



## Catalase from larvae of the camel tick *Hyalomma dromedarii*



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### ABSTRACT

Catalase plays a major role in protecting cells against toxic reactive oxygen species. Here, Catalase was purified from larvae of the camel tick *Hyalomma dromedarii* and designated TLCAT. It was purified by ammonium sulfate precipitation and chromatography on DEAE-cellulose, Sephacryl S-300 and CM-cellulose columns. Gel filtration and SDS-PAGE of the purified TLCAT indicated that the protein has a native molecular weight of 120 kDa and is most likely a homodimer with a subunit of approximately 60 kDa. The  $K_m$  value of TLCAT is 12 mM  $H_2O_2$  and displayed its optimum activity at pH 7.2.  $CaCl_2$ ,  $MgCl_2$ ,  $MnCl_2$  and  $NiCl_2$  increased the activity of TLCAT, while  $FeCl_2$ ,  $CoCl_2$ ,  $CuCl_2$  and  $ZnCl_2$  inhibited the activity of TLCAT. Sodium azide inhibited TLCAT competitively with a  $K_i$  value of 0.28 mM. The presence of TLCAT in cells may play a role in protecting *H. dromedarii* ticks against oxidative damage. This finding will contribute to our understanding of the physiology of these ectoparasites and the development of untraditional methods to control them.

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### 1. Introduction

All aerobic organisms during the course of metabolism form reactive oxygen species (ROS) as by products. Superoxide ( $\bullet O_2^-$ ), nitric oxide ( $\bullet NO$ ), hydroxyl ion radicals ( $\bullet OH$ ) and hydrogen peroxide ( $H_2O_2$ ) are the common ROS [9]. ROS are maintained under certain levels by a battery of enzymatic and non-enzymatic molecules with antioxidant capacity. The enzymatic defense against oxidative stress primarily comprises of superoxide dismutase, catalase and glutathione peroxidases [26]. Catalase (CAT,  $H_2O_2$ :  $H_2O_2$  oxidoreductase; EC 1.11.1.6) plays a key role in protecting cells against toxic ROS [17]. It is an antioxidant and hydroperoxidase enzyme that protects the cellular environment from harmful effects of  $H_2O_2$  by facilitating its degradation to oxygen and water [5]. Aerobic organisms benefit substantially from the high energy yields obtained via controlled conversion of molecular oxygen to water, yet reactive intermediates are burden that cause cellular damage. Catalase is one of the antioxidant enzymes, which deals with removal of the oxidative damage in cells. It has a double function; it catalyzes the decomposition of  $H_2O_2$  into oxygen and water (catalase activity) and also oxidizes electron donors such as

ethanol, methanol, or phenols (peroxidative activity) [27,28]. Catalases are classified into four groups: monofunctional heme (typical) catalases, catalase-peroxidases, manganese catalases and catalase-phenol oxidases (CATPO). CATPOs are bifunctional enzymes being capable of  $H_2O_2$  decomposition (catalase activity) and phenolic oxidation in the absence of  $H_2O_2$  (phenol oxidase activity) [18]. Catalase is a common enzyme found in nearly all living organisms. It was the first antioxidant enzyme to be characterized. Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide [22]. Each of these four protein subunits also contains a molecule of NADPH [16]. Catalase has one of the highest turnover rates of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen [2].

Ticks are members of the arthropod class Arachnida, subclass Acari, and order Parasitiformes. Ticks live on all continents of the world. Many of the 899 or so species of ticks are associated with disease in humans, livestock and wild life [4]. Studies about ecology, behavior and physiology of ticks afford a better understanding of these organisms and therefore become important tools to develop new control methods. Due to the rapid increase in pesticide-resistant tick populations [6], the study of tick physiology has gained increasing importance regarding the mechanisms involved in detoxification of toxins [11,19]. These mechanisms, in general, not only act against specific toxic molecules, but also help in the maintenance of physiologic homeostasis, e.g. in avoiding oxidative damage generated by ROS [7]. Catalase was purified from

Abbreviations: BSA, bovine serum albumin; CAT, catalase; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; SOD, superoxide dismutase; TLCAT, tick larvae catalase

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developing embryo and cuticle of the camel tick *Hyalomma dromedarii* [14,8] and three SOD isoenzymes were purified from its larvae [13]. This study aims at purification and characterization of CAT from the larvae of the camel tick *H. dromedarii* to investigate its role as antioxidant enzyme.

