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Characterization of an interesting selenium-dependent glutathione peroxidase (Se-GPx) protecting cells against environmental stress: The *Camelus dromedarius* erythrocytes Se-GPx

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ABSTRACT

Camel lives most of its life under high environmental stress in the desert. Glutathione peroxidase plays a key role in protecting cells against oxidative stress. For the first time, selenium-dependent glutathione peroxidase (Se-GPx) was purified from camel erythrocytes, biochemically characterized, and some of its properties were studied. The enzyme was purified using ethanol-chloroform treatment, acetone precipitation and ion exchange chromatography. A purification fold of 33.72 with 0.19% yield were obtained. The native molecular weight of the enzyme was estimated to be about 69 kDa. On SDS-PAGE, the enzyme was composed of two different subunits with a molecular weight of approximately 53 and 21 kDa. An optimum temperature of 47 °C and an optimum pH of 7.2 were found. The activation energy was 41.71 kJ/mol. This enzyme was inhibited by thiol reagents, D,L-Dithiothreitol and β-Mercaptoethanol, and was sensitive to bivalent cations. The enzyme had a general specificity toward hydroperoxides, and high specificity for reduced glutathione. The purified enzyme contained 3.06 mol of selenium per mol of protein. The K_m and V_{max} values for hydrogen peroxide and reduced glutathione were 0.72 and 1.58 mM, and 25.33 and 31.03 U/mg, respectively. The biochemical properties of camel Se-GPx were different comparing to those of mammalian species. Lower molecular weight, heterodimeric structure, higher optimum temperature, relatively lower optimum pH, lower content of selenium and higher affinity for hydrogen peroxide at low reduced glutathione concentration, these could be explained by the fact that camel is able to live in the intense environmental stress in the desert.

1. Introduction

All living organisms are exposed to reactive oxygen species (ROS) during the normal metabolic processes. Oxidative stress occurs when there is an imbalance in the generation and metabolism of ROS in the cells (Birben et al., 2012). The excessive ROS accumulation will lead to cellular injury, such as damage to DNA, proteins and lipid membranes (Halliwell, 1991). The major antioxidant enzymes that are found to provide protection against the deleterious actions of ROS are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Birben et al., 2012).

GPx (EC 1.11.1.9) was first reported by Mills (1957) in bovine erythrocytes as an enzyme that protect hemoglobin from oxidative degradation by catalyzing the reduction of hydrogen peroxide (H_2O_2) to water using reduced glutathione (GSH) as a hydrogen donor. Besides its decomposition of H_2O_2, the GPx enzyme was later characterized in various mammalian tissues and also found to decompose lipid hydroperoxides (Little and O'Brien, 1968). CAT is also among the principle antioxidant enzymes responsible for reducing H_2O_2, but, it had no effect on the lipid hydroperoxides (O'Brien and Frazer, 1966). However, due to its unique properties in the reduction of H_2O_2 and lipid hydroperoxides, GPx constitutes the first line of defense against oxidative damage due to lipid peroxidation (Christophersen, 1968).

The characterization studies of GPx have been investigated in mammalian cells; eight GPxs have been identified and differed in many properties, including their amino acid sequence, structure, substrate

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specificity, subcellular localization and selenium content (Brigelius-Flohé and Maiorino, 2013). Depending on its requirement for selenium, two types of GPx activities are generally present (Brigelius-Flohé and Maiorino, 2013): selenium-dependent GPx (Se-GPx) and non-selenium-dependent GPx (non-Se-GPx) which are distinguished on the basis of their substrate specificity (Lawrence and Burk, 1976), and sensitivity to cyanide (Kraus and Ganther, 1980) and cadmium (Jamall and Smith, 1985). Se-GPx has been purified and characterized from many mammalian organs (Awasthi et al., 1975; Bergad et al., 1982; Nakamura et al., 1974; Takahashi et al., 1987), plants (Sabeh et al., 1993; Shigeoka et al., 1991), fishes (Bell et al., 1984; Thompson et al., 2006) and microorganisms (Singh and Rathaur, 2005). However, much less is known on the biochemical characterization of Se-GPx in camel *(Camelus dromedarius)*.

The one-humped camel is the most useful domestic animal to humans in the desert life. In fact, the camel represents an important source of animal proteins (meat and milk), besides its cultural, agricultural and economical values. Unlike other domestic mammalian species, the camel has some biochemical, anatomical and physiological peculiarities that allow it to live in the hot and arid conditions (Alhadrami and Faye, 2016). In these conditions, camel is continuously exposed to high environmental stress (Wu et al., 2015), which induces overproduction of ROS that are known to cause damage of important biomolecules (Halliwell, 1991). However, camel Se-GPx enzyme may play an important role in the cellular defense mechanisms against the damaging and deleterious effects of oxidative stress caused by the excessive production of ROS.

Recently, we have found that the Se-GPx purified from camel liver exhibits different properties comparing to those reported from mammalian species (Chafik et al., 2018). Therefore, more information on the biochemical properties of Se-GPx in camel would be helpful to better understand the role of this enzyme in the mechanisms involved in the resistance to stress induced by life in the desert, especially with current climatic changes and increasing temperatures (Rogelj et al., 2016). To gain more insights into the biochemical characterization of Se-GPx, in this study, this enzyme was purified from camel erythrocytes and characterized on its biochemical properties. The obtained properties were compared with those of other Se-GPxs.

2. Materials and methods

2.1. Chemicals

DEAE-Sepharose, Sephacryl S-200, gel filtration markers kit for protein molecular weights 12–200 kDa, reduced glutathione (GSH), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), D,L-Dithiothreitol (DTT), phenylmethylsulfonylfluoride (PMSF), cumene hydroperoxide (CHP), *tert*-Butyl hydroperoxide (TBH), benzoyl peroxide (BP) and L-Cysteine (Cys) were purchased from Sigma Aldrich. PageRuler™ Plus Prestained Protein Ladder 10–250 kDa was purchased from ThermoFisher Scientific. All other chemicals were of analytical grade.

2.2. Purification of Se-GPx from camel erythrocytes

Purification of Se-GPx from camel erythrocytes was carried out using the methods described by Wendel (1981), with slight modifications. Unless stated otherwise, all purification steps were performed at low temperatures between 0 and 4 °C.

