

Purification and Characterization of a Cu,Zn-SOD from Garlic (*Allium sativum* L.). Antioxidant Effect on Tumoral Cell Lines

Imen Hadji · M. Nejib Marzouki · Daniela Ferraro · Elena Fasano · Hafedh Majdoub · Giovanni Pani · Ferid Limam

Published online: 12 June 2007
© Humana Press Inc. 2007

Abstract Crude garlic extract contains one Mn-superoxide dismutase designated as SOD1 and two Cu,Zn superoxide dismutases as SOD2 and SOD3. The major isoform SOD2 was purified to homogeneity by Sephacryl S200-HR gel filtration, DEAE Sepharose ion exchange chromatography, and chromatofocusing using PBE 94. SOD2 was purified 82-fold with a specific activity of 4,960 U/mg protein. This enzyme was stable in a broad pH range from 5.0 to 10.0 and at various temperatures from 25 to 60°C. The native molecular mass of SOD2 estimated by high performance liquid chromatography on TSK gel G2000SW column was 39 kDa. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis showed a single band near 18 kDa, suggesting that native enzyme was homodimeric. The isoelectric point as determined by chromatofocusing was 5. Analysis of its N terminal amino acid sequence revealed high sequence homology with several other cytosolic Cu,Zn-SODs from plants. Exposure of cancer cell lines to garlic Cu,Zn-SOD2 led to a significant decrease in superoxide content with a concomitant rise in intracellular peroxides, indicating that the enzyme is active in mammalian cells and could, therefore, be used in pharmacological applications.

Keywords Cu,Zn-SOD · Antioxidant activity · Garlic · Purification · Tumoral cell lines · Reactive oxygen species

I. Hadji · M. N. Marzouki
National Institute of Applied Sciences and Technology, BP 676-1080, Tunis Cedex, Tunisia

D. Ferraro · E. Fasano · G. Pani
Institute of General Pathology, Catholic University of Rome, Rome, Italy

H. Majdoub
Faculty of Sciences of Sfax, Sfax, Tunisia

F. Limam (✉)
Center of Biotechnology, Borj-Cédria, BP 901-2050, Hammam-Lif, Tunisia
e-mail: ferid.limam@cbbc.rnrt.tn

Introduction

Superoxide dismutase enzymes EC1.15.1.1 (SOD) are ubiquitous metalloenzymes involved in the first step of the reactive oxygen species (ROS) scavenging system by catalyzing the dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen [1]. SOD can be classified into three major distinct types on the basis of their metal cofactor: the copper/zinc (Cu,Zn-SOD), the iron (Fe-SOD), and the manganese (Mn-SOD) isozymes. These enzymes can be identified according to their sensitivity to inhibitors. Cu,Zn-SOD are inhibited by potassium cyanide (KCN) and peroxide hydrogen (H_2O_2), Fe-SOD by KCN, whereas Mn-SOD are insensitive to both inhibitors [2]. Another SOD type containing Nickel as metal cofactor (Ni-SOD) has been described only in soil aerobic bacteria [3]. This type is structurally and immunologically distinct from other SOD classes [4].

SOD enzymes are located in different cell compartments. Fe-SOD are found in the chloroplast of several plant species, Mn-SOD in mitochondria [5], and Cu,Zn-SODs in cytosol, chloroplast, peroxisome [6] glyoxysomes [7], and even in extracellular space [8]. However, they are all nuclear-encoded and targeted to their respective subcellular compartments by amino terminal targeting sequence [9]. It is generally admitted that SOD activity is regulated by environmental [10] or xenobiotic stresses [11].

Cu,Zn-SOD, which is the most prevalent SOD in plant tissue, has been purified from a variety of plant sources including spinach [12], cabbage [13], maize [14], *Nicotiana glauca* [15], *Radix leithospermi* [16], *Olea europaea* [17]. Garlic (*Allium sativum* L.) is an extensively studied medicinal plant with a wide range of pharmacological effects, such as hypolipidemic, antihypertensive, antibacterial, and antitumoral [18]. Garlic extracts have also been shown to possess free radical scavenging and antioxidant activities [19]. Garlic extracts increase SOD [20], glutathione peroxidase and catalase (CAT) activities [21], and even prevent the decrease in hepatic SOD activities observed in diabetic rats [22]. However, the level of intrinsic antioxidant enzymes found in garlic has been only poorly investigated.

ROS accumulation is highly deleterious particularly in high-energy expenditure tissue as heart or brain where expression of antioxidant enzymes is low [23]. Dereglulation of SOD activity has been linked to a large number of human and experimental diseases including chronic neurodegenerative inflammation [24] and cancer [25]. Overexpression of targeted SOD and CAT or the use of SOD and CAT mimetics protects against Parkinson disease [26] and aging [27].

We investigated in the present paper the SOD antioxidant enzyme content of fresh garlic. A major Cu,Zn isoform has been purified at homogeneity, and biochemical characterization showed high physico-chemical stability. This purified enzyme has been used in preliminary experiments as an exogenous ROS scavenging activity in a model of cancer cell lines in culture.

Materials and Methods

Materials

Fresh garlic bulbs (*A. sativum* L) were purchased from local market.

