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Research article

Superoxide dismutases from the camel plasma: Purification and characterization of two copper/zinc isoforms

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Key words: Superoxide dismutase, Camel plasma, Purification, Characterization, isoenzymes, copper/zinc.

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Abstract

Superoxide dismutase (SOD) is a metalloenzyme that represents the initial defense line versus oxidative stress as it dismutates the superoxide ions to hydrogen peroxide and molecular oxygen. SODs have broad medical applications in clinical nutrition, cosmetics and pharmaceuticals. Here, two superoxide dismutases designated camel plasma superoxide dismutase 1 (CPSOD1) and camel plasma superoxide dismutase 2 (CPSOD2) were purified from camel plasma using ammonium sulfate fractionation and chromatography on anion exchanger and gel filtration columns. CPSOD1 had a native molecular weight of about 240 kDa, whereas two bands with molecular weights of 65 kDa and 55 kDa were found on SDS–PAGE suggesting it to be heterotetramer. CPSOD2 exhibited monomeric structure with molecular weight of 60 kDa. The *p*I values are evaluated at pH 6.9 and pH 6.2 for the two SODs. $CoCl_2$, $CuCl_2$, $MgCl_2$, $NiCl_2$ and $ZnCl_2$ activated CPSOD1 and CPSOD2 while $CaCl_2$, $FeCl_2$ and MnCl2 inhibited them. The activity of both isoenzymes is inhibited with KCN and H_2O_2. CPSOD1 and CPSOD2 are proposed to be copper/zinc containing isoenzymes.

Introduction

Free radicals are a set of highly active chemical molecules with one or more unpaired electrons and can cause oxidative modifications for biomolecules. The most serious free radicals are the oxygen derivatives recognized as reactive oxygen species (ROS). The antioxidant defense systems save cells from poisonous influences of ROS and all organisms maintain equilibrium between their own free radicals and antioxidants. When this equilibrium break, a state called oxidative stress arises that the antioxidants cannot dispose of free radicals and causes too much increase in ROS that harms biological systems [1-3]. ROS may cause lipid peroxidation, membrane fluidity disruption, apoptosis initiation in the mitochondria, amino acid modifications, peptide chains fragmentation, enzymes inactivation, DNA deletions, mutations, base degeneration, single-strand breaking and cross-linking of proteins [4-6]. ROS can undergo neutralization process by the antioxidant defense system either by enzymatic or non-enzymatic molecules. The enzymatic antioxidant system comprises enzymes as superoxide dismutase, catalase and glutathione peroxidase [7, 8].

Superoxide dismutase (SOD) is broadly propagated metalloenzyme that protects against oxidative stress from superoxide radicals [9-11]. SODs stimulate the transformation of superoxide ions to O_2 and H_2O_2 to keep tissues from poisonous oxidants yielded through oxidative operations [12, 13]. Thereafter, catalase is securely breaks down the hydrogen peroxide into H_2O and O_2 [14]. SODs are existed in almost whole organisms which consume oxygen [10, 15]. The SODs are categorized into four kinds on the basis of the metal ions existing in the active site [16]. Copper-zinc SOD (CuZnSOD), manganese SOD (MnSOD), iron SOD (FeSOD) and nickel SOD (NiSOD) [13, 17, 18]. The four kinds can be found in prokaryotes, while in eukaryotes FeSOD is found in chloroplasts, MnSOD is found in mitochondria and peroxisomes, and CuZnSOD is exist in chloroplasts, cytosol and extracellular spaces [15, 19, 20, 21]. It was demonstrated that SOD activity is dispensable for normal animal life-span and needed to survive acute stresses [22]. SOD had wide commercial uses in clinical nutrition, cosmetics and pharmaceuticals [23]. SOD was used as candidate antioxidant drug in different diseases and augmentation of antioxidant defenses [24]. SOD has been administered as therapeutic agent in hypoxic/ischemic cerebral injury, ischemic reperfusion and cerebral edema [13. 25]. SOD derivative drugs are efficient in controlling hypertension [26], reducing hypoxic-ischemic brain damage [27, 28] and treating endothelial cell dysfunction [29] and are likely useful antitumor drug targets [30]. Therefore, the aim of this study is purification and characterization of SOD from the camel plasma for its wide therapeutic applications.

