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PURIFICATION AND CHARACTERIZATION OF TWO GLUTATHIONE PEROXIDASES FROM EMBRYO OF THE CAMEL TICK *HYALOMMA DROMEDARII*

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Two glutathione peroxidase isoenzymes were purified from 24-day old embryos of the camel tick *Hyalomma dromedarii* and designated tick embryo glutathione peroxidase 1 and 2 (TEGPx1 and TEGPx2). The purification procedure involved ammonium sulfate precipitation, as well as ion exchange and gel filtration column chromatography. Glutathione peroxidase isoenzymes subunit molecular mass was determined by SDS-PAGE to be 36 ± 2 and 59 ± 1.5 kDa for TEGPx1 and TEGPx2, respectively. TEGPx1 isoenzyme exhibited a dimeric structure with native molecular mass of 72 kDa while TEGPx2 was a monomeric protein. TEGPx1 and TEGPx2 displayed their pH optima at 7.6 and 8.2. Both isoenzymes cleaved preferentially H_2O_2 with K_m values of 24 and 49 μM. Iodoacetamide competitively inhibited TEGPx1 with K_i value of 0.45 mM and 1,10-phenanthroline competitively inhibited TEGPx2 with K_i value of 0.12 mM. These results indicate the presence of two different forms of glutathione peroxidase in the developing camel tick embryos. This finding enhances our knowledge and understanding of the physiology of these ectoparasites and will encourage the development of new and untraditional control methods.

Keywords: glutathione peroxidase, purification, characterization, camel tick, Hyalomma dromedarii.

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INTRODUCTION

Antioxidant protection is mediated by molecules that are capable of removing, neutralizing, or scavenging reactive oxygen species (ROS), including hydroxyl radical, superoxide anion, hydrogen peroxide (H_2O_2), and singlet oxygen. The protection mechanism may also include the inhibition of ROS formation, the binding of metal ions needed for catalysis of ROS generation, and the up-regulation of antioxidant defense activity. This suggests that the removal of free radicals is being accomplished by several cascades of intricately related events [1].

A number of enzymes are involved in the antioxidant system including superoxide dismutase, catalase, glutathione peroxidase [2] and glutathione reductase [3], glutathione transferase [4], and ceruloplasmin [5]. Glutathione peroxidase (EC 1.11.1.9) is a critical antioxidant enzyme in organisms that protects cells and prevents oxidative stress by catalyzing the reduction and thus inactivation of a number of potentially harmful organic hydroperoxides [6, 7]. The enzyme utilizes glutathione as a source of electrons and protons to eliminate toxic H_2O_2 and other hydroperoxides generated in the course of tissue metabolism [8–12]. Moreover, H_2O_2 can be utilized as a possible substrate for glutathione peroxidase [13]. Glutathione peroxidases are a family of enzymes; they have been described as six different multiple isozymes in mammals [14]. These glutathione peroxidases are classified into two subgroups; selenium-dependent glutathione peroxidase and non-selenium-dependent glutathione peroxidase. The selenium dependent glutathione peroxidase is characterized by the presence of selenium in the active site as selenocysteine and consists of four identical subunits; it is active with both organic hydroperoxides (ROOH) and H_2O_2. The non-selenium dependent glutathione peroxidase consists of proteins that do not depend on selenium for catalysis and have negligible activity with H_2O_2 [9, 15]. Functionally, the glutathione peroxidase family is detoxifying lipids and H_2O_2 that are produced during phagocytosis or physi-

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hyrdogen peroxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; PAGE, polyacrylamide gel electrophoresis; PMS, phenazine methosulfate; PMSF, phenylmethylsulfonylfluoride; ROOH, hydroperoxides; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TEGPx, tick embryo glutathione peroxidase.

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Table 1. Purification scheme of glutathione peroxidase isoenzymes from the camel tick 24-day old embryos

Purification step	Total mg proteins	Total Units	Recovery (%)	Specific activity	Fold purification
Crude extract	532.8	780.0	100.0	1.5	1.0
80% $(NH_4)_2SO_4$ fraction	384.0	644.0	82.6	1.7	1.2
DEAE-cellulose fraction					
0.05 M NaCl (TEGPx1)	35.1	322.0	42.6	9.5	6.5
0.1 M NaCl (TEGPx2)	56.0	200.0	25.6	3.6	2.4
Sephacryl S-300 fraction					
TEGPx1	4.8	270	34.6	56.3	37.5
TEGPx2	3.7	156	20.0	42.2	28.1

ological metabolisms. They catalyze the reduction of organic ROOH and H_2O_2 to fatty alcohol and/or water by oxidizing glutathione, which gets recycled later by glutathione reductase [16].

Ticks are widespread in all continents and are associated with disease in humans, livestock, and wildlife [17]. The different ecological, behavioral, and physiological studies on ticks gave a better understanding of these organisms and the development of new control strategies against them. As a result of the rapid increase in pesticide-resistant tick populations [18], the study of tick physiology has gained considerable attention to understand the mechanisms involved in toxins detoxifications [19, 20]. The aim of this study was to purify and characterize glutathione peroxidase from the 24-day old embryos of the camel tick *H. dromedarii* to understand its role as an antioxidant enzyme at this early stage of the tick growth. Such study will establish the basis for developing new and effective biochemical control strategies of ticks.

RESULTS AND DISCUSSION

A novel approach that might be effective, in comparison to the traditional pesticides, for the biochemical control of the camel tick, is the inhibition of the detoxification enzymes during embryogenesis and larval stage. We have previously studied superoxide dismutase from tick larvae and characterized its different forms [21]. The present study is looking into the isolation and characterization of glutathione peroxidase during the late stage of embryogenesis. Glutathione peroxidase is one of the key enzymes in the antioxidant defense system of living cells [22].