## 2. Materials and methods

### 2.1. Tick material

Engorged camel tick *H. dromedarii* females were collected from a Camel market near Cairo and held at 28 °C and 85% relative humidity. Eggs were collected daily from fertilized oviposition female ticks and incubated under the same condition until hatching larvae at day 27 then frozen immediately at –40 °C.

### 2.2. Chemicals

Phenylmethylsulfonyl fluoride (PMSF), carboxymethyl-cellulose (CM-cellulose), diethylaminoethyl-cellulose (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.

### 2.3. Assay of catalase activity

The assay of CAT activity was carried out according to the method described by Aebi [1]. The assay reaction mixture contained in 3.0 ml total volume of 0.05 M potassium phosphate buffer pH 7.0 containing 0.02 M H<sub>2</sub>O<sub>2</sub> and the reaction was started by addition of enzyme solution. The decomposition of H<sub>2</sub>O<sub>2</sub> was followed as a decline in absorbance at 240 nm for 1 min. One unit of CAT activity was defined as the calculated consumption of 1 μmol of H<sub>2</sub>O<sub>2</sub>/min at 25 °C. The extension coefficient of H<sub>2</sub>O<sub>2</sub> was taken to be 43.6 M<sup>-1</sup> cm<sup>-1</sup>.

### 2.4. Staining of CAT activity on native PAGE

Activity staining of CAT was determined as described by Harris and Hopkinson [10]. After electrophoresis, the gel is incubated in 3% H<sub>2</sub>O<sub>2</sub> for about 15 min. Rinse the gel with distilled water and then immerse it in a 1:1 mixture of 2% Potassium ferricyanide and 2% Ferric chloride. Gently agitate the tray containing the gel for few minutes. Yellow bands of CAT activity appear on a blue green background.

### 2.5. Purification of camel tick larval catalase

#### 2.5.1. Preparation of crude extract

Two grams of camel tick larvae were homogenized in 10 ml 0.02 M K-phosphate buffer pH 7.0, using a Teflon-pestled homogenizer. Cell debris and insoluble materials were removed by centrifugation at 12,000 × g for 20 min and the supernatant was saved and designated as crude extract.

#### 2.5.2. Ammonium sulfate precipitation

The crude extract was brought to 70% saturation by gradually adding solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and stirred for 30 min at 4 °C. The pellet was obtained by centrifugation at 12,000 × g for 30 min and dissolved in 0.02 M K-phosphate buffer pH 7.0 and dialyzed extensively against the same buffer.

#### 2.5.3. DEAE-cellulose column chromatography

The dialyzed sample was chromatographed on a DEAE-cellulose column (12 × 2.4 cm<sup>2</sup> i.d.) previously equilibrated with 0.02 M

K-phosphate buffer pH 7.0. 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/h. 5 ml fractions were collected and the fractions containing CAT activity were pooled and lyophilized.

#### 2.5.4. Sephacryl S-300 column chromatography

The concentrated solution containing the CAT activity was applied onto a Sephacryl S-300 column (142 cm × 1.75 cm i.d.). The column was equilibrated and developed with 0.02 M K-phosphate buffer pH 7.0 at a flow rate of 30 ml/h and 2 ml fractions were collected.

#### 2.5.5. CM-cellulose column chromatography

The concentrated solution containing the CAT activity obtained from the Sephacryl S-300 column was chromatographed on a CM-cellulose column (4 × 1.6 cm i.d.) previously equilibrated with 0.02 M Na-acetate buffer pH 5.6. The adsorbed proteins were eluted with stepwise NaCl gradient ranging from 0 to 0.3 M prepared in the equilibration buffer at a flow rate of 30 ml/h and 2 ml fractions were collected.