2.2.1. Hemolysate

Fresh camel blood was obtained from the municipal slaughterhouse of Casablanca–Morocco. Blood was collected with sodium citrate solution (4%) and centrifuged immediately at 3500 × *g* for 10 min. The erythrocytes were washed three times in 3 volumes of 0.9% NaCl by centrifugation at 3500 × *g* for 10 min. The cells were lysed by addition of an equal volume of distilled water.

2.2.2. Ethanol-chloroform treatment

Hemoglobin was precipitated from the hemolysate by chloroform-ethanol treatment according to Tsuchihashi (1923). With stirring, 0.25 volume of ethanol and 0.15 volume of chloroform, pre-cooled in a freezer (−24 °C), were added rapidly to the lysate. Stirring was continued for 15 min during which time the hemoglobin was rendered insoluble. The mixture was diluted with 0.10 volume of distilled water and centrifuged at 3500 × *g* for 60 min.

2.2.3. Acetone precipitation

To the supernatant recovered, 0.75 volume of cold acetone was added under stirring to precipitate the proteins. After 15 min, the mixture was centrifuged at 4000 × *g* for 10 min. The precipitate was dissolved in an equal volume of 10 mM potassium phosphate buffer at pH 7.0 and dialyzed against four changes of the same buffer, 20 volumes each, over a period of 8 h.

2.2.4. First DEAE-Sepharose column chromatography

The dialyzed solution was clarified by centrifugation at 3500 × *g* for 10 min. The clear supernatant was loaded onto a DEAE-Sepharose column (2.5 × 10 cm), which was previously equilibrated with the dialysis buffer. After washing the column with the dialysis buffer, the adsorbed proteins were eluted at room temperature with a linear gradient of 0–0.5 M KCl prepared in the same buffer. Fractions of 2 ml were collected at a flow rate of 60 ml/h. The fractions containing Se-GPx activity were pooled and concentrated by lyophilization.

2.2.5. Second DEAE-Sepharose column chromatography

The concentrated enzyme solution was loaded on a second DEAE-Sepharose column and run with the same conditions as described in the first DEAE-Sepharose column chromatography.

2.3. Homogeneity of the purified Se-GPx

Homogeneity of the purified camel erythrocytes Se-GPx was confirmed by electrophoretic analyses using a vertical slab gel apparatus. Native gel electrophoresis was carried out with 7.5% polyacrylamide gel electrophoresis (PAGE) according to Davis (1964). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12% polyacrylamide gel according to Laemmli (1970).

2.4. Characterization of the purified Se-GPx

2.4.1. Molecular weight determination

The native molecular weight of camel erythrocytes Se-GPx was determined by gel filtration chromatography on a Sephacryl S-200 column (1.75 × 37 cm) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.1 M KCl. The column was calibrated with cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-Amylase (200 kDa). The purified enzyme was eluted at room temperature with the equilibration buffer at a flow rate of 0.5 ml/min. The enzyme activity was determined in the collected fractions of 1 ml.

The molecular weight of the subunits of camel erythrocytes Se-GPx was estimated by SDS-PAGE, as described by Weber and Osborn (1969). The purified enzyme was incubated in boiling water for 3 min in the presence of 2% SDS and 5% β-Mercaptoethanol (β-ME). After incubation, the enzyme was loaded onto the wells of SDS-PAGE gel. Molecular weight marker proteins used for SDS-PAGE were from commercial grade with molecular mass ranging from 10 to 250 kDa. The proteins were stained with Coomassie brilliant blue.

2.4.2. Optimum temperature and pH

The optimum temperature for the activity of camel erythrocytes Se-

GPx was determined in a temperature range of 22–80 °C. From the temperature profile of camel erythrocytes Se-GPx activity, an Arrhenius plot was used to determine the activation energy (E_a) of the enzyme by plotting the logarithm of the rate constant, k, versus the inverse temperature, 1/T. The optimum pH for camel erythrocytes Se-GPx activity was determined at different pH values using two different buffer systems: 0.05 M potassium phosphate buffer (pH 6.0–8.0) and 0.05 M Tris-HCl buffer (pH 8.6–9.6). Se-GPx activity was measured as described in assay system.

2.4.3. Effect of various chemicals and metal ions

The effect of some chemicals on the camel erythrocytes Se-GPx activity was tested at final working concentrations of 2 and 5 mM. The following chemicals were tested: β-ME, D,L-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), sodium azide (NaN_3), phenylmethylsulfonylfluoride (PMSF) and sodium dodecyl sulfate (SDS). In addition, the effect of various metal ions ($AlCl_3$, $BaCl_2$, $CaCl_2$, $CoCl_2$, $FeCl_2$, $MgCl_2$, $NiCl_2$ and $ZnCl_2$) on the camel erythrocytes Se-GPx activity were also tested at final working concentrations of 2 and 5 mM. Se-GPx activity was measured as described in assay system.

2.4.4. Substrate specificity

Hydroperoxide substrate specificity was determined by substitution of 2.5 mM hydrogen peroxide (H_2O_2), in assay system that contained reduced glutathione (GSH) as the unique sulfhydryl compound, with 2.5 mM cumene hydroperoxide (CHP), *tert*-butyl hydroperoxide (TBH) and benzoyl peroxide (BP). Sulfhydryl substrate specificity was determined by substitution of 2 mM GSH, in assay system that contained H_2O_2 as the unique peroxide, with 2 mM β-ME, DTT and cysteine (Cys).

2.4.5. Selenium analysis

The selenium content in the purified GPx was estimated using quadrupole inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7800) with a set of standards ranging from 10 to 1000 ppb of selenium. For checking the inhibition of Se-GPx with cyanide and cadmium, the purified enzyme was incubated with final working concentrations of 2 and 5 mM of potassium cyanide (KCN) and cadmium chloride ($CdCl_2$).

2.4.6. Kinetic parameters

Kinetic parameters, K_m and V_{max}, of camel erythrocytes Se-GPx for H_2O_2 and GSH were determined. Experiments for H_2O_2 were carried out by varying concentrations from 0.25 to 2 mM and keeping the GSH concentration constant at 2 mM. Experiments for GSH were carried out by varying concentrations from 0.25 to 3 mM and keeping the H_2O_2 concentration constant at 2.5 mM. Se-GPx activity was measured as described in assay system. The data were analyzed with non-linear regression using GraphPad Prism software version 7.

