Bioorganic Chemistry 86 (2019) 428–436

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Purification and biochemical characterization of a novel copper, zinc superoxide dismutase from liver of camel *(Camelus dromedarius)*: An antioxidant enzyme with unique properties

Abdelbasset Chafik[a,*], Abdelkhalid Essamadi[a], Safinur Yildirim Çelik[b], Ahmet Mavi[c]

[a] *Laboratory of Biochemistry and Neuroscience, Team of Applied Biochemistry and Toxicology, Faculty of Science and Technology, University Hassan First, 577 Settat, Morocco*

[b] *College of Education, Bayburt University, 69000 Bayburt, Turkey*

[c] *Chemistry Education, Kazim Karabekir Education Faculty, Atatürk University, 25240 Erzurum, Turkey*

ARTICLE INFO

Keywords:
Copper, zinc superoxide dismutase
Camel
Camelus dromedarius
Purification
Biochemical characterization
Unique properties
Stressful desert conditions

ABSTRACT

A novel copper, zinc superoxide dismutase (CuZnSOD) was purified to homogeneity from the liver of an animal well adapted to the stressful living conditions of the desert, the camel *(Camelus dromedarius)*. The biochemical properties of camel liver CuZnSOD were examined. The purified enzyme had a native molecular weight of 28 kDa, as judged by gel filtration chromatography, and showed a single band at 27 kDa on SDS-PAGE, indicating that it is a monomeric protein. Optimal activity of the purified enzyme occurred at 43 °C and pH 6.0, and the activation energy was 1.42 kJ/mol. CuZnSOD activity was strongly inhibited by β-ME, DTT, H_2O_2 and SDS and slightly inhibited by EDTA, NaN_3 and PMSF. Al^{3+}, Ca^{2+}, Cd^{2+}, Mg^{2+} and Zn^{2+} stimulated CuZnSOD activity, whereas Ba^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+} inhibited it. The purified enzyme contained 0.010 µg of Cu and 0.69 µg of Zn per mg of protein. K_m, V_{max}, k_{cat} and k_{cat}/K_m values for NBT and riboflavin were 16.27 and 0.16 µM, 20.85 and 21.54 U/mg, 9.65 and 9.97 s^{-1}, and 0.59 and 62.33 s^{-1} μM^{-1}, respectively. Camel liver CuZnSOD exhibited unique biochemical properties compared to those of other CuZnSODs, including lower molecular weight with a monomeric structure, higher optimum temperature, very low E_a, very low optimum pH, very low contents of Cu and Zn, and higher affinity, turnover number and catalytic efficiency for riboflavin. These unique properties of camel liver CuZnSOD might be related to the ability of this animal to inhabit stressful desert conditions.

1. Introduction

In living organisms, reactive oxygen species (ROS) are generated as byproducts of normal metabolic processes. Increased production of ROS results in oxidative stress if antioxidant defense systems are unable to eliminate these species. Generally, cells are equipped with both enzymatic and nonenzymatic antioxidant defense mechanisms to protect against the deleterious effects of ROS, which can damage DNA, proteins and lipids. The antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) play a major role in protecting cells from oxidative damage from accumulating ROS [1,2].

SOD (EC 1.15.1.1) constitutes the first line of defense against oxidative cell damage by catalyzing the dismutation of superoxide anion radicals ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). SOD enzyme reportedly exists in multiple organisms, both prokaryotes and eukaryotes, and differs in many respects between these lifeforms, including with respect to its amino acid sequence, structure, subcellular localization and metallic cofactors. Depending on its metal cofactor, SOD is classified into one of four groups: copper, zinc SOD (CuZnSOD), manganese SOD (MnSOD), iron SOD (FeSOD) and nickel SOD (NiSOD) [3]. Three types of SOD isoenzymes have been identified in mammals and can be distinguished by their sensitivity to cyanide and H_2O_2 [3]: (i) cytoplasmic CuZnSOD is sensitive to cyanide and H_2O_2, (ii) mitochondrial MnSOD is insensitive to cyanide and H_2O_2 but is inhibited by chloroform-ethanol [4], (iii) and extracellular CuZnSOD is extremely sensitive to cyanide and H_2O_2. CuZnSOD has been purified and characterized from diverse mammalian species, including humans [5,6], bovines [7–9], horses [10], pigs [11,12], monkeys [13], rats [14–16] and mice [17]. However, there has never been a thorough investigation to purify and characterize the CuZnSOD from camels *(Camelus dromedarius)*.

Camels are the most useful animals to humans in desert areas. They

* Corresponding author.
E-mail address: a.basset.chafik@live.fr (A. Chafik).

https://doi.org/10.1016/j.bioorg.2019.02.024
Received 16 December 2018; Received in revised form 8 February 2019; Accepted 9 February 2019
Available online 11 February 2019

are used for production, leisure, transport and agricultural work [18]. In addition, camel products, such as milk and urine, have been used to treat cancer, diabetes, ulcers, allergies, autism and infections [19]. Camels differ from other mammals in some key biochemical, anatomical and physiological ways due to their adaptation to desert life [18]. Camels are able to survive in the stressful desert environment, enduring challenges such as direct exposure to intense heat and dryness [20]. The continuous exposure of camels to these environmental conditions perturbs the balance between the production of ROS and antioxidant defenses, leading to oxidative stress [2,21]. Consequently, overproduction of ROS causes damage to biomolecules, such as DNA, proteins and lipids [1]. In the specific biochemistry of camels, CuZnSOD may play an important role in protecting cells from oxidative damage due to the accumulation of $O_2^{\cdot -}$.

We recently purified, biochemically characterized and studied the properties of CAT [22] and GPx [23] enzymes from camel liver. Both studies revealed the interesting and unique properties of these enzymes compared with those reported in the literature, which contributed to a better understanding of the biochemical mechanisms involved in the resistance of camels to their specific ecosystem. The aim of the present study was to purify, biochemically characterize and study the properties of CuZnSOD from camel liver. CuZnSOD properties were compared to those of other CuZnSODs. Understanding the properties of camel CuZnSOD will contribute to increasing our understanding of the biochemical mechanisms involved in the adaptation of camels to stressful desert conditions, particularly in light of current climatic changes and increasing temperatures [24].

2. Materials and methods

2.1. Chemicals

DEAE-Sepharose, Sephacryl S-200, gel filtration markers kit for protein molecular weights 12–200 kDa, 6-hydroxydopamine (6-OHDA), nitroblue tetrazolium (NBT), riboflavin, D,L-dithiothreitol (DTT) and phenylmethylsulfonylfluoride (PMSF) 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. SOD activity assays and protein determination

2.2.1. SOD spectrophotometric assays

SOD activity was measured using two types of assay. The first was used throughout all purification steps and during characterization analysis of the purified enzyme, including for determination of optimum pH and assessing the effect of metal ions and inhibitors on enzyme activity. The second assay was used in additional characterization analyses of the purified enzyme, including determination of optimum temperature and kinetic parameters.

Assay 1: SOD activity was measured using the method of Crosti et al. [25]. This method is based on SOD's inhibitory effect on the spontaneous autoxidation of 6-OHDA. A stock 6-OHDA solution (10 mM) was prepared daily in 10 mM HCl solution containing KCl at a final concentration of 1 mM. The enzyme sample (50 µl) was mixed in a cuvette with 680 µl of 50 mM potassium phosphate buffer (pH 7.4) and immediately used to determine SOD activity. The enzymatic reaction was initiated by addition of 30 µl 6-OHDA (10 mM), and the absorbance change at 490 nm was immediately recorded for 30 sec at room temperature. A nonenzymatic reaction containing just the substrate was also run as a control, wherein potassium phosphate buffer was added instead of the enzyme solution. One unit of SOD activity is defined as the amount of SOD required to inhibit the rate of 6-OHDA autoxidation by 50%. Percent inhibition was calculated using the following formula: Percent inhibition = $[(A_{490}$ Control $- A_{490}$ Sample$)/A_{490}$ Control$] \times 100$. All experiments were performed in triplicate.

Assay 2: SOD activity was measured by inhibition of the photochemical reduction of NBT as described by Beauchamp and Fridovich [26]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 4.3 mM N,N,N′,N′-tetramethylethylenediamine (TEMED), 112 µM NBT, 60 µM riboflavin and 50 µl enzyme solution in a total volume of 600 µl. The enzymatic reaction was initiated by illuminating the reaction mixture with a fluorescent light source for 6 min at room temperature, and absorbance was recorded at 560 nm. A nonenzymatic reaction containing only the substrates was also run as a control in which potassium phosphate buffer was added instead of the enzyme solution. One unit of SOD activity is defined as the amount of SOD required for 50% inhibition of the rate of NBT reduction in one minute at room temperature. Percent inhibition was calculated using the following formula: Percent inhibition = $[(A_{560}$ Control $- A_{560}$ Sample$)/A_{560}$ Control$] \times 100$. All experiments were performed in triplicate.