### 2.6. Electrophoretic analysis

Native gel electrophoresis was carried out with 7% PAGE according to Smith [30]. SDS-PAGE was performed with 12% polyacrylamide gel according to Laemmli [21]. The subunit molecular weight of the purified CAT enzyme was determined by SDS-PAGE as described by Weber and Osborn [32]. The proteins were stained with 0.25% coomassie brilliant blue R-250.

### 2.7. Protein determination

Protein was determined by the dye binding assay method of Bradford [3] using BSA as a standard protein.

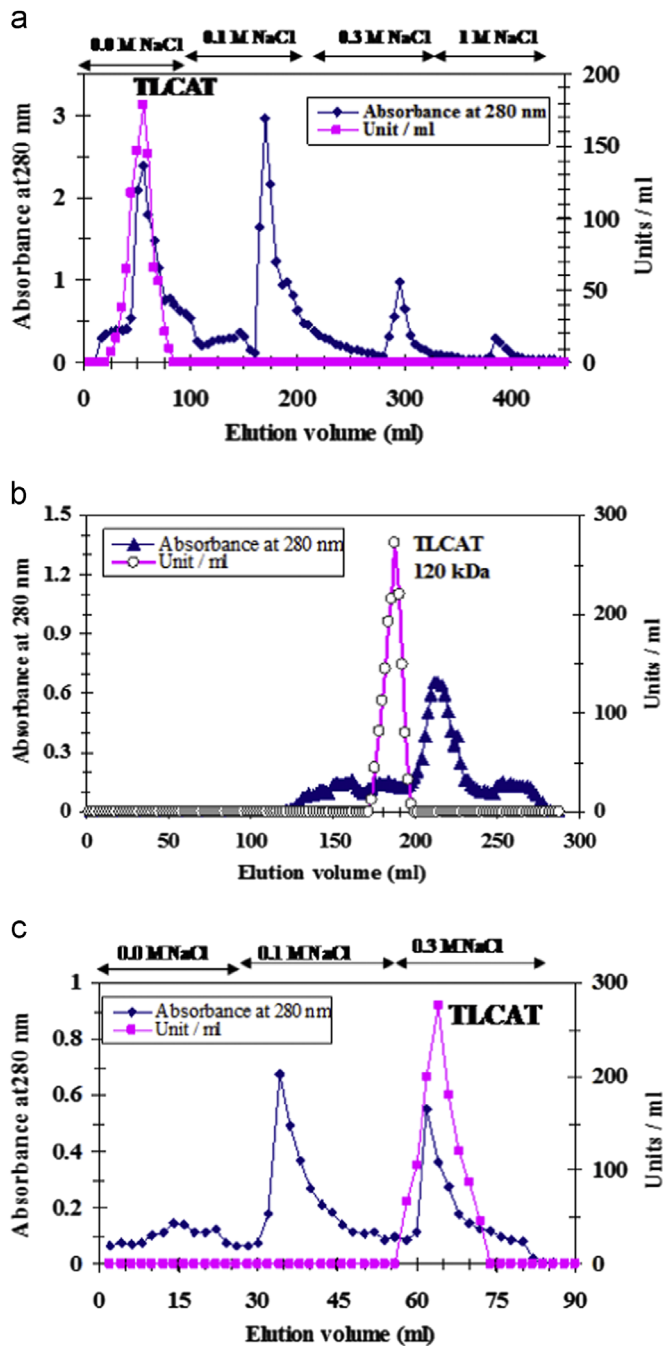
## 3. Results

### 3.1. Purification of CAT from camel tick larvae

The CAT specific activity of the larval crude extract was found to be 25.9 units/mg protein. A typical purification scheme of CAT from the camel tick *H. dromedarii* larvae is presented in Table 1. After ammonium sulfate precipitation, most of the CAT activity was precipitated so that 89.7% of the activity was recovered. The DEAE-cellulose elution profile (Fig. 1a) revealed the presence of one major peak containing CAT activity designated TLCAT and eluted with 0.0 M NaCl. The DEAE-cellulose fractions were pooled, concentrated by lyophilization and applied onto a Sephacryl S-300 column. The elution profile of TLCAT on the Sephacryl S-300

