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Apyrase from Embryo of the Camel Tick *Hyalomma dromedarii.*

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ABSTRACT

Apyrase is an enzyme that inhibits the aggregation of platelets by hydrolyzing the molecules ATP and ADP. Apyrase was purified from 18-days old embryos of the camel tick *Hyalomma dromedarii* and designated camel tick apyrase (CTA). The purification procedure involved anion exchange and gel filtration column chromatography. The purified CTA turned out to be homogeneous as judged by native polyacrylamide gel electrophoresis and its molecular weight was determined by gel filtration and SDS polyacrylamide gel electrophoresis to be 67 kDa. CTA displayed its maximum activity at pH 8.0 and cleaved preferentially ATP with Km value of 1.8 mM. $CoCl_2$ and $CaCl_2$ increased the activity of CTA while $MnCl_2$, $NiCl_2$, $FeCl_2$ and $ZnCl_2$ inhibited its activity. EDTA inhibits CTA noncompetitively with Ki value of 1.1 mM.
Keywords: Apyrase, Purification, Characterization, Camel tick, *Hyalomma dromedarii.*

Abbreviations

ATP:	Adenosine triphosphate
ADP:	Adenosine diphosphate
AMP**:**	Adenosine monophosphate
G6P:	Glucose-6-phosphate
p-NPP*:*	*p*-Nitrophenyl phosphate
Pi:	Inorganic phosphate
PPi:	Pyrophosphate
CTA:	Camel tick apyrase

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Introduction

Apyrases are (ATP-diphosphohydrolases, EC 3.6.1.5) a class of enzymes found in all animals, which hydrolyze ATP and ADP to AMP and phosphate [1, 2]. Animal apyrases form three distinct families; the 5′-nucleotidase family [3], the Cimex-type apyrases family that first discovered in the bed bug *Cimex lectularius* [4-6] and the cell-surface apyrase family that is homologues of the human B cell antigen CD39 [7]. It is present in the epithelial linings of the circulation system of mammals where it is involved in the regulation of hemostasis by inhibition of platelet aggregation and control of blood pressure [8]. Apyrases can be classified as endoapyrases that act intracellularly and ectoapyrases enzymes that have their catalytic domain outside the cell. Ectoapyrases present in the saliva of many insects that feed on blood can prevent blood platelet aggregation directly by decreasing the ADP concentration in damaged tissues [8]. Apyrases have been proposed to function in very diverse roles including neurotransmission [9, 10], protein glycosylation [11] and phosphate metabolism [12]. Apyrases have been reported in some invertebrates such as triatomine bugs [13, 14], ticks [15, 16], fleas [17], mosquitoes [3, 18], sand flies [19] and tse-tse fly [20].

Ticks are blood-sucking arthropods that infest a wide array of species and live on all continents of the world and are associated with disease in humans, livestock and wild life [21]. Blood-sucking arthropods have evolved special mechanisms to overcome host hemostatic systems to successfully obtain the blood meal. Antihemostatic factors from hematophagous animals include vasodilators, platelet aggregation inhibitors and anticoagulants [22, 23]. Prostaglandin D2 and I2 found in tick saliva can cause vasodilation and can inhibit platelet aggregation [24, 25]. Various platelet aggregation inhibitors have been identified in tick salivary glands or whole body such as apyrase, disagregin, moubatin and variabilin [26]. Ticks have developed a diverse array of anti-hemostatic agents that are considered to be essential for successful feeding and tick survival. These anti-hemostatics have been found in salivary glands, saliva, eggs and hemolymph appearing not only to prevent blood clot formation in the host, as well as the ingested blood meal, but also to regulate hemolymph coagulation in the tick itself [27]. The nymphs of the camel tick *Hyalomma dromedarii* were found to contain inhibitors for the major blood factors thrombin and Factor Xa [28, 29]. The present study describes the isolation and characterization of apyrase from developing embryos of the camel tick *Hyalomma dromedarii*. Elucidation of the characteristics of these components may lead to a better understanding of tick–host interactions and how blood feeding organisms modulate the host's hemostatic response. This in turn could lead to the development of anti-tick feeding strategies.