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Materials and methods

Preparation of camel plasma

The camel plasma was obtained by centrifugation of mixture of 900 ml of camel blood and 100 ml of 0.11 M trisodium citrate solution at 2700 *x g* for 15 min at 4°C. If the plasma was not used immediately, it was dispended into eppendorf tubes and stored at -40°C [31].

Chemicals

Phenylmethylsulfonylfluoride (PMSF), xanthine sodium salt, xanthine oxidase enzyme, diethylaminoethyl cellulose (DEAE-cellulose), Nitroblue tetrazolium chloride (NBT), Phenazine methosulfate (PMS), isoelectric focusing (IEF) standard markers mixture *p*I 3.6 - 9.3, molecular weight marker kits for gel filtration and Sephacryl S-300 were purchased from Sigma Chemical Co. All other chemicals were of analytical grade.

Assay of superoxide dismutase activity

The SOD activity assay reaction mixture contained in a total volume of 1.0 ml of 0.05 M potassium phosphate buffer pH 7.8, containing 0.01 mM cytochrome C, 0.1 mM EDTA and 0.05 mM sodium xanthine. The reaction was started by adding 21 mU xanthine oxidase enzyme. One unit of SOD activity is defined as the amount of enzyme giving 50% inhibition on cytochrome C reduction at 550 nm [32].

Staining of SOD activity

Detection of SOD activity is usually achieved by a system containing nitroblue tetrazolium salt (NBT) and phenazine methosulfate (PMS) that generate superoxide anions when reoxidized in daylight. The superoxide anions in turn, reduce NBT to an insoluble formazan. Achromatic zones indicate where the deficiency of superoxide radicals, due to the SOD activity, prevented the reduction of NBT. After electrophoresis, the gel was submerged in 50 ml 0.1 M Tris-HCl pH 8.6, containing 20 mg NBT and traces of PMS. Gels are then exposed for several minutes to daylight until achromatic zones appear on a blue background, indicating the presence of SOD activity [33].

Purification of camel plasma superoxide dismutases

Ammonium sulfate precipitation

The camel plasma was brought to 80% saturation by gradually adding solid $(NH_4)_2SO_4$ and stirred for 30 min at 4°C. The pellet was obtained by centrifugation at 12000 *x g* for 30 min and dissolved in 0.02 M potassium phosphate buffer pH 7.4 and dialyzed extensively against the same buffer.

DEAE-cellulose column chromatography

The dialyzed sample was chromatographed on DEAE-cellulose column (12 x 2.4 cm i.d.) previously equilibrated with 0.02 M potassium phosphate buffer pH 7.4. The adsorbed proteins were eluted with a stepwise NaCl gradient ranging from 0 to 1 M prepared in the equilibration buffer at a flow rate of 60 ml/hour. 5 ml fractions were collected and the fractions containing SOD activity were pooled and concentrated by lyophilization.

Sephacryl S-300 column chromatography

The concentrated solution containing the SOD activity was applied onto Sephacryl S-300 column (142 cm x 1.75 cm i.d.). The column was equilibrated and developed with 0.02 M potassium phosphate buffer pH 7.4 at a flow rate of 30 ml/hour and 2 ml fractions were collected.

Electrophoretic analysis

Native gel electrophoresis was carried out with 7% PAGE [34]. SDS-PAGE was performed with 12% polyacrylamide gel [35]. The subunit molecular weights of the purified SOD isoenzymes were determined by SDS-PAGE [36]. Electrofocusing was performed and the isoelectric point (*p*I) values were calculated from a calibration curve [37, 38]. The proteins were stained with 0.25% Coomassie Brilliant Blue R-250.