2.4.7. Assay of Se-GPx activity

The activity of Se-GPx was assayed based on procedure 2 of Mills (1959) as it has been modified by Rotruck et al. (1973) and Hafeman et al. (1974). The reaction mixture consisted of 0.2 ml of 0.8 mM ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2), 0.1 ml of 10 mM NaN_3, 0.2 ml of 2 mM GSH, 0.4 ml of 0.4 M phosphate buffer, pH 7.0, and 0.2 ml of sample. The mixture was pre-incubated at 37 °C for 2 min, then 0.1 ml of 2.5 mM H_2O_2 (pre-warmed to 37 °C) was added and the mixture was incubated at 37 °C for 10 min. The reaction was always initiated by addition of H_2O_2. After incubation, the reaction was arrested by the addition of 0.5 ml of 10% trichloroacetic acid (TCA) and then centrifugation at 2000 × *g* for 5 min was performed. To the supernatant, 2.5 ml of 0.1 M sodium phosphate tribasic (Na_3PO_4) and 1.0 ml of 0.04% 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were added. After 2 min, the absorbance of the developed color was measured at 412 nm. The enzyme activity was determined by measuring the residual GSH concentration in the sample using GSH standard curve, as described by Sedlak and Lindsay (1968) based on Ellman's reagent (Ellman, 1959), with slight modifications. One unit of enzyme activity is defined as micromoles of GSH per minute per milligram of protein at 37 °C. A blank (the enzyme source substituted with distilled water) was carried through the incubation simultaneously with the samples, since non-enzymatic GSH oxidation by H_2O_2 occurred during incubation. All experiments were performed in triplicates.

Staining of Se-GPx activity on native PAGE was performed according to the procedure of Lin et al. (2002). After electrophoresis the gel was cut in two parts. One part was stained for protein with Coomassie brilliant blue. The other part was soaked in 50 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 200 mg GSH and 8 µl of 30% H_2O_2 for 20 min, the Se-GPx activity was then stained by 50 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 25 mg nitro blue tetrazolium (NBT) and 25 mg phenazine methosulphate (PMS).

2.4.8. Protein determination

Protein concentrations were measured by the dye binding assay method of Bradford (1976) using bovine serum albumin as a standard.

3. Results and discussion

3.1. Purification and homogeneity

For the first time, Se-GPx has been purified from camel erythrocytes. The purification procedure was carried out by ethanol-chloroform treatment, acetone precipitation and ion exchange chromatography on DEAE-Sepharose columns. A typical purification process of the Se-GPx from camel erythrocytes is summarized in Table 1. The ethanol-chloroform treatment step is beneficial because it precipitates a large amount of hemoglobin protein (Tsuchihashi, 1923). It has been suggested that there is a presence of a strong protein-protein interaction between hemoglobin and GPx (Awasthi et al., 1975). Thus, after the removal of hemoglobin, the enzyme was easily bound to anion exchangers. Although there was a loss of about 36% of the specific activity during the acetone precipitation, this step served to partially purify as well as concentrate the sample for application onto chromatography columns. The use of ion exchange chromatography on DEAE-Sepharose columns allowed us to obtain a good purity of Se-GPx preparation. The elution pattern of the first DEAE-Sepharose column showed one enzyme

Table 1
Summary of purification of Se-GPx from camel erythrocytes.

Step	Total proteins	Se-GPx activity		Specific activity	Purification	Yield
	mg/ml	U[a]	U/ml	U/mg	-fold	%
Hemolysate	1307.46	224,972.60	468.69	0.36	1.00	100.00
Ethanol-Chloroform treatment	144.69	23,359.96	95.74	0.66	1.85	10.38
Acetone precipitation	407.65	3252.36	98.56	0.24	0.67	1.45
First DEAE-Sepharose	7.23	1381.55	62.80	8.69	24.25	0.61
Second DEAE-Sepharose	1.25	423.60	15.13	12.09	33.72	0.19

[a] Enzyme activity (U) was expressed as 1 µmol/min of GSH at 37 °C.

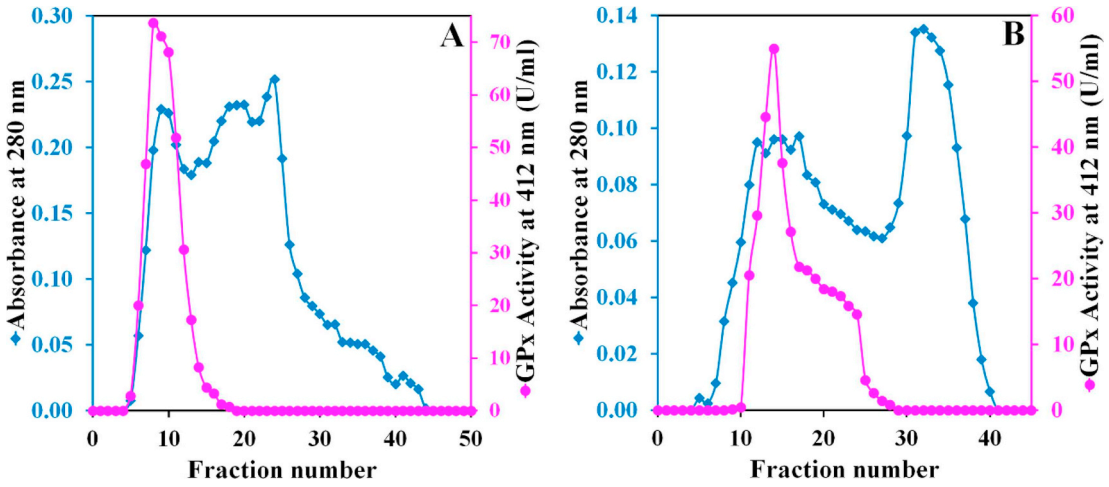

Fig. 1. Elution profiles of Se-GPx on the first (A) and second (B) DEAE-Sepharose chromatography.

activity peak that eluted prior to several contaminating proteins (Fig. 1A). This step resulted in a 24.25-fold purification with 0.61% yield of enzyme. The residual contaminants were removed by the ion exchange chromatography through the second DEAE-Sepharose column, which increased the purification fold of the enzyme. The elution pattern of the second DEAE-Sepharose column also showed one peak of enzyme activity (Fig. 1B). The five-step purification procedure described in this study resulted in a 33.72-fold purification with 0.19% yield of enzyme and final specific activity of 12.09 U/mg protein.