MATERIALS AND METHODS

Tick material

The engorged camel tick *Hyalomma dromedarii* females were collected from a Camel market near Cairo and held at 28ºC and 85% relative humidity. Eggs were collected daily from fertilized oviposition female ticks and either frozen immediately (-40˚C) or incubated under the same condition until the appropriate age and transferred to frozen storage at intervals of three days (0, 3, 6, 9 etc.). The hatched larvae were collected at day 27.

Chemicals

DEAE-cellulose, molecular weight marker kits for gel filtration and Sephacryl S-300 were purchased from Sigma Chemical Co. All other chemicals were of analytical grade.

Assay of apyrase activity

The assay of apyrase activity [4] was carried out by measuring the release of Pi from ATP or ADP in 1 ml 50 mM Tris-HCl buffer, pH 8, containing 0.1 M NaCl, 5 mM CaCl2, and 2 mM nucleotides. The reaction was allowed to proceed for 30 minutes at 37 °C followed by addition of ammonium molybdate to stop the reaction. The Fiske-Subbarrow reagent (1-amino-2-naphthol-4-sulfonic acid, sodium bisulfite, and sodium sulfite) was then added and color was allowed to develop for 20 minutes. The activity was determined colorimetrically at 680 mm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of orthophosphate/min at 37 °C. The concentrations of Pi were determined from a standard curve prepared using a serially diluted phosphorous standard solution.

Purification of camel tick embryonic Apyrase

Preparation of crude extract

Two grams of 18 days-old camel tick embryos were homogenized in 10 ml 0.02 M Tris-HCl buffer, pH 8.0, using a Teflon-pestled homogenizer. Cell debris and insoluble materials were removed by centrifugation at 12000 xg for 20 min and the supernatant was saved and designated as crude extract.

DEAE-cellulose column chromatography

The dialyzed sample was chromatographed on a DEAE-cellulose column (12 x 2.4 cm i.d.) previously equilibrated with 0.02 M Tris-HCl buffer, pH 8.0. The adsorbed proteins were eluted with a stepwise NaCl gradient ranging from 0 to 1 M prepared in the equilibration buffer at a flow rate of 60 ml/hour. 5 ml fractions were collected and the fractions containing apyrase activity were pooled and lyophilized.

Sephacryl S-300 column chromatography

The concentrated solution containing the apyrase activity was applied onto a Sephacryl S-300 column (142 cm x 1.75 cm i.d.). The column was equilibrated and developed with 0.02 M Tris-HCl buffer, pH 8.0 at a flow rate of 30 ml/hour and 2 ml fractions were collected.

Electrophoretic analysis

Native gel electrophoresis was carried out with 7% PAGE [30]. SDS-PAGE was performed with 12% polyacrylamide gel [31] and the subunit molecular weight of the purified enzyme was determined by SDS-PAGE [32]. The proteins were stained with 0.25% Coomassie brilliant blue R-250.