Protein determination

Protein was determined by the dye binding assay method using BSA as a standard protein [39].

Results

Purification of SOD isoenzymes from camel plasma

A purification scheme of camel plasma SODs is given in table (1). The ammonium sulfate fraction elution profile on DEAE-cellulose column (Figure 1a) manipulated two major SOD activity peaks which designated CPSOD1 eluted with 0.0 M NaCl and CPSOD2 eluted with 0.1 M NaCl. The fractions comprising SOD activity of each peak were collected, lyophilized and loaded through Sephacryl S-300 column that revealed one SOD activity peak for CPSOD1 and CPSOD2 (Figure 1b and 1c). The CPSOD1 specific activity is 75.9 units / mg protein representing 27.7% recovery, while CPSOD2 specific activity is 17.5 units / mg protein representing 17.5% recovery (Table 1).

Molecular weight determination and Electrophoretic analysis of CPSODs

The intact molecular weights of CPSOD1 and CPSOD2 obtained from Sephacryl S-300 column are concluded from a standardization curve as 240 ± 2.4 kDa and 60 ± 1.6 kDa. Electrophoretic analysis of samples from the various purification phases; camel plasma, $(NH_4)_2SO4$

fraction, DEAE-cellulose fraction and Sephacryl S-300 fraction of CPSOD1 and CPSOD2 on 7 % native PAGE was carried out (Figure 2). Odd protein bands met the SOD isoenzymes bands affirming purity of the two molecules. Native and denatured purified CPSOD1 and CPSOD2 isoforms were analyzed electrophoretically on SDS-PAGE in comparison with standard proteins (Figure 3a and 3b). The subunit molecular weights were deduced from a standardization curve as 65 and 55 kDa for CPSOD1 and 60 kDa for CPSOD2. The purified CPSOD1 and CPSOD2 isoenzymes were analyzed on isoelectrofocusing PAGE (Figure 3c) and the isoelectric points (*p*I) were deduced from a standard curve. The two isoforms displayed single molecular species with *p*I values of 6.9 for CPSOD1 and 6.2 for CPSOD2.

Effect of divalent cations and various inhibitors

The purified camel plasma CPSOD1 and CPSOD2 were preincubated with various divalent cations and different inhibitors for 5 min at 37˚C. The inhibition % was calculated as a ratio of a control lacking the cation or the inhibitor. $CoCl_2$, $CuCl_2$, $MgCl_2$, $NiCl_2$ and $ZnCl_2$ increased the activity of CPSOD1 and CPSOD2 while $CaCl_2$, $FeCl_2$ and $MnCl_2$ inhibited them (Table 2). KCN, H_2O_2, SDS and PMSF inhibited the activity of CPSOD1 and CPSOD2 isoenzymes (Table 3).