Se-GPx from camel erythrocytes was purified to homogeneity, which was confirmed by electrophoretic and chromatographic analyzes. On electrophoresis in PAGE, a single protein band was observed when the gel was stained for Se-GPx activity (Fig. 2A) and for protein with Coomassie brilliant blue (Fig. 2B). SDS-PAGE of the purified enzyme, pretreated with 2% SDS and 5% β-ME, revealed two protein bands (Fig. 2C, lane 6). In addition, on a Sephacryl S-200 column chromatography, one single peak of protein corresponding to the

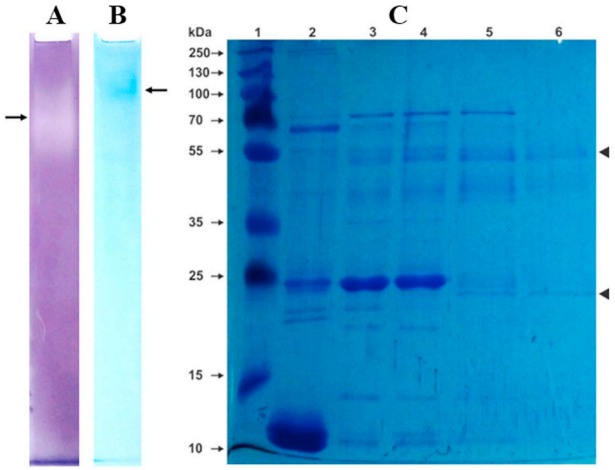

Fig. 2. Electrophoresis analyses of camel erythrocytes Se-GPx using native PAGE (A and B) and SDS-PAGE (C). After native PAGE electrophoresis, the gel was stained for Se-GPx activity (A) and proteins with Coomassie brilliant blue (B), the arrow indicates the Se-GPx enzyme. PAGE analyses (A and B) were performed in different electrophoresis analyses. (C) SDS-PAGE analysis of the purified camel erythrocytes Se-GPx. Lane 1, marker proteins; lane 2, hemolysate; lane 3, ethanol-chloroform treatment; lane 4, acetone precipitation; lane 5, first DEAE-Sepharose; lane 6, second DEAE-Sepharose. The triangle indicates the subunits of Se-GPx enzyme. The experimental procedures were detailed under "Materials and methods". (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

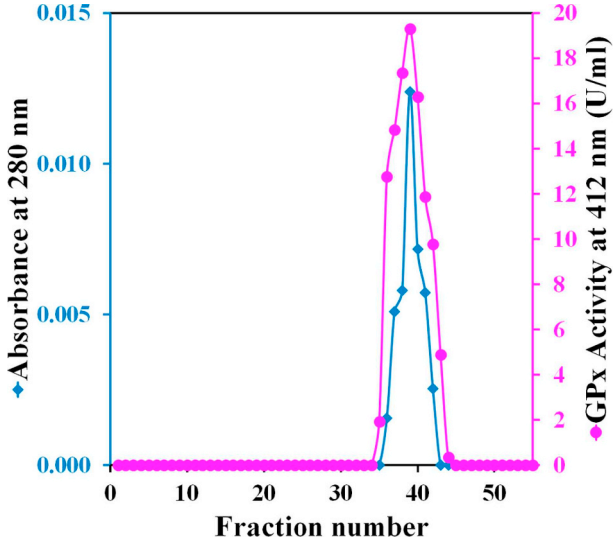

Fig. 3. Elution profile of Se-GPx on Sephacryl S-200 chromatography.

enzyme activity of Se-GPx was obtained (Fig. 3).

3.2. Characterization of Se-GPx from camel erythrocytes

3.2.1. Molecular weight determination

The molecular weight of the native enzyme was determined by Sephacryl S-200 gel filtration chromatography. A plot of the elution volumes against the logarithm of the molecular weights of standard proteins gave a molecular weight for Se-GPx of 68629 Da (Fig. 4A), which is the same as that found in camel liver Se-GPx (Chafik et al., 2018). On the other hand, SDS-PAGE analysis of the purified enzyme, pre-incubated with 2% SDS and 5% β-ME in boiling water for 3 min, revealed two protein bands with a molecular weight of approximately 52814 and 21150 Da. These molecular weights were estimated from comparison of the electrophoretic mobility of Se-GPx with the mobilities of marker proteins (Fig. 4B). These results indicate that the purified Se-GPx from camel erythrocytes consists of two subunits with different molecular weight, it was heterodimeric. This structure is similar to that of the purified Se-GPx from camel heart, which has a heterodimeric structure (Unpublished data), and different from that of the partially purified Se-GPx from camel liver, which has a homodimeric structure (Chafik et al., 2018).

The native molecular weight of camel erythrocytes Se-GPx is lower than that of other purified Se-GPx, reported to have tetrameric