2.2.2. SOD zymography assay

SOD activity was also investigated using zymography as described by Beauchamp and Fridovich [26]. Native gel electrophoresis was performed using 7.5% polyacrylamide gel electrophoresis (PAGE) according to Davis [27]. After electrophoresis, the gel was incubated in 50 mM Tris-HCl buffer (pH 8.0) containing 10 mg NBT, 1 mg ethylenediaminetetraacetate (EDTA) and 2 mg riboflavin for 15 min in the dark at room temperature. The gel was then exposed to a fluorescent light source until it became uniformly violet except in regions containing the SOD activity, which remained colorless. To identify the nature of the specific metal ion that combines with camel liver SOD, staining for SOD activity on PAGE was also conducted in the presence of 5 mM H_2O_2 in the incubation solution. In mammalian species, CuZnSOD activity is sensitive to H_2O_2, while MnSOD is insensitive to H_2O_2 [3].

2.2.3. Protein determination

Protein concentrations were measured using the Bradford assay [28] using bovine serum albumin as a standard.

2.3. Purification of CuZnSOD from camel liver

Purification of CuZnSOD from camel liver was performed using a modification of the procedure reported for purification of CuZnSOD from bovine heart [8]. Unless otherwise stated, all purification steps were performed at temperatures between 0 and 4 °C.

2.3.1. Homogenate

Fresh camel liver was obtained from the municipal slaughterhouse of Casablanca–Morocco. Liver was homogenized in 2 volumes of potassium phosphate buffer (50 mM, pH 7.8) using an Ultra–Turrax homogenizer. The mixture was then centrifuged at 3500*g* for 20 min, and the supernatant was recovered.

2.3.2. Ethanol–chloroform treatment

Hemoglobin was precipitated from the homogenate by ethanol–chloroform treatment according to Tsuchihashi [29]. With stirring, 0.25 volumes of ethanol and 0.15 volumes of chloroform, precooled in a freezer (−24 °C), were rapidly added to the lysate. Stirring was continued for 15 min, during which time the hemoglobin was rendered insoluble. The mixture was then centrifuged at 3500*g* for 60 min, and the supernatant was recovered.

2.3.3. Salting out effect

The supernatant was warmed to room temperature, and solid dibasic potassium phosphate (K_2HPO_4) (300 g/l) was slowly added under continuous stirring, resulting in separation of the two phases, an ethanol–water upper phase containing little salt and a denser aqueous phase containing most of the salt. The upper phase was collected and

centrifuged at 4000*g* for 15 min, and the resultant supernatant was cooled to 4 °C.

2.3.4. Acetone precipitation

0.75 volumes of cold acetone were added to the recovered supernatant while stirring to precipitate the proteins. After 15 min, the mixture was centrifuged at 4000*g* for 10 min. Thereafter, 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 eight hours.

2.3.5. DEAE-Sepharose ion exchange chromatography

The precipitate that formed during dialysis was removed by centrifugation, and the supernatant was adsorbed onto a column of DEAE-Sepharose (2.5 × 10 cm) which had been equilibrated with the same buffer as that used for dialysis. Adsorbed proteins were eluted at room temperature with a linear gradient of 0–0.5 M KCl prepared in equilibration buffer at a flow rate of 60 ml/h. Fractions of 2 ml were collected, and fractions containing CuZnSOD activity were pooled and subjected to gel filtration chromatography.

2.3.6. Sephacryl S-200 gel filtration chromatography

Pooled active fractions from the previous step were applied to a Sephacryl S-200 gel filtration column (1.75 × 37 cm) that had been equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.1 M KCl. Proteins were eluted at room temperature with the abovementioned buffer at a flow rate of 0.5 ml/min, and CuZnSOD activity was determined in the collected 1 ml fractions. Fractions containing CuZnSOD activity were pooled and stored at −24 °C until use.

2.3.7. Homogeneity of purified CuZnSOD

Homogeneity of the purified CuZnSOD was confirmed by electrophoretic analysis using a vertical slab gel apparatus. Native PAGE was performed on 7.5% polyacrylamide gels as described by Davis [27]. Sodium dodecyl sulfate-PAGE (SDS-PAGE) was performed with a 4% stacking gel and a 12% separation gel as described by Laemmli [30]. Proteins were stained with Coomassie brilliant blue G-250.

2.4. Characterization of purified CuZnSOD from camel liver

2.4.1. Determination of molecular weight

The native molecular weight of camel liver CuZnSOD was determined by gel filtration chromatography on a Sephacryl S-200 column pre-equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.1 M KCl. The purified enzyme was applied to the column and chromatographed at a flow rate of 0.5 ml/min. The column was calibrated with the following standard proteins: cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β-Amylase (200 kDa). Molecular weight determination was performed using a plot of V_e/V_o versus log molecular weight of standard proteins, where V_e is the elution volume for each protein and V_o is the void volume determined by blue dextran (2000 kDa).

SDS-PAGE was performed according to the method of Laemmli [30]. Camel liver CuZnSOD 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 into SDS-PAGE gel wells. Molecular weight marker proteins used for SDS-PAGE were commercial grade with molecular weights ranging from 10 to 250 kDa. Protein bands in gels were visualized by staining with Coomassie brilliant blue G-250.

2.4.2. Effect of temperature and pH on CuZnSOD activity

The optimum temperature for camel liver CuZnSOD activity was determined at a range of temperatures from 20 to 80 °C. After 10 min incubation, samples were immediately cooled in ice water and assayed for enzyme activity as described in assay procedure 2. From the temperature profile of enzyme activity, the activation energy (E_a) of camel liver CuZnSOD was determined using an Arrhenius plot by plotting the logarithm of the rate constant, k, versus the inverse temperature, 1/T.

The optimum pH of camel liver CuZnSOD was determined by assaying enzyme activity at different pH values using two different buffer systems: 50 mM potassium phosphate buffer (pH 5.6–7.6) and 50 mM Tris-HCl buffer (pH 8.0–8.6). Enzyme activity was measured using the assay procedure 1.

2.4.3. Effect of various inhibitors and metal ions on CuZnSOD activity

The effect of various inhibitors on the activity of camel liver CuZnSOD was investigated. The inhibitors β-ME, EDTA, DTT, H_2O_2, sodium azide (NaN_3), PMSF and SDS were all tested at a final working concentration of 2 and 5 mM. Similarly, various metal ions (chlorides of Al^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Ni^{2+} and Zn^{2+}) were also used at a final working concentration of 2 and 5 mM to test their effect on camel liver CuZnSOD activity. The activity of the enzyme, in the presence of each concentration of inhibitor or metal ion, was measured as described in assay procedure 1. As a control, enzyme activity was also measured without an inhibitor or a metal ion.

2.4.4. Determination of metal content

Copper and zinc contents of the purified enzyme was determined by quadrupole Inductively Coupled Plasma Mass Spectrometry (Agilent 7800 Quadrupole ICP-MS, Agilent Technologies, USA) using standard solutions for the corresponding metals.

2.4.5. Determination of kinetic parameters

Kinetic parameters of camel liver CuZnSOD for NBT and riboflavin were determined. Experiments for NBT were performed using varying concentrations from 5 to 100 μM while keeping riboflavin concentration constant at 60 μM. In the same manner, experiments for riboflavin were performed using varying concentrations from 0.5 to 10 μM while keeping NBT concentration constant at 112 μM. K_m and V_{max} were determined by nonlinear regression using GraphPad Prism software version 7. Subsequently, turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) of the enzyme were also calculated. Enzymatic activity was measured as described in assay procedure 2.