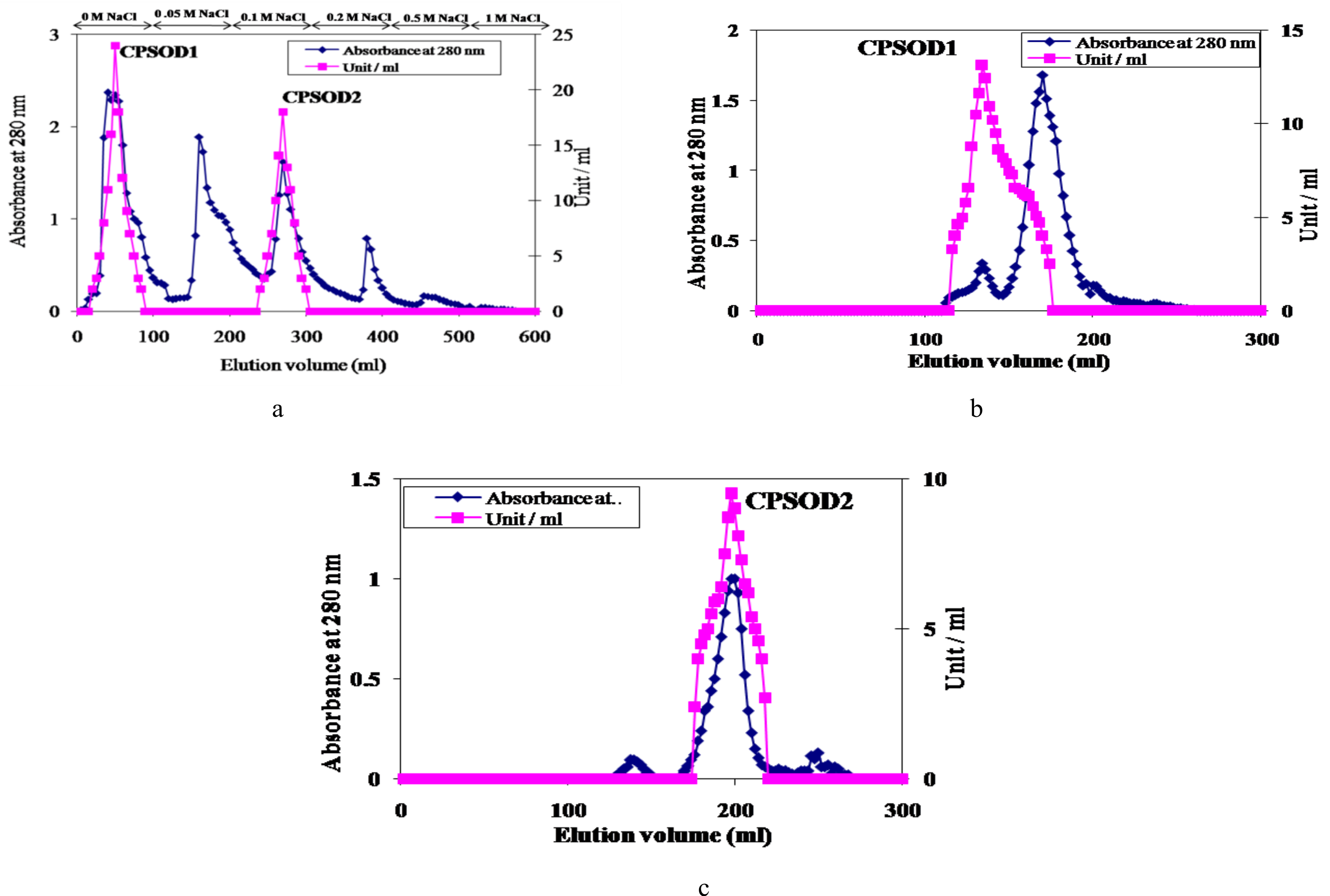

Figure 1. (a) A typical elution profile for the camel plasma ammonium sulfate fraction on DEAE-cellulose column (12 cm x 2.4 cm i.d.) previously equilibrated with 0.02 M K-phosphate buffer pH 7.4. (b) Typical elution profile for the chromatography of the concentrated pooled DEAE-cellulose fractions CPSOD1 on Sephacryl S-300 column (142 cm x 1.75 cm i.d.) previously equilibrated with 0.02 M K-phosphate buffer pH 7.4. (c) Typical elution profile for the chromatography of the concentrated pooled DEAE-cellulose fractions CPSOD2 on Sephacryl S-300 column (142 cm x 1.75 cm i.d.) previously equilibrated with 0.02 M K-phosphate buffer pH 7.4.

Table 1. A typical purification scheme of SOD isoenzymes from camel plasma.

Purification step	Total mg proteins	Total units	Recovery (%)	Specific activity	Fold purification
Camel plasma	525	534	100.0	2.9	**1.0**
80% $(NH_4)_2SO_4$ fraction	360	266	82.5	3.5	**1.2**
DEAE-cellulose fractions					
0.0 M NaCl (CPSOD1)	28.6	90	38.5	20.6	**7.1**
0.1 M NaCl (CLSOD2)	16.4	85	31.6	29.5	**10.2**
Sephacryl S-300 fractions					
CPSOD1	5.6	25	27.7	75.9	**26.2**
CPSOD2	3.8	69	17.5	70.8	**24.4**

Table 2. Effect of divalent cations on the purified camel plasma CPSOD1 and CPSOD2.