Preparation of Crude Extract

Garlic bulbs were peeled, weighed, minced with an electric blender, powdered in a mortar with liquid nitrogen, and homogenized in buffer A: 25 mM Tris-HCl pH 7.5 containing 2 mM ethylenediamine tetraacetic acid and 0.1 mM phenylmethylsulfonyl fluoride. The

homogenate was filtered and centrifuged at $10,000\times g$ for 30 min at 4°C . Supernatant was brought to 80% saturation with ammonium sulfate and stirred overnight at 4°C . The precipitate was collected by centrifugation at $10,000\times g$ for 25 min at 4°C and dissolved in buffer A. The solution was dialyzed three times against 1 l of the same buffer at 4°C . The obtained crude extract was stored at -20°C until use.

Protein Determination

Protein content was determined using a Bio-Rad protein assay with bovine serum albumin as standard [28].

Enzyme Assay

SOD activity was determined using modified epinephrine assay [29]. At alkaline pH, superoxide anion O_2^- causes the oxidation of epinephrine to adrenochrome; SOD competes with this reaction by decreasing the adrenochrome formation. One SOD unit is defined as the amount of extract that inhibits the rate of adrenochrome formation by 50%. Enzyme extract was added in 2-ml reaction mixture containing 10 μl bovine catalase (0.4 U/ μl), 20 μl epinephrine (5 mg/ml), and 62.5 mM $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (pH 10.2). Changes in the absorbance were recorded with a spectrophotometer (Beckman DU 640 B) at 480 nm.

Enzyme Purification

All steps were carried out at 4°C except for high performance liquid chromatography (HPLC) analysis performed at 20°C .

Gel Filtration Chromatography

Crude extract was filtered on a column (100 \times 2.2 cm) of Sephacryl S200-HR gel (Pharmacia, Uppsala, Sweden) previously equilibrated with buffer A. The flow rate was 10 ml/h, and fractions of 3 ml were collected. Fractions 50 to 60 corresponding to peak B were pooled and subjected to ion exchange chromatography.

DEAE Sepharose Chromatography

Pooled fractions of peak B were further submitted to DEAE Sepharose CL6B column (1.2 \times 9 cm; Pharmacia) previously equilibrated in buffer A. SOD2 activity bound to DEAE Sepharose was eluted with a linear gradient of NaCl (0 to 250 mM) in buffer A at a flow rate of 10 ml/h. Fractions of 2.5 ml were collected.

Chromatofocusing Column

The active fractions from the DEAE step were pooled and dialyzed overnight against starting buffer: 25 mM imidazole-HCl pH 7.4, applied onto a chromatofocusing column (1 \times 50 cm) which was packed with a gel exchanger PBE94 and equilibrated with starting buffer. SOD2 activity was eluted with a pH gradient from 7 to 4, formed with eluent buffer pH 4 composed of 10% (v/v) polybuffer 74 (Pharmacia) adjusted with HCl according to the manufacturer's instructions. Fractions of 1.2 ml were collected at a flow rate of 15 ml/h and protein's absorbance monitored at 280 nm. After pH adjustment, SOD activity was measured.

Gel Filtration on TSK Gel G2000SW HPLC Column

HPLC gel filtration was used to remove ampholytes from SOD2 extract. Active fractions from chromatofocusing step were pooled and concentrated by ultrafiltration (Amicon PM 10 membrane) and applied onto TSK gel G2000SW column (60 cm×7.5 mm) pre-equilibrated in 50 mM phosphate buffer pH 7 containing 100 mM NaCl. Elution was performed at a flow rate of 0.8 ml/min and protein's absorbance recorded at 280 nm. SOD activity was tested for all collected peaks. Active fraction was concentrated by ultrafiltration on Amicon PM 10 membrane and stored at -20°C .

Effects of Temperature and pH on SOD2 Stability

Thermostability of SOD2 was studied by pre-incubating enzyme samples (8 U corresponding to 3.2 μg of purified enzyme) at various temperatures from 25 to 80°C in 50 mM Tris-HCl buffer pH 7.5 during 30 min. Residual activity was determined under standard conditions.

SOD2 stability towards pH was also examined in the following 50 mM buffers with pH values ranging from 3.0 to 10.0: glycine-HCl (pH 3.0); sodium acetate (pH 4.0–5.0), potassium phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0), and $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (pH 10.0). Enzyme samples (8 U corresponding to 3.2 μg of purified enzyme) were pre-incubated in the above-cited buffers at 4°C for 24 h before adding the substrate. After pH adjustment, SOD activity was assayed under standard conditions.

Stability of SOD2 Toward Organic Solvents

The effects of acetone and ethanol on SOD2 activity were also investigated. One volume of purified enzyme (8 U corresponding to 3.2 μg protein) in 50 mM Tris-HCl buffer pH 7.5 was mixed with four volumes of acetone or nine volumes of ethanol. Incubation of both solutions was carried out at -20°C during 2 h. After centrifugation, pellet was dried and dissolved in 50 mM Tris-HCl buffer pH 7.5. Residual activity was determined as previously described.

Effect of Storage Duration on SOD2 Stability

Purified SOD2 extract was stored at 25°C during 5 weeks. Storage stability of SOD2 enzyme was evaluated by measuring residual activity once a week under standard conditions.

Molecular Mass Determination

The molecular weight of native SOD2 was determined by HPLC on a TSK SW2000 gel filtration column in 50 mM phosphate buffer pH 7 containing 100 mM NaCl. The column was calibrated with the following standard markers (Bio-Rad): thyroglobulin (670 kDa), bovine δ -globulin (158 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The molecular mass of SOD2 subunits was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) carried out in a discontinuous system according to Laemmli [30]. Dual slab unit (Model DSG-125 apparatus) was used with 5%

concentrating and 15% resolving gels. Subunit size was estimated from the curve $\text{Log molecular weight} = f(d)$ using low range SDS-PAGE markers from Bio-Rad: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Proteins were stained with coomassie brilliant blue G250.