3. Results and discussion

3.1. Purification to homogeneity of camel liver CuZnSOD

Herein, CuZnSOD was purified from camel liver for the first time. A purification procedure using ethanol-chloroform and K_2HPO_4 treatments, acetone precipitation, ion exchange chromatography on DEAE-Sepharose column and gel filtration chromatography on Sephacryl S-200 column was applied. Results of this purification procedure are summarized in Table 1. Ethanol-chloroform treatment is beneficial because it denatures and precipitates hemoglobin and other unwanted proteins. In addition, this treatment eliminates mitochondrial SOD (MnSOD) and facilitates isolation of cytoplasmic SOD (CuZnSOD) [4]. K_2HPO_4 treatment resulted in the salting out of an ethanol-water upper phase containing little salt from a denser aqueous phase containing most of the salt. CuZnSOD partitioned preferentially into the ethanol-water upper phase due to the salting out effect produced by salts in the denser aqueous phase. A similar partition behavior was reported for CuZnSOD from different sources [4,8,11,14]. K_2HPO_4 treatment resulted in a 3.40-fold purification with a 55.19% yield of enzyme. A small increase in purification fold of the enzyme (3.63-fold) was obtained after acetone precipitation. This step serves to partially purify, as well as concentrate, the sample for application onto chromatography columns. The most successful step in the purification procedure was ion exchange chromatography on DEAE-Sepharose column, which eliminated most extraneous proteins. As shown in Fig. 1A, chromatography on the DEAE-Sepharose column yielded a single peak of protein and enzyme activity. In this step, CuZnSOD was purified up to 6.10-fold

Table 1
Summary of purification of CuZnSOD from camel liver.

Step	Total proteins	CuZnSOD activity		Specific activity	Purification	Yield
	mg/ml	U	U/ml	U/mg	-fold	%
Homogenate[a]	673.45	n.d.	n.d.	n.d.	n.d.	n.d.
Ethanol-chloroform treatment	138.23	10880.43	111.02	0.80	1.00	100.00
Salting out effect	95.73	6004.64	261.07	2.73	3.40	55.19
Acetone precipitation	84.11	3680.12	245.34	2.92	3.63	33.82
DEAE-Sepharose column	6.14	1443.41	30.07	4.90	6.10	13.27
Sephacryl S-200 column	1.57	139.86	12.95	8.25	10.27	1.29

[a] The enzymatic activity could not be determined in the homogenate. It was determined in the ethanol-chloroform treatment step after the removal of hemoglobin.
n.d., not determined.

with a 13.27% yield of enzyme. Active fractions from the DEAE-Sepharose column were pooled and loaded for additional purification using a Sephacryl S-200 column to obtain final purification. Camel liver CuZnSOD was eluted from a Sephacryl S-200 column as a single peak clearly separated from minor contaminating proteins (Fig. 1B). The purification fold of the enzyme additionally increased at the gel filtration stage through the Sephacryl S-200 column. The specific activity of the final preparation was 8.25 U/mg protein, which represents 10.27-fold purification with a 1.29% yield.

CuZnSOD from camel liver was purified to homogeneity. The final enzyme preparation was subjected to electrophoretic analysis. In SDS-PAGE, the final enzyme preparation produced a single protein band when the gel was stained with Coomassie brilliant blue G-250 (Fig. 2A, lane 6). A single protein band was also obtained when the final enzyme preparation was subjected to PAGE, both on protein and SOD activity staining (Fig. 2B and C, respectively). Since ethanol-chloroform treatment eliminates MnSOD [4], PAGE was also employed to evaluate SOD activity. However, a single protein band disappeared in the presence of 5 mM H_2O_2 (data not shown), a specific inhibitor of CuZnSODs [3]. This result indicates that the purified SOD from camel liver is a CuZnSOD.

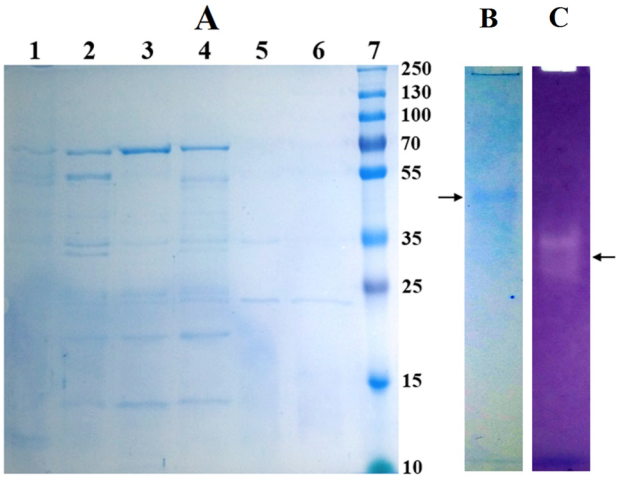

Fig. 2. Electrophoretograms showing homogeneity of the purified CuZnSOD from camel liver. (A) SDS-PAGE analysis for each purification step of CuZnSOD. Lane 1, homogenate; lane 2, ethanol-chloroform treatment; lane 3, salting out effect; lane 4, acetone precipitation; lane 5, fractions from DEAE-Sepharose ion exchange chromatography; lane 6, fractions from Sephacryl S-200 gel filtration chromatography; lane 7, molecular weight marker. (B and C) PAGE analyses of the purified camel liver CuZnSOD. The gel was stained for (B) proteins with Coomassie brilliant blue and (C) CuZnSOD activity. PAGE analyses (B and C) were performed in different electrophoresis analyses. The arrow indicates CuZnSOD. The experimental procedures were detailed under "Materials and methods".

3.2. Characterization and properties of purified CuZnSOD from camel liver

3.2.1. Molecular weight of camel liver CuZnSOD

The native molecular weight of camel liver CuZnSOD was found to be 28 kDa, determined by Sephacryl S-200 gel filtration chromatography using an elution calibration curve of standard proteins (Fig. 3A). In addition, SDS-PAGE analysis of the purified CuZnSOD preparation, preincubated with 2% SDS and 5% β-ME in boiling water for 3 min,

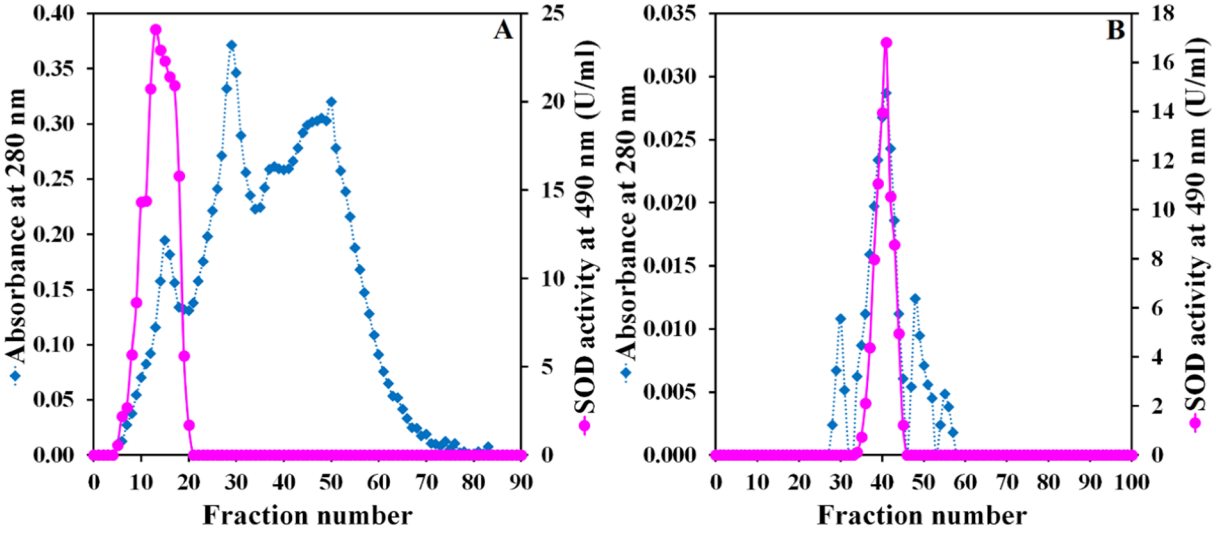

Fig. 1. Chromatogram of camel liver CuZnSOD elution from (A) DEAE-Sepharose ion exchange chromatography and (B) Sephacryl S-200 gel filtration chromatography. The experimental procedures were detailed under "Materials and methods".

