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Purification and some properties of Cu,Zn superoxide dismutase from *Radix lethospermi* seed, kind of Chinese traditional medicine

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Abstract

Copper–zinc superoxide dismutase (Cu,Zn SOD) has been extracted, purified and characterized from *Radix lethospermi* seed (RLS), a kind of Chinese traditional medicine. Before extraction, the lipid was removed by super critical fluid extraction (SCF). Partial protein fractionation in the crude extract was affected by using 50–75% $(NH_4)_2SO_2$. Subsequently, superoxide dismutase was fractionated by column chromatographies on DEAE-52, Sephadex G-200 and DEAE-52 again. Pure Cu,Zn SOD had a specific activity of 4843 U/mg protein and was purified 267.2-fold, with a yield of 23.55%. The purified enzyme has a molecular weight of about 30,500 ± 100 and is composed of two non-covalently joined equal subunits. Purity was confirmed by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), HPLC and mass spectroscopy. Amino acid content has been investigated. The enzyme was found to remain stable in the pH range 6.0–9.0 at 25 °C and up to 45 °C at pH 7.8 for a 30 min incubation period. RLS Cu,Zn SOD appeared to have significant thermal stability lower than other Cu,Zn SODs, as revealed by irreversible heat inactivation at 60 °C. The enzyme was not inhibited by DTT, NaN_3 and β-mercaptoethanol, but was inhibited by cyanide and hydrogen peroxide. Finally, in the presence of 2 mM ethylendiamine tetra acetic acid (EDTA) and sodium dodecyl sulphate (SDS), the enzyme showed approximately 18 and 34% activity loss.

Keywords: Superoxide dismutase; *Radix lethospermi* seed; Purification; pH stability; Thermal stability; Amino acid analysis

1. Introduction

The superoxide radicals ($O_2^{\bullet -}$) has been the subject of much research in recent years, due to its role as an agent of oxygen toxicity to cells [1]. Oxygen radicals are byproducts of many biological oxidation occurring in different subcellular locations [2]. In general, superoxide can be arisen when electrons are misdirected and denoted to oxygen. The cytotoxicity of this free radical is well documented [3].

Through the metal-catalyzed Haber–Weiss reaction, superoxide anions are further converted to reactive hydroxyl radicals ($OH^{\bullet}$), which attack and oxidize cell membrane, DNA and others [4].

The presence of superoxide dismutase (SOD) enzymes in aerobic organisms appears to be strictly related to their catalytic property in removing superoxide radicals, thus controlling oxidative risk in the cell [5]. This enzyme is a metalloprotein with a redox metal in its active site. Three distinct types of SOD have been reported. The Cu and Zn enzyme, the most studied form Cu,Zn SOD, is usually present in the cytosol of eukaryotes, fungi, mammalian cells and in higher plants. It was reported to also be present in mitochondria, chloroplast and glyoxysomes [6–9]. Cu,Zn SODs apparently have an evolutionary origin independent from Mn SOD and Fe SOD, which shows a close evolutionary relationship. Mn SODs are widely distributed among prokaryotes, and in eukaryotes are mainly localized in mitochondria. However, in higher plants Mn SODs also occur in different types of peroxisomes [10,11]. Fe SOD has been found in bacteria, blue-green

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algae and protozoa [12,13]. In higher plants Fe SODs are found in three families Ginkoaceae, Nymphaceae and Cruciferae [14] and the subcellular localization is nuclear.

Discrimination of the three classes of SODs is based on different inhibition or inactivation by selective chemicals. Cyanide inhibits Cu,Zn SOD [15], hydrogen peroxide irreversibly inhibits both, Fe SOD and Cu,Zn SOD [16,17], while azide inhibits these enzymes in the following order; Fe SOD > Mn SOD > Cu,Zn SOD [18]. SODs play an important role that they are able to survive in the presence of superoxide radicals. This enzyme has been shown to influence cancer, arteriosclerosis, cataract ischemia, hypertension, and age-depend immune deficiency disease [19].

Radix lethospermi is a kind of perennial Chinese herb. It has been shown to have activity as an anti-inflammatory, anticancer, and anticoagulant medicine [20]. The oil extract from RLS is used to cure burn injuries and radioactive rhinitis [20]. In this study, we describe the isolation, purification and characterization of Cu,Zn SOD from RLS, and compare it with the properties of other Cu,Zn SODs.