The developmental profile of the specific activity of glutathione peroxidase during embryogenesis of the camel tick *H. dromedarii* revealed a gradual increase in the specific activity of glutathione peroxidase in every stage from day 1 to reach its highest level in 24-day old embryos (1.32 ± 0.2 units/mg proteins) (Fig. 1*a*). This was further confirmed by glutathione peroxidase isoenzyme pattern on native 7% polyacrylamide gel electrophoresis (PAGE) (Fig. 1*b*). This was investigated in order to determine the embryonic stage that has the highest enzyme activity, which was subsequently used for enzyme isolation and characterization. Based on that, we used the 24-day old embryos in the subsequent study stages.

We introduced a simple and reproducible purification method of glutathione peroxidase isoenzymes from the embryo of the camel tick *H. dromedarii*. The purification procedure was carried out by ammonium sulfate precipitation, ion exchange chromatography on DEAE-cellulose column, and gel filtration chromatography on Sephacryl S-300 column. The specific activity of glutathione peroxidase of the 24-day old embryonic crude extract was found to be 1.5 units/mg protein. A typical scheme of glutathione peroxidase purification from the camel tick *H. dromedarii* embryos is presented in Table 1. After ammonium sulfate precipitation, most of the glutathione peroxidase activity was in the precipitated fraction, which contained 82.6% of the activity. The DEAE-cellulose elution profile revealed the presence of two peaks of glutathione peroxidase activity. These two peaks were eluated by 0.05 M and 0.1 M NaCl and designated as TEGPx1 and TEGPx2, respectively. The elution fractions corresponding to these two peaks were pooled, concentrated by lyophilization, and applied onto a Sephacryl S-300 column. The elution profiles of TEGPx1and TEGPx2 on the Sephacryl S-300 column revealed the presence of a single peaks of the enzyme activity. The specific activity of TEGPx1 was increased to 56.3 units/mg protein, which represents 37.5-fold purification over the crude extract with 34.6% yield. The specific activity of TEGPx2 was increased to 42.2 units/mg protein, which represents 28.1-fold purification over the crude extract with 20.0% yield (Table 1). The native molecular mass of TEGPx1 and TEGPx2 eluted from Sephacryl S-300 column were deduced from a calibration curve to be 72 ± 1.6 and 60 ± 1.8 kDa, respectively.

Similar purification procedures of glutathione peroxidases were reported from pig liver [23], human plasma [24], human blood platelets [25], human liver [26], and bovine plasma [27]. The chromatography on DEAE-cellulose resolved the glutathione peroxidase activity conveniently into two isoenzymes, TEGPx1 and TEGPx2. In addition, a large variety of purification fold and recovery percentage of glutathione peroxidase