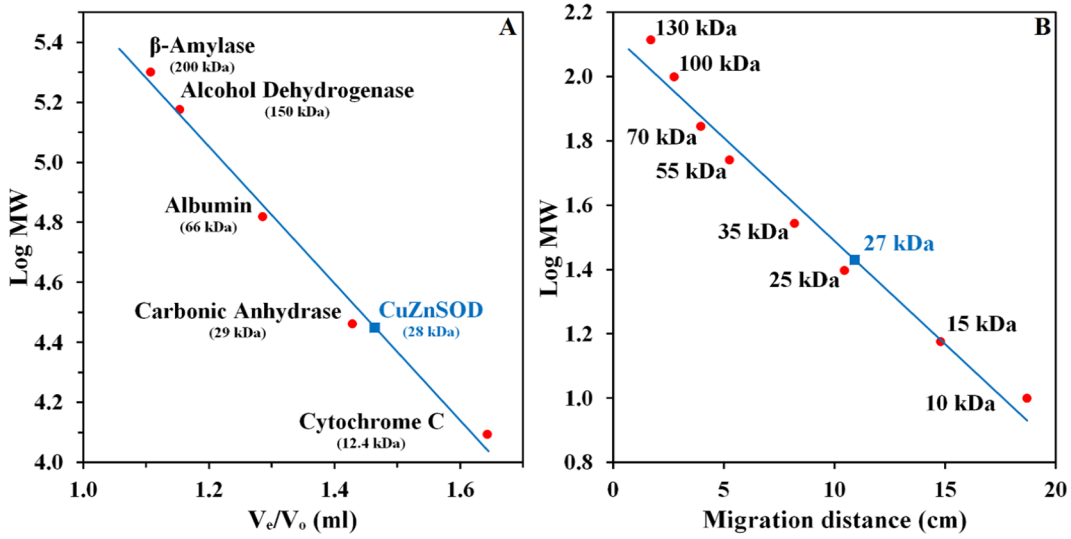

Fig. 3. Molecular weight determination of purified CuZnSOD from camel liver. Calibration curve for determination of CuZnSOD molecular weight by (A) gel filtration chromatography on Sephacryl S-200 column and (B) SDS-PAGE. The square indicates CuZnSOD. The experimental procedures were detailed under "Materials and methods".

revealed a single protein band with a molecular weight of approximately 27 kDa (Fig. 2A, lane 6). This molecular weight was estimated from comparison of the electrophoretic mobility of CuZnSOD with the mobilities of marker proteins (Fig. 3B). These results indicate that purified CuZnSOD from camel liver has a monomeric structure in its native form.

Before the discovery of the enzymatic activity of SOD, this enzyme was known as a copper-containing protein. It has been purified from the erythrocytes of human [31] and bovine [32] and called erythrocuprein, from the liver of human [33] and horse [34] and called hepatocuprein, and from the brain of human [35] and bovine [36] and called cerebrocuprein. The native molecular weight of erythrocupreins, hepatocupreins and cerebrocupreins is in the range of 30–40 kDa. After its enzymatic activity was discovered, a native molecular weight ranging from 31 to 39 kDa was also identified for purified CuZnSOD, reported to have a dimeric structure, from placenta [5] and erythrocytes [6] of human; erythrocytes [7] and heart [8] of bovine; horse liver [10]; pig liver [11]; rat liver [16]; and mouse liver [17]. In addition, the native molecular weight of purified CuZnSOD, reported to have a dimeric structure, from other animals [37,38], plants [39–42], insects [43] and microorganisms [44,45] was found to be in the range of 30–39 kDa. In contrast, very few studies have found that CuZnSOD exists in a monomeric form, e.g., purified CuZnSOD from bovine erythrocytes (16 kDa [46]), insects (40 and 67 kDa [47]), plants (15.7 kDa [48] and 23 kDa [49]) and microorganisms (17 kDa [50]).

Compared to the literature cited above, the native molecular weight of CuZnSOD purified from camel liver is lower than that of other CuZnSODs purified from different sources. Moreover, camel liver CuZnSOD is a monomeric protein, which is very rare among members of the CuZnSOD family in mammalian species.

3.2.2. Effect of temperature and pH on CuZnSOD activity

The effect of temperature on the activity of camel liver CuZnSOD is shown in Fig. 4A. The optimum temperature for enzyme activity was found to be 43 °C. The enzyme was active over the full temperature range tested, where more than 80% of its activity was retained between 20 and 50 °C. However, a further increase in temperature above 50 °C resulted in decreased enzyme activity. The optimum temperature of camel liver CuZnSOD was higher than that of purified CuZnSOD from human erythrocytes (15 °C [51]), pig spermatozoa (20 °C [12]), chicken heart (35 °C [37]), fish (40 °C [52]) and bacteria (37 °C [53]) but lower than that reported for plants (50 °C [54,55]). Similarly, in our previous studies, we found that the optimum temperatures of purified CAT (47 °C [22]) and GPx (47 °C [23]) from camel liver were higher compared to those reported in the literature. The higher optimum temperature of camel liver CuZnSOD might be attributed to the camel's adaptation to hot and dry environments of desert life, where it is continually exposed to high temperature and direct solar radiation. Therefore, this enzyme might play an important role in protecting cells against oxidative stress caused by high environmental temperatures.

Data from the 20–43 °C temperature range in camel liver CuZnSOD activity were transformed into an Arrhenius plot to calculate E_a of the enzyme (insert of Fig. 4A), which was 1.42 kJ/mol. This value is very low compared with that obtained for purified CuZnSOD from human erythrocytes (16.86 kJ/mol [51]) and plants (14.9 kJ/mol [56], 16.0 kcal/mol [42], 20.62 kJ/mol [57], 143.5 kJ/mol [49] and 224.0 kJ/mol [40]). This low value indicates that the enzymatic dismutation of $O_2^{\cdot -}$ by camel liver CuZnSOD proceeds faster, preventing the accumulation of $O_2^{\cdot -}$ generated by stressful living conditions in the desert, thus protecting cells from oxidative damage caused by this free radical.

Camel liver CuZnSOD activity was measured in the pH range of 5.6–8.6. As shown in Fig. 4B, the enzyme was optimally active at pH 6.0 and retained greater than 60% of its activity between pH 5.6 and 7.0. However, enzyme activity was less than 50% above pH 7.0 and was completely lost at pH 8.6. From the pH profile, camel liver CuZnSOD is more active in acidic than in alkaline environments. In contrast, optimum pH values for purified CuZnSOD from different sources has been reported to vary from neutral to alkaline pH. In mammals, optimum pH of purified CuZnSOD was shown to be 7.8–8.2 in rat liver [15] and 10.0 in pig spermatozoa [12]. Additionally, purified CuZnSOD from non-mammals [37,52], insects [47], plants [40,41,54,55] and microorganisms [44,53] exhibits an optimum pH in the range of 7.0–11.0, 7.6–8.0, 7.0–8.0 and 7.5–8.8, respectively. However, an acidic optimum pH was reported for purified CuZnSOD from hen eggs (pH 6.0 [58]) and plants (pH 5.0 [59]). Most of the purified CuZnSODs from the sources cited above were reported to have a dimeric structure and exhibited low enzyme activity at acidic pH, likely due to dissociation of the dimer into inactive monomers [60–62]. In contrast, camel liver CuZnSOD exhibited an acidic optimum pH (pH 6.0), which was very low compared to the optimum pH of other CuZnSODs and is consistent with the monomeric structure of the purified enzyme identified in the present study.

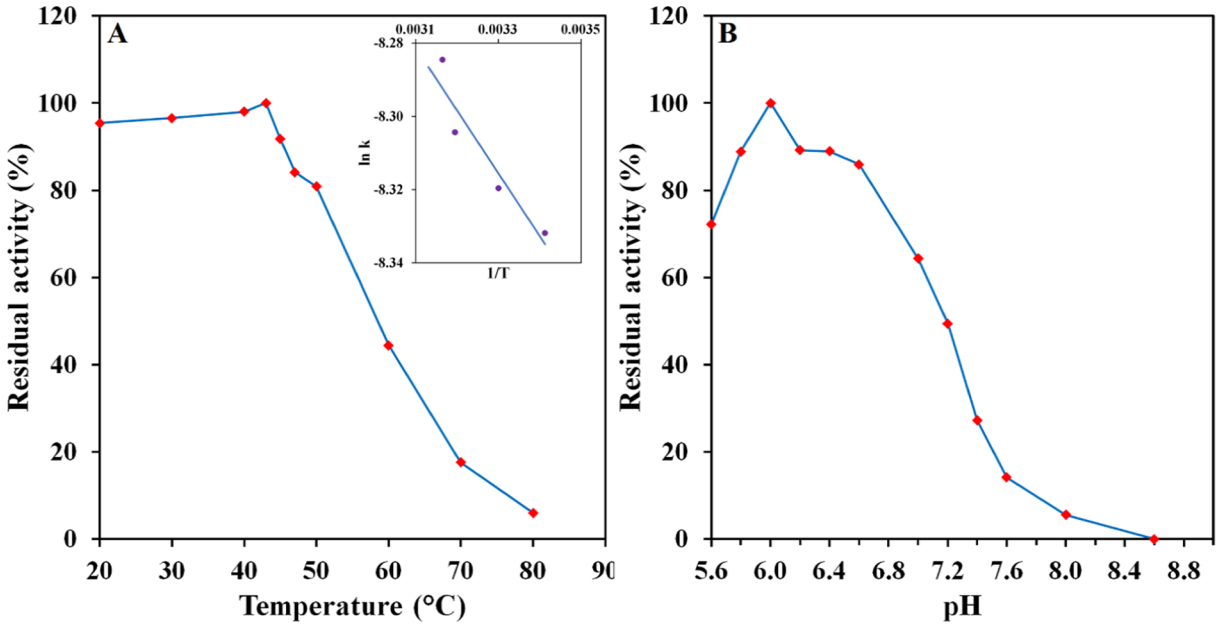

Fig. 4. Optimum temperature and pH of camel liver CuZnSOD. (A) Effect of temperature on the activity of camel liver CuZnSOD. The insert shows Arrhenius plot for determination of E_a of camel liver CuZnSOD. (B) Effect of pH on the activity of camel liver CuZnSOD. The experimental procedures were detailed under "Materials and methods".