Reagent	Concentration (mM)	Residual activity (%)	
		CPSOD1	**CPSOD2**
Control	-----	100.0	100.0
$CaCl_2$	2.0	85.2	81.4
	5.0	71.6	57.2
$CoCl_2$	2.0	108.4	112.5
	5.0	122.3	128.8
$CuCl_2$	2.0	112.2	123.5
	5.0	136.7	140.6
$FeCl_2$	2.0	93.5	90.8
	5.0	72.9	76.2
$MgCl_2$	2.0	110.0	121.1
	5.0	126.7	145.4
$MnCl_2$	2.0	55.8	46.3
	5.0	33.2	26.6
$NiCl_2$	2.0	112.6	109.4
	5.0	126.3	114.9
$ZnCl_2$	2.0	136.2	133.8
	5.0	148.5	152.3

*** These values represent % of the control and the means of triplicate experiments.**

Table 3. Effect of inhibitors on the purified camel plasma CPSOD1 and CPSOD2.

Reagent	Concentration (mM)	Inhibition (%)	
		CPSOD1	CPSOD2
Control	-----	0.0	0.0
Potassium cyanide (KCN)	2.0	66.2	59.7
	5.0	83.4	86.1
Hydrogen peroxide (H_2O_2)	2.0	32.8	35.9
	5.0	50.0	54.8
Sodium Azide (NaN_3)	2.0	7.1	4.4
	5.0	16.2	12.9
Sodium dodecyl sulphate (SDS)	2.0	27.1	24.3
	5.0	48.5	43.6
Ethylenediamine tetra acetic acid (EDTA)	2.0	2.1	3.7
	5.0	5.6	10.4
DL-Dithiothreitol (DTT)	2.0	16.9	29.3
	5.0	26.5	36.8
β-Mercaptoethanol	2.0	13.3	6.9
	5.0	21.8	14.4
Phenylmethylsulfonyl fluoride (PMSF)	2.0	42.7	49.0
	5.0	55.4	66.1
Iodoacetamide	2.0	8.3	11.6
	5.0	14.8	28.2