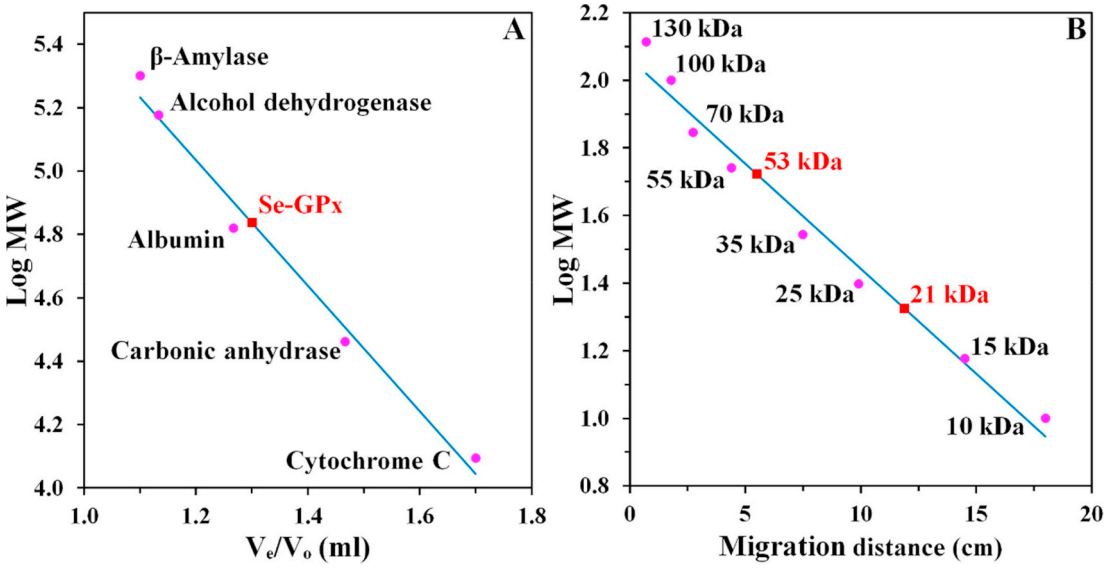

Fig. 4. Molecular weight estimation of camel erythrocytes Se-GPx. (A) To estimate the native molecular weight of Se-GPx, a Sephacryl S-200 column was calibrated with the molecular weight of standard proteins. V_e is the elution volume of each protein and V_0 is the void volume determined using blue dextran. (B) Estimation of the subunit molecular weight of Se-GPx from the standard curve for the electrophoretic mobilities of marker proteins on SDS-PAGE. The red square indicates the Se-GPx enzyme. The experimental procedures were detailed under "Materials and methods". (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structure, from erythrocytes of human (95 ± 3 kDa (Awasthi et al., 1975)), bovine (84 kDa (Flohé et al., 1973)), ovine (89 kDa (Oh et al., 1974)) and rat (90 kDa (Rotruck et al., 1973); 113 kDa (Stýblo, 1992)). Also, our molecular weight is lower than that of purified Se-GPx, reported to have tetrameric structure, from liver of rat (75 ± 6 kDa (Nakamura et al., 1974)), human (90 kDa (Miwa et al., 1983)) and hamster (92 kDa (Chaudiere and Tappel, 1983)), human plasma (86 kDa (Maddipati and Marnett, 1987); 92 kDa (Broderick et al., 1987); 100 kDa (Takahashi et al., 1987)), human placenta (85 kDa (Awasthi et al., 1979)), human milk (92 kDa (Bhattacharya et al., 1988)) and bovine lens (140 kDa (Bergad et al., 1982)). On the other hand, the molecular weight of the purified Se-GPx reported to have monomeric structure was found to be 16.1 kDa in human liver (Chambers et al., 1994), 20 kDa (Maiorino et al., 1990) and 23 kDa (Ursini et al., 1985) in pig heart. However, according to the authors knowledge, the dimeric structure of the purified Se-GPx from mammalian species has not been reported in the literature. Therefore, the heterodimeric structure of camel erythrocytes Se-GPx is first time evidence about Se-GPx in mammals.

3.2.2. Optimum temperature and pH

The study of dependence of enzyme activity versus temperature from 22 to 80 °C showed that the optimum temperature for camel erythrocytes Se-GPx is 47 °C (Fig. 5A). Maximum activity of the enzyme was recorded in the temperature range of 40–50 °C. Further increase of the temperature over 50 °C resulted in a decrease of relative activity, this is explained by the denaturation and inactivation of the enzyme. In our previous study, we have also found an optimum temperature of 47 °C for camel liver Se-GPx (Chafik et al., 2018). The optimum temperatures reported in literature of GPx purified from erythrocytes showed lower values, with 40 °C in rat (Choe and Choi, 1986) and 42 °C in bovine (Schneider and Flohé, 1967; Wendel, 1981). Also, our value is higher than purified GPx from rat liver (32 °C (Shulgin et al., 2008) and 42 °C (Yoshimura et al., 1980)). The optimum temperature value of camel erythrocytes Se-GPx is higher than that reported for mammalian species. This finding could be explained by the fact that camel is highly adapted to live in the hot and dry desert conditions using a variety of adaptation mechanisms.

The data on the temperature dependence of camel erythrocytes Se-GPx, from 22 to 47 °C, were transformed into an Arrhenius plot to determine the E_a of the enzyme (insert of Fig. 5A). The E_a of camel erythrocytes Se-GPx was found to be 41.71 kJ/mol. The reported E_a values of the purified Se-GPx from mammalian species were found to be: 34.31 kJ in human erythrocytes (Awasthi et al., 1975), 33.47 kJ in human platelets (Ramos-Martinez et al., 1980), 83.68 kJ/mol in ovine erythrocytes (Prohaska et al., 1977) and 12.55 kJ/mol in hamster liver (Chaudiere and Tappel, 1983). The E_a of rat liver GPx was found to be 29.10 kJ/mol (Shulgin et al., 2008). In comparison with these reported results, camel erythrocytes Se-GPx showed a high E_a, indicating that the reduction reaction of H_2O_2 by Se-GPx proceeds slower.

The influence of pH upon the activity of camel erythrocytes Se-GPx was investigated under various pH conditions from 6.0 to 9.6. As shown in Fig. 5B, the optimum pH of the enzyme was 7.2, while the enzyme retained more than 60% of the original activity at pH values between 7.0 and 7.6. The enzyme showed about 30% of its maximal activity in the pH range of 6.0–9.0 suggesting its wide working pH range. The optimum pH of the purified Se-GPx from camel liver was found to be 7.8 (Chafik et al., 2018). Our value for camel erythrocytes Se-GPx was lower than that of the purified Se-GPx from erythrocytes of human (pH 8.5 (Awasthi et al., 1975)), bovine (pH 8.0 (Mills, 1959); pH 8.8 (Wendel, 1981)) and rat (pH 7.5 (Choe and Choi, 1986)). Likewise, the optimum pH of camel erythrocytes Se-GPx was lower than that of the purified Se-GPx from liver of human (pH 8.5 (Miwa et al., 1983)) and hamster (pH 8.0 (Chaudiere and Tappel, 1983)), human platelets (pH 8.5 (Ramos-Martinez et al., 1980)), bovine lens (pH 7.9 (Bergad et al., 1982)) and rat lung (between pH 8.8 and 9.1 (Chiu et al., 1976)). From these results, it is revealed that the optimum pH for the catalytic reaction of camel erythrocytes Se-GPx was relatively lower compared with that of well-known mammals. This relatively lower optimum pH may play an important role in the regulation of this enzyme.