Protein determination

Protein was determined by the dye binding assay method using bovine serum albumin as a standard protein [33].
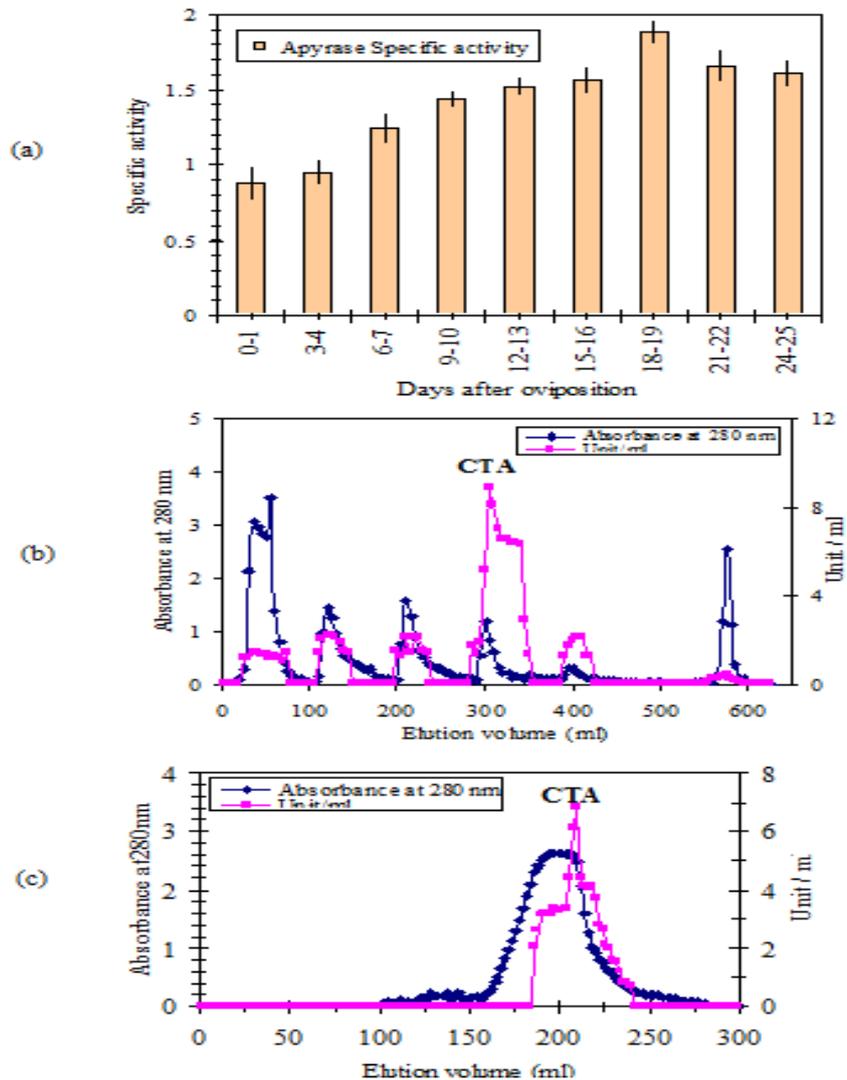
RESULTS

Changes in apyrase specific activity during embryogenesis

A developmental profile of apyrase specific activity was constructed during embryogenesis of the camel tick *H. dromedarii* which revealed that the apyrase specific activity was increased gradually and reached its highest level at the 18 days-old embryos (1.88 ± 0.075 units/mg proteins) (Fig. 1a).

Purification of apyrase enzyme from camel tick embryos

The apyrase specific activity of the embryonic crude extract was found to be 1.9 units/mg protein. A typical purification scheme of apyrase from the camel tick *H. dromedarii* embryos is presented in table (1). The DEAE-cellulose elution profile (Fig. 1b) revealed the presence of one peak containing apyrase activity eluted with 0.2 M NaCl designated CTA. The DEAE-cellulose fractions of the peak were pooled, concentrated and applied onto a Sephacryl S-300 column. The elution profile of CTA activity on the Sephacryl S-300 column (Fig. 1c) revealed the presence of one peak of apyrase activity. The specific activity of CTA activity was increased to 46.7 units / mg protein which represent 24.5 fold purification over the crude extract with 28.8 % yield.

Fig. 1. (a) Developmental profile of apyrase specific activity during embryogenesis of the camel tick *Hyalomma dromedarii.* (b) A typical elution profile of camel tick embryonic crude extract on DEAE-cellulose column (12 cm x 2.4 cm i.d.) previously equilibrated with 02 M Tris-HCl buffer, pH 8.0. (c) Typical elution profiles for the chromatography of the concentrated DEAE-cellulose CTA fractions on Sephacryl S-300 column (142 cm x 1.75 cm i.d.).
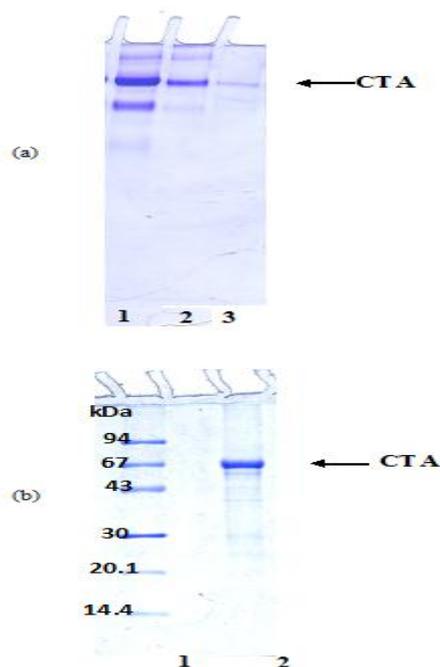