*** These values represent % of the control and the means of triplicate experiments.**

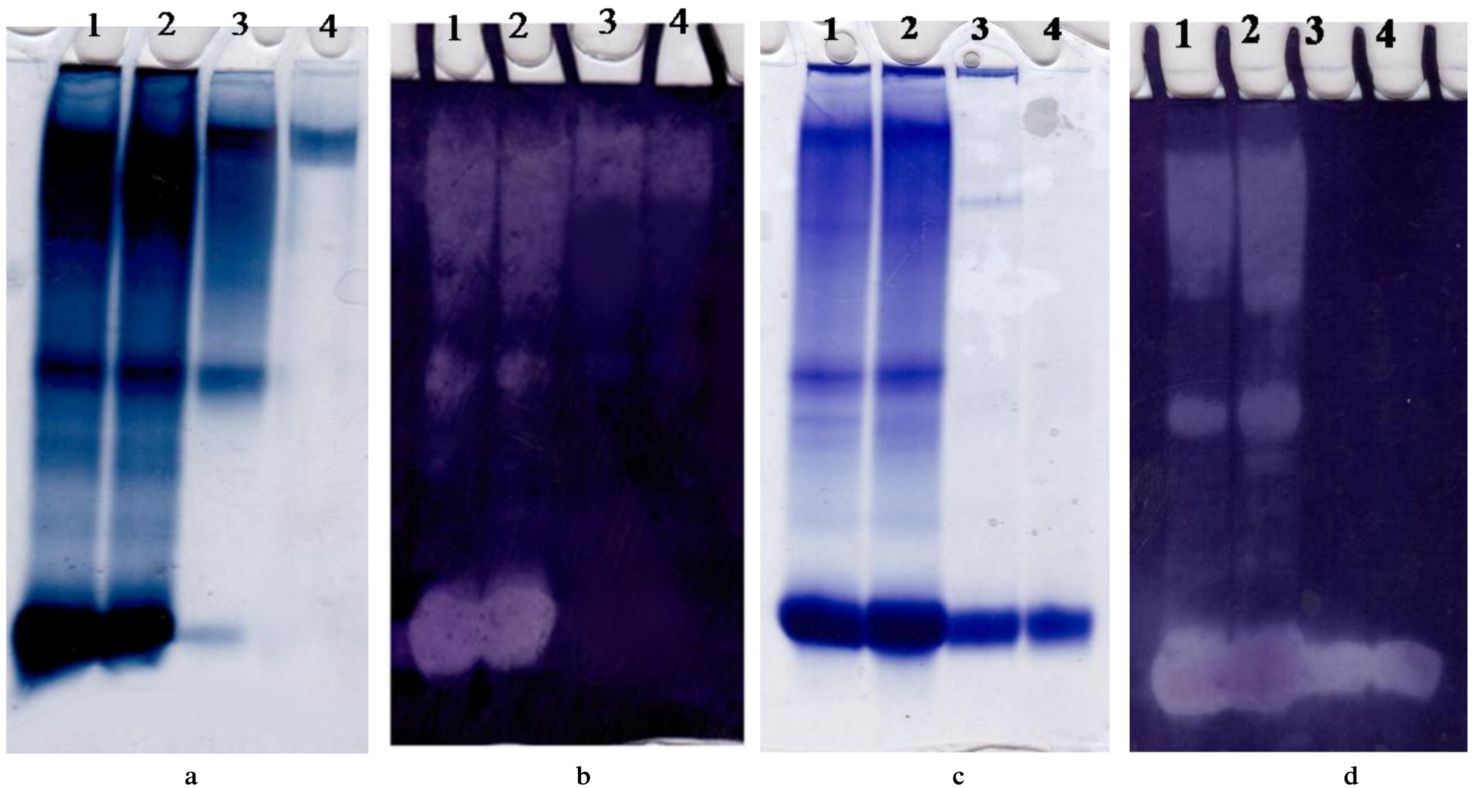

Figure 2. Electrophoretic analysis of protein and SOD isoenzyme patterns on 7% native PAGE. (a) CPSOD1 protein pattern, (b) CPSOD1 isoenzyme pattern, (c) CPSOD2 protein pattern and (d) CPSOD2 isoenzyme pattern: (1) Camel plasma, (2) Ammonium sulfate fraction, (3) DEAE-cellulose fraction and (4) Sephacryl S-300 fraction.