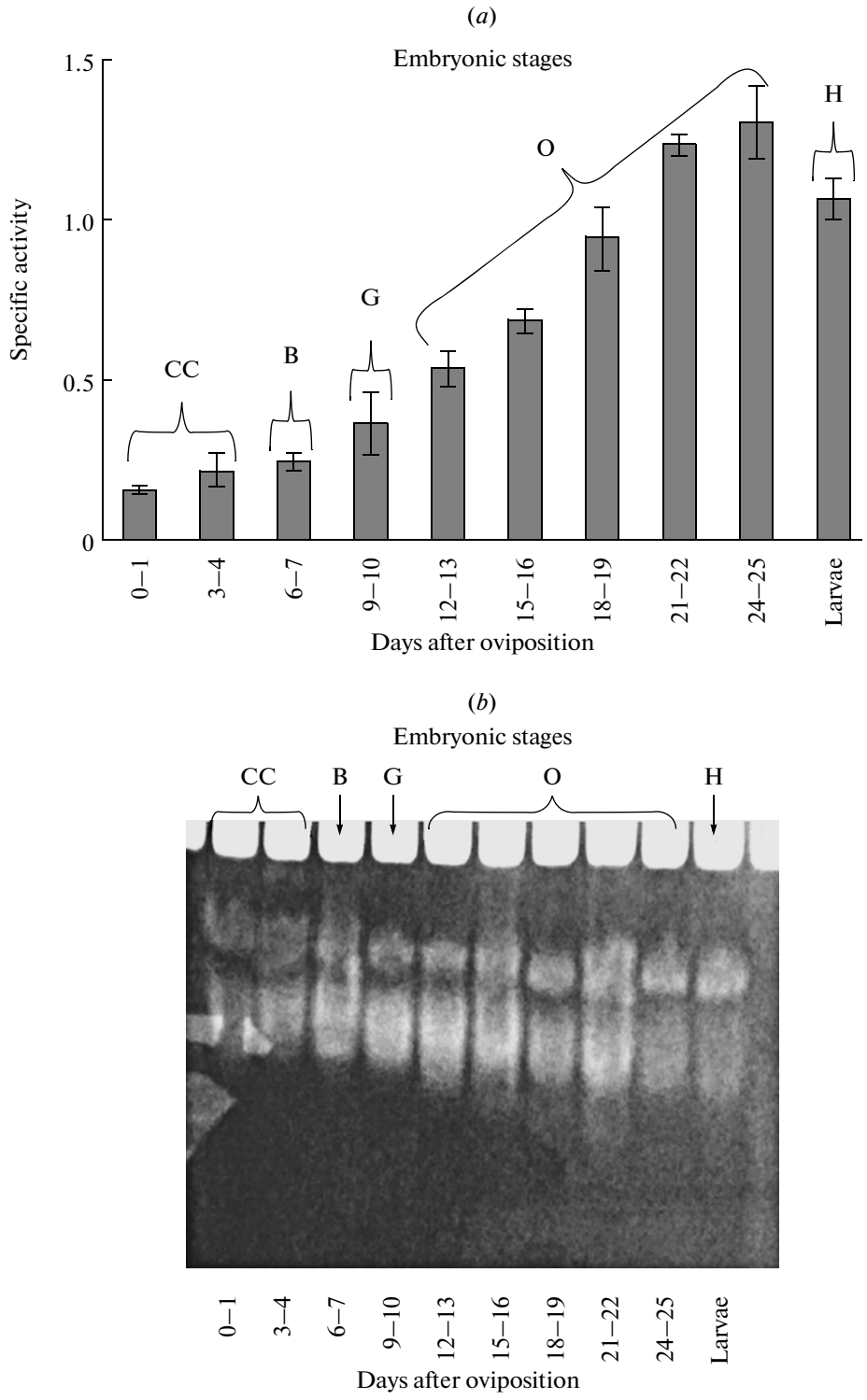

Fig. 1. (*a*) Developmental profile of the specific activity of glutathione peroxidase during embryogenesis of the camel tick *Hyalomma dromedarii*. Each point represents the mean of at least 4 runs for each developmental stage ± S.E. (*b*) Electrophoretic analysis of glutathione peroxidase isoenzyme pattern of the camel tick *H. dromedarii* during embryogenesis on native 7% PAGE. CC, cell cleavage; B, blastula formation; G, gastrulation; O, organogenesis; H, hatching.

were previously reported. Thus, glutathione peroxidase was purified from human blood platelets with 712-fold purification and 23% yield [25]; from human plasma with 6800-fold purification and 2.8% yield [24]; from human liver with 11200-fold purification and 9% yield [26]; and from Southern bluefin tuna liver with 1071-fold pu-

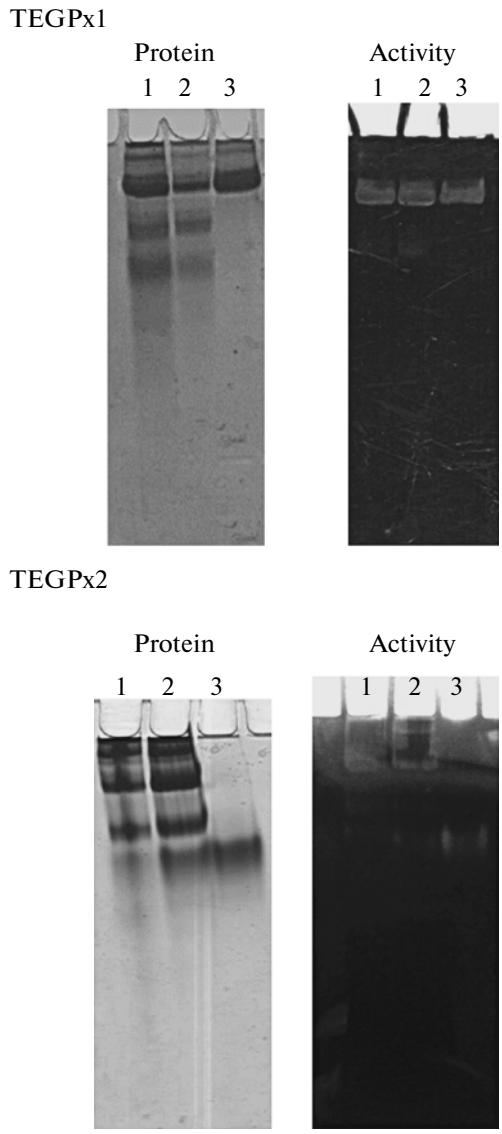

Fig. 2. Electrophoretic analysis of protein and glutathione peroxidase activity isoenzyme patterns of (*a*) TEGPx1 and (*b*) TEGPx2 in 7% native PAGE: (1) crude extract, (2) DEAE-cellulose fraction, (3) Sephacryl S-300 fraction.

rification and 1% yield [28]. The recovered yield is similar to that of glutathione peroxidase of human blood platelets, which is also characterized by age-related changes in the enzymatic activity [25].

The samples of the crude extract and the DEAE-cellulose, and Sephacryl S-300 elution fractions were analyzed electrophoretically in 7% native PAGE (Fig. 2). Single protein bands coincided with the enzyme activity bands of the two isoenzymes indicating the purity of TEGPx1 and TEGPx2preparations. Sodium dodecyl sulfate (SDS)-PAGE of the denatured purified TEGPx1 and TEGPx2 isoenzymes (Fig. 3) was used to calculate the subunit molecular mass; they were found to be 36 ± 2 and 59 ± 1.5 kDa. Comparison of subunit molecular mass obtained from SDS-PAGE to that of the native intact protein as determined by gel filtration revealed that TEGPx1 exhibited dimeric structure with two identical subunits of 36 kDa each (Fig. 3*a*). On the other hand, TEGPx2 is a monomeric isoenzyme with molecular mass of 60 kDa (Fig. 3*b*). Glutathione peroxidase was reported to have a monomeric structure, e.g. the glutathione peroxidase of pig liver that has a molecular mass of 20 kDa [23]. In addition, many glutathione peroxidase isoenzymes were reported to have tetrameric structure consisting of four protein subunits, for exampe the 112-kDa glutathione peroxidase of the bovine ciliary body with a subunit molecular weight of 29 kDa [29], the 85-kDa glutathione peroxidase of the Southern bluefin tuna liver with a subunit molecular weight of 24 kDa [28], the 92-kDa glutathione peroxidase of the human blood platelets with a subunit molecular weight of 23 kDa [25], and the 100-kDa glutathione peroxidase of the human plasma with a subunit of 23 kDa [24]. The higher activity of TEGPx1 (33.4% higher) compared to TEGPx2 might pertain to its dimeric nature. On the other hand, the monomeric TEGPx2 has 35.8% higher catalytic activity towards *t*-butyl hydroperoxide than the dimeric TEGPx1 isoenzyme that can indicate higher tolerance to oxidative stress by the dimeric enzyme and may explain the need to have both isoenzyme forms.