Molecular weight determination by gel filtration

The native molecular weight CTA eluted from Sephacryl S-300 column was deduced from a calibration curve to be 67 ± 1.5 kDa.

Electrophoretic analysis of CTA

Samples from crude extract, DEAE-cellulose and Sephacryl S-300 fractions of CTA were analyzed electrophoretically on 7% native PAGE (Fig. 2a). Single protein band of the enzyme was obtained indicating the tentative purity of the preparation. SDS-PAGE of denatured purified CTA enzyme (Fig. 2b) calculated the subunit molecular weight to be 67 ± 1.8 kDa.

Fig. 2. (a) Electrophoretic analysis of CTA on 7% native PAGE: (1) crude extract, (2) DEAE-cellulose fraction (3) Sephacryl S-300 fraction. (b) Subunit molecular weight determination by electrophoretic analysis of CTA on 12 % SDS-PAGE: (1) molecular weight marker proteins and (2) denatured purified CTA.

Substrate specificity and Km values of CTA

The substrate specificity of the purified CTA was screened toward ATP, ADP, AMP, PPi, *p*-NPP and G-6-P. The purified CTA cleaved preferentially ATP (Table 2). A Lineweaver-Burk plot for the reciprocal of the reaction velocity (1/v) and substrate concentration (1/[S]) was constructed and Km value was found to be 0.18 mM ATP (Fig. 3a).

Determination of optimum pH

The effect of pH on the activities of CTA was examined in 50 mM Tris-HCl buffer, pH (7 - 9) containing 0.1 M NaCl, 5 mM $CaCl_2$ and 2 mM nucleotides. The pH profile of CTA displayed its optimum activity at pH 8 (Fig. 3b).

Effect of divalent cations on CTA

The purified CTA was preincubated with 5 mM of each cation at 37°C and the activity was assayed. The data presented in Table (3) show the activity of CTA in the presence of various cations. A control test without any cation was taken as 100 % relative activity. $CaCl_2$ and $CoCl_2$ increased the activity of CTA while $MnCl_2$, $NiCl_2$, $FeCl_2$ and $ZnCl_2$ inhibited the enzyme activity.

Effect of various inhibitors on CTA activity

The purified CTA was preincubated with each inhibitor for 5 min at 37°C and the inhibition % was calculated as a ratio of a control lacking inhibitor. EDTA is found to be the most potent inhibitor of CTA (Table 4).

Kinetics of CTA inhibition by EDTA

EDTA was found to be the most potent inhibitor of camel tick embryos apyrase. The effect of EDTA concentrations on the purified enzyme activity indicted that the maximum inhibition of the enzyme (85.8%)

was achieved by 5 mM EDTA (Fig. 4a). A linear relationship was observed by constructing the Hill plot for the inhibition of purified enzyme activity by EDTA. The slope of the Hill plot was found to be 0.8 indicating the existence of one binding site for EDTA on the purified enzyme (Fig. 4b). The type of inhibition of CTA by EDTA was found to be noncompetitive (Fig. 4c) where the presence of EDTA did not alter the Km value but increased the Vmax value. The Ki value of the enzyme activity inhibition by EDTA is determined to be 1.1 mM (Fig. 4d).