3.2.3. Effect of various chemicals and metal ions

The effect of various chemicals, at final concentration of 2 and 5 mM, on the activity of camel erythrocytes Se-GPx was examined (Table 2). The enzyme was strongly inhibited by DTT and was moderately inhibited by β-ME, whereas EDTA, NaN_3, PMSF and SDS

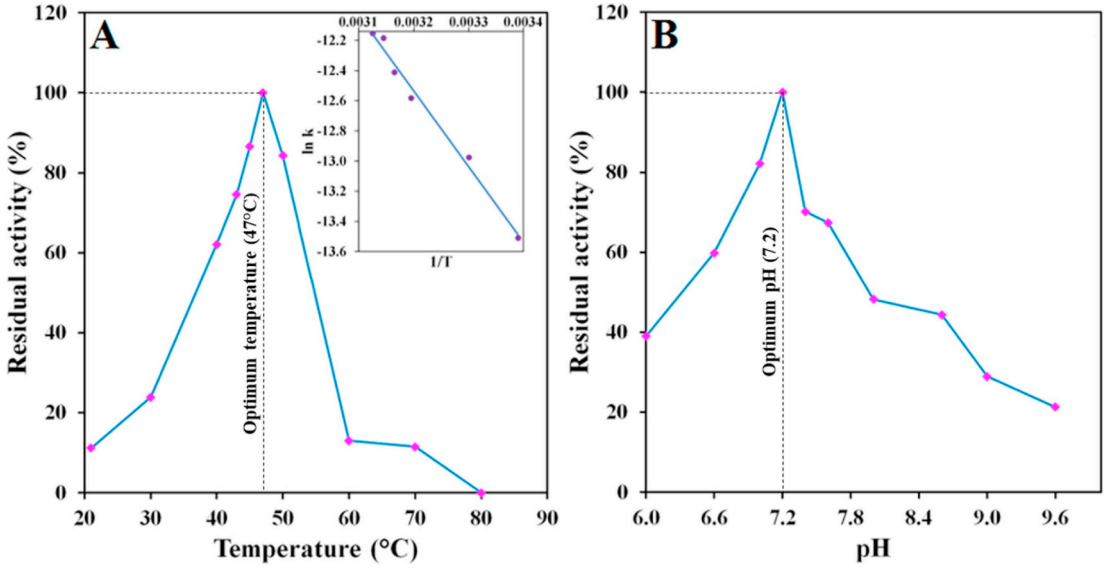

Fig. 5. Properties of camel erythrocytes Se-GPx. (A) Effect of temperature on activity of camel erythrocytes Se-GPx. The insert shows an Arrhenius plot for determination of E_a of camel erythrocytes Se-GPx. Straight line correlation coefficient $r^2 = 0.9869$. (B) Effect of pH on activity of camel erythrocytes Se-GPx. The influence of temperature and pH on Se-GPx activity was determined as described under "Materials and methods".

Table 2
Effect of various chemicals on the activity of camel erythrocytes Se-GPx.

Reagent	Final concentration (mM)	Residual activity (%)
Control	–	100
EDTA	2.0	128
	5.0	138
DTT	2.0	0
	5.0	0
β-ME	2.0	23
	5.0	31
NaN_3	2.0	131
	5.0	141
SDS	2.0	154
	5.0	140
PMSF	2.0	167
	5.0	181

enhanced the enzyme activity. Inhibition of the enzyme by reducing agents, DTT and β-ME, suggests that the −SH groups in the enzyme structure play an important role in the enzymatic activity. These findings are in agreement with the findings of other workers (Nakamura et al., 1974; Shigeoka et al., 1991). While, the enzyme activity was not significantly affected by the increasing concentration of EDTA, suggesting that the metal ions are not directly involved in enzyme activity. Although, the increase in the Se-GPx activity was observed with the increase in concentration of NaN_3 and PMSF. The observation of the effect of PMSF indicated that serine residue has no important role in the Se-GPx activity. From the effect of denaturing agent, SDS enhanced the enzyme activity, suggesting the presence of disulfide bonds which inhibit the denaturation by SDS (Pitt-Rivers and Impiombato, 1968). However, most enzymes are denatured by SDS and lose their functions when complexed with this denaturant (Weber and Kuter, 1971).

The effect of metal ions, at final concentration of 2 and 5 mM, on the activity of camel erythrocytes Se-GPx is given in Table 3. Fe^{2+} was found to be the greatest inhibitor of enzyme activity. Co^{2+}, Mg^{2+}, Ni^{2+}, and Zn^{2+} were also found to be inhibitory to the enzyme activity, at final concentration of 5 mM. Our results show that Se-GPx enzyme is sensitive to bivalent cations. However, this enzyme was activated by Al^{3+}, Ba^{2+} and Ca^{2+}. The purified Se-GPx from different sources was also inhibited by Fe^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, and Zn^{2+} (Awasthi et al., 1975; Takahashi et al., 1987; Shigeoka et al., 1991). These findings are the same as our results.

Table 3
Effect of metal ions on the activity of camel erythrocytes Se-GPx.

Metal ions	Final concentration (mM)	Residual activity (%)
Control	–	100
Al^{3+}	2.0	96
	5.0	100
Ba^{2+}	2.0	147
	5.0	160
Ca^{2+}	2.0	150
	5.0	148
Co^{2+}	2.0	184
	5.0	89
Fe^{2+}	2.0	21
	5.0	44
Mg^{2+}	2.0	154
	5.0	93
Ni^{2+}	2.0	105
	5.0	48
Zn^{2+}	2.0	166
	5.0	91

Table 4
Substrate specificity of camel erythrocytes Se-GPx.

Substrate	Residual activity (%)
Hydroperoxide	
H_2O_2	100
CHP	83
TBH	87
BP	27
Sulfhydryl	
GSH	100
β-ME	0
DTT	0
Cys	0

3.2.4. *Substrate specificity*

Substrate specificity of Se-GPx purified from camel erythrocytes was determined for several hydroperoxide and sulfhydryl substrates (Table 4). H_2O_2 was the most suitable substrate for camel erythrocytes Se-GPx. Residual activity with CHP, TBH and BP was 83%, 87%, and

27%, respectively, of that seen with H_2O_2. The enzyme efficiently reduces H_2O_2 and can also reduce organic hydroperoxides. This substrate specificity suggests that camel erythrocytes Se-GPx may not be responsible for detoxification of H_2O_2 but can also act to protect the membranes from damage due to organic hydroperoxides. On the other hand, the enzyme was very specific for GSH, and exhibited no activity with β-ME, DTT and Cys. Therefore, only GSH was able to serve as substrate for the enzyme, while, none of β-ME, DTT and Cys can be served as the substrate. Se-GPx enzyme purified from bovine lens (Bergad et al., 1982) and rat lung (Chiu et al., 1976) was highly specific for H_2O_2 and GSH comparing to other hydroperoxide and sulfhydryl substrates, respectively. These results are in agreement with the findings of our work.