Native Polyacrylamide Gel Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was performed at 4°C using a 4% concentrating and 10% resolving gels in 125 mM Tris–HCl buffer pH 8.3. SOD activity was revealed on gel according to [31]. For identification of various SOD isoforms, gels were treated with inhibitors as KCN (3 mM) or H₂O₂ (5 mM) in pre-equilibrating buffer.

N Terminal Sequencing

The amino acid terminal sequence of the purified SOD2 protein was determined by sequential degradation using the Edman method (model 610 A protein/peptide sequencer coupled with the on line model A PTH analyzer, Applied Biosystems, Foster City, CA). The native enzyme was blotted onto a polyvinylidene difluoride membrane using microorb system. The membrane containing proteins was washed with 0.1% trifluoroacetic acid, ethanol, and dried. About 400 pmol of purified protein was sequenced and analysis was carried out for 29 Edman degradation steps.

Culture of Cell Lines

Dulbecco's modified eagle medium (DMEM), glutamine, antibiotics, sodium pyruvate, amino acids, trypsin, HAM F12 medium, and fetal bovine serum (FBS) were purchased from Eurobio. Dichlorodihydrofluorescein diacetate (DCF-DA) was obtained from Molecular Probes. Nitrobluetetrazolium chloride (NBT) and isopropanol were purchased from Sigma. The murine melanoma B16 F0 cell line was maintained in DMEM supplemented with 1% glutamine, 2% penicillin–streptomycin, 1% pyruvate, 1% amino acids, and 10% FBS. The porcine aortic endothelial (PAE) cells were grown in HAM F12 medium supplemented with 10% FBS, 1% antibiotics, and 1% glutamine. Both cell lines were cultured at 37°C in a humidified 5% CO₂ atmosphere and harvested by trypsinization upon confluency.

Intracellular H₂O₂ Measurement

Intracellular content of peroxides (mainly hydrogen peroxide) was assessed by flow cytometry of cells loaded with the redox-sensitive fluorescent dye DCF-DA. Briefly cells were treated with purified SOD2 (8 U corresponding to 3.2 µg protein) during 24 h. After medium removal, the dye (1 µg/ml) was added to cell cultures and incubation conducted during 10 min. Cells were then trypsinized, transferred to new tubes, and subjected to flow cytometry [32].

Anion Superoxide Measurement

Adherent cells were washed with Hank's balanced salt solution (HBSS) (containing Ca⁺⁺ and Mg⁺⁺) and incubated with 2 µg/ml NBT in HBSS for 2 h until formation of visible blue aggregates. Cells were then lysed with isopropanol and NBT aggregates solubilized in

pyridine at 100°C [32]. Absorbance of the samples was recorded at 570 nm using a Bio-Rad multi-well plate reader.

Statistical Analysis

All experiments were carried out in triplicate. All data were expressed by mean values \pm SEM. Statistical analysis was carried out using student's test. *P* value less than 0.05 was considered significant.

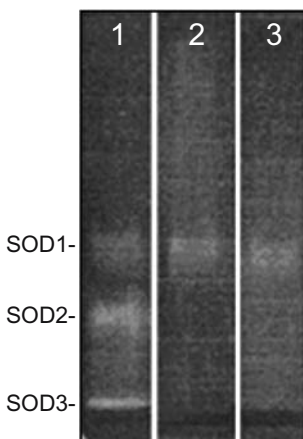
Results and Discussion

Analysis of Garlic SOD Isoforms

Crude extract from garlic bulbs exhibited SOD activity of 60 U/mg protein. Stajner et al. [33] found SOD activity of 52 U/mg protein in fresh garlic leaves. These values ranged from 2.50 U/mg protein in *A. ursinum* to 77 U/mg protein in *A. schenoprasum*. SOD isoforms were revealed by activity staining after native PAGE (Fig. 1). At least three distinct active bands were detected. They were designated as SOD1, SOD2, and SOD3 in the order of their migration towards the anode. SOD1, which is insensitive to KCN and H_2O_2 , was identified as a Mn-SOD. SOD2 and SOD3, which were inhibited by both KCN and H_2O_2 , were identified as Cu,Zn isoforms. However, we did not find Fe-SOD isoform probably because of the non-chloroplastic nature of garlic cloves. Garlic extract expressed mainly Cu,Zn isoforms like various organisms as *Pinus sylvestris* [34] or *Arabidopsis thaliana* [35]. In *P. sylvestris*, two classes of SODs have been reported, four Cu,Zn-SODs, and five Mn-SODs. The Cu,Zn-SODs were predominantly found in the cytosol, chloroplasts, and in the mitochondrial matrix. *A. thaliana* contained three types of SODs. The Cu,Zn-SODs were located in the cytosol and in the plastids. Fe-SOD was found in the chloroplasts and Mn-SOD in the mitochondrial matrix.

From this study, one can deduce that Cu,Zn-SODs are (1) the most abundant isoforms and that (2) the number of SOD isoforms depends on both the organ source (leaves, seeds, or bulbs...) and the extraction method. In this respect, it has been reported that the number of SOD isoforms found in Scots pine needles was increased by nine when acetone was used [36].