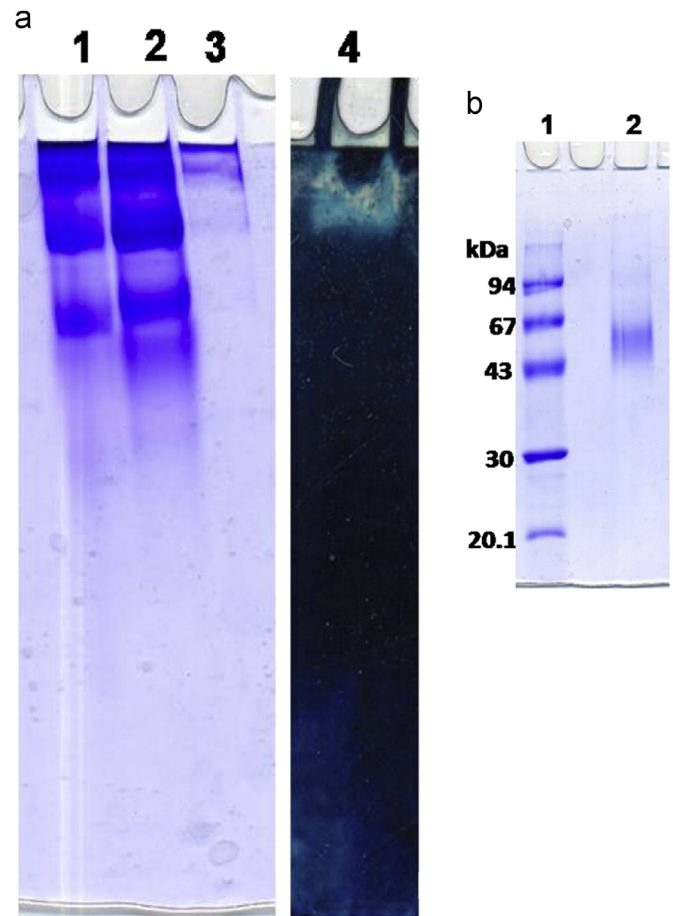
**Table 1**  
A typical purification scheme of catalase from the camel tick *H. dromedarii* larvae.

Purification step	Total mg	Total units	Recovery (%)	Specific activity	Fold purification
Crude extract	242	6260	100.0	25.9	1.0
70% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction	192	5616	89.7	29.2	1.13
DEAE-cellulose fraction	25.8	4170	66.6	161.6	6.2
Sephacryl S-300 fraction	8.6	2988	47.7	347.4	13.4
CM-cellulose fraction	1.7	2120	33.8	1247.0	48.1



**Fig. 1.** (a) A typical elution profile for the ammonium sulfate containing CAT activity fraction of the camel tick *H. dromedarii* larvae crude extract on DEAE-cellulose column (12 cm × 2.4 cm i.d.). (b) A typical elution profile for the chromatography of the concentrated pooled DEAE-cellulose fractions containing TLCAT on Sephacryl S-300 column (142 cm × 1.75 cm i.d.). (c) A typical elution profile for the concentrated pooled Sephacryl S-300 fractions containing TLCAT on CM-cellulose column (4 cm × 1.6 cm i.d.).

column (Fig. 1b) revealed the presence of one peak of the enzyme activity. The Sephacryl S-300 fractions were pooled, lyophilized, dissolved in 0.02 M sodium acetate buffer pH 5.6 and dialyzed extensively against the same buffer and applied onto a CM-cellulose column. The CM-cellulose elution profile (Fig. 1c) revealed the presence of one major peak containing CAT activity eluted with 0.3 M NaCl. The specific activity of TLCAT was increased to 1247 units/mg protein which represent 48.1 folds over the crude extract with 33.8% yield (Table 1).



**Fig. 2.** (a) Electrophoretic analysis of TLCAT on 7% native PAGE: (1) crude extract, (2) DEAE-cellulose fraction, (3) CM-cellulose fraction and (4) TLCAT activity. (b) Subunit molecular weight determination by electrophoretic analysis of TLCAT on 12% SDS-PAGE: (1) molecular weight marker proteins and (2) denatured purified TLCAT.