Substrate specificity of TEGPx1 and TEGPx2 was screened toward three substrates: hydrogen peroxide (H_2O_2), cumene hydroperoxide, and *t*-butyl hydroperoxide. The both isoenzymes preferentially cleaved H_2O_2 and had lower activity to cumene hydroperoxide and *t*-butyl hydroperoxide (data not shown). Similarly, glutathione peroxidase had greater affinity for hydrogen peroxide in Southern bluefin tuna liver [28], rat lung [30], and bovine lens [31]. A Lineweaver–Burk plot for the reciprocal of the reaction velocity (1/v) and the substrate concentration (1/[S]) was constructed and K_m values were found to be 24 and 49 μM H_2O_2; the corresponding V_{max} values were calculated to be 100 and 84 units/mg protein for TEGPx1 and TEGPx2, respectively (Fig. 4). This indicates the high affinity of both isoenzymes toward H_2O_2, especially when compared to the K_m value found for Southern bluefin tuna liver glutathione peroxidase (12 μM H_2O_2) [28] and for the bovine ciliary body (25 μM H_2O_2) [29]. The effect of pH on the activities of camel tick embryo TEGPx1 and TEGPx2 was examined in 0.05 M potassium phosphate buffer, pH 5.7 to pH 8.0, and 0.05 M Tris-HCl buffer, pH 8.2 to pH 9.6. The pH profiles of TEGPx1 and TEGPx2 displayed optimum activity around pH 7.6 and 8.2, respectively (Fig. 4). These optimum pH values are in the same range as observed for other glutathione peroxidases including those from Southern bluefin tuna liver [28], hamster liver [32], and the carp hepatopancreas [33].

The purified camel tick embryos TEGPx1 and TEGPx2 were pre-incubated for 5 min at 37°C with 2 and 5 mM of each of the following salts: $CaCl_2$, $CoCl_2$,

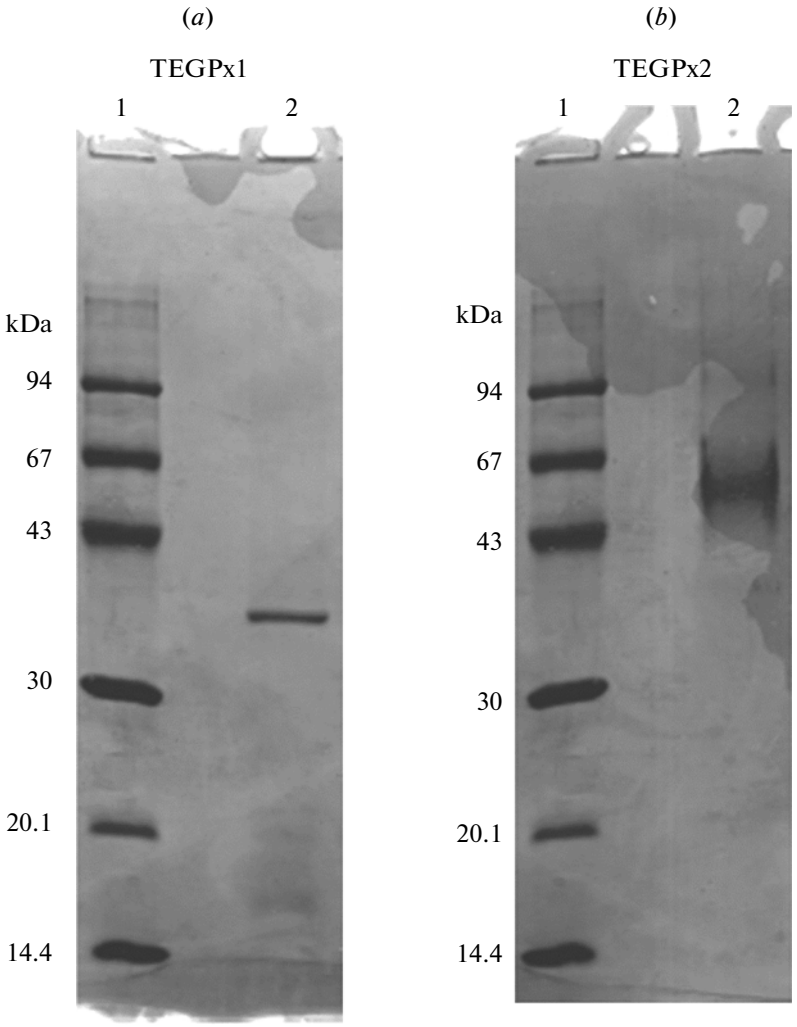

Fig. 3. Subunit molecular weight determination by electrophoretic analysis of (*a*) TEGPx1 and (*b*) TEGPx2 in 12% SDS-PAGE: (1) molecular weight marker proteins and (2) denatured purified isoenzyme.

$CuCl_2$, $FeCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$. Then, the activity of each isoenzyme was assayed. The obtained data are presented in Table 2 and show the activity of TEGPx1 and TEGPx2 in the presence of the various cations. A control sample without any cation was assayed and the measured activity was taken as 100% relative activity. $MgCl_2$ increased the activity of TEGPx1 and TEGPx2; $NiCl_2$ increased only the activity of TEGPx2; while $FeCl_2$ and $MnCl_2$ inhibited the activity of both isoenzymes (more than 30% reduction in relative activity). Thus, $FeCl_2$ and $MnCl_2$ may be used to develop new specific pesticides for the tick biological control. Copper, mercury, and zinc exhibited no significant effect on both isoenzymes in spite of their documented strong inhibitory effect on glutathione peroxidase from human plasma [24].