2. Materials and methods

R. lethospermi seed (RLS) was purchased from (Dalian city, PR China). The Experiments were performed by using 6-hydroxydopamine, Commassie brilliant blue G, Folin–Ciocalteau reagent, gel filtration and electrophoresis molecular weight marker kits, *o*-diethylaminoethyl (DEAE)-52 (Sigma Chemical Co., MO, USA), and Sephadex G-200 (Pharmacia Fine Chemicals, Upsala, Sweden). These and all other chemicals reagents used were of analytical grade.

Optical measurements were achieved with a spectrophotometer (UNICO UV-2100, Shanghai, China), electrophoresis (BIO-RAD, Protean XII, CA, USA), and atomic absorption (Varian, Spectr AA-300 Plus, CA, USA).

2.1. Super critical fluid extraction (SCF)

R. lethospermi seed was ground using an electrical blender machine. Seeds were treated with super critical fluid extraction processes [21], under extraction pressure, 30 MPa; separation pressure, 5.8 MPa; extraction temperature, 37.5 °C; and flux, 100 kg/h.

2.2. Isolation and purification of RLS Cu,Zn SOD

2.2.1. Extraction

Five hundred grams of *R. lethospermi* seed, which was obtained from SCF processes was sieved, using 40 μm sieve size. Mixed with 500 ml of 50 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM ethylendiamine tetra acetic acid (EDTA), using a mechanical stirrer for 8 h. The homogenate was filtered through six layers of cheesecloth and centrifuged at 12,000 × *g* for 30 min.

2.2.2. Ammonium sulphate fractionation

The homogenate was brought to 50% saturation with solid ammonium sulphate. After 2 h of stirring, the precipitate was removed by centrifugation at 13,000 × *g* for 15 min. The supernatant was brought to 75% saturation with ammonium sulphate, and stirred for 2 h, centrifuged at 10,000 × *g* for 20 min. The precipitate was dissolved in a 5 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 5 mM β-mercaptoethanol.

2.2.3. Ion exchange chromatography

The sample was dialyzed against 5 mM potassium phosphate buffer, pH 7.8, and applied to a column 1.5 cm × 25 cm of DEAE-52 equilibrated five times (column volume) with the same buffer, Cu,Zn SOD was eluted by washing with 500 ml of the same buffer with a gradient of 0–500 mM NaCl, at a flow rate of 3 ml h^{-1} cm^{-1}.

2.2.4. Chromatography on Sephadex G-200

SOD fractions from the previous step were put on a 2.6 cm × 86 cm column, of a Sephadex G-200 equilibrated with 5 mM potassium phosphate buffer, pH 7.8. Sample volume of 2 ml was applied and the column was run at a flow rate of 5 ml h^{-1} cm^{-1}. Samples containing SOD activity were collected.

2.2.5. Second step of ion exchange chromatography

Concentrated and dialyzed fractions of Cu,Zn SOD were applied to DEAE-52 column 1.5 cm × 25 cm equilibrated with 5 mM potassium phosphate buffer, pH 7.8. Activity was eluted by washing with 400 ml of the same buffer with a gradient of 0–250 mM NaCl. The column was run at a flow rate of 3 ml h^{-1} cm^{-1} and 2 ml fractions were collected. The samples containing Cu,Zn SOD activity were pooled, concentrated by ultrafiltration, and stored at −20 °C.

2.3. Electrophoretic procedure

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli using a vertical slab gel apparatus [22]. Electrophoresis was performed in 12% polyacrylamide gel with a current of 50 mA for approximately 4 h. Samples were solubilized at 100 °C for 5 min in 10 mM Tris–HCl, pH 7.6, containing 5% β-mercaptoethanol, 2.5% sodium dodecyl sulphate (SDS), and 1 mM EDTA. Protein bands were visualized in gels by staining with Commasie brilliant blue G-250 according to Reisner [23].