3.2.3. Effect of various inhibitors and metal ions on CuZnSOD activity

The effect of various inhibitors on the activity of camel liver CuZnSOD is shown in Table 2. Enzyme activity was completely inhibited by 2 and 5 mM DTT. Similarly, 2 mM of β-ME, H_2O_2 and SDS inhibited enzyme activity by 87, 91 and 38%, respectively, while these inhibitors all completely inhibited enzyme activity at 5 mM. Furthermore, 2 mM EDTA and PMSF inhibited enzyme activity by approximately 42 and 68%, respectively, and inhibition increased to greater than 80% at 5 mM for both inhibitors. In contrast, slowly decreasing inhibition of enzyme activity was observed (from 39 to 35%) with increasing NaN_3 concentrations (from 2 to 5 mM). Camel liver CuZnSOD activity was inhibited by β-ME, DTT and PMSF, indicating that cysteine, threonine and serine amino acid residues play an important role in enzyme activity. Inhibition of CuZnSOD activity by β-ME, DTT and PMSF has been reported for other purified CuZnSODs [5,47], whereas other reports suggest that β-ME has no effect on enzyme activity [12,41]. The chelating agent EDTA inhibited activity of camel liver CuZnSOD, indicating that the metal ions are involved in enzyme activity. As reported in the literature, CuZnSODs are generally more sensitive to H_2O_2 [3]. In the present study, the purified enzyme from camel liver was inhibited by H_2O_2. Therefore, based on the effects of EDTA and H_2O_2, we conclude that camel liver SOD contains Cu and Zn ions at its active site. Similarly, purified CuZnSOD from different sources is inhibited by both EDTA and H_2O_2 [12,39,41,47,52]. NaN_3 also inhibited the activity of camel liver CuZnSOD. This inhibition is related to the ability of the anion azide to binds Cu atoms in the active site of the enzyme, provoking changes in the conformation of the active site and resulting in enzyme inhibition. Azide has been reported to act as an inhibitor of metalloenzymes, especially copper-containing enzymes [63–65]. Purified CuZnSOD from insects [47] and plants [41] is also inhibited by NaN_3. From the effect of the denaturing agent, SDS inhibited the activity of camel liver CuZnSOD, which can be explained by SDS binding to the enzyme and subsequently inducing conformational changes in its active site. Previous studies have reported the same inhibitory effect of SDS on the activity of CuZnSOD [39,47,59].

The effect of various metal ions on camel liver CuZnSOD activity was also investigated. The metal ions tested exerted differential effects on enzyme activity, which is presented in Table 3 that shows means of

Table 2
Effect of various inhibitors on the activity of camel liver CuZnSOD.

Inhibitors	Final concentration (mM)	Inhibition (%)
Control	–	0 ± 0.0024
β-ME	2.0	87 ± 0.0018
	5.0	100 ± 0.0000
EDTA	2.0	42 ± 0.0034
	5.0	80 ± 0.0040
DTT	2.0	100 ± 0.0000
	5.0	100 ± 0.0000
H_2O_2	2.0	91 ± 0.0012
	5.0	100 ± 0.0000
NaN_3	2.0	39 ± 0.0045
	5.0	35 ± 0.0028
PMSF	2.0	68 ± 0.0046
	5.0	81 ± 0.0033
SDS	2.0	38 ± 0.0078
	5.0	100 ± 0.0000

Table 3
Effect of various metal ions on the activity of camel liver CuZnSOD.

Metal ions	Final concentration (mM)	Residual activity (%)
Control	–	100 ± 0.0027
Al^{3+}	2.0	156 ± 0.0031
	5.0	142 ± 0.0046
Ba^{2+}	2.0	99 ± 0.0035
	5.0	0 ± 0.0000
Ca^{2+}	2.0	109 ± 0.0064
	5.0	117 ± 0.0039
Cd^{2+}	2.0	127 ± 0.0039
	5.0	96 ± 0.0064
Co^{2+}	2.0	9 ± 0.0061
	5.0	40 ± 0.0055
Fe^{2+}	2.0	36 ± 0.0028
	5.0	0 ± 0.0000
Mg^{2+}	2.0	149 ± 0.0043
	5.0	97 ± 0.0050
Ni^{2+}	2.0	21 ± 0.0029
	5.0	93 ± 0.0021
Zn^{2+}	2.0	88 ± 0.0019
	5.0	103 ± 0.0031

triplicate values. Ba^{2+} at 2 mM had no effect on enzyme activity, while at 5 mM it completely inhibited enzyme activity. Similarly, Fe^{2+} reduced enzyme activity to 36% at 2 mM and caused complete inhibition at 5 mM. The enzyme was inhibited by 2 mM Co^{2+} (9%), Ni^{2+} (21%) and Zn^{2+} (88%), while increasing the concentration of these metal ions (up to 5 mM) resulted in increasing enzyme activity to 40, 93 and 103%, respectively. Al^{3+}, Cd^{2+} and Mg^{2+} at 2 mM increased enzyme activity by 156, 127 and 149%, respectively; however, at 5 mM, these metal ions decreased enzyme activity to 142, 96 and 97%, respectively. Finally, Ca^{2+} increased enzyme activity by 109% at 2 mM and by 117% at 5 mM. Among the metal ions tested, Al^{3+}, Ca^{2+}, Cd^{2+}, Mg^{2+} and Zn^{2+} were found to increase the activity of camel liver CuZnSOD, suggesting that these metal ions might serve as cofactors for this enzyme. Similarly, it has been reported that Ca^{2+} [59], Mg^{2+} [41] and Zn^{2+} [47,54] increase CuZnSOD activity. However, in contrast to our study, Ca^{2+}, Mg^{2+} and Zn^{2+} exert an inhibitory effect on CuZnSOD activity as reported in an earlier study [39]. On the other hand, the activity of camel liver CuZnSOD was inhibited by Ba^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+}, possibly due to the displacement of active metal ions from their binding site on the enzyme by these inhibitor metal ions. In agreement with our results, purified CuZnSOD from plants was also inhibited by Ba^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+} [39,54]. In contrast, Co^{2+} and Ni^{2+} activated purified CuZnSOD from plants [41] and insects [47].

3.2.4. Metal content

The purified enzyme from camel liver was assayed for Cu and Zn using ICP-MS. Results revealed that camel liver CuZnSOD contained 0.010 μg Cu and 0.69 μg Zn per mg of protein, corresponding to 0.0044 mol Cu and 0.30 mol Zn per mol of protein based on the native molecular weight of the enzyme (28 kDa). The metal content results are in agreement with the inhibitory effect of H_2O_2 on CuZnSOD activity and confirm the copper–zinc nature of the enzyme. However, camel liver CuZnSOD displayed lower Cu content and higher Zn content and differs from other CuZnSODs. In fact, Cu and Zn contents of the dimeric CuZnSOD purified from mammals [5,6,8,10,11,16], non-mammals [37,38], insects [43], plants [40,42] and microorganisms [44,45] falls in the range of 1.0–2.8 mol Cu and 1.0–2.2 mol Zn per mol of protein. In contrast, Cu and Zn contents of the monomeric CuZnSOD was found to be 2.0 mol of each Cu and Zn per mol of protein in bovine erythrocytes [46] and 1.0 mol Cu and 0.5 mol Zn per mol of protein in plants [49].

Compared to results reported in the literature, camel liver CuZnSOD exhibits very low Cu and Zn contents, but this does not affect its activity or stability, as Cu is essential for the catalytic activity and Zn stabilizes the protein structure [3]. This finding might be a unique property of camel liver CuZnSOD related to its reactional mechanism and ability to catalyze the dismutation of $O_2^{\cdot -}$ at low metal content in the stressful conditions of the desert. Moreover, the enzymatic activities of SOD [66], GPx [67] and ceruloplasmin [68] in camels were not affected by low copper-zinc, selenium and copper concentrations, respectively. Furthermore, the metabolism of trace element in camels is certainly very remarkable and could explain the low contents of Cu and Zn identified in our study. It has been reported that camels regulate Cu and Zn concentrations at low levels compared to cattle [66,69]. In addition, the tissue distribution of Cu and Zn in camels demonstrate that this animal contains low concentrations of Cu and Zn compared to other domestic animals [70].