Fig. 3. (a) Effect of concentration of the substrate ATP in mM on the reaction velocity of the purified CTA. (b) Effect of pH on the activity of purified CTA using 50 mM Tris-HCl buffer, pH (7 - 9) containing 0.1 M NaCl, 5 mM CaCl$_2$ and 2 mM ATP.
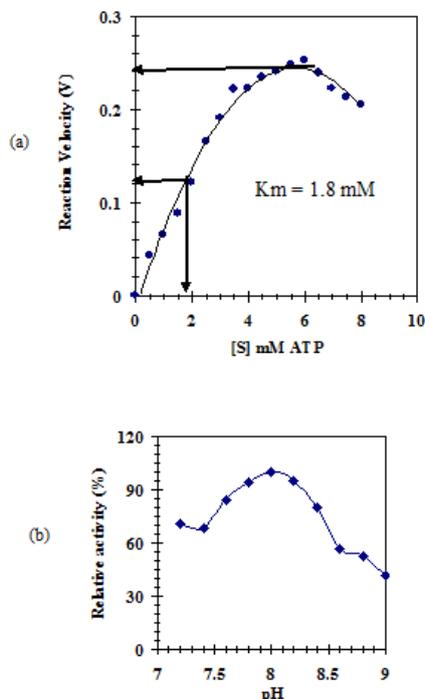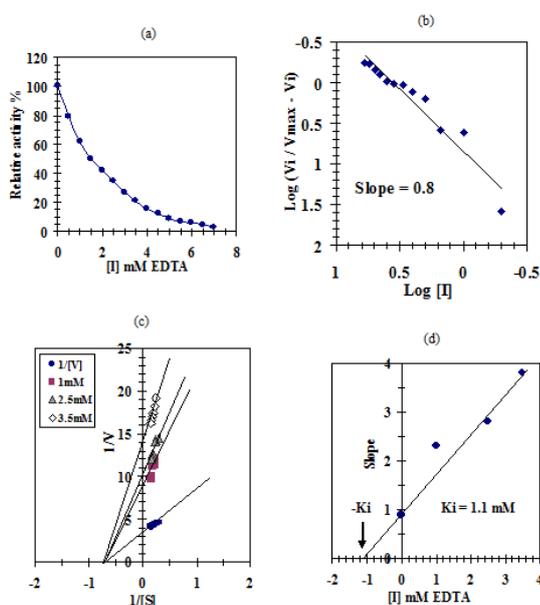

Fig. 4. (a) Inhibition of CTA by varying concentrations of EDTA. (b) Hill plot for inhibition of CTA by varying concentrations of EDTA. (c) Lineweaver-Burk plots showing the type of inhibition of CTA by EDTA. The activity of CTA was measured with varying concentrations of the substrate ATP in absence and presence of three various concentrations of EDTA. (d) Determination of the inhibition constant (Ki) value for the inhibition of the CTA by EDTA.

DISCUSSION

Apyrase cleaves ATP and ADP to AMP and Pi. Since ADP is necessary for platelet activation and aggregation, cleavage of ADP by apyrase prevents the hemostatic effects of platelets [5]. This study presents a simple and reproducible purification method for apyrase from the embryo of the camel tick *H. dromedarii*. A developmental profile of apyrase specific activity during embryogenesis of the camel tick *H. dromedarii* revealed that the apyrase specific activity exhibited the highest level at the 18 days old embryos (Fig. 1a). The purification procedure was carried out by a combination of anion exchange and gel filtration chromatography and the overall yield of CTA from the Sephacryl S-300 column is 28.8 % (Table 1). Apyrase from tick *Ornithodoros savignyi* was purified with a similar purification procedure [34] and large variety of recovery percent of apyrases were reported; apyrase from the mosquito *Aedes aegypti* has a yield of 26.3% [3] and from the soft tick *Ornithodoros savignyi* has a yield of 16 % [34].

