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Research article

Isolation and activation of factor X from camel plasma

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Abstract

This study presents the purification of a well-characterized activated FX from the camel plasma. Also, due to the unavailability of Russell viper venom, this study presents *Cerastus cerastus* viper venom as a new alternative activator of FX. Camel factor X was purified by barium ion precipitation, ion exchange and affinity chromatography columns and activated to FXa with venom from the viper *Cerastus cerastus*. The specific activity of FXa was found to be 699.3 units/mg protein. It turned out to be homogenous on both native PAGE and 12% SDS PAGE with a molecular weight of 59kDa. The molecular weights of the two heavy and light chains of camel FX were determined to be 42kDa and 17kDa respectively. The Km value was found to be 91µM of N-Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide HCl. Soybean trypsin inhibitor was the most potent inhibitor of camel FXa. The activated Fx displayed its optimum activity at pH 7.4.

Key words: Activated factor X (FXa), Camel plasma, Purification.

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1. Introduction

The clotting factors II, VII, IX and X as well as the most important inhibitor protein C and protein S belong to the group of vitamin K-dependent proteins. All vitamin K-dependent proteins, which play a part in the clotting cascade, are synthesized in the liver. Prothrombin or factor II (FII), factor VII (FVII), factor IX (FIX), factor X (FX) and protein C (PC) circulate in blood as zymogen molecules and are converted to active serine proteases during the coagulation cascade[1,2]. Activated factor X (FXa) is found in plasma as a zymogen (FX) that consists of two polypeptide chains linked by a disulfide bond. In the liver, FX is synthesized as a single-chain precursor that is subsequently processed to give the two-chain form found in plasma[3]. The carboxylation status of FX influences the intracellular trafficking of the FX precursor [4]. The light chain of FX contains two epidermal growth factor-like domains (E1 and E2) that are probably involved in protein–protein interactions during the activation of FX *in vivo* [5]. The heavy chain of FX is made up of an activation peptide and a serine protease domain; on activation of FX by factor IXa in the presence of factor VIIIa, the activation peptide is removed

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proteolytically, resulting in an active protease [6].

Factor X is a predominant molecule of coagulation cascade[7]. FXa holds a pivotal position in blood coagulation as the only known physiological activator of prothrombin. Factor X activates prothrombin efficiently on a phospholipid surface in the presence of a cofactor; factor Va[8]. The FX is activated to FXa in the presence of calcium by complexes in the intrinsic (fIXa/fVIIIa) [9] and extrinsic (fVIIa/TF) pathways and RVV-X [10,11]. Factor Xa (FXa) was purified from human plasma [12;13], bovine plasma [14,15] and mouse plasma [16]. A factor Xa-like activity metalloprotease 'trimarin' was purified from the *Trimeresurus malabaricus* snake venom clotted the factor X deficient human plasma [17].

In previous studies in our laboratory, the nymphs of camel tick *Hyalomma dromedarii* contain a potent inhibitor of bovine FXa and prolonged both the activated partial thromboplastin time (APTT) and the prothrombin time (PT) of the camel plasma in a concentration-dependent manner[18].The activity of the reported anticoagulant was assayed toward bovine FXa. Therefore, this study aims at purification of the camel FXa, the proper target enzyme for the purified FXa inhibitor from the camel tick *Hyalomma dromedarii.* This study also describes a simple and reliable method for the complete separation and characterization of factor X from the camel plasma and its conversion to the activated form (FXa) with venom from the viper *Cerastus cerastus* as a new locally available alternative activator of factor X instead of the Russell's viper venom which is reported as the most common activator of factor X and it is not commercially available or may be out of production.

2. Materials and Methods

Preparation of the camel plasma

The camel plasma was obtained by centrifugation of a mixture of 900 ml of camel blood and 100ml of 0.11M trisodium citrate solution at 2700 *x g* for 15 min at 4°C. If the plasma was not used immediately, it was dispensed and stored at -40^{o}C.

Chemicals

Factor Xa from bovine plasma, Fibrinogen from bovine plasma, N- Benzoyl- Ile- Glu- Gly- Arg-*p*-nitroanilide HCl, DEAE-cellulose, EGTA, SBTI, PMSF, BSA, and Heparin-agarose were purchased from Sigma Chemical Co. All other chemicals were of analytical grade.