3.2.5. Kinetic parameters

Next, the effect of NBT and riboflavin concentrations on the activity of camel liver CuZnSOD was examined (Fig. 5). As shown in Fig. 5A, camel liver CuZnSOD reaction rate displays a sigmoidal dependence on NBT concentrations. The enzyme exhibited no activity at NBT concentration lower than 10 μM, whereas increasing NBT concentrations above 10 μM resulted in increased enzyme activity to a maximum at 100 μM. In contrast, camel liver CuZnSOD reaction rate displayed a hyperbolic dependence on riboflavin concentrations (Fig. 5B). The enzyme activity increased in a manner dependent on riboflavin concentrations. Moreover, the enzyme requires lower concentrations of riboflavin (0.5–10 μM) for maximum activity. These results indicate that the dependence of the reaction rate catalyzed by camel liver CuZnSOD on concentrations of NBT and riboflavin follows non-Michaelian and Michaelian kinetics, respectively.

K_m and V_{max} of camel liver CuZnSOD for NBT and riboflavin were determined from nonlinear regression using GraphPad Prism software version 7. k_{cat} and k_{cat}/K_m were then calculated. Kinetic parameters are summarized in Table 4. K_m values of camel liver CuZnSOD for NBT and riboflavin were 16.27 and 0.16 μM, respectively. K_m value for NBT was 100 times higher for riboflavin, indicating that camel liver CuZnSOD exhibits more affinity for riboflavin than for NBT. However, V_{max} values of camel liver CuZnSOD for NBT (20.85 U/mg) and riboflavin (21.54 U/mg) were similar. Moreover, the k_{cat} value of camel liver CuZnSOD for riboflavin (9.97 s^{-1}) was slightly higher than for NBT (9.65 s^{-1}). In addition, camel liver CuZnSOD exhibited significantly higher catalytic efficiency, as indicated by the k_{cat}/K_m ratio, for riboflavin (62.33 s^{-1} μM^{-1}) compared to NBT (0.59 s^{-1} μM^{-1}), indicating very high specificity for riboflavin.

K_m values of camel liver CuZnSOD for NBT and riboflavin were very low compared to those of other purified CuZnSODs from different sources. In plants, K_m values for NBT and riboflavin were found to be 25.0 and 1.7 μM [41], 62.414 and 27.389 M [59], 310×10^{-6} and 41×10^{-6} M [71], and 57.31 and 1.51 M [72], respectively. In addition, purified CuZnSOD from bovine erythrocytes and microorganism had a K_m value of 6.3 μM for riboflavin [73] and 371.2 μM for NBT [74], respectively. Similarly, higher K_m values were reported for other substrates for SOD, such as pyrogallol (11.5 μM [72]), dianisidine (83 μM [73]) and xanthine (6.198 mM [74]). In contrast, the k_{cat} and k_{cat}/K_m values of other purified CuZnSODs were reported to be different: 1667 s^{-1} and 5.6×10^5 s^{-1} mM^{-1} for xanthine in human erythrocytes [51], 1.4×10^{-1} s^{-1} and 43.75 s^{-1} mM^{-1} for xanthine in crocodiles [75], 107,000 s^{-1} and 9300 s^{-1} μM^{-1} for pyrogallol in plants [55], and 1.358 s^{-1} and 3.7×10^{-3} s^{-1} μM^{-1} for NBT in microorganisms [74], respectively.

Kinetic data revealed that camel liver CuZnSOD displayed hyperbolic behavior and higher affinity, turnover number and catalytic efficiency for riboflavin, even at very low concentrations. These findings likely indicate that under the stressful living conditions of the desert, camel liver CuZnSOD accelerates dismutation of $O_2^{\cdot -}$, potentially playing a key role in the protection of cells from the deleterious effect of this free radical.

3.2.6. Unique properties of camel liver CuZnSOD

Biochemical characterization of purified CuZnSOD from camel liver revealed that this enzyme has unique properties compared with those of other purified CuZnSODs from different sources. These unique properties are summarized as follows: lower molecular weight with a monomeric structure, higher optimum temperature, very low E_a, very low optimum pH, very low Cu and Zn contents, and higher affinity, turnover number and catalytic efficiency for riboflavin. These unique properties of camel liver CuZnSOD could be attributed to the unique behavior of camels, which are well adapted to living in hot and dry desert conditions due to their biochemical, anatomical and physiological peculiarities [18]. These new findings on the biochemical properties of camel liver CuZnSOD are not surprising, since we previously found that the properties of CAT [22] and GPx [23] purified from camel liver were also different compared to other species. In addition, the enzymes glutathione transferase [76,77], arginase [78] and ceramidase [79] from camels were also found to have unique molecular and biochemical properties.

4. Conclusion

A novel CuZnSOD was successfully purified to homogeneity from camel liver. The purified enzyme was biochemically characterized,

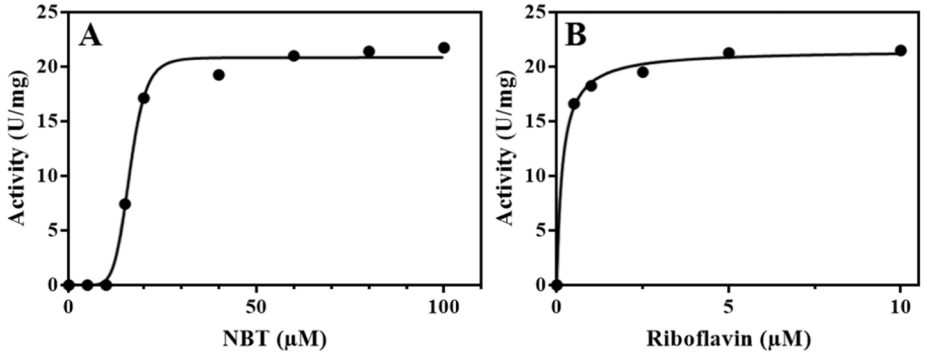

Fig. 5. Nonlinear regression plots for determination of kinetic parameters of camel liver CuZnSOD. K_m and V_{max} of CuZnSOD were determined from the plots for (A) NBT and (B) riboflavin. The experimental procedures were detailed under "Materials and methods".

Table 4
Kinetic parameters of camel liver CuZnSOD.

Substrate	K_m (μM)	V_{max} (U/mg)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} μM^{-1})
NBT	16.27 ± 0.33	20.85 ± 0.41	9.65	0.59
Riboflavin	0.16 ± 0.02	21.54 ± 0.36	9.97	62.33

revealing that camel liver CuZnSOD is unique in its biochemical properties compared to other CuZnSODs. These unique biochemical properties provide new insight into the antioxidant defense systems against the deleterious effects of oxidative stress induced by environmental stress conditions. Moreover, the results of the present study could help explain camels' ability to adapt their biochemical properties to stressful living conditions in the desert. Further studies are needed to fully understand the biochemical mechanisms involved in the resistance of camels to their specific ecosystem.

Acknowledgments

This work was supported by Atatürk University (Turkey) and University Hassan First (Morocco).

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 Abdelkhalid Essamadi conceived and supervised project, and wrote paper with contributions from all authors.