### 3.2. Molecular weight determination by gel filtration

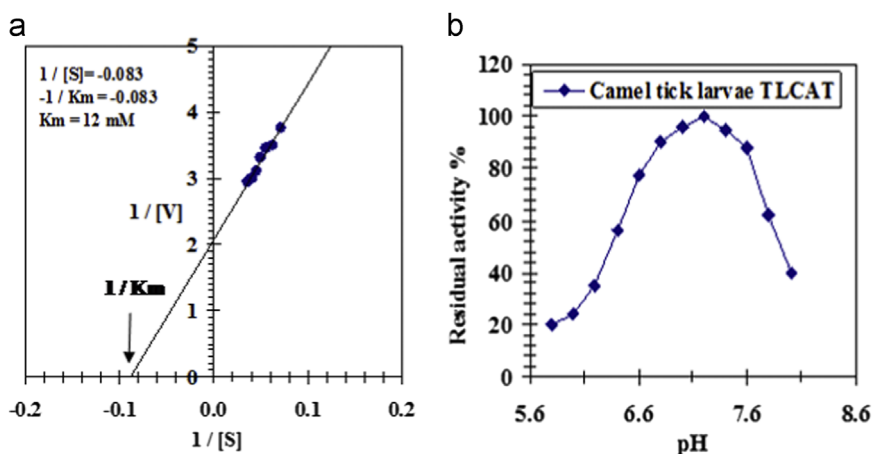
The native molecular weight of TLCAT eluted from Sephacryl S-300 column was deduced from a calibration curve to be  $120 \pm 2.4$  kDa.

### 3.3. Electrophoretic analyses of TLCAT

Samples from the different purification steps; crude extract, DEAE-cellulose and CM-cellulose fractions of TLCAT was analyzed electrophoretically on 7% native PAGE (Fig. 2a). Single protein band coincided with the enzyme activity band of TLCAT indicating the tentative purity of the preparation. Electrophoretic analysis of denatured purified TLCAT on SDS-PAGE was compared with molecular weight marker proteins (Fig. 2b). The subunit molecular weight was calculated from a molecular weight calibration curve to be  $60 \pm 1.8$  kDa.

### 3.4. Determination of $K_m$ of TLCAT

In order to determine  $K_m$  of the enzyme we used different concentrations of substrate and measured its rate of decomposition. A Lineweaver–Burk plot for the reciprocal of the reaction velocity ( $1/v$ ) and substrate concentration ( $1/[S]$ ) was constructed and the  $K_m$  value was found to be 12 mM and the corresponding  $V_{max}$  was calculated to be 760 units/mg protein for TLCAT (Fig. 3a).



**Fig. 3.** (a) Lineweaver–Burk plot relating the reciprocal of the reaction velocity of the purified TLCAT to  $\text{H}_2\text{O}_2$  concentration in mM. (b) Effect of pH on the purified TLCAT using 0.05 M potassium phosphate buffer, pH (5.7–8.0).

### 3.5. Determination of optimum pH

The effect of pH on the activity of camel tick larvae TLCAT was examined in 0.05 M potassium phosphate buffer, pH (5.7–8.0). The pH profile of TLCAT displayed its optimum activity at pH 7.2 (Fig. 3b).

### 3.6. Effect of divalent cations

The purified camel tick larvae TLCAT was preincubated with 2 mM and 5 mM of each cation at 37 °C and the activity was assayed. A control test without any cation was taken as 100% relative activity.  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{MnCl}_2$  and  $\text{NiCl}_2$  increased the activity of TLCAT, while  $\text{FeCl}_2$ ,  $\text{CoCl}_2$ ,  $\text{CuCl}_2$  and  $\text{ZnCl}_2$  inhibited the activity of TLCAT (Table 2).