Enzyme assays

Assay of FXa activity

The chromogenic substrate assay of FXa was carried out in a 96-well microtiter plate at 25°C [19]. The buffer is consisted of 0.02M Tris-HCI, 0.15M NaCI, and 0.1% BSA, pH 7.4. The substrate N-Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide HCI was dissolved in DMSO at a concentration of 1mM. The assay reaction mixture of FXa activity contained in 125µl total volume: 75µl buffer, 25µl of the enzyme and 25µl of the substrate (0.2mM final concentration). The reaction was initiated by the addition of the substrate and the absorbance was recorded every 5 min for 30 min at 25^{O}C by microtiter plate reader at 405nm against control lacking enzyme.

Assay FXa clotting activity

The FXa level was determined from coagulation time assay by using factor X-deficient plasma [16]. 50µl containing definite units of FXa from bovine plasma were added to 50µl of factor X-deficient

plasma and incubated for 2 min at 37°C. Following incubation, 100µl of pre-warmed thromboplastin solution were added while simultaneously starting the timer to record the clotting time. A standard curve was constructed with FXa unit on the abscissa and the clotting time on the ordinate. The clotting time for the camel FXa was measured and the FXa value was determined by using the standard curve.

Isolation of FX from camel plasma

Fractionation of camel plasma

To one liter citrated camel plasma treated with protease inhibitors (50µg/ml SBTI and 1.6 mg/ml benzamidine HCl), 80 ml of 1M $BaCl_2$ was slowly added with stirring, followed by additional stirring for 20 min and centrifugation at 6000 xg for 20 min. The pellet was washed by resuspension in 600ml of 0.02M sodium citrate containing 0.15M NaCl, 10mM benzamidine HCl and 50µg/ml SBTI, stirred for 30 min, then centrifuged for 20 min at 6000 xg. The washed pellet was resuspended in 100 ml of 35% $(NH4)_2SO_4$ containing 50mM benzamidine HCl and the pH was adjusted at 6.0 using 3.5M citric acid, gently stirred for 30 min, then centrifuged for 30 min at 9000 xg and the obtained supernatant was kept. The pellet was extracted again in the same way by resuspension and centrifugation to obtain a second supernatant. The two supernatants were combined for recovery of the desorbed proteins by addition of solid $(NH4)_2SO_4$ (75% saturation) and centrifugation for 30 min at 9000 *x g*. The resulting pellet was dissolved in less volume of 0.025M sodium citrate, pH 6.0 containing 0.1M NaCl and 1mM benzamidine HCl, and dialysed in the same buffer. The dialysate was referred to as the barium adsorbate.

Activation of camel FX

The method was adapted from a snake venom protease digest protocol [20]. Two viper venoms and tissue thromboplastin were compared for activation of camel factor X. Samples of the barium adsorbate containing factor X were subjected to activation with venom from the viper *Cerastus cerastus* (1:20 w/w), venom from the viper *Echus carinatus* (1:20w/w), tissue thromboplastin (1:40 and 1:20w/w) in 0.025M Tris-HCl buffer, pH 8.0 and stock $CaCl_2$ was added to a final concentration of 0.025M $CaCl_2$ at 37°C for 60 min.

Chromatography on DEAE- cellulose column

The barium adsorbate was applied to a DEAE-cellulose column (9.5 cm x 2.6 cm i.d.) previously equilibrated with 0.025M sodium citrate, pH 6.0 containing 0.1M NaCl and 1mM benzamidine HCl. The proteins were eluted with NaCl gradient ranging from 0.1 to 0.6M prepared in the equilibration buffer. 2ml fractions were collected at a flow rate of 30ml/hr. Fractions collected were screened using the chromogenic assay after the activation with the *Cerastus cerastus* viper venom and a pool of selected fractions with FX activity was precipitated with $(NH4)_2SO_4$, 0.532g/ml, to recover the protein. The precipitated protein was recovered by centrifugation at 8000g for 30 min and was dissolved and equilibrated by dialysis with 0.02M sodium citrate, pH 7.5 containing 1mM benzamidine HCl. The dialysate was referred to as the DEAE–cellulose concentrate.