References

[1] B. Halliwell, Reactive oxygen species in living systems: source, biochemistry, and role in human disease, Am. J. Med. 91 (1991) 14–22.
[2] E. Birben, U.M. Sahiner, C. Sackesen, S. Erzurum, O. Kalayci, Oxidative stress and antioxidant defense, World Allergy Organ. J. 5 (2012) 9–19.
[3] Y. Sheng, I.A. Abreu, D.E. Cabelli, M.J. Maroney, A.F. Miller, M. Teixeira, J.S. Valentine, Superoxide dismutases and superoxide reductases, Chem. Rev. 114 (2014) 3854–3918.
[4] R.A. Weisiger, I. Fridovich, Superoxide dismutase. Organelle specificity, J. Biol. Chem. 248 (1973) 3582–3592.
[5] M. Sugiura, T. Adachi, H. Inoue, Y. Ito, K. Hirano, Purification and properties of two superoxide dismutases from human placenta, J. Pharmacobiodyn. 4 (1981) 235–244.
[6] R.G. Briggs, J.A. Fee, Further characterization of human erythrocyte superoxide dismutase, Biochim. Biophys. Acta 537 (1978) 86–99.
[7] D.P. Malinowski, I. Fridovich, Subunit association and side-chain reactivities of bovine erythrocyte superoxide dismutase in denaturing solvents, Biochemistry 18 (1979) 5055–5060.
[8] B.B. Jr, J.M. Keele, I. Fridovich McCord, Further characterization of bovine superoxide dismutase and its isolation from bovine heart, J. Biol. Chem. 246 (1971) 2875–2880.
[9] R.E. Bensinger, J.W. Crabb, C.M. Johnson, Purification and properties of superoxide dismutase from bovine retina, Exp. Eye Res. 34 (1982) 623–634.
[10] V. Albergoni, A. Cassini, A cupro-zinc protein with superoxide dismutase activity from horse liver. Isolation and properties, Comp. Biochem. Physiol. B 47 (1974) 767–777.
[11] M.L. Salin, W.W. Wilson, Porcine superoxide dismutase. Isolation and characterization of a relatively basic cuprozinc enzyme, Mol. Cell. Biochem. 36 (1981) 157–161.
[12] A. Orzołek, P. Wysocki, J. Strzeżek, W. Kordan, Superoxide dismutase (SOD) in boar spermatozoa: Purification, biochemical properties and changes in activity during semen storage (16°C) in different extenders, Reprod. Biol. 13 (2013) 34–40.
[13] M. Manohar, K.A. Balasubramanian, Studies on cytosolic superoxide dismutase from intestinal mucosa, Indian J. Biochem. Biophys. 28 (1991) 52–57.
[14] J.D. Crapo, J.M. McCord, Oxygen-induced changes in pulmonary superoxide dismutase assayed by antibody titrations, Am. J. Physiol. 231 (1976) 1196–1203.
[15] U. Reiss, D. Gershon, Rat-liver superoxide dismutase. Purification and age-related modifications, Eur. J. Biochem. 63 (1976) 617–623.
[16] S. Yano, Multiple isoelectric variants of copper, zinc-superoxide dismutase from rat liver, Arch. Biochem. Biophys. 279 (1990) 60–69.
[17] K. Asayama, R.A. Sharp, I.M. Burr, Purification and radioimmunoassays for superoxide dismutases in the mouse: Tissue concentrations in different strains, Int. J. Biochem. 17 (1985) 1171–1178.
[18] G. Alhadrami, B. Faye, Animals that produce dairy foods: Camel, in: Reference module in food science, Elsevier, Amsterdam, Academic Press, 2016, pp. 1–12.
[19] A.G.M. Abdel Gader, A.A. Alhaider, The unique medicinal properties of camel products: A review of the scientific evidence, J. Taibah Univ. Med. Sci. 11 (2016) 98–103.
[20] H. Wu, X. Guang, M.B. Al-Fageeh, J. Cao, S. Pan, H. Zhou, L. Zhang, M.H. Abutarboush, Y. Xing, Z. Xie, A.S. Alshanqeeti, Y. Zhang, Q. Yao, B.M. Al-Shomrani, D. Zhang, J. Li, M.M. Manee, Z. Yang, L. Yang, Y. Liu, J. Zhang, M.A. Altammami, S. Wang, L. Yu, W. Zhang, S. Liu, L. Ba, C. Liu, X. Yang, F. Meng, S. Wang, L. Li, E. Li, X. Li, K. Wu, S. Zhang, J. Wang, Y. Yin, H. Yang, A.M. Al-Swailem, J. Wang, Camelid genomes reveal evolution and adaptation to desert environments, Nat. Commun. 6 (2015) 1–9.
[21] P. Schröder, J. Krutmann, Environmental oxidative stress – environmental sources of ROS, in: T. Grune (Ed.), Reactions Processes. The Handbook of Environmental Chemistry, Springer, Berlin, Heidelberg, 2005, pp. 19–31.
[22] A. Chafik, A. Essamadi, S.Y. Çelik, A. Mavi, Purification of camel liver catalase by zinc chelate affinity chromatography and pH gradient elution: An enzyme with interesting properties, J. Chromatogr. B 1070 (2017) 104–111.
[23] A. Chafik, A. Essamadi, S.Y. Çelik, K. Solak, A. Mavi, Partial purification and some interesting properties of glutathione peroxidase from liver of camel *(Camelus dromedarius)*, Russ. J. Bioorg. Chem. 44 (2018) 41–51.
[24] J. Rogelj, M. den Elzen, N. Höhne, T. Fransen, H. Fekete, H. Winkler, R. Schaeffer, F. Sha, K. Riahi, M. Meinshausen, Paris Agreement climate proposals need a boost to keep warming well below 2 °C, Nature 534 (2016) 631–639.
[25] N. Crosti, T. Servidei, J. Bajer, A. Serra, Modification of the 6-hydroxydopamine technique for the correct determination of superoxide dismutase, J. Clin. Chem. Clin. Biochem. 25 (1987) 265–266.
[26] C. Beauchamp, I. Fridovich, Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels, Anal. Biochem. 44 (1971) 276–287.
[27] B.J. Davis, Disc electrophoresis. II. Method and application to human serum proteins, Ann. N. Y. Acad. Sci. 121 (1964) 404–427.
[28] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[29] M. Tsuchihashi, Zür Kenntnis der Blutkatalase, Biochem. Z 140 (1923) 65–74.
[30] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[31] H. Markowitz, G.E. Cartwright, M.M. Wintrobe, Studies on copper metabolism. XXVII. The isolation and properties of an erythrocyte cuproprotein (erythrocuprein), J. Biol. Chem. 234 (1959) 40–45.