Fig. 1 Identification of SOD isoenzymes from garlic extract. Crude garlic extract was subjected to native PAGE, which was stained for SOD activity; 1 Control, no inhibitor 2 with 3 mM KCN, 3 with 5 mM H_2O_2 . Thirty-three micrograms of SOD protein was loaded on gels



Enzyme Purification

Table 1 summarizes all the steps (carried out at 4°C) involved in SOD2 enzyme purification. Soluble proteins from garlic homogenates were concentrated by ammonium sulfate precipitation at 80% saturation. SOD isoenzymes were fractionated by gel filtration chromatography on Sephacryl S200-HR column. Three peaks called (A, B, and C) displaying SOD activity were separated. Peaks A and C contained, respectively, mainly SOD1 and SOD3 isoenzymes (Fig. 2). Peak B, containing mainly SOD2 in which 54% of overall SOD activity was recovered, exhibited a specific activity of 236 U/mg protein. SOD2 was efficiently separated from other SOD isoforms by ion exchange chromatography on DEAE Sepharose column. This enzyme was eluted with a linear gradient of NaCl at 100 mM (Fig. 3) and displayed a specific activity of 415 U/mg protein. Further purification was achieved by chromatofocusing on gel exchanger PBE94 column, giving rise to one active peak (Fig. 4) with a specific activity of 2,566 U/mg protein. Ampholytes were removed from SOD2 protein solution by HPLC on TSK G2000SW gel filtration column. A single peak was recovered (Fig. 5a) with a specific activity of 4,960 U/mg protein.

Molecular Characteristics

Molecular mass of native SOD2 was 39 kDa as determined by HPLC gel filtration column (Fig. 5a). The purified enzyme was further characterized by native (Fig. 4, inset) and SDS PAGE (Fig. 5b). A single band was observed on both gels, indicating that SOD2 has been purified at homogeneity. Subunit size calculated on the basis of SOD2 mobility in SDS-PAGE was near 18 kDa, which suggests that SOD2 is composed of two non-covalently linked subunits of equal size. Molecular mass of SOD2 enzyme is close to several plants [37, 38] and even human [39] Cu,Zn-SODs which are also homodimeric with a molecular mass near 32 kDa.

As determined by chromatofocusing, isoelectric point (*pI*) of SOD2 protein was close to 5 (Fig. 4) which is similar to most cytosolic or chloroplastic Cu,Zn-SODs isolated so far. One Cu,Zn-SOD with *pI* of 6.5 was also identified in extracellular washing fluid from *P. sylvestris* L. needles [40]. On the other hand, one basic Cu,Zn,-SOD with high *pI* of 10 isolated from Scots pine has been located in Golgi apparatus of albuminous cells [41].

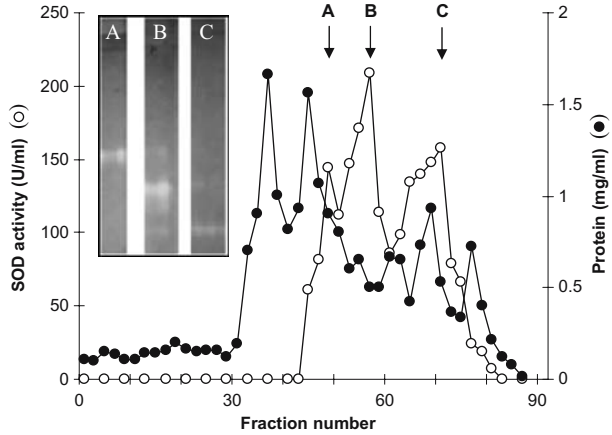
The N terminal amino acid sequence of SOD2 was determined until 29 residues (VKAVAVLNSAEGVKGHVFFFTQEGDGPTAVV). This sequence was compared with known sequences of cytosolic Cu,Zn-SODs from other plants using Blosum62 integrated matrix in BioEditT program. Garlic SOD2 exhibited strong homologies with several other Cu,Zn-SODs, reaching 90% with maize [42] and rice [43], 86% with populus [44] and rice [45], 83% with soybean [46], 79% with cabbage [13], and 76% with citrus [47], pepper

Table 1 Purification steps of SOD2 enzyme.

Steps	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification factor
(NH ₄) ₂ SO ₄ precipitation	9,480	157	60	100	1
Sephacryl S200-HR PeakB	5,094	22	236	54	4
DEAE CL-6B	1,260	3	415	13	7
Chromatofocusing PBE 94 ^a	647	0.25	2,566	7	42
TSK gel G2000-SW ^a	556	0.11	4,960	6	82

^aProteins were concentrated by ultrafiltration on Amicon PM 10 membrane before protein assay.

Fig. 2 Gel filtration chromatography on Sephacryl S-200HR column. The column was equilibrated in 25 mM Tris-HCl buffer (pH 7.5). Flow rate was 10 ml/h and collected fraction was 3 ml/tube. Protein content (filled circle) and SOD activity (open circle) were determined as described in text. *Inset:* SOD activity detection on native PAGE, fraction no. 48 (lane A); fraction no. 56 (lane B); fraction no. 70 (lane C)



[48], tomato [49], and spinach [50] (Table 2). Multiple sequence alignment using Clustal W showed that all plants Cu,Zn-SODs harbored methionine as the initial residue, except garlic and cabbage SODs. Cu,Zn-SODs from plants contained a conserved domain KAVAVL (positions 2–7), except in cabbage where A was substituted by G at position 3 and in spinach where A was replaced by V at position 5. Although all cytosolic Cu,Zn-SODs have V at position 1, spinach and cabbage showed, respectively, G and A at position 1. Conserved amino acids VKG and F which are also present in garlic SOD2 at positions 13–15 and 19, respectively, are essential for catalytic function of Cu,Zn-SOD enzymes [51]. High homology between cytosolic Cu,Zn-SODs from plants suggested that these enzymes have evolved from a single ancestral protein. Characterization of other garlic SOD isoforms and comparison with known sequences might ascertain this hypothesis. Although subcellular localization of SOD2 enzyme has not been done, all molecular characteristics of this garlic protein are close to cytosolic Cu,Zn-SODs isolated either from other plants or humans [37, 39], i.e., molecular mass, subunit composition, acidic *pI*, and high sequence homology.