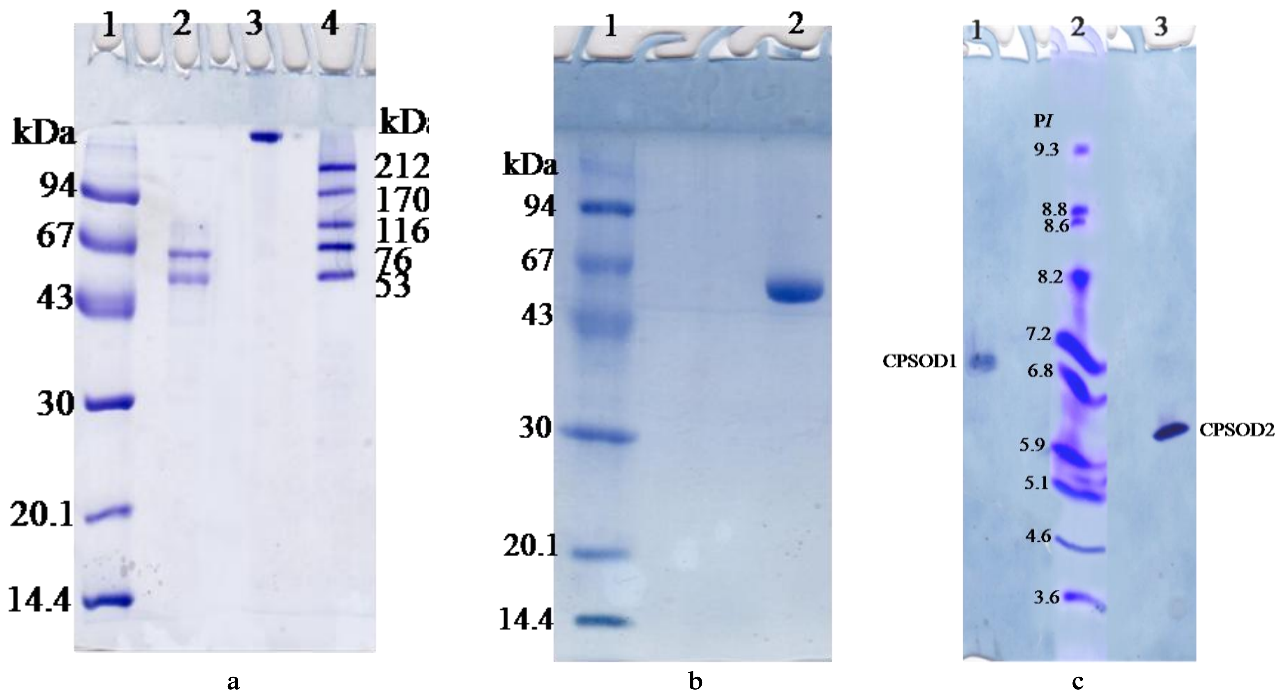

Figure 3. (a) Subunit molecular weight determination of CPSOD1 on 12 % SDS-PAGE: (1) Low molecular weight marker proteins, (2) denatured purified CPSOD1, (3) Native purified CPSOD1 and (4) High molecular weight marker proteins. (b) Subunit molecular weight determination of CPSOD2 on 12 % SDS-PAGE: (1) molecular weight marker proteins and (2) denatured purified CPSOD2. (c) Isoelectrofocusing PAGE: (1) CPSOD1, (2) Isoelectric point (*pI*) marker proteins and (3) CPSOD2.

Discussion

Superoxide dismutases are important enzymes with different industrial and medical applications. SOD was used as a drug in cerebral and hypertension diseases, treating inflammation and preventing the alcohol induced hangover. It proliferated in industry of cosmetics and supplementary products utilized in skin protection. SOD is also utilized as a preservative of transplanted organs, sperms, food stuffs and laundry ingredients [11, 22, 40, 41]. This study outlines an easy purification method for SOD isoenzymes from the camel plasma. The separation technique was achieved by ammonium sulfate deposition, anion exchange chromatography on DEAE-cellulose matrix and gel-filtration chromatography on Sephacryl S-300 resins. Comparable purification processes of SODs were stated like tea clone SOD [42], *Leishmania infantum* SOD [43], shrimp muscle tissue SOD [10], hens eggs SOD [44] and mangrove tree SOD [45]. In this study, the DEAE-cellulose chromatographical separation analyzed the SOD activity into two isoenzymes named CPSOD1 and CPSOD2. The Sephacryl S-300 resin analyzed each isoform as a single SOD activity peak and deduced their molecular weights as 240 ± 2.4 kDa for CPSOD1 and 60 ± 1.6 kDa for CPSOD2 (Fig. 1). The total yield of the two isoenzymes after size exclusion is 45.2% recovery. CPSOD1 and CPSOD2 were purified 26.2 and 24.4 folds over the crude plasma (Table 1). A big diversity of purification folds and recovery percentages for SOD were stated; from *Leishmania peruviana* 103.7folds and 70% yield, from *Leishmania amazonensis* 101.6 folds and 86% yield [46], from tea clone 51 folds with 3.77% yield [42], from yeast 28.5 folds and 53% yield [47] and from black soybean 15.4 folds and 29.4% yield [48].