Chromatography on heparin-agarose column

The DEAE-cellulose concentrate was applied to a column of heparin-agarose (7

x 1.4 cm i.d.) previously equilibrated with 0.02M sodium citrate, pH 7.5 containing 1mM benzamidine HCl. The protein fractions were eluted with stepwise NaCl gradient ranging from 0 to 0.5M prepared in the equilibration buffer. 1ml fractions were collected at a flow rate of 20ml/h.

Electrophoretic analysis

Native gel electrophoresis was carried out with 7% polyacrylamide gel [21]. SDS-PAGE was performed with 12% polyacrylamide [22]. The molecular weight of the native purified camel FX and its subunits were determined by SDS-PAGE [23]. The proteins were stained with 0.25% coomassie brilliant blue R-250.

Protein determination

Protein was determined by the dye binding assay method [24] using BSA as a standard protein.

3. Results

Activation of FX from camel plasma

Two viper venoms and tissue thromboplastin were compared for activation of camel factor X. Venom from the viper *Cerastus cerastus* (1:20w/w) was found to be the most efficient activator of factor X (Figure 1a).

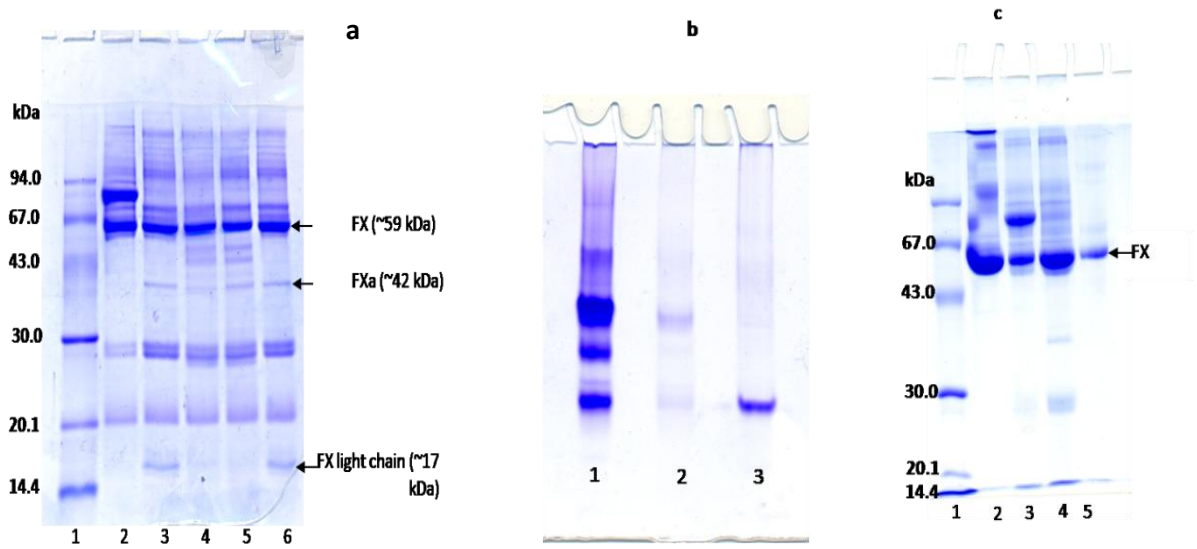


Figure 1. (a) 12% SDS PAGE analysis of barium adsorbate containing FX samples subjected to activation, (1) molecular weight markers, (2) sample before activation (3) activated sample with *Cerastus cerastus* viper venom (4) activated sample with *Echus carinatus* viper venom (5) activated sample with thromboplastin (1:40 w/w) (6) activated sample with thromboplastin (1:20 w/w). (b) Native 7% PAGE for purification steps of camel FX: (1) the barium adsorbate fraction, (2) DEAE–cellulose fraction, (3) heparin-agarose fraction. (c) 12% SDS PAGE (1) molecular weight markers, (2) plasma, (3) the barium adsorbate fraction, (4) DEAE–cellulose fraction, (5) heparin-agarose fraction.