[32] J. Bannister, W. Bannister, E. Wood, Bovine erythrocyte cupro-zinc protein. 1. Isolation and general characterization, Eur. J. Biochem. 18 (1971) 178–186.
[33] R.J. Carrico, H.F. Deutsch, Isolation of human hepatocuprein and cerebrocuprein. Their identity with erythrocuprein, J. Biol. Chem. 244 (1969) 6087–6093.
[34] M.S. Mohamed, D.M. Greenberg, Isolation of purified copper protein from horse liver, J. Gen. Physiol. 37 (1954) 433–439.
[35] H. Porter, S. Ainsworth, The isolation of the copper-containing protein cerebrocuprein I from normal human brain, J. Neurochem. 5 (1959) 91–98.
[36] H. Porter, J. Folch, Cerebrocuprein I. A copper-containing protein isolated from brain, J. Neurochem. 1 (1957) 260–271.
[37] L.A. Demirel, L. Tarhan, Dismutation properties of purified and GDA modified CuZnSOD from chicken heart, Artif. Cells Blood Substit. Immobil. Biotechnol. 32 (2004) 609–624.
[38] M.L. Rhoads, *Trichinella spiralis*: identification and purification of superoxide dismutase, Exp. Parasitol. 56 (1983) 41–54.
[39] S. Wang, B. Shao, S. Liu, X. Ye, P. Rao, Purification and characterization of Cu, Zn-superoxide dismutase from black soybean, Food Res. Int. 47 (2012) 374–379.
[40] J. Kwiatowski, Z. Kaniuga, Isolation and characterization of cytosolic and chloroplast isoenzymes of Cu, Zn-superoxide dismutase from tomato leaves and their relationships to other Cu, Zn-superoxide dismutases, Biochim. Biophys. Acta 874 (1986) 99–115.
[41] S. Kumar, S. Dhillon, D. Singh, R. Singh, Partial purification and characterization of superoxide dismutase from tomato (*Lycopersicon esculentum*) fruit, J. Food Sci. Nutr. 9 (2004) 283–288.
[42] A. Padiglia, R. Medda, E. Cruciani, A. Lorrai, G. Floris, Purification and properties of *Oryza sativa* Cu-Zn superoxide dismutase, Prep. Biochem. Biotechnol. 26 (1996) 135–142.
[43] Y.M. Lee, F.J. Ayala, H.P. Misra, Purification and properties of superoxide dismutase from *Drosophila melanogaster*, J. Biol. Chem. 256 (1981) 8506–8509.
[44] Z. Wang, Z. He, S. Li, Q. Yuan, Purification and partial characterization of Cu, Zn containing superoxide dismutase from entomogenous fungal species *Cordyceps militaris*, Enzyme Microb. Technol. 36 (2005) 862–869.
[45] C.S. Hwang, G. Rhie, S.T. Kim, Y.R. Kim, W.K. Huh, Y.U. Baek, S.O. Kang, Copper- and zinc-containing superoxide dismutase and its gene from *Candida albicans*, Biochim. Biophys. Acta 1427 (1999) 245–255.
[46] U. Weser, E. Bunnenberg, R. Cammack, C. Djerassi, L. Flohé, G. Thomas, W. Voelter, A study on purified bovine erythrocuprein, Biochim. Biophys. Acta 243 (1971) 203–213.
[47] M.A. Ibrahim, M.M. Mohamed, A.H. Ghazy, H.M. Masoud, Superoxide dismutases from larvae of the camel tick *Hyalomma dromedarii*, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 164 (2013) 221–228.
[48] Y.C. Liu, M.R. Lee, C.J. Chen, Y.C. Lin, H.C. Ho, Purification of Cu/Zn superoxide dismutase from *Piper betle* leaf and its characterization in the oral cavity, J. Agric. Food Chem. 63 (2015) 2225–2232.
[49] L. Sheng, X. Zheng, H. Tong, S. Liu, J. Du, Q. Liu, Purification and characterization of cytosolic isoenzyme III of Cu, Zn-superoxide dismutase from tobacco leaves, Plant Sci. 167 (2004) 1235–1241.
[50] A. Battistoni, G. Rotilio, Isolation of an active and heat-stable monomeric form of Cu, Zn superoxide dismutase from the periplasmic space of *Escherichia coli*, FEBS Lett. 374 (1995) 199–202.
[51] H. Karadag, R. Bilgin, Purification of copper-zinc superoxide dismutase from human erythrocytes and partial characterization, Biotechnol. Biotechnol. Equip. 24 (2010) 1653–1656.
[52] H. Ben Khaled, N. Ktari, R. Siala, N. Hmidet, A. Bayoudh, M. Nasri, S. Ghorbel, A heat-stable Cu/Zn superoxide dismutase from the viscera of sardinelle (*Sardinella aurita*): purification and biochemical characterization, Biologia 69 (2014) 1770–1776.
[53] B. Petkar Medha, M. Pillai Meena, A. Kulkarni Amarja, H. Bondre Sushma, K.R.S.S. Rao, Purification and characterization of superoxide dismutase isolated from sewage isolated *E. coli*, J. Microb. Biochem. Technol. 5 (2013) 102–106.
[54] J. Liu, J. Wang, M. Yin, H. Zhu, J. Lu, Z. Cui, Purification and characterization of superoxide dismutase from garlic, Food Bioprod. Process. 89 (2011) 294–299.
[55] F. Zeinali, A. Homaei, E. Kamrani, Identification and kinetic characterization of a novel superoxide dismutase from *Avicennia marina*: An antioxidant enzyme with unique features, Int. J. Biol. Macromol. 105 (2017) 1556–1562.
[56] A. Vuleta, B. Tucić, Thermal dependence of the antioxidant enzymes superoxide dismutase, catalase and peroxidase in foliage of *Iris pumila* L, Arch. Biol. Sci. 61 (2009) 441–446.
[57] B. Asthir, A. Koundal, N.S. Bains, Kinetic properties of cell wall bound superoxide dismutase in leaves of wheat (*Triticum aestivum L.*) following stripe rust (*Puccinia striiformis*) infection, Indian J. Biochem.Biophys. 48 (2011) 341–345.
[58] J. Wawrzykowski, M. Kankofer, Partial biochemical characterization of Cu, Zn-superoxide dismutase extracted from eggs of hens *(Gallus gallus domesticus*), Food Chem. 227 (2017) 390–396.
[59] P. Niyomploy, R. Boonsombat, A. Karnchanatat, P. Sangvanich, A superoxide dismutase purified from the roots from *Stemona tuberosa*, Prep. Biochem. Biotechnol. 44 (2014) 663–679.
[60] C.T. Lin, M.T. Lin, Y.T. Chen, J.F. Shaw, Subunit interaction enhances enzyme activity and stability of sweet potato cytosolic Cu/Zn-superoxide dismutase purified by a His-tagged recombinant protein method, Plant Mol. Biol. 28 (1995) 303–311.
[61] C.T. Lin, T.J. Kuo, J.F. Shaw, M.C. Kao, Characterization of the dimer-monomer equilibrium of the papaya Copper/Zinc superoxide dismutase and its equilibrium shift by a single amino acid mutation, J. Agric. Food Chem. 47 (1999) 2944–2949.
[62] C.T. Lin, T.L. Lee, K.J. Duan, J.C. Su, Purification and characterization of black porgy muscle Cu/Zn superoxide dismutase, Zool. Stud. 40 (2001) 84–90.
[63] G. Curzon, The inhibition of caeruloplasmin by azide, Biochem. J. 100 (1966) 295–302.
[64] C. Shi, Y. Dai, X. Xu, Y. Xie, H. Han, Q. Liu, The interaction of azide with polyphenol oxidase II from tobacco, J. Protein Chem. 20 (2001) 463–468.
[65] H. Patel, S. Gupte, M. Gahlout, A. Gupte, Purification and characterization of an extracellular laccase from solid-state culture of *Pleurotus ostreatus* HP-1, 3, Biotech. 4 (2014) 77–84.
[66] M. Bengoumi, A. Essamadi, J.P. Chacornac, J.C. Tressol, B. Faye, Comparative relationship between copper-zinc plasma concentrations and superoxide dismutase activity in camel and cow, Vet. Res. 29 (1998) 557–565.
[67] M. Bengoumi, A. Essamadi, J.C. Tressol, J.P. Chacornac, B. Faye, Comparative effects of selenium supplementation on the plasma selenium concentration and erythrocyte glutathione peroxidase activity in cattle and camels, Animal Sci. 67 (1998) 461–466.
[68] A. Essamadi, M. Bengoumi, J.P. Chacornac, B. Faye, Relationship between plasma copper concentration and caeruloplasmin activity in camel, Trends Comp. Biochem. Physiol. 5 (1998) 211–220.
[69] M. Bengoumi, A. Essamadi, J.C. Tressol, B. Faye, Comparative study of copper and zinc metabolism in cattle and camel, Biol. Trace Elem. Res. 63 (1998) 81–94.
[70] A. Chafik, R. Eddoha, A. Bagri, B. Nasser, A. Essamadi, Copper and zinc contents in different organs of animals slaughtered in Casablanca city-Morocco, J. Mater. Environ. Sci. 5 (2014) 1737–1741.
[71] V. Pruthi, *Alternaria brassicae* induced alterations in enzymatic activities and polypeptide patterns in *Brassica juncea*, PhD Thesis Chaudhary Charan Singh Haryana Agricultural University, Hisar, India, 2002.
[72] W. Moon-ai, P. Niyomploy, R. Boonsombat, P. Sangvanich, A. Karnchanatat, A superoxide dismutase purified from the rhizome of *Curcuma aeruginosa* Roxb. as inhibitor of nitric oxide production in the macrophage-like RAW 264.7 cell line, Appl. Biochem. Biotechnol. 166 (2012) 2138–2155.
[73] H.P. Misra, I. Fridovich, Superoxide dismutase: a photochemical augmentation assay, Arch. Biochem. Biophys. 181 (1977) 308–312.
[74] A. Indrayati, S. Asyarie, T. Suciati, D.S. Retnoningrum, Study on the properties of purified recombinant superoxide dismutase from *Staphylococcus equorum*, A local isolate from Indonesia, Int. J. Pharm. Pharm. Sci. 6 (2014) 440–445.
[75] P. Sujiwattanarat, P. Pongsanarakul, Y. Temsiripong, T. Temsiripong, C. Thawornkuno, Y. Uno, S. Unajak, Y. Matsuda, K. Choowongkomon, K. Srikulnath, Molecular cloning and characterization of Siamese crocodile (*Crocodylus siamensis*) copper, zinc superoxide dismutase (CSI-Cu, Zn-SOD) gene, Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 191 (2016) 187–195.
[76] A. Malik, D. Fouad, N.E. Labrou, A.M. Al-Senaidy, M.A. Ismael, H.M. Saeed, F.S. Ataya, Structural and thermodynamic properties of kappa class glutathione transferase from Camelus dromedarius, Int. J. Biol. Macromol. 88 (2016) 313–319.
[77] F. Perperopoulou, F.S. Ataya, D. Fouad, A. Malik, H.M. Saeed, N.E. Labrou, Biochemical characterization of the detoxifying enzyme glutathione transferase P1–1 from the camel *Camelus dromedarius*, Cell Biochem. Biophys. 74 (2016) 459–472.
[78] T.M. Maharem, W.E. Zahran, R.E. Hassan, M.M. Abdel, Fattah, Unique properties of arginase purified from camel liver cytosol, Int. J. Biol. Macromol. 108 (2018) 88–97.
[79] S. Chathoth, F. Thayyullathil, A. Galadari, M. Patel, S. Galadari, Purification and biochemical characterization of membrane-bound neutral ceramidase from camel brain (*Camelus dromedarius*), Int. J. Biochem. Mol. Biol. 4 (2013) 54–66.