2.4. Determination of molecular weight (M_r and subunit size)

The native molecular weight of the purified Cu,Zn SOD was determined by gel filtration on a Sephadex G-100 column 2.6 cm × 86 cm (Pharmacia). The column was calibrated

with the following standard proteins: bovine serum albumin (66 kDa), ovalbomine (43 kDa), trypsine (20.1 kDa), hen white lysozyme (14.4 kDa). Subunit size was determined by SDS-PAGE after heating the proteins at 100 °C for 5 min in the presence of 2% SDS and 5% 2-mecaptoethanol. Electrophoresis was carried out on 12% acrylamide gels, using a Bio-Rad Mini-protein Π slab cell. Standards used were rabbit phosphorylase b (97.4 kDa), bovine serum albumine (66 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20.1 kDa), hen white lysozyme (14.4 kDa). Proteins were visualized by staining with Commasie brilliant blue G-250.

2.5. SOD activity assay

Superoxide dismutase activity was determined in triplet using pyrogallol autooxidation procedure by Marklund and Marklund [24]. The assay was run in triplicate and the mean values were obtained. One unit of activity is defined as the amounts of SOD required to inhibit the autooxidation of pyrogallol to 50%. Specific activity is defined as the units of activity per milligram of protein.

2.6. Protein determination

Protein determination between the purification steps was estimated spectrophotometrically using Follin–Ciocalteau reagent, by Lowry et al. [25]. Crystalline bovine serum albumin was used as a standard. Protein concentrations in the fractions from the column were determined from the absorbance value at 280 nm.

2.7. Metal analysis

Metal content of the purified enzyme was determined by the atomic absorption spectrophotometry with a Perkin-Elmer 503 apparatus equipped with heated graphite atomizer. The enzyme sample was dialyzed exhaustively in a metal-free dialysis membrane, first against 5 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, and then against this buffer lacking EDTA.

2.8. Mass spectrometric analysis and spectroscopic characterization

The purity and molecular weight of the purified RLS Cu,Zn SOD was analyzed by matrix-assisted laser desorption mass spectroscopy (MALDI-TOF, Reflex-III, Bruker, Germany). The samples (10 pmol) were dissolved in 0.1% (v/v) TFA and applied onto a target. Analysis was carried out in α-cyano-4-hydroxy-cinnamic acid. Solutions of trypsinogen (M_r = 23,982 Da) and bovine serum albumin (M_r = 66,431 Da) were used to calibrate the mass scale.

2.9. High-performance liquid chromatography (HPLC)

The purity of the RLS Cu,Zn SOD was analyzed by high-performance liquid chromatography (Waters 510, MA, USA), column used was Nucleosil RPC18 (250 mm × 10 mm, Macherey–Nagel (MN), Düren, Germany). The conditions used for HPLC separation were as follows: eluent A, 0.058% trifluoroacetic acid (TFA); eluent B, 80% acetonitrile in A. The gradient was run from 5 to 100% B within 60 min at a flow rate of 1 ml min^{-1}.

2.10. Amino acid analysis

Amino acid determination of the purified enzyme was performed on a waters HPLC model 510. Protein samples were dialyzed extensively against water, hydrolyzed for 24 h at 110 °C in 6 M HCl under vacuum. Half cystine and methionine were determined as cysteic acid and methionine sulphone, respectively, after performic acid oxidation [26]. Tryptophan was determined after hydrolysis in 6 M HCl plus 2% (v/v) thioglycolic acid under the same conditions. Acid-hydrolyzed samples were derivatized with phenylthiocyanate and the phenylthiocarbamil derivate were detected at 254 nm.

2.11. Effect of temperature and pH on SOD activity

Thermostability was studied by incubating the enzyme at different temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85 °C) in 5 mM potassium phosphate, pH 7.8. Aliquots required for the assays were removed at different time intervals and kept immediately on ice, for the determination of residual enzymatic activity.

The effect of pH on the stability of pure Cu,Zn SOD was examined by performing enzyme incubation during different times (0.5–24 h) in 50 mM buffers at different pH values (4–12) (pH 4.0–6.0, citric-citrate; pH 6.0–7.8, potassium phosphate; pH 7.8–8.8, Tris–HCl; pH 8.8–12, glycine–NaOH). The pH of the incubation mixtures was measured immediately after the addition of the enzyme and after different incubation times. The activity of the samples was assayed under standard conditions.

