Carretero et al. (1976).

Characteristics of Kallikrein enzyme:

1. Effect of the enzyme inhibitors.

3 µg aliquots of kallikrein enzyme were added to the inhibitors listed in table 1, for 15 minutes at 4°C. After incubation, the residual enzyme activity was determined as described in the above step (Carretero et al., 1976). A control experiment was concurrently run where buffer was used instead of inhibitor. % of enzyme activity inhibited = $\frac{\text{activity of the enzyme alone} - \text{activity of enzyme plus inhibitor}}{\text{activity of the enzyme alone}} \times 100$

2. Effect of different pH-values on enzyme activity.

Aliquots of 3 µg enzyme and 1ml Phosphate buffer were added in test tubes and were then adjusted at many pH (s) listed in table 2. Mixtures were incubated overnight at 4°C and specific enzyme activity was determined as described by Carretero and Scicli (1992).

3. Effect of detergents:

Aliquots of 3µg of the enzyme were incubated with various concentrations of detergents (Table 3) for 15 minutes and then residual enzyme activity was determined as published by Carretero et al. (1976)

4. Effect of temperature on the stability of the enzyme.

Aliquots of 3µg enzyme in phosphate buffer pH 10.0, were incubated at various temperatures (Table 5) and at time intervals, samples were withdrawn and residual activity was determined as published by Carretero et al.(1976). The thermal inactivation of the enzyme and the order rate constant as well as decay constants (Table 5) were obtained as published by Woodley et al. (1986).

5. Effect of cations on kallikrein activity:

0.54 µg of the enzyme was incubated with various cations (Table 4) for 15 min. at 4°C. The residual activity was determined by Carretero et al. (1976).

Kinetics of Kallikrein enzyme:

Apparent Michaelis-Menten constant, Km and Vmax were determined by least-square regression analysis (Pallock et al., 1986). The kinetic data against N-α-benzol arginine ethyl ester (BAEE) and Val-Leu-Arg-pNA as enzyme substrates (Okamoto & Greenbourn, 1983) were determined with concentrations of 0.005mM to 0.6 mM for BAEE and concentrations (Table 6) of 0.002 mM to 0.022 mM for Val-Leu-Arg-PNA respectively. Km & Vmax were calculated from Linweaver Burk plot (Okamoto and Greenbourn, 1983).

Results and Discussion

The enzyme concentrations used in this work and the concentrations of inhibitors, detergents, cations were recommended in a similar published work to that studied herein in this paper (Carretero and Scicli, 1992).

From table 1, it can be observed that the enzyme was inhibited by urea, PMSF, leupeptin, antipain and aprotinin and this corroborate the work published by Roman et al. (1988). The enzyme was not inhibited by LBTI and slightly inhibited by SBTI. This finding

Table 1. Effect of inhibitors on the activity of camel pancreatic kallikrein.

Inhibitor	Inhibitor concentration	% inhibition
Control	0.0	0
SBTI	2.48 μ M	0
SBTI	4.96 μ M	14.3
LBTI	5.56 μ M	0
LBTI	11.11 μ M	0
Aprotinin	5.54 μ M	33
Aprotinin	11.08 μ M	71.4
Antipain	17.8 μ M	33
Leupeptin	23.13 μ M	66.7
PMSF	0.96 mM	71.4
EDTA	1.0 mM	0
Urea	0.1 M	50

Table 2. Effect of pH on the activity of camel pancreatic kallikrein.

pH	SPA μ moles/min/mg protein
3.0	0
4.0	0
5.0	0.58
6.0	0.87
6.5	1.16
7.0	1.45
7.5	1.74
8.0	2.32
9.0	2.99
10.0	3.33
11.0	2.61

is in agreement with earlier reports (Fiedler, 1979). Consequently, the enzyme is a serine protease and the lack of any noticeable inhibition by LBTI indicated the absence of trypsin or chymotrypsin contaminant in the enzyme.