Fig. 3 DEAE chromatography on Sepharose CL-6B column. Active pooled fractions of SOD2 (peak B) from gel filtration were fractionated on DEAE Sepharose column previously equilibrated in 25 mM Tris-HCl (pH 7.5) buffer. Flow rate was adjusted at 9 ml/h and collected volume was 2.5 ml/tube. Elution of proteins was carried out by linear gradient 0–250 mM NaCl (filled diamond). Protein content (filled circle) and SOD activity (open circle) were determined as described in text. *Inset:* SOD activity detection on native PAGE 1 crude extract 2, fraction no. 38 3, fraction no. 40, 4 fraction no. 42, 5 fraction no. 44, 6 fraction no. 46, 7 fraction no. 48

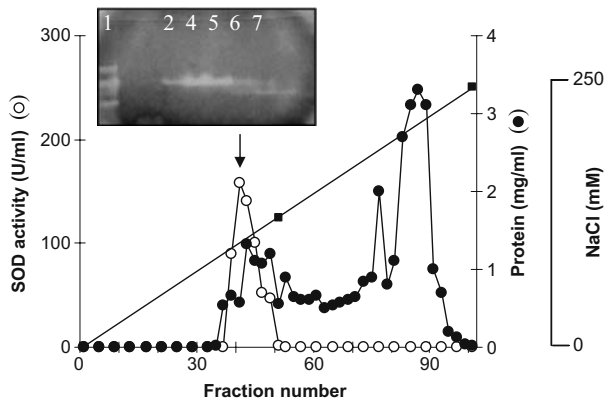
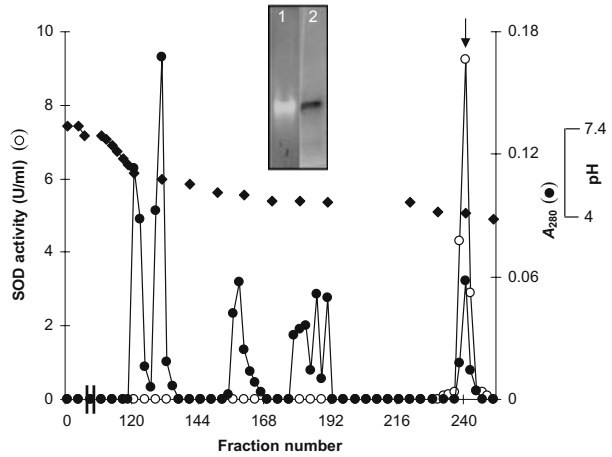


Fig. 4 Purification of SOD2 on chromatofocusing column. Active pooled fractions from DEAE Sepharose were fractionated on chromatofocusing column packed with a polybuffer gel exchanger PBE 94 equilibrated with starting buffer 25 mM Imidazole-HCl pH 7.4. Flow rate was adjusted to 15 ml/h, and fractions of 1.2 ml were collected. Elution of proteins was carried out by pH gradient (filled diamond) between 7 and 4. Protein content (filled circle) was monitored at 280 nm and SOD activity (open circle) was determined as described in text. *Inset:* Native PAGE analysis of purified SOD2 1 activity staining (0.8 μg), 2 protein staining with brilliant blue coomassie G-250 (8 μg)



Physicochemical Properties

Thermostability of SOD2 was studied over a wide range of temperature from 25 to 80°C in 50 mM Tris-HCl buffer pH 7.5 during 30 min (Fig. 6). Enzyme activity was unaltered till 50°C, decreased by 20% at 70°C, and was drastically inactivated at 80°C.

SOD2 stability was also examined over a wide pH range from 3 to 10 (Fig. 6). Enzyme activity was highly stable between pH 6 and 10. It decreased by 15% at pH 5, by 27% at pH 4, and more drastically (64%) at pH 3.

We also studied the effect of storage duration at 25 °C on SOD2 stability. SOD activity from *Pisum sativum* decreased by 24% after 1 month at 4 °C [52]. In this respect, it is generally recognized that Mn-SOD is more labile than Cu,Zn-SOD [53].

Acetone and ethanol treatment slightly affected SOD2 stability, as only 10% of SOD activity was lost after solvent treatment (data not shown). The use of such mild solvents allowed the purification of large quantities of Cu,Zn-SODs from garlic with a high yield

Fig. 5 Determination of native SOD2 molecular weight. **a** HPLC gel filtration profile of purified SOD2 (11 μg) TSK gel G2000SW column (60 cm× 7.5 mm) was used in 50 mM phosphate buffer pH 7 containing 100 mM NaCl. Flow rate was of 0.8 ml/min and protein’s absorbance recorded at 280 nm. **b** SDS-PAGE analysis 1 purified SOD2 from HPLC (2 μg), 2 standard molecular weights. Proteins were submitted to 15% polyacrylamide gels and stained with coomassie brilliant blue G-250