The effect of different previously reported peroxidase inhibitors was examined. These include EDTA, D,L-dithiothreitol (DTT), iodoacetamide, β-mercaptoethanol, sodium azide (NaN_3), sodium dodecyl sulfate (SDS), 1,10-phenanthroline, phenazine methosulfate (PMSF), potassium cyanide (KCN), and potassium dichromate ($K_2Cr_2O_7$) [34–40]. The purified camel tick embryonic TEGPx1 and TEGPx2 were pre-incubated for 5 min at 37°C with each of these inhibitors. Then, the activity of isoenzymes was assayed and the percentage of inhibition was calculated as a ratio to a control inhibitor. Iodoacetamide inhibits the activity of TEGPx1 and TEGPx2 (Table 2), which indicates that methionine, cysteine, and histidine residues have important effects on the structure and activity of these isoenzymes similar to glutathione peroxidase from hamster liver [32] and rat liver mitochondria [41]. Iodoacetamide and 1,10-phenanthroline were found to be the most potent inhibitors of TEGPx1 and TEGPx2, respectively.

The effect of iodoacetamide concentrations on TEGPx1 indicted that the 50% inhibition (I50) was 0.68 mM iodoacetamide and the maximum inhibition

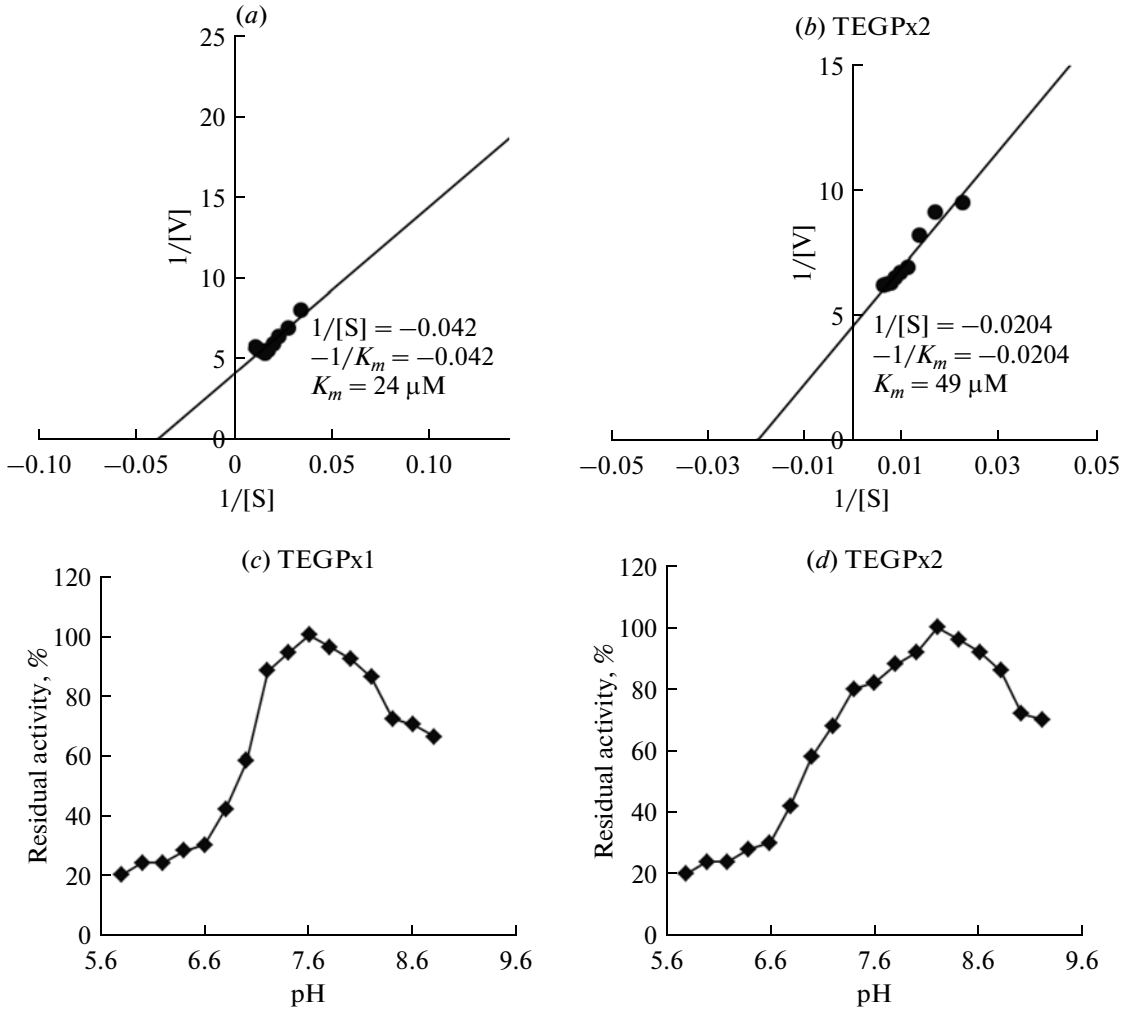

Fig. 4. (*a*, *b*) Lineweaver–Burk plot relating the reciprocal of the reaction velocity of the purified (*a*) TEGPx1 and (*b*) TEGPx2 to H_2O_2 concentration in μM. The reaction velocity is the change in absorbance at 340 nm per min. (*c*, *d*) Effect of pH on the purified camel tick embryo (*c*) TEGPx1 and (*d*) TEGPx2 using 0.05 M potassium phosphate buffer, pH 5.8 to pH 8.0, and 0.05 M Tris-HCl buffer, pH 8.0 to pH 9.6.