The enzyme optimum pH of about 10.0 (Table 2), this compared with that it was obtained for porcine pancreatic kallikrein (pH 10.0) and close to the urinary tract kallikrein (Rabio et al., 1985). The enzyme was inactive at pH 3.0 and 4.0. This loss of activity at these pH values and the decrease of activity might be due to changes in enzyme ionization groups at or near the enzyme active site(s), and such affect the binding of substrate and/or subsequent decomposition of

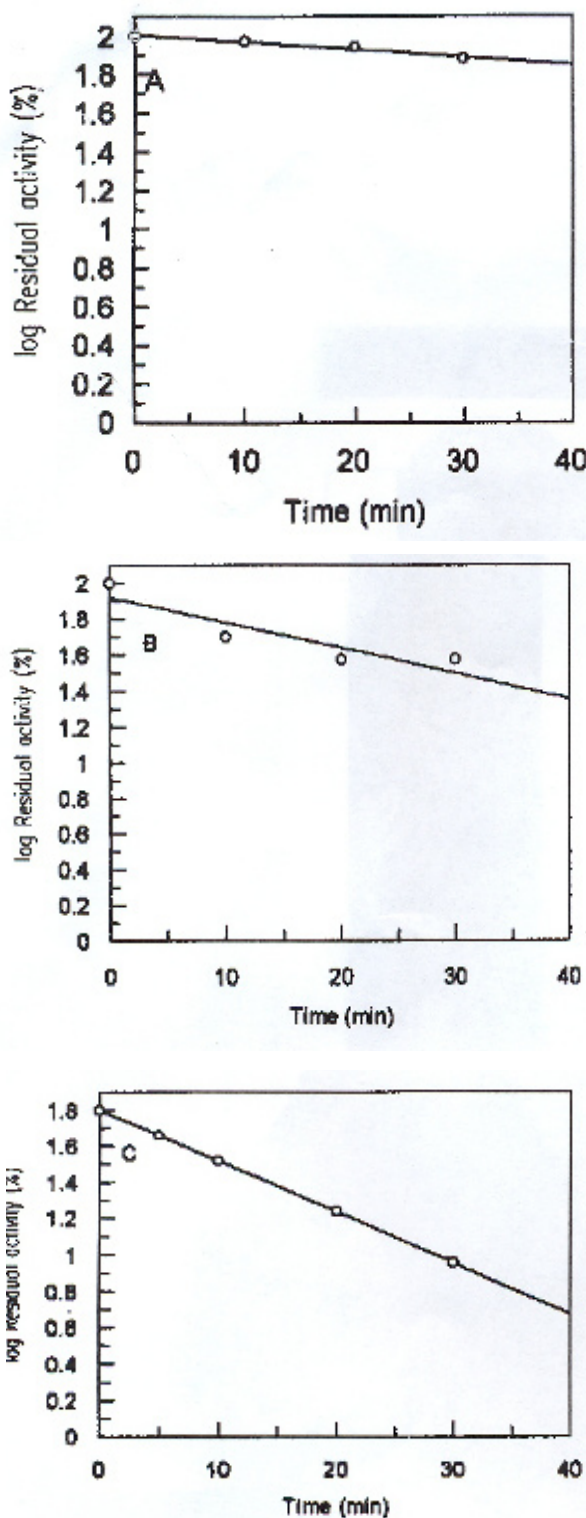


Fig 1. First order rate constant of thermal inactivation camel pancreatic kallikrein at (A) 50, (B) 60, (C) 70 °C.

Table 3. Effect of detergents on the activity of camel pancreatic kallikrein.

Detergent	Concentration in % (mg/100 ml)	% relative activity
Control	0	100
Deoxycholate	0.01	107.45
Deoxycholate	0.02	125.0
Deoxycholate	0.03	120.0
Triton X100	0.01	100
Triton X100	0.02	67.0
Triton X100	0.03	67.0
Tween 20	0.01	100
Tween 20	0.02	100

Table 4. Effect of cations on the activity of camel pancreatic kallikrein

Ions	Concentration (M)	% Inhibition
Ca ²⁺	0.02	0.0
	0.04	33.0
	0.06	33.0
	0.08	33.0
	0.1	33.0
Mg ²⁺	0.02	6.7
	0.04	6.7
	0.06	6.7
	0.08	20.0
	0.1	20.0
Mn ²⁺	0.005	26.7
	0.01	60.0
	0.02	73.0
	0.03	73.0
	0.04	73.0
Sr ²⁺	0.02	0.0
	0.04	9.5
	0.06	33.0
	0.08	33.0
	0.1	33.0
Cd ²⁺	0.01	43.0
	0.02	43.0
	0.04	43.0
	0.05	43.0

Table 5. Effect of temperature on the stability of the enzyme.