**Table 2**  
Effect of divalent cations on the purified camel tick larvae TLCAT.

Reagent	Final concentration (mM)	TLCAT residual activity (%)
Control	–	100.0
$\text{CaCl}_2$	2.0	104.5
	5.0	112.5
$\text{CoCl}_2$	2.0	76.6
	5.0	55.0
$\text{CuCl}_2$	2.0	88.6
	5.0	74.3
$\text{FeCl}_2$	2.0	52.2
	5.0	29.5
$\text{MgCl}_2$	2.0	122.3
	5.0	141.4
$\text{MnCl}_2$	2.0	125.1
	5.0	156.7
$\text{NiCl}_2$	2.0	114.6
	5.0	133.7
$\text{ZnCl}_2$	2.0	87.6
	5.0	62.2

### 3.7. Effect of various inhibitors

The purified camel tick larvae catalase TLCAT was preincubated with each inhibitor for 5 min at 37 °C and the inhibition % was calculated as a ratio of a control lacking inhibitor. Sodium azide ( $\text{NaN}_3$ ) is found to be the most potent inhibitor of TLCAT (Table 3).

### 3.8. Mechanism of TLCAT inhibition by sodium azide

The effect of varying concentration of  $\text{NaN}_3$  on the TLCAT activity is shown (Fig. 4a). In the Hill plot (Fig. 4b); a straight line was obtained with slope of about 1.07. The type of inhibition of TLCAT by  $\text{NaN}_3$  was found to be competitive type (Fig. 5a). The  $K_i$  value for TLCAT inhibition by  $\text{NaN}_3$  was determined to be 0.28 mM (Fig. 5b).

## 4. Discussion

Catalase is an antioxidant and hydroperoxidase enzyme that protects the cellular environment from harmful effects of  $\text{H}_2\text{O}_2$  by facilitating its degradation to oxygen and water [5]. This study presents a simple and reproducible purification method for CAT from the larvae of the camel tick *H. dromedarii*. The purification procedure was carried out by ammonium sulfate precipitation and chromatography on DEAE-cellulose column, Sephacryl S-300 column and on CM-cellulose column. Similar purification procedures

**Table 3**  
Effect of inhibitors on the purified camel tick larvae TLCAT.

Reagent	Final concentration	TLCAT inhibition (%)
Control	–	0.0
Ethylenediamine tetraacetic acid (EDTA)	5 mM	50.6
DL-Dithiothreitol (DTT)	10 mM	3.4
Iodoacetamide	10 mM	13.6
<i>p</i> -Hydroxymercuribenzoic acid ( <i>p</i> HMBBA)	10 mM	2.2
Iodo acetic acid	5 mM	77.4
$\beta$ -Mercaptoethanol	10 mM	5.2
N-Ethylmaleimide	10 mM	8.6
1,10 Phenanthroline	5 mM	88.2
Phenylmethylsulfonyl fluoride (PMSF)	10 mM	66.3
Potassium cyanide (KCN)	5 mM	83.3
Potassium dichromate ( $\text{K}_2\text{Cr}_2\text{O}_7$ )	10 mM	59.6
Sodium azide ( $\text{NaN}_3$ )	2 mM	100
Sodium dodecyl sulfate (SDS)	10 mM	6.4
soya bean trypsin inhibitor	15 $\mu\text{g}$	32.5

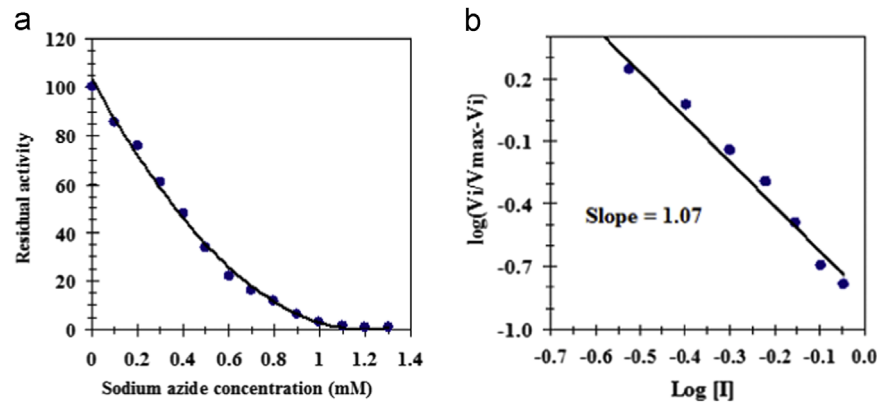


Fig. 4. (a) Inhibition of the purified TLCAT by varying concentrations of  $\text{NaN}_3$ . (b) Hill plot for inhibition of the purified TLCAT by varying concentrations of  $\text{NaN}_3$ .