of the enzyme (97.8%) was achieved by 2.4 mM iodoacetamide (Fig. 5*a*). A linear relationship was observed by constructing the Hill plot of the inhibition of TEGPx1, and the slope was found to be 1.04 indicating the presence of one binding site for iodoacetamide (Fig. 5*b*). The inhibition of TEGPx1 by iodoacetamide was competitive, since the presence of the inhibitor did not alter the V_{max} value but increased the K_m value (Fig. 5*d*) and the K_i value of the TEGPx1 inhibition by iodoacetamide was determined to be 0.45 mM (Fig. 5*c*). On the other hand, the effect of 1,10-phenanthroline concentrations on TEGPx2 indicated that the I50 was 0.2 mM and the maximum inhibition of the enzyme (98.4%) was achieved by 0.6 mM 1,10-phenanthroline (Fig. 6*a*). A linear relationship was observed by constructing the Hill plot for the inhibition of TEGPx2 by 1,10-phenanthroline and the slope was found to be 0.93 indicating the presence of one binding site for 1,10-phenanthroline on TEGPx2 (Fig. 6*b*). The of inhibition of TEGPx2 by 1,10-phenanthroline was competitive, since the presence of the inhibitor did not alter the V_{max} value but increased the K_m value (Fig. 6*d*) and the K_i value of the TEGPx2 inhibition by 1,10-phenanthroline was determined to be 0.12 mM (Fig. 6*c*).

Both inhibitors exert a competitive inhibitory effect on two isoenzymes (Fig. 5*d* and 6*d*) and have a single binding site (Fig. 5*b* and 6*b*). This is the most important finding in characterization of both isoenzymes, since it provides new sights into the development of effective and new tick-specific pesticides that might be efficient against current pesticide-resistant ticks. The inhibition of TEGPx1 and TEGPx2 isoenzymes by EDTA and 1,10-phenanthroline (Table 2) indicates that both isoenzymes are metalloenzymes. Furthermore, the strong inhibition of both isoenzymes by β-mercaptoethanol and dithiothreitol indicates that the –SH groups in

Table 2. Effect of divalent cations and inhibitors on the purified camel tick *H. dromedarii* 24-day old embryos glutathione peroxidases TEGPx1 and TEGPx2

Reagent	Final concentration, mM	Residual activity, %		Reagent	Final concentration, mM	Inhibition, %	
		TEGPx1	TEGPx2			TEGPx1	TEGPx2
Control	–	100.0	100.0	Control	–	0.0	0.0
$CaCl_2$	2.0	98.2	98.5	Ethylenediaminetetraacetic acid (EDTA)	2.0	4.8	5.7
	5.0	92.4	93.9		5.0	8.9	10.1
$CoCl_2$	2.0	96.6	100.4	D,L-Dithiothreitol (DTT)	2.0	17.3	19.1
	5.0	100.3	102.3		5.0	55.0	48.2
$CuCl_2$	2.0	96.1	90.3	Iodoacetamide	2.0	82.7	49.1
	5.0	100.1	98.2		5.0	96.4	83.6
$FeCl_2$	2.0	82.9	88.4	β-Mercaptoethanol	2.0	21.4	28.2
	5.0	58.1	63.2		5.0	62.7	53.4
$MgCl_2$	2.0	115.6	108.2	Sodium azide (NaN_3)	2.0	7.6	11.5
	5.0	133.5	117.5		5.0	33.7	42.2
$MnCl_2$	2.0	83.8	85.6	Sodium dodecyl sulfate (SDS)	2.0	16.2	14.5
	5.0	61.5	67.5		5.0	47.5	53.6
$NiCl_2$	2.0	96.2	100.0	1,10-Phenanthroline	2.0	39.6	76.2
	5.0	89.4	109.8		5.0	68.2	93.3
$ZnCl_2$	2.0	94.1	96.4	Phenylmethylsulfonylfluoride (PMSF)	2.0	2.2	5.8
	5.0	90.4	89.2		5.0	8.6	11.6
				Potassium cyanide (KCN)	2.0	4.6	6.3
					5.0	10.2	16.1
				Potassium dichromate ($K_2Cr_2O_7$)	2.0	57.3	43.6
					5.0	78.1	73.8

* These values represent percentage from the control and the mean of triplicate experiments.

the enzyme structure play an important role in the enzymatic activity. In addition, the inhibitory effect of $K_2Cr_2O_7$ on both TEGPx1 and TEGPx2 was probably due to strong oxidizing power of $K_2Cr_2O_7$ that may cause oxidation of metal prosthetic groups that are important to the enzyme activity.

The present study was the first one to report camel tick glutathione peroxidase activity during embryonic stages. We introduced a simple and reproducible purification procedure. Analysis and characterization of the purified isoenzymes revealed some important information regarding their behavior. Iodoacetamide and 1,10-phenanthroline might be useful in development of control methods that are targeted on ticks at the embryonic stage to efficiently eradicate them. As these glutathione peroxidase isoenzymes might be essential for avoiding oxidative damage generated by reactive oxygen species or by the wide use of pesticides, targeting on these glutathione peroxidase isoenzymes might be useful in developing new methods to control the rapid increase in pesticide-resistant tick populations.