Temperature °C	Incubation Time (min)	% residual activity
Control (25)		100
30	10	100
30	20	100
30	30	100
40	10	100
40	20	100
40	30	100

Table 5 cont.

50	10	93.5
50	20	87.5
50	30	75
60	10	50
60	20	37.5
60	30	37.5
70	5	37.5
70	10	25
70	20	12.5
70	30	12.5

Table 6. Decay constants of camel pancreatic kallikrein.

Temperature °C	Time (min)	K_D (min ⁻¹)
30	30	9.55×10^{-3}
60	30	3.3×10^{-2}
70	30	6.93×10^{-2}

Table 7. Kinetics parameters of the purified camel pancreatic kallikrein.

Substrate	K_m (μM)	V_{max} (μmole/min)
BAEE	40.0	8.53
Val-Leu-Arg-pNA	2.7	1.07

enzyme-substrate complex. This is in agreement with Al-Hamidi (1992).

Concerning the effect of detergents (Table 3), the enzyme was found to be stimulated by the concentrations of deoxycholate and inhibited by triton X100. This was similar to that was observed with sheep kallikrein (Fiedler, 1979). However inhibition of the enzyme by triton X100 was contrary to what was reported for rat submandibular gland and rat urinary Kallikreins (Chao, 1978). The mechanism of the activation of inhibition was not known, however, stimulation of the enzyme by deoxycholate suggested binding of the detergent to the hydrophobic region of the enzyme which might be accompanied by conformational change.

Results in table 4 showed that the kallikrein activity was inhibited by ions employed in concentration dependent manner viz Ca^{+2} , Mg^{+2} , Mn^{+2} , Sr^{+2} , Gd^{+2} (Table 4). This result is in agreement with that of Chao et al, (1983) and Lawton et al

1980. Study of the interactions between cations and kallikrein may be relevant not only to the regulation of the enzyme activity and structure, but also to the role of the enzyme in the membrane ion-transporting events. The nature of interaction between kallikrein and the cations is not clearly understood but it could be possible that the inhibition is accompanied by the conformational change. The enzyme was reported to be inhibited by low concentration of cationic detergent that was accompanied by conformational change.

Kallikrein enzyme is usually heat labile enzyme. It can be seen from table 5 that the enzyme retained 75% of its activity for 30 min. at 50°C. The temperature below 5°C was not effective on enzyme activity (Table 5). Temperature above 50°C affected partially enzyme activity as % residual activity decreased to be 12.5% at 70°C after incubation of the enzyme at 30 min. Chao et al., (1983) reported that dog renal kallikrein retained more than 80% of its esterolytic activity for 60 minutes at 50°C. The degree of heat stability of camel tissue kallikrein differs from rat urinary kallikrein (Chao et al., 1987). Rat urinary kallikrein retained 70% of its activity for 18 hours at 55°C and 40% for 5 minutes at 100°C. The rate of thermal inactivation of the enzyme were measured over a temperature range of 50 - 70°C. The inactivation process was irreversible at the temperature studied, that is, no activity could be recovered by prolonged incubation of partially inactivated sample at room temperature. The rate of the thermal inactivation of the enzyme at 50, 60 and 70°C follows first order kinetics (Fig. 1) with first order rate constants of