3.2.5. Selenium analysis

The selenium content of the purified GPx from camel erythrocytes contained 3.52 μg per mg of protein, which corresponds to 3.06 mol of selenium per mol of protein, based on the estimated molecular weight of 68629 Da for the native enzyme. Camel erythrocytes Se-GPx consists of two subunits with different molecular weight. Accordingly, the subunits of 52814 and 21150 Da of the enzyme contain 2 and 1 mol of selenium, respectively. In camel liver, the selenium content of the purified GPx was found to be 0.053 mol of selenium per mol of protein (Chafik et al., 2018). It has been reported that the GPx activity is a good indicator of selenium status in a variety of mammalian species, including camel (Faye and Seboussi, 2009). In fact, the specific activity of the purified GPx from camel erythrocytes is 12.09 U/mg, and that of the partially purified GPx from camel liver is 1.27 U/mg (Chafik et al., 2018). Therefore, the very low amount of selenium found in camel liver GPx compared to camel erythrocytes GPx is due to the very low activity of GPx in liver. The same finding was reported in human plasma GPx (Broderick et al., 1987). In addition, a highly significant correlation between selenium concentration and GPx activity in camel erythrocytes has been demonstrated by our laboratory (Bengoumi et al., 1998) and other laboratories (Faye and Seboussi, 2009).

The selenium content of the purified GPx from mammalian tissues (Flohé et al., 1973; Nakamura et al., 1974; Oh et al., 1974; Awasthi et al., 1975, 1979; Bergad et al., 1982; Miwa et al., 1983; Broderick et al., 1987; Maddipati and Marnett, 1987; Takahashi et al., 1987; Bhattacharya et al., 1988) and other sources (Bell et al., 1984; Shigeoka et al., 1991; Thompson et al., 2006) falls in the range from 3.5 to 4 mol of selenium per mol of protein. According to these reported results, camel erythrocytes GPx described in this work is selenoenzyme containing low amount of selenium. Moreover, the stoichiometric relationship of selenium to GPx purified from camel erythrocytes differs from GPx purified from mammalian species. Therefore, the camel erythrocytes Se-GPx is distinct from previously described Se-GPxs.

It has been reported that Se-GPx catalyzes the reduction of both H_2O_2 and organic hydroperoxides, but that non-Se-GPx catalyzes the reduction of organic hydroperoxides and has no effect on H_2O_2 (Lawrence and Burk, 1976). As shown in Table 4, camel erythrocytes Se-GPx catalyzes the breakdown of both H_2O_2 and organic hydroperoxides. In addition, the non-Se-GPx was not inhibited by high concentration of KCN (33.3 mM) (Awasthi et al., 1980). Although, several works reported that Se-GPx was inhibited by KCN at high concentrations in mammalian species (33 mM (Awasthi et al., 1979) or 10 mM (Kraus and Ganther, 1980; Bergad et al., 1982)) and microorganisms (10 mM (Singh and Rathaur, 2005)), which resulted in the release of selenium from the enzyme. In the present study, treatment of the camel erythrocytes Se-GPx by KCN, at a final concentration of 2 and 5 mM, resulted in the loss of enzyme activity, which confirm the presence of selenium in the active site of the enzyme (Fig. 6). However, KCl experiments did not exhibit significant loss of enzyme activity (Fig. 6). Another difference between the Se-GPx and non-Se-GPx is the effect of Cd^{2+} on the enzyme activity. Cd^{2+} treatment resulted in an increase in the activity of non-Se-GPx and a reduction in the activity of Se-GPx

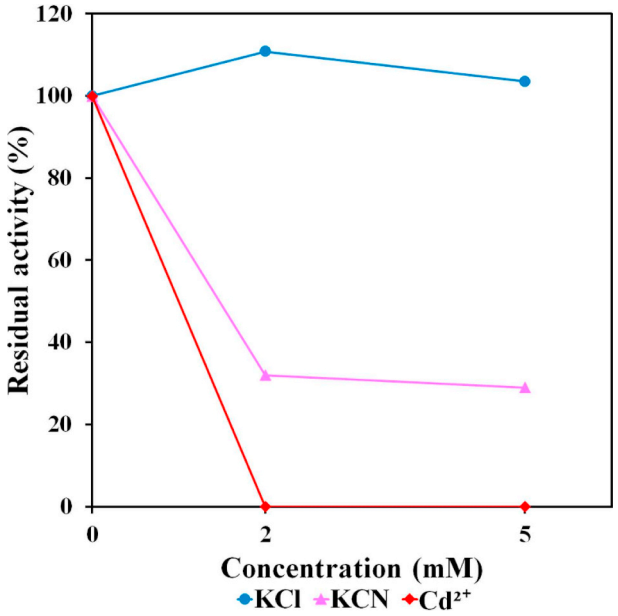

Fig. 6. Effect of KCN and Cd^{2+} on activity of camel erythrocytes Se-GPx. The results of Se-GPx activity were given as percentage of control (100%) without any compound. The experimental procedures were detailed under "Materials and methods".

(Meyer et al., 1982; Jamall and Smith, 1985). As indicated by kinetic data, Cd^{2+} inhibition of Se-GPx was competitive with respect to the donor substrate, GSH (Splittgerber and Tappel, 1979). As shown in Fig. 6, the effect of Cd^{2+}, at final concentration of 2 and 5 mM, on camel erythrocytes Se-GPx resulted in the total inhibition of enzyme activity.

3.2.6. Kinetic parameters

As shown in Fig. 7A, a hyperbolic curve was obtained with H_2O_2, and the rate of reaction strongly increased in a manner dependent on the H_2O_2 concentration. For GSH, the curve obtained was sigmoidal (Fig. 7B). At concentrations of GSH lower than 1 mM, a slower acceleration of H_2O_2 destruction was obtained, but as the concentration of GSH increases the acceleration of H_2O_2 destruction increases. These results indicate that the reaction is first order with respect to GSH concentration and zero order with respect to H_2O_2 concentration. It has been reported that the dependence of the rate of the reaction catalyzed by GPx on the concentration of H_2O_2 obeyed Michaelian kinetics and on GSH obeyed non-Michaelian kinetics (Flohé et al., 1972). These findings are the same as our report.