of CATs were reported, CAT was purified from acatalasemic beagle dog liver [24], from Sprouted blackgram (*Vigna mungo*) seeds [15] and from the bacterium *Deinococcus radiodurans* [17]. In the present study, TLCAT eluted from a Sephacryl S-300 column as a single enzyme activity peak and the deduced molecular weight from its elution volume was found to be  $120 \pm 2.4$  kDa (Fig. 1b). The overall yield of the enzyme from the CM-cellulose column is 33.8% (Table 1). A large variety of purification fold and recovery percent of CAT were reported; the CAT was purified from acatalasemic dog liver 147.6-fold with 5.8% yield [24], from Sprouted blackgram (*V. mungo*) seeds 106.8-fold with 39.7% yield [15], from the radioresistant bacterium *D. radiodurans* 20.8-fold with 41% yield [17] and from the cyanobacterium *Anacystis nidulans* 178-fold with 11.8% yield [25].

On SDS-PAGE, TLCAT enzyme showed the presence of a major protein band which coincided with the enzyme activity band confirming that the single protein band is the enzyme band (Fig. 2a). Comparison of subunit molecular weight with that of native intact protein determined by gel filtration revealed that TLCAT is a dimer protein composed of two identical subunits of 60 kDa each (Fig. 2b). Some CATs reported to have a dimer structure such as *Bifidobacterium asteroides* CAT [12], bacteria *Pseudomonad* EF group 70B CAT [20] and cyanobacterium *A. nidulans* CAT [25].

The  $K_m$  value of the purified camel tick larvae TLCAT was found to be 12 mM  $\text{H}_2\text{O}_2$  (Fig. 3a) indicating the high affinity of TLCAT toward  $\text{H}_2\text{O}_2$ .  $K_m$  values were found to be 48 mM  $\text{H}_2\text{O}_2$  for CAT from culture broth of *Thermoascus aurantiacus* [31], 4.3 mM for CAT from cyanobacterium *A. nidulans* [25], 16.2 mM for CAT from Sprouted blackgram (*V. mungo*) seeds [15] and 100 mM for rice

plant CAT [29]. The camel tick larvae TLCAT displayed its optimum activity at pH 7.2 (Fig. 3b). Similarly, the optimum pH of CAT was found at pH 7.0 in sprouted blackgram (*V. mungo*) seeds [15], at pH 6.0 in the bacterial strain, *Pseudomonad* EF group 70B [20] and at pH between 6.5 and 7.5 in the cyanobacterium *A. nidulans* [25].  $\text{CaCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{MnCl}_2$  and  $\text{NiCl}_2$  increased the activity of camel tick larvae TLCAT, while  $\text{FeCl}_2$ ,  $\text{CoCl}_2$ ,  $\text{CuCl}_2$  and  $\text{ZnCl}_2$  inhibited it (Table 2). This was consistent with CAT of Sprouted blackgram (*V. mungo*) seeds which was enhanced by the ions of  $\text{Ni}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  and inhibited by ions of  $\text{Fe}^{3+}$  and  $\text{Cu}^{2+}$  [15]. In this study, TLCAT activity was strongly inhibited by  $\text{NaN}_3$  and KCN which indicates that TLCAT is a heme-containing catalase. PMSF and soya bean trypsin inhibitor inhibited TLCAT indicating that the enzyme active site contains a serine residue. Also, iodoacetic acid strongly inhibited the purified TLCAT activity indicating that the enzyme belongs to the thiol enzymes. EDTA and 1,10 phenanthroline inhibited TLCAT which indicates that it is metalloenzyme. The inhibition of TLCAT activity with  $\text{K}_2\text{Cr}_2\text{O}_7$  was probably due to strong oxidizing power of  $\text{K}_2\text{Cr}_2\text{O}_7$  that may cause oxidation of metal prosthetic groups that are important to enzyme activity. Iodoacetamide inhibited the purified TLCAT enzyme activity which indicates that methionine, cysteine and histidine residues have important effects on the structure and activity of the enzyme (Table 3).