FX isolation from camel plasma

A typical purification scheme of camel FX is presented in (Table 1). The Chromatography of barium adsorbate of camel plasma on DEAE-cellulose column (Figure 2a) revealed the presence of one peak showing FX activity eluted with 0.25M NaCl. The FX fractions were pooled, recovered and chromatographed on a heparin agarose column (Figure 2b) revealed the presence of one peak eluted with 0.1M NaCl showing FX activity. The specific activity of camel FX was increased to 699.3units/mg protein, which represent 47.6 folds purification with 68.7 % yield.

Homogeneity and molecular weight determination of FX

The molecular weights of FXa and the light chain of the FX were obviously detectable on 12% SDS-PAGE (Figure 1a) with 42kDa and 17kDa. The purified camel FX eluted from the heparin-agarose column turned out to be homogeneous on 7% native-PAGE (Figure 1b) and 12% SDS-PAGE (Figure 1c). The molecular weight of the purified camel FX was determined to be 59kDa.

Effect of substrate concentration on the camel FXa activity

The Michaelis-Menten kinetics was applied to the camel FXa. The Km value was found to be 91µM of N-Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide HCl for purified camel FXa (Figure 3a).

Effect of various inhibitors on the camel FXa activity

The effect of different inhibitors on the activity of the purified camel FXa was tested. The SBTI was the most potent inhibitor of factor Xa. The benzamidine HCl, and PMSF showed significant inhibition, while β-mercaptoethanol and EDTA displayed moderate inhibition (Table 2).

Effect of pH on the camel FXa activity

The effect of pH on the camel factor Xa was examined in 0.05 M Tris-HCl buffer of various pH values from 7.2 to 9.0 using N-Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide HCl as substrate. The camel FXa enzyme displayed an optimum activity at pH 7.4 (Figure 3b).

Table 1. A typical purification scheme of the purified camel FXa

Purification step	Total protein (mg)	Activity (unit)	Specific activity	Recovery (%)	Fold Purification
Barium adsorbate fraction	96.8	1423.5	14.7	100	1.0
DEAE-cellulose fraction	4.7	1236.8	236	86.8	16
Heparin-agarose fraction	1.4	979	699.3	68.7	47.6