The purified camel erythrocytes Se-GPx was assayed to determine its K_m and V_{max} values for H_2O_2 and GSH. The kinetic parameters of enzyme derived from the non-linear regression, using GraphPad Prism software version 7, are summarized in Table 5. The K_m and V_{max} values of the enzyme for H_2O_2 and GSH were calculated to be 0.72 and 1.58 mM, and 25.33 and 31.03 U/mg, respectively. The K_m value for H_2O_2 is lower than that for GSH, suggesting that camel erythrocytes Se-GPx exhibits more affinity for H_2O_2 than for GSH. Moreover, as indicated by the V_{max}/K_m ratio, camel erythrocytes Se-GPx displayed a higher catalytic efficiency towards H_2O_2 and a weaker catalytic efficiency towards GSH, indicating that this enzyme is an efficient scavenger of H_2O_2 and plays a major role in protecting cells against oxidative damage caused by H_2O_2.

The K_m exhibited by camel erythrocytes Se-GPx for H_2O_2, when keeping GSH concentration constant at 2 mM, is higher than that reported for Se-GPx from other mammalian organisms. The reported K_m values for H_2O_2, when keeping GSH concentration constant, were found to be: 0.045 mM with 10 mM GSH concentration in bovine lens (Bergad

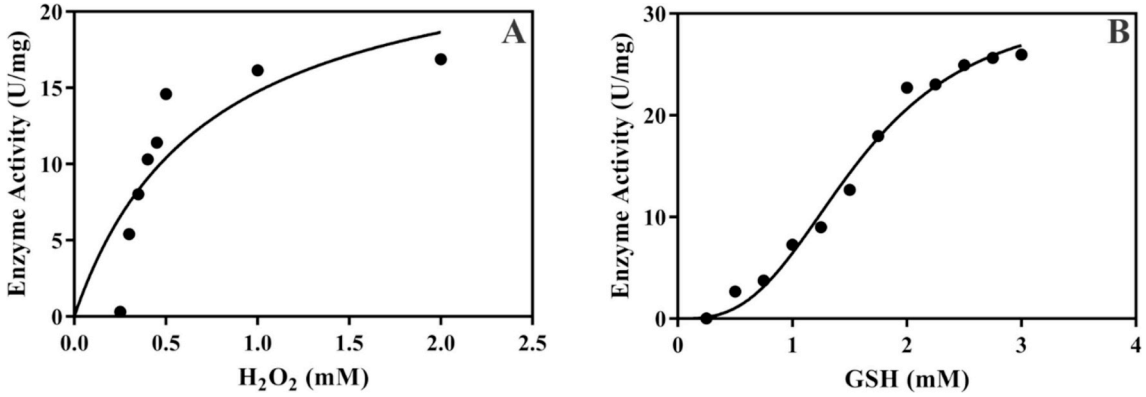

Fig. 7. Kinetic analysis of camel erythrocytes Se-GPx. Nonlinear regression plots to determine K_m and V_{max} of Se-GPx for H_2O_2 (A) and GSH (B). The experimental procedures were detailed under "Materials and methods". The kinetic parameters were calculated using GraphPad Prism software version 7.

Table 5
Kinetic parameters for camel erythrocytes Se-GPx.

Substrate	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m
H_2O_2	0.72 ± 0.45	25.33 ± 7.82	35.18
GSH	1.58 ± 0.13	31.03 ± 2.78	19.64

et al., 1982), 0.0833 mM with 4 mM GSH concentration in human liver (Miwa et al., 1983) and 0.2 mM with 10 mM GSH concentration in bull seminal plasma (Kantola et al., 1988). Also, our value is higher than that found in fish liver (0.012 and 0.010 mM) (Bell et al., 1984; Thompson et al., 2006) and plant (0.24 mM) (Shigeoka et al., 1991).

The kinetic properties of camel erythrocytes Se-GPx have shown that this enzyme displays a hyperbolic dependence on H_2O_2 concentration, a higher catalytic efficiency for H_2O_2 compared to GSH and a higher affinity for H_2O_2 at low GSH concentration. These findings probably mean that under the high environmental stress in the desert, camel erythrocytes Se-GPx plays an important role in the protection of cells from oxidative damage due to the accumulation of H_2O_2, and therefore, participates in the adaptation of camel to its specific ecosystem.

3.3. Properties of camel erythrocytes Se-GPx

The purified Se-GPx enzyme from camel erythrocytes presents different biochemical properties compared to those reported from other mammalian species. Differences such as lower molecular weight, heterodimeric structure, higher optimum temperature and E_a, relatively lower optimum pH, lower content of selenium, and higher catalytic efficiency and affinity for H_2O_2 at low GSH concentration. All these could be explained by the fact that camel has adapted to life in the specific ecosystem of the desert. In the same way, we have previously found that the properties of Se-GPx (Chafik et al., 2018) and catalase (Chafik et al., 2017) purified from camel liver were different comparing to those of mammalian species. Moreover, the unique molecular and biochemical properties were reported for glutathione transferase (Malik et al., 2016; Perperopoulou et al., 2016), arginase (Maharem et al., 2018) and ceramidase (Chathoth et al., 2013) from camel.

The unique properties of Se-GPx from camel suggest that this enzyme could bring promising prospects in several applications. Exposure to ultraviolet radiation is a major risk factor that causes skin cancers (Godic et al., 2014). Camel Se-GPx showed a higher optimum temperature. Therefore, this enzyme has a wide range of applications in dermatology and cosmetics, and can be further implicated in the development and prevention of many diseases, including cancer.

4. Conclusion

We purified, biochemically characterized and studied some properties of a Se-GPx enzyme from camel erythrocytes and showed that it was different from Se-GPx enzymes purified from other mammalian species. Due to its unique biochemical properties, camel erythrocytes Se-GPx provides novel findings in the cellular defense against H_2O_2-induced oxidative stress and in the understanding of some biochemical mechanisms involved in the resistance of camel to the stressful living conditions in the desert. However, the stress response mechanisms in camel had not been entirely characterized. Future studies are needed to deepen our understanding on the adaptation mechanisms of camel to its specific ecosystem by studying some of the enzymes implicated in the metabolism of ROS and oxidative stress.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

Abdelbasset Chafik and Safinur Yildirim Çelik purified enzymes and characterized their properties, Ahmet Mavi and Kübra Solak helped with interpretation of data, Ahmet Mavi and Abdelkhalid Essamadi conceived and supervised project, and wrote paper with contributions from all authors.

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