The effect of  $\text{NaN}_3$  concentrations on the purified camel tick larvae TLCAT indicated an  $I_{50} = 0.4$  mM of  $\text{NaN}_3$  and the maximum inhibition of the enzyme (98.9%) was achieved by 1.2 mM  $\text{NaN}_3$  (Fig. 4a). A linear relationship was observed by constructing the Hill plot for the inhibition of TLCAT by  $\text{NaN}_3$  (Fig. 4b). The slope of the Hill plot was found to be 1.07 indicating the existence of one

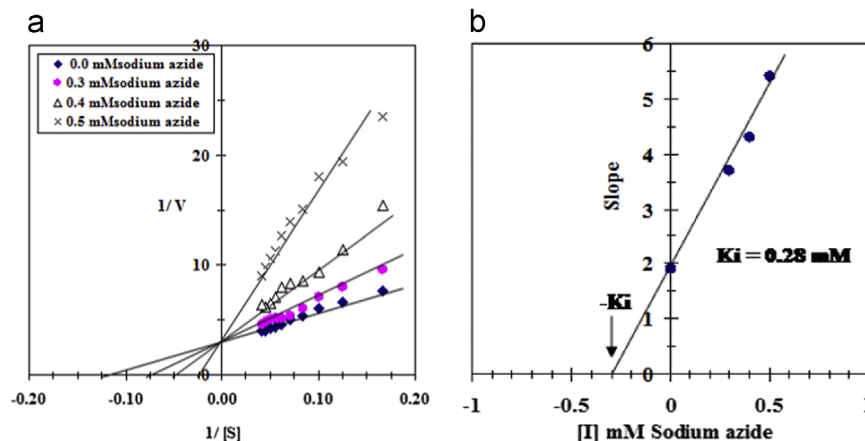


Fig. 5. (a) Lineweaver–Burk plots showing the type of inhibition of purified TLCAT by  $\text{NaN}_3$ . (b) Determination of the inhibition constant ( $K_i$ ) value for the inhibition of TLCAT by  $\text{NaN}_3$ .

binding site for  $\text{NaN}_3$  on the purified TLCAT. The type of inhibition of the camel tick larvae TLCAT by  $\text{NaN}_3$  was found to be competitive (Fig. 5a) where the presence of  $\text{NaN}_3$  did not alter the  $V_{\text{max}}$  value but increased the  $K_m$  value. For the determination of the  $K_i$  value, the slopes of the reciprocal plots lines were plotted against the  $\text{NaN}_3$  concentration (Fig. 5b). The  $K_i$  value of the TLCAT inhibition by  $\text{NaN}_3$  is determined to be 0.28 mM directly from the intercept of the X axis of the plot. In agreement with these data, it is well known that  $\text{NaN}_3$  and  $\text{NaCN}$  inhibited CAT from the radioresistant bacterium *D. radiodurans* [17], from the yeast *Trigonopsis variabilis* [23], from sprouted blackgram (*V. mungo*) seeds [15] and from the bacteria *Pseudomonad* EF group 70B [20]. In conclusion, this study presents a simple and convenient method for the purification of CAT from the camel tick larvae. This CAT enzyme might be essential for avoiding oxidative damage generated by reactive oxygen species or by the wide use of pesticides. Therefore, targeting TLCAT might be an important tool to develop new control methods for the rapid increase in pesticide-resistant tick populations.

### Conflicts of interest

The authors declare that there are no conflicts of interest. The article represents original work that is not being considered for publication, in whole or in part, in another journal, book, conference proceedings, or government publication with a substantial circulation. All previously published work cited in the manuscript has been fully acknowledged.

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