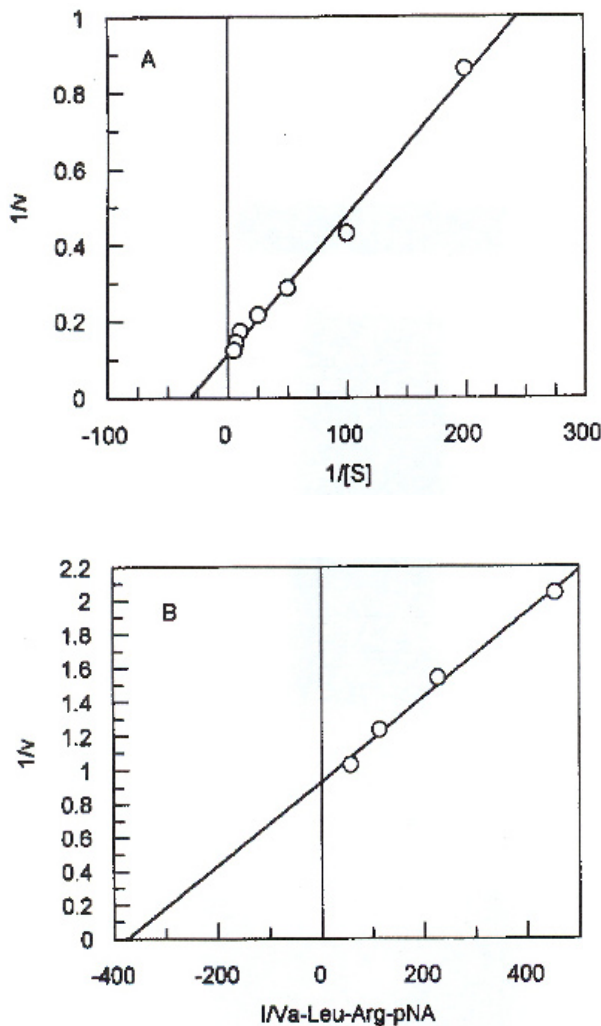


Fig 2. Lineweaver-Burk plot of initial velocity of camel pancreatic kallikrein, (A) BAEE as substrate and (B) Val-Leu-Arg-pNA as substrate.

8.98×10^{-3} , 3.22×10^{-2} and 9.60×10^{-2} /min respectively. It was also observed that the rate of thermal inactivation of the enzyme did not depend on the initial concentration of the sample. This showed that there were no intermediates of partial catalytic activity on the reaction pathway. Since the thermal inactivation of the enzyme followed first order kinetics, it is possible to determine the decay constant of the inactivation at each temperature as follows, $k_D = 2.303/t \log E_0/E_t$, k_D = decay constant, t = time, E_0 = enzyme activity at $t = 0$ and E_t = enzyme activity

at time t (Table 6). The K_m (μM); V_{max} (μ mole/min) were 40.0; 8.53 respectively using BAEE as substrate and were 2.7; 1.07 using Val-Leu-Arg-PNA as substrate (Table 7). Consequently, the Lineweaver-Burk plot of initial velocity of camel tissue kallikrein was designed at each case (Fig. 2). Such curve is standard and similar to the one published by few authors (Carretero & Scicli, 1992).

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السمات المختلفة وحركات التفاعل لأنزيم الكالكريين المستخلص من بنكرياس الجمل العربي

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ملخص: بدراسة السمات الفيزيوكيميائية لأنزيم الكالكريين المستخلص من بنكرياس الجمل العربي أتضح أن نشاط هذا الأنزيم يزداد بعد معاملته بتركيزات مختلفة من مادة الديوكسيكولات، وينقص بنسب مختلفة بعد معاملته بمواد اليوريا، سلفونيل الميثيل فلورايد، بعض الكاتيونات وبعض البيبتيدات. وكذلك دراسة النشاط الانزيمي عند درجات مختلفة من تركيز الاس الهيدروجيني (pH) حيث اتضح ان الاس الهيدروجيني 10.0 pH هو الأمثل لنشاط هذا الأنزيم، وبدراسة ثبات الأنزيم عند درجات الحرارة المختلفة تبين أن نشاط هذا الانزيم ثابت عند درجات الحرارة حتى 50 م بينما يقل نشاطه بسبب تكسره عند معاملته وتخزينه عند درجات الحرارة الأعلى من 50 م. كما دلت الدراسة على ان نشاط هذا الانزيم يمثل معادلة كيميائية حركية من الدرجة الاولى.