EXPERIMENTAL

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

Chemicals. Phenylmethylsulfonylfluoride (PMSF), oxidized glutathione (GSSG), reduced glutathione (GSH), glutathione reductase, DEAE-cellulose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenazine methosulfate (PMS), Sephacryl S-300, and Gel Filtration Markers Kit for Protein Mo-

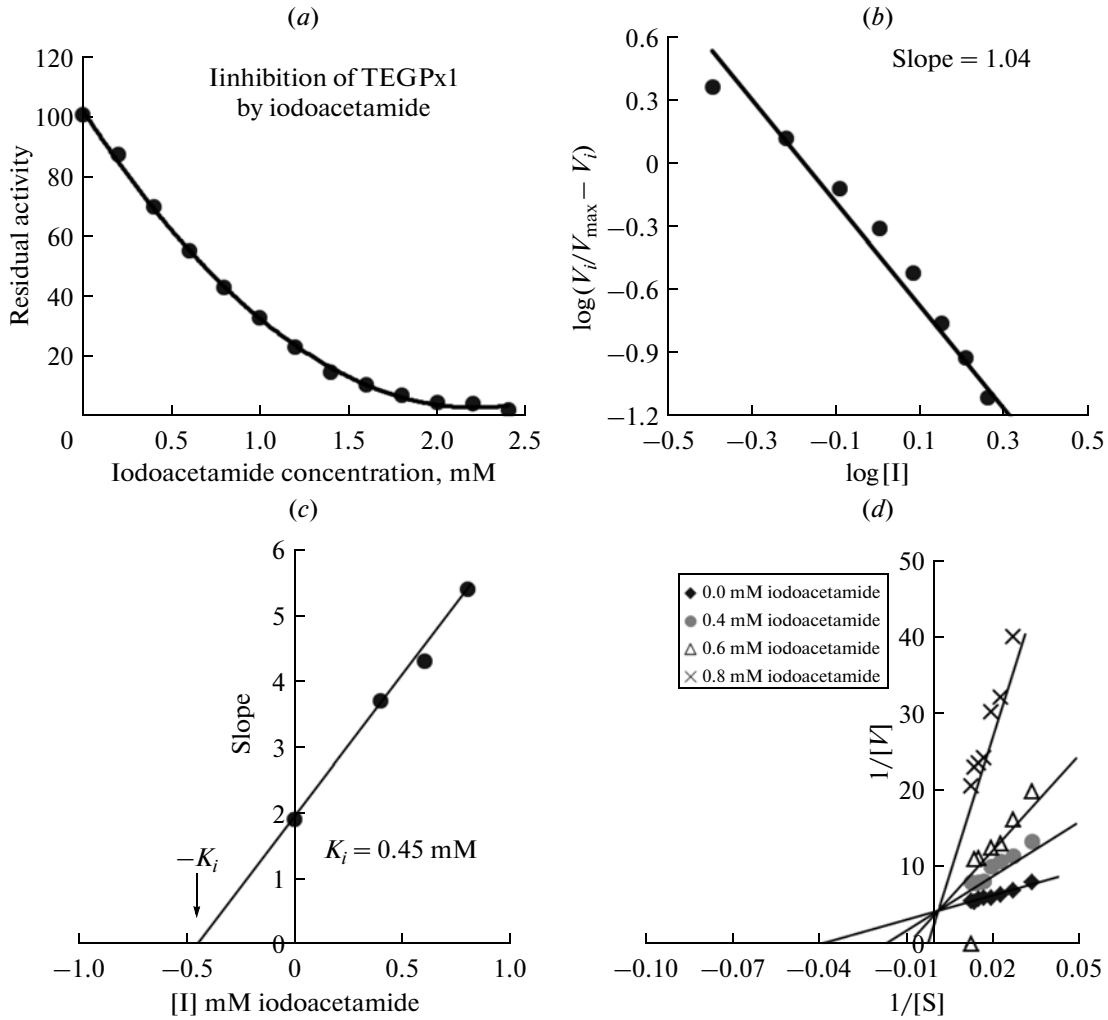

Fig. 5. (*a*) Inhibition of TEGPx1 by varying concentrations of iodoacetamide. (*b*) Hill plot for inhibition of TEGPx1 by iodoacetamide. (*c*) Determination of the inhibition constant (K_i) value for the inhibition of the TEGPx1 by iodoacetamide. (*d*) Lineweaver–Burk plots showing the type of inhibition of TEGPx1 by iodoacetamide.

lecular Weights 12000–200000 Da were purchased from Sigma-Aldrich. All other chemicals were of analytical grade.

Assay of glutathione peroxidase activity. Glutathione peroxidase activity assay reaction mixture contained 5 mM EDTA, 0.075 mM H_2O_2, 5 mM GSH, 0.28 mM nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), 1 IU glutathione reductase, and the enzyme solution in 1 mL 0.05 M potassium phosphate buffer, pH 7.0. One unit is equivalent to the oxidation of 1 μmole of NADPH in 1 min at 25°C. The extinction coefficient of NADPH was taken to be 6.22 mM^{-1} cm^{-1}. The decrease in absorbance at 340 nm was monitored against control lacking the enzyme [42].

Staining of glutathione peroxidase activity on native PAGE. After electrophoresis, the gel was submerged in 50 mM Tris-HCl buffer, pH 7.9, containing 13 mM GSH and 0.004% H_2O_2 with gentle shaking for 10 to 20 min. Glutathione peroxidase activity was stained with 1.2 mM MTT and 1.6 mM PMS. The active band showed a clear zone against backgrounds [43].

Preparation of crude extract. Two grams of camel tick embryos were homogenized in 10 mL of 0.01 M potassium phosphate buffer, pH 7.3, containing 0.05 mM EDTA, using a Teflon-pestled homogenizer. Cell debris and insoluble materials were removed by centrifugation at 12000 *g* for 20 min and the supernatant was saved and designated as crude extract.