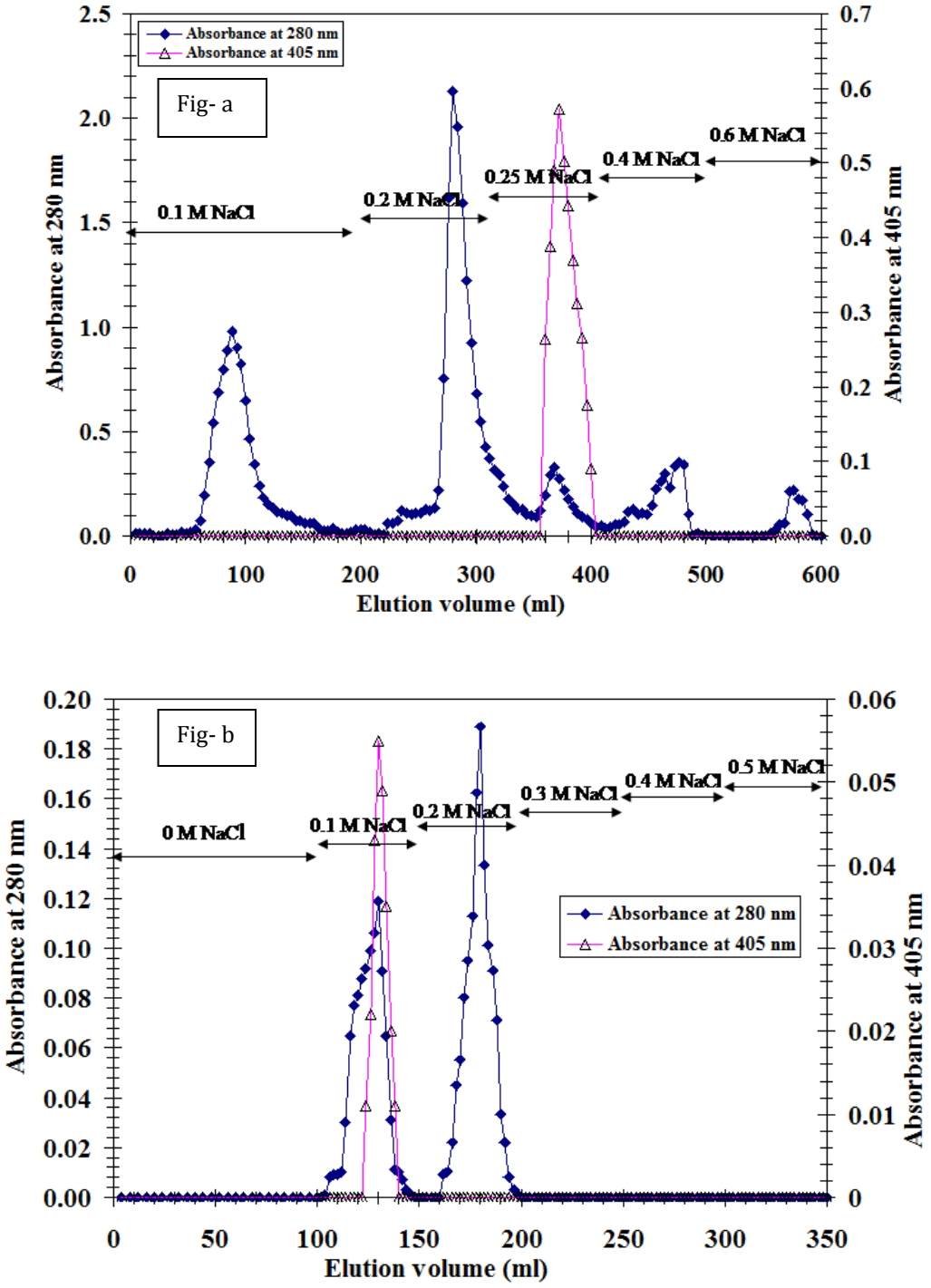


Figure 2. (a) A typical elution profile for the chromatography of barium adsorbate of camel plasma on a DEAE–cellulose column previously equilibrated with 0.025 M sodium citrate, pH 6.0, containing 0.1 M NaCl and 0.001M benzamidine HCl. (b) A typical elution profile for the chromatography of DEAE–cellulose pooled fractions on a column of heparin-agarose previously equilibrated with 0.02 M sodium citrate, pH 7.5, containing 0.001M benzamidine HCl.

Table 2. Effect of different inhibitors on the purified camel FXa

Inhibitor	Concentration	Residual camel FXa activity (%)	Camel FXa inhibition %
No inhibitor	-	100.0	00.0
Benzamidine HCl	5mM	15.4	84.6
Cysteine	5mM	85.6	14.4
EDTA	5mM	30.8	69.2
EGTA	5mM	79.0	21.0
β-Mercaptoethanol	5mM	66.7	33.3
PMSF	5mM	16.4	83.6
Soybean trypsin inhibitor	50µM	2.5	97.5