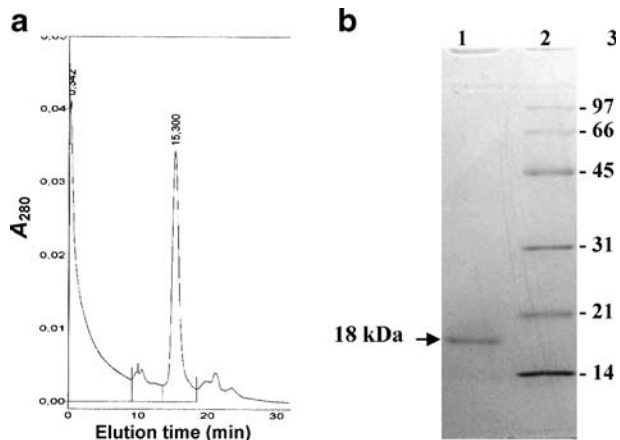


Table 2 Comparison of N-terminal amino acid sequence of garlic Cu,Zn-SOD2 with Cu,Zn-SODs from other species

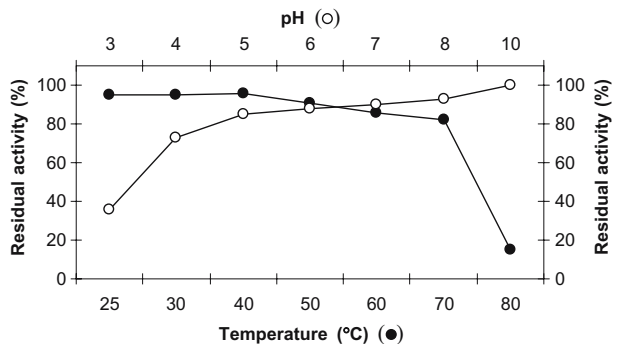
Source	NH ₂ -terminal sequence	Similarity (%)	References
	10 20 30		
		
Garlic SOD2	VKAVAVLNSAEGVKGHVFFFTQEGDGPTAV		
Maize SOD4	M. G. SD. TI.	90	[42]
Rice SODIII	M. A. S. TI. . S. T.	90	[43]
Populus SODI	M. S. . . . S. TI. T.	86	[44]
Rice SODII	M. A. S. TI. . S. S.	86	[45]
Soybean	M. G. S. . . . T. TI. N. . . T.	83	[46]
Cabbage	A. G. S. TI. . H. . N. A. T.	79	[13]
Citrus	M. GGT. T. S. T.	76	[47]
Pepper	M. S. S. C. S. TIL. S. D. . A. . T.	76	[48]
Tomato	M. S. . . S. TIL. . . D. AA. . T.	76	[49]
Spinach SODI	MG. . V. . S. S. . . S. T. Y. A. T.	76	[50]
Consensus	k v v l v g f g t v		

Sequence alignment was performed using Clustal W. For similarity comparison between garlic and other species, we used Blossum62-integrated matrix in BioEditT program.

extraction of 80% useful for physico-chemical studies. Another interesting feature is the putative use of garlic SOD2 enzyme as immobilized detecting system even in the presence of organic solvents.

Although not as stable as Cu,Zn-SOD isolated from Egyptian mummy [54], SOD2 protein from garlic exhibited high thermochemical stability. It is noteworthy that high thermal stability of Cu,Zn-SOD has been linked to metallation and intracellular disulfide formation of SOD polypeptide. For instance, the fully metallated and disulfide intact

Fig. 6 Effects of temperature (filled circle) and pH (open circle) on SOD2 stability. Purified SOD2 (8 U or 3.2 µg) was incubated at various temperatures in 50 mM Tris–HCl buffer pH 7.5 during 30 min. Residual activity was determined under standard conditions. For pH dependency, purified SOD2 (8 U or 3.2 µg) was incubated at 4°C for 24 h in various buffers with pH ranging from 3 to 10 as indicated in “Materials and Methods”. After pH adjustment, residual SOD activity was assayed under standard conditions

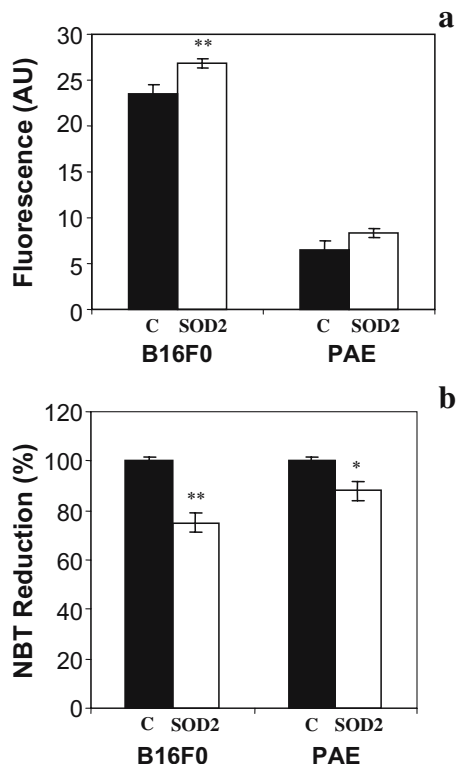


protein ($\text{Cu}_2\text{Zn}_2\text{-SOD}^{\text{S-S}}$) which is highly stable has a melting temperature (T_m) of 95°C [55], although the metal-free and disulfide-reduced SOD protein (SOD^{SH}) is more unstable and exhibits a T_m of 42°C [56]. Moreover, high stability of Cu,Zn-SOD has also been linked to net negative charge at physiological pH, and most mutations that decreased the net negative charge showed a propensity to lower SOD stability [57].