The two camel plasma SOD isoenzymes showed the appearance of one protein band that matched the SOD activity band affirmed the purity of CPSOD1 and CPSOD2 preparations (Figure 2). Comparing the subunit molecular weights of CPSOD1 and CPSOD2 with their native intact protein masses obtained from the gel-filtration columns revealed that CPSOD1 is heterotetramer protein consisted of four polypeptides while CPSOD2 is monomer protein (Fig. 3). Various SODs were stated with different molecular weights like; 28 kDa garlic SOD [49], 31 kDa black soybean SOD [48], 33 kDa hens eggs SOD [44], 31 kDa mangrove tree SOD [45], 34.8 kDa pumpkin pulp SOD [50], 66.1 kDa oriental river prawn SOD [51] and 40 kDa, 67 kDa and 90 kDa camel tick larvae SODs [52]. High molecular weights tetrameric SODs were stated in bacteria ranging from 110 to 140 kDa [53] 125 kDa yeast SOD [54] and 169 kDa tea leaves SOD [42]. The purified camel plasma CPSOD1 and CPSOD2 isoenzymes have isoelectric point

(*p*I) values of 6.9 and 6.2 (Fig. 3b). Analogous *p*I values were stated as bacteria SOD isoenzymes *p*I values of 5.9, 6.15, 6.35, 6.6 and 7.5 [54], fish SODs isoenzymes *p*Is of 5.9, 6.0 and 6.2 [55], black soybean SOD *p*I of 5.6 [48] and camel tick larvae SODs *p*Is of 8, 7.2 and 6.6 [52].

$CoCl_2$, $CuCl_2$, $MgCl_2$, $NiCl_2$ and $ZnCl_2$ increased the activity of both preparations CPSOD1 and CPSOD2 while $CaCl_2$, $FeCl_2$ and $MnCl_2$ decreased it (Table 2) affirming the involvement of Cu^{+2} and Zn^{+2} for CPSOD1 and CPSOD2 action. $ZnCl_2$ is involved for SOD of bacteria [56] and shrimp muscle tissue [10]. Recognition of various SODs types is depending on the characteristic inhibition by certain chemical compounds. The SODs classes can be specified by their sensitivity to potassium cyanide and hydrogen peroxide inhibition [9, 10, 48]. Also, existence of Cu^{+2} and Zn^{+2} in the SOD active site was affirmed by KCN and H_2O_2 inhibition [54]. It was stated that, CuZnSOD isoenzymes are sensitive to both KCN and H_2O_2 [58-60]. Here, KCN and H_2O_2 inhibited the activity of CPSOD1 and CPSOD2 potently (Table 3) affirming that both preparations are powerfully proposed to be copper-zinc SOD isoenzymes. Various SOD types were purified; Cu/Zn-SOD from eggs hens [44], *Kluyveromyces marxianus* yeast [47], black soybean [48], pumpkin [50], haemolymph of oriental river prawn [51], camel tick larvae [52], Japanese flounder [55] and bay scallop [12]. Mn-SOD was purified from tea leaves [42], muscle tissue of the shrimp [10] and camel tick larvae [52]. Also, Fe-SOD was purified from *Leishmania peruviana* and *Leishmania amazonensis* [43]. β-Mercaptoethanol and dithiothreitol inhibited both CPSOD1 and CPSOD2 isoenzymes activity affirming involvement of thiol groups in their structure and action as in case of Radix lethospermi seed SOD [61]. PMSF inhibited both isoenzymes activity suggesting the presence of serine residue in both isoenzymes active sites. In conclusion, this is the first study reporting SOD from camel plasma. It provides an appropriate purification method for two Cu/Zn-SODs that could be fundamental for averting oxidative stress. These SOD isoenzymes may be used as important agents in cosmetics and supplementary products industry and as candidate drugs for treating of various diseases.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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