*These values represent % of the control and the means of triplicate experiments

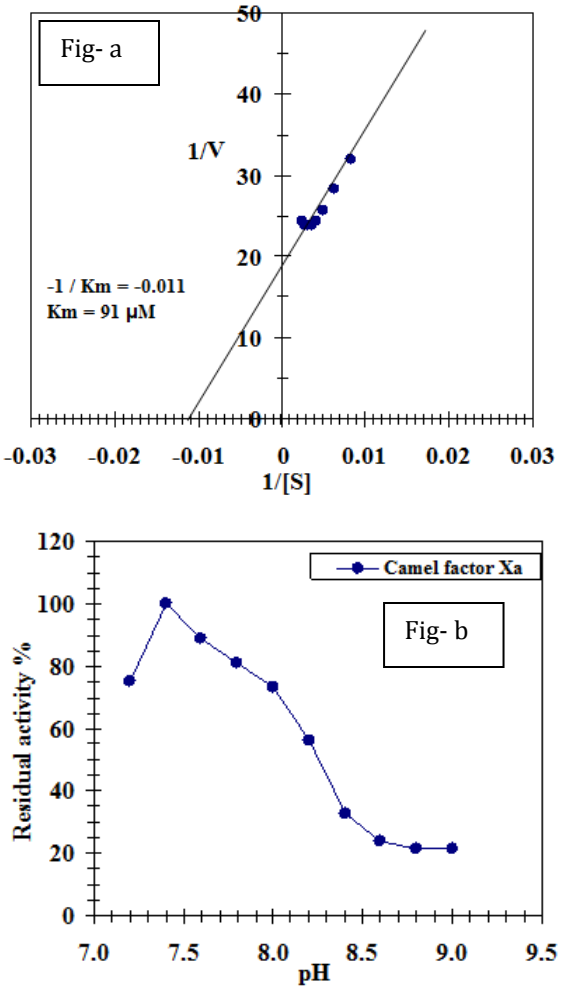


Figure 3. (a) Lineweaver-Burk plot for the reciprocal of the reaction velocity of the purified camel FXa (1/V) and substrate concentration (1/[S]). (b) Effect of pH on the purified camel FXa activity using N-Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide HCl as substrate in 0.05 M Tris-HCl buffer.

4. Discussion

The activation of FX to FXa marks the penultimate step in the coagulation cascade [25]. The sole site of thrombin formation in the vasculature is comprised of FXa assembled with the factor Va in the presence of Ca^{2+} on the surface of activated platelets [26]. Inhibition of FXa activity by low molecular weight heparins has been found to be an effective antithrombotic therapy, with lowered bleeding consequences [27]. The plasma-derived FXa is commercially available in spite of this form of the protease is expensive to produce as it is found in plasma at a low concentration and requires purification and subsequent activation from FX to FXa [28]. Factor Xa was purified from human plasma by affinity chromatography and activated with Russel's viper venom [12,13]. Bovine FXa was purified by DEAE-cellulose chromatography with a specific activity of 1420 units/mg protein [14] and was converted to its active enzymatic form with Russell's viper venom[15]. FX was also highly purified from mouse plasma which activated with rabbit thromboplastin and Russell's viper venom [16].

In this study, the locally available venom from the viper *Cerastus cerastus* was used as a new alternative activator of factor X

instead of the Russell's viper venom which is reported as the common activator of factor X and is not commercially available or may be out of production. The purification of camel FX was carried out by barium ion precipitation and a combination of anion-exchange chromatography on a column of DEAE-cellulose (Figure 2a) and affinity chromatography on heparin-agarose column (Figure 2b). The specific activity of purified camel FX was found to be 699.3units/mg protein with 47.6 fold over the sample before chromatography and 68.7 % recovery (Table 1).The purified native camel FX eluted from heparin-agarose column turned out to be homogeneous on 7% native-PAGE (Figure 1b) and 12% SDS-PAGE with a molecular weight of 59kDa as indicated in (Figure 1c). The molecular weights of the camel FXa and the light chain of the FX were found to be 42kDa and 17kDa as indicated in (Figure 1a). Human FX was reported to be 59kDa [9], bovine FX was 48kDa [15], the intact mouse FX and its heavy and light chains were 59kDa, 43kDa, and 16kDa [16] and the rabbit FX was 75kDa [25]. The molecular weights of camel FXa and the light chain are consistent with that of mouse [16] and human [9]. The Km values of human FXa 255, 63 and 135Mm (Bz-Ile-Glu (γ-OR)-Gly-Arg-*p*NA HCl), (Z-D-Arg-Gly-Arg-*p*NA HCl) and Spectrozyme Xa[25]. The Km value of human FXa was 422 nM chromozym X, while that of bovine FXa was 450nM [29].In this study, the Km value of camel FXa is 91μM of N-Benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide HCl (Figure 3a). The purified camel FXa is inhibited with β-mercaptoethanol indicating the presence of disulfide bond in the FXa molecule and EDTA indicating that the enzyme is metaloenzyme. The camel FXa was strongly inhibited with the protease inhibitors benzamidine HCl, PMSF and SBTI which indicating that the enzyme is a serine protease (table 2). The camel FXa displayed its optimum activity at pH at 7.4 (Figure 3b).

In conclusion, this study presents simple, convenient and reproducible method for the purification of a well characterized activated FX from the camel plasma which is clinically very useful as a hemostatic agent.

Acknowledgements

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