Use of SOD2 as Exogenous Antioxidant in Tumoral Cell Lines

B16F0 and PAE cell lines exposed to garlic SOD2 enzyme showed a significant increase in intracellular hydrogen peroxide levels, as revealed by cytofluometric analysis of cells loaded with the ROS-sensitive fluorescent dye DCF-DA (Fig. 7a). We also observed a decrease in superoxide content as measured by the NBT reduction method (Fig. 7b). These results showed that exogenous SOD2 enzyme is able to dismutate anion superoxide to hydrogen peroxide in both cell lines. SOD2 enzyme appeared as a more efficient antioxidant towards B16FO than PAE cells. This discrepancy could result from the difference in the “transformation state” between the two cell lines. However, it is not yet clear how SOD2 crosses the cell membrane. The enzyme could be taken up by cells and directly dismutate the intracellular pool of anion superoxide. Alternatively, the enzyme might act extracellularly (adsorbed on the cell surface) to eliminate anion superoxide released into the medium. Hydrogen peroxide might cross the cell membrane to oxidize intracellular dye. Additional experiments are needed to distinguish among these different ways.

Fig. 7 Effect of purified SOD2 on hydrogen peroxide generation (a) and anion superoxide dismutation (b) in eukaryotic cell lines. Melanoma B16FO and PAE cells were used in control conditions (filled square) or in the presence of $3.2\ \mu\text{g}$ of purified SOD2 (open square). All experiments were carried out in triplicate. Data were expressed by mean values \pm SEM. Statistical analysis was carried out using Student's *t* test. * $P < 0.05$ and ** $P < 0.01$ versus control (without SOD2)



Although preliminary, our data showed that purified SOD2 could be envisaged as a putative antioxidant and ROS scavenging activity. In this respect, Cu,Zn-SOD was shown to affect oxidative damage in amyotrophic lateral sclerosis [58]. The cloning of this Cu,Zn-SOD from garlic will allow the comparison with other Cu,Zn-SODs in which mutations were shown to affect diversely and unexpectedly the structure, activity, and native state stability of Cu,Zn-SOD [59].

Acknowledgments We thank Dr. Ezzedine Aouani for valuable discussion and critical reading of the manuscript. The authors acknowledge the support of Dr Tommaso Galeotti head of department of the Institute of General Pathology of Rome. The Tunisian Ministry of High Education, Scientific Research and Technology is gratefully acknowledged for supporting this research.

References

1. Fridovich, I. (1986). *Advances in Enzymology and Related Areas in Molecular Biology*, 58, 61–97.
2. Beyer, W. F., & Fridovich, I. (1987). *Analytical Biochemistry*, 161, 559–566.
3. Youn, H. D., Kim, E. J., Roe, J. H., Hah, Y. C., & Kang, S. O. (1996). *Biochemical Journal*, 358, 889–896.
4. Youn, H. D., Youn, H., Lee, J. W., Yim, Y. I., Lee, J. K., Hah, Y. C. et al. (1996). *Archives of Biochemistry and Biophysics*, 334, 341–348.
5. White, J. A., & Scandalios, J. G. (1987). *Biochimica Et Biophysica Acta*, 962, 16–25.
6. Bueno, P., Varela, J., Gimenez, G., & Del Rio, L. A. (1992). *Plant Physiology*, 108, 1151–1160.
7. Bueno, P., & Del Rio, L. A. (1992). *Plant Physiology*, 98, 331–336.
8. Alscher, R. G., Erturk, N., & Heath, L. S. (2002). *Journal of Experimental Botany*, 53, 1331–1341.
9. Scandalios, J. G. (1990). *Progress in Clinical and Biological Research*, 344, 515–544.
10. Elkahoui, S., Hernandez, J. A., Abdelly, C., Ghir, R., & Limam, F. (2005). *Plant Science*, 168, 607–613.
11. Scandalios, J. G. (2005). *Brazilian Journal of Medical and Biological Research*, 38, 995–1014.
12. Kitagawa, Y., Tsunawa, S., Tanaka, N., Katsuk, Y., & Asada, K. (1986). *Journal of Biochemistry*, 99, 1289–1298.
13. Steffens, G. J., Michelson, A. M., Otting, F., Puget, K., & Flohe, L. (1986). *Biological Chemistry*, 367, 1007–1016.
14. Cannon, R. E., & Scandalios, J. G. (1989). *Mol. Gen. Genet*, 219, 1–8.
15. Ragusa, S., Cambria, M. T., Scarpa, M., Di Paolo, M. L., Falconi, Rigo, A., et al. (2001). *Protein Expression and Purification*, 23, 261–269.
16. Haddad, N. I., & Yuan, Q. (2005). *Journal of Chromatography B*, 818, 123–131.
17. Butteroni, C., Afferni, C., Barletta, B., Iacovacci, P., Corinti, S., Brunetto, B. et al. (2005). *International Archives of Allergy and Immunology*, 137, 9–17.
18. Li, M., Ciu, J. R., Ye, Y., Min, J. M., Zhang, L. H., Wang, K. et al. (2002). *Carcinogenesis*, 23, 573–579.
19. Marzouki, S., Limam, F., Smaali, M. I., & Marzouki, M. N. (2005). *Applied Biochemistry and Biotechnology*, 127, 201–214.
20. Geng, Z., & Lau, B. H. S. (1997). *Phytotherapy Research*, 11, 54–56.
21. Wei, Z., & Lau, B. H. S. (1998). *Nutrition Research*, 18, 61–70.
22. Augusti, K. T., & Sheela, C. G. (1996). *Experientia*, 52, 115–119.
23. Simmons, R. A., Suponitsky-Kroyter, I., & Selak, M. A. (2005). *Journal of Biological Chemistry*, 280, 28785–28791.
24. Lynch, S. M., Boswell, S. A., & Colon, W. (2004). *Biochemistry*, 43, 16525–16531.
25. Ding, W. Q., Liu, B., Vaught, J. L., Yamauchi, H., & Lind, S. E. (2005). *Cancer Research*, 65, 3389–3395.
26. Peng, J., Stevenson, F. F., Doctrow, S. R., & Andersen, J. K. (2005). *Journal of Biological Chemistry*, 280, 29194–29198.
27. Morten, K. J., Ackrell, B. A., & Melov, S. (2006). *Journal of Biological Chemistry*, 281, 3354–3359.
28. Bradford, M. M. (1976). *Analytical Biochemistry*, 72, 241–254.
29. Misra, H. P., & Fridovich, I. (1972). *Journal of Biological Chemistry*, 247, 3170–3175.
30. Laemmli, U. K. (1970). *Nature*, 227, 680–685.
31. Donahue, J. L., Okpodu, C. M., Cramer, C. L., Grabau, E. A., & Alscher, R. G. (1997). *Plant Physiology*, 113, 249–257.
32. Pani, G., Bedogni, B., Anzevino, R., Colavitti, C., Palazzotti, B., Borrello, et al. (2000). *Cancer Research*, 60, 4654–4660.