Ammonium sulfate precipitation. The crude extract was brought to 80% saturation by gradually adding solid $(NH_4)_2SO_4$ and stirred for 30 min at 4°C. The pellet was obtained by centrifugation at 12000 *g* for 30 min and dissolved in 0.01 M potassium phosphate buffer, pH 7.3, containing 0.05 mM EDTA and dialyzed extensively against the same buffer.

DEAE-cellulose column chromatography. The dialyzed sample was chromatographed on a DEAE-cellulose column (12 × 2.4 cm i.d.) previously equilibrated

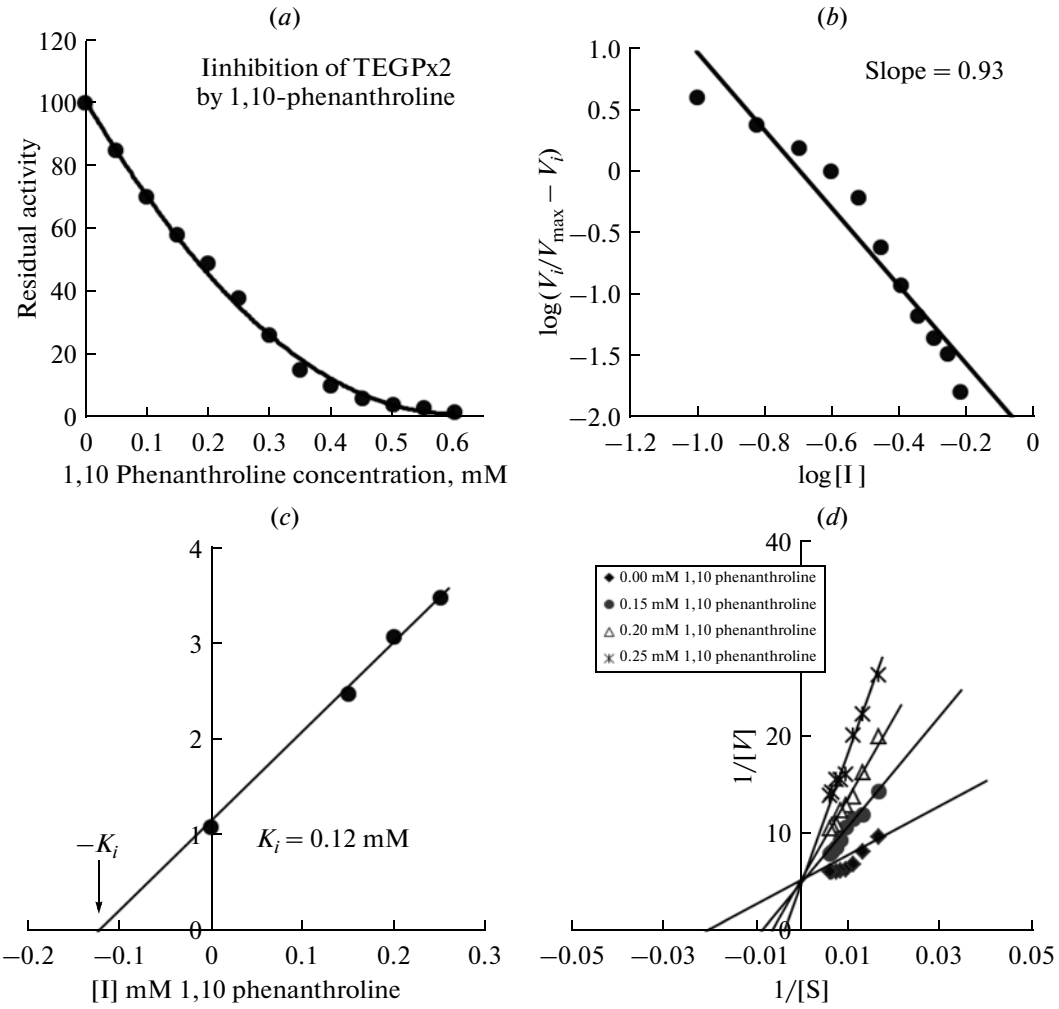

Fig. 6. (*a*) Inhibition of TEGPx2 by varying concentrations of 1,10-phenanthroline. (*b*) Hill plot for inhibition of TEGPx2 by 1,10-phenanthroline. (*c*) Determination of the inhibition constant (K_i) value for the inhibition of the TEGPx2 by 1,10-phenanthroline. (*d*) Lineweaver–Burk plots showing the type of inhibition of TEGPx2 by 1,10-phenanthroline.

with 0.01 M potassium phosphate buffer, pH 7.3, containing 0.05 mM EDTA. 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. Five-milliliter fractions were collected and the fractions with glutathione peroxidase activity were pooled and lyophilized.

Sephacryl S-300 column chromatography. The concentrated pooled solution of fractions with glutathione peroxidase activity was applied onto a Sephacryl S-300 column (142 cm × 1.75 cm i.d.). The column was equilibrated and developed with 0.01 M potassium phosphate buffer, pH 7.3, containing 0.05 mM EDTA at a flow rate of 30 mL/h and 2-mL fractions were collected.

Electrophoretic analysis. Native gel electrophoresis was carried out with 7-% PAGE [44]. SDS-PAGE was performed with 12-% polyacrylamide gel [45]. The subunit molecular weights of the purified glutathione peroxidase isoenzymes were determined by SDS-PAGE [46]. Proteins were stained with 0.25% Coomassie Brilliant Blue R-250.

Protein concentration determination. Protein concentration was determined by the dye binding assay [47] using bovine serum albumin (BSA) as a standard protein.

Statistical analysis. All statistical analysis was performed in Excel (Microsoft). Student *t*-test was utilized in this study to calculate the significance. P values of <0.05 were considered significant.

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