Table (1): A typical purification scheme of CTA from the camel tick embryos

Purification step	Total mg proteins	Total Units	Recovery (%)	Specific activity	Fold purification
Crude extract	201	388	100.0	1.9	1.0
DEAE-cellulose fraction	11.3	195	50.3	17.3	9.1
Sephacryl S-300 fraction	2.4	112	28.8	46.7	24.5

CTA turned out to be tentatively homogenous as indicated by both native and SDS-PAGE (Fig. 2). The molecular weight of CTA was deduced from its elution volume from the Sephacryl S-300 column to be 67 ± 1.5 kDa and determined by SDS-PAGE to be 67 ± 1.8 kDa indicating the monomeric structure of this protein. The molecular weight of apyrase from the soft tick *Ornithodoros savignyi* was 67 kDa [34], from *Rhodnuis prolixus* was 83 kDa [9] and from the mosquito *Aedes aegypti* was 68 kDa [3]. The substrate specificity showed that CTA cleaved nucleotides in the following order; ATP > ADP > AMP > PPi > *p*-NPP > G6P (Table 2). The Km value of CTA was found to be 0.18 mM ATP (Fig. 3 a). Human apyrase cleaved the neocleotides in the order of UDP > GDP > CDP > ADP > GTP > CTP > UTP > ATP [35]. Km values of apyrase from human lymphocytes for ATP and ADP were found to be 77.6 and 106.8 ± µM respectively [36]. The camel tick embryo CTA displayed its optimum activity at pH 8.0 (Fig. 3 b). Similarly, the optimum pH of apyrase was found at pH 8.0 in human lymphocytes [36]. Apyrase from salivary glands of the cat flea *Ctenocephalides felis* was optimal at pH 7 - 9 [37] and that from starfish ovarian follicle cells has optimum activity at pH 6 - 7.5 [38].

Table (2): Effect of substrate specificity of the purified CTA

Substrate	Concentration	Rate of hydrolysis	Relative activity
ATP	2 mM	0.253	100 %
ADP	2mM	0.221	87 %
AMP	2mM	0.125	49 %
PPi	2mM	0.116	46 %
p-NPP	2mM	0.079	31 %
G6P	2mM	0.048	19 %

$CoCl_2$ and $CaCl_2$ increased the activity of CTA while the metal ions Mn^{2+}, Ni^{2+}, Fe^{2+} and Zn^{2+} have great inhibitory effects (Table 3). The activation order for two isoapyrases from potatoes was: Ca^{2+} > Mn^{2+} > Mg^{2+} > Co^{2+} > Zn^{2+} [39]. The purified CTA is resistant to the serine protease inhibitor PMSF indicating that the enzyme active site does not contain a serine residue. Also, CTA doesn't belong to thiol proteases since iodoacetic acid did not affect it. CTA isn't cysteine protease due to its resistance to the cysteinyl protease inhibitors *p*-hydroxymercuribenzoate and N-ethylmaleimide. The inhibition of CTA by EDTA and EGTA indicates that it is a metalloenzyme (Table 4). EDTA inhibited CTA noncompetitively and has one binding site on it with Ki value of 1.1 mM (Fig. 4). Apyrase from human lymphocytes was inhibited by EDTA [36]. In conclusion, this study presents a simple, convenient and reproducible method for the purification of a well characterized apyrase from the camel tick embryo which plays important roles in feeding, egg hatching and embryogenesis.

Table (3): Effect of Divalent captions on purified CTA

Reagent	Final concentration (mM)	Residual activity (%)
$CoCl_2$	100	217.9
$MnCl_2$	100	52.8
$FeCl_2$	100	57.5
$ZnCl_2$	100	61.3
$CuCl_2$	100	92.6
$NiCl_2$	100	55.6
$MgCl_2$	100	102
$CaCl_2$	100	189.6
$FeCl_3$	100	93.3

Table (4): Effect of inhibitors on purified CTA

inhibitor	concentration	Inhibition %
Control		0.0
NaN3	2 mM	0.0
Puromycin	1 mM	12
Ethylene glycol tetraacetic acid (EGTA)	5 mM	76.9
p-Hydroxymercuribenzoate (pHMB)	0.2 mM	0.0
β - Mercaptoethanol	0.4 mM	5.7
DL-Dithiothreitol (DTT)	0.1 mM	2.0
L-Cysteine	1 mM	14.6
Iodoacetic acid	5 mM	2.1
N-Ethylmaleimide	1 mM	22.5
Iodoacetamide	5 mM	3.7
EDTA	5 mM	85.8
1,10 Phenanthroline	10 mM	0.0
Phenylmethylsulfonylfluoride (PMSF)	1 mM	8.9

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