33. Stajner, D., Milić, N., Canadanovic-Brunet, J., Kapor, A., Stajner, M., & Popovic, B. M. (2006). *Phytotherapy Research*, *20*, 581–584.
34. Wingsle, G., Gardestrom, P., Hallgren, J. E., & Karpinski, S. (1991). *Plant Physiology*, *95*, 21–28.
35. Kliebenstein, D. J., Dietrich, R. A., Martin, A. C., Last, R. L., & Dangl, J. L. (1999). *Molecular Plant–Microbe Interactions*, *12*, 1022–1026.
36. Schulz, H. (1986). *Biochemie und Physiologie der Pflanzen*, *181*, 241–256.
37. Tanaka, K., Stakio, S., Yamamoto, I., & Sotoh, T. (1996). *Plant and Cell Physiology*, *37*, 523–529.
38. Baum, J. A., & Scandalios, J. G. (1981). *Archives of Biochemistry and Biophysics*, *206*, 249–264.
39. Strange, R. W., Antonyuk, S., Hough, M. A., Doucette, P. A., Rodriguez, J. A., Hart, P. J. et al. (2003). *Journal of Molecular Biology*, *328*, 877–891.
40. Streller, S., & Wingsle, G. (1994). *Planta*, *182*, 195–201.
41. Karpinska, B., Karlsson, M., Schinkel, H., Streller, S., Suss, K. H., Melzer, M., et al. (2001). *Plant Physiology*, *126*, 1668–1677.
42. Kernodle, S. P., & Scandalios, J. G. (1996). *Genetics*, *144*, 317–328.
43. Kanematsu, S., & Asada, K. (1990). *Plant and Cell Physiology*, *31*, 383–390.
44. Akkapeddi, A. S., & Podila, G. K. (1999). *Plant Science*, *143*, 151–162.
45. Sakamoto, A., Ohsuga, H., & Tanaka, K. (1992). *Plant Molecular Biology*, *19*, 323–327.
46. Arahira, M., Nong, V. H., & Fukazawa, C. (1998). *Bioscience, Biotechnology, and Biochemistry*, *62*, 1018–1021.
47. Lin, C. T., Lin, M. T., & Lin, M. W. (2002). *Journal of Agricultural and Food Chemistry*, *50*, 7264–7270.
48. Kim, Y. K., Kwon, S. I., & An, C. S. (1997). *Molecular Cell*, *7*, 668–673.
49. Galun, E., & Ori, N. (1995). *Plant Physiology*, *108*, 1747–1749.
50. Sakamoto, A., Ohsuga, H., Wakaura, M., Mitsukawa, N., Hibino, T., Masumura, T. et al. (1990). *Nucleic Acids Research*, *18*, 4923.
51. Kanematsu, S., & Asada, K. (1991). *Free Radical Research Communications*, *12–13*, 383–390.
52. Sevilla, F., Lopez-Gorge, J., Gomez, M., & del Rio, L. A. (1980). *Planta*, *15*, 153–157.
53. Pedraza-Chaveri, J., Maldonado, P. D., Medina-Campos, O. N., Olivares-Corichi, I. M., Granados-Silvestre, M. A., Hernandez-Pando, R. et al. (2000). *Free Radical Research Communications*, *29*, 602–611.
54. Weser, U., Miesel, R., & Hartman, H. J. (1989). *Nature*, *341*, 696.
55. Rodriguez, J. A., Valentine, J. S., Eggers, D. K., Roe, J. A., Tiwari, A., Brown Jr, R. H. et al. (2002). *Journal of Biological Chemistry*, *277*, 15932–15937.
56. Rodriguez, J. A., Shaw, B. F., Durazo, A., Sohn, S. H., Doucette, P. A., Nersissian, A. M. et al. (2005). *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 10516–10521.
57. Calamai, M., Taddei, N., Stefani, M., Ramponi, G., & Chiti, F. (2003). *Biochemistry*, *42*, 15078–15083.
58. Lee, M., Hyun, D., Jenner, P., & Halliwell, B. (2001). *Journal of Neurochemistry*, *76*, 957–965.
59. Shaw, B. F., & Valentine, J. S. (2006). *Trends in Biochemical Sciences*, *32*, 78–85.