2.12. Effect of chemicals and inhibitions on SOD activity

The effect of some compounds known to be selective inhibitors on SOD activity, were investigated. The enzyme solution containing each compounds was incubated in 50 mM phosphate buffer, pH 7.8 and 25 °C for different time intervals. SOD control reactions were carried out in the presence of each concentration of inhibitor to ensure that the compound added along with enzyme did not interfere with the SOD assay. The generation rate of $O_2^{\bullet -}$ radicals ($\Delta A min^{-1}$) was kept constant by adding an appropriate dilution of XOD. The following inhibitors were tested, DTT, NaN_3, β-mercaptoethanol, SDS, EDTA, H_2O_2 and KCN.

3. Results

3.1. Purification of enzyme

R. lethospermi seed is one of the Chinese traditional medicines which contains about 23% of its dry weight as a fat, so the first process was the super critical fluid extraction, to separate the fat content from the seed and to prevent the interferences which may occur during the purification processes due to production of turbidity caused from a creamy fat layer [21].

The first purification step of crude extract was achieved by fractional precipitation of proteins using $(NH_4)_2SO_4$ and $(NH_4)_2SO_4$ methanol. We have optimized the ammonium sulphate method by two major steps, the first is to optimize the best ratio to remove the unwanted proteins. 30, 35, 40, 45, 50 and 60% (w/v) saturation with ammonium sulphate were used and after centrifugation at 15,000 × *g* for 20 min, the precipitate was removed. SOD activity and protein content were determined from supernatant. As can be seen from Fig. 1A. The highest SOD activity 27.53 U/mg was obtained with 50% ammonium sulphate precipitation, which was the best ratio to remove the unwanted proteins. The same experiments have been repeated to optimize the second step of ammonium sulphate precipitation, to collect the highest amount of SOD protein from crude extract. Experiments have been started from 50% (w/v) to a series of ratios, 60, 65, 70, 75, 80, 85 and 90% (w/v) saturation with ammonium sulphate. After centrifugation at 15,000 × *g* for 30 min, the precipitate was dissolved in the initial volume of 5 mM phosphate buffer, pH 7.8. SOD activity and protein content were determined. As can be seen from Fig. 1B, the highest SOD activity was obtained from 75% (w/v) saturation with ammonium sulphate. The above-mentioned precipitation procedures were also repeated for each concentration of $(NH_4)_2SO_4$ in the presence of methanol. The results achieved indicated that the activity yielded from the precipitation processes was not influenced by the addition of methanol.

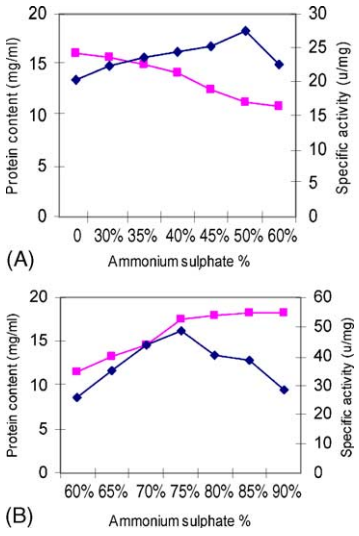

Fig. 1. Variation of specific activity and protein content with different ammonium sulphate %, (■) protein content, (♦) specific activity. (A) First step of optimization of protein precipitation by ammonium sulphate. (B) Second step of optimization of protein precipitation by ammonium sulphate.

After the ammonium sulphate precipitation processes, further purification of SOD was achieved with DEAE-52. The dialyzed solution was applied to a column 1.5 cm × 25 cm of DEAE-52, which had been equilibrated three times (column volume) with 5 mM potassium phosphate buffer, pH 7.8.

Cu,Zn SOD was adsorbed on the column, and washed with the same buffer. Then a linear gradient (0–500 mM, 500 ml) of NaCl in the same buffer. Fig. 2, gives the profile of SOD on the DEAE, ion exchanger column, revealing a single peak. The SOD was found in the fractions 25–31, Fig. 2. Seventy-one percent of SOD activity was recovered in the pooled fractions with a 35.8-fold increase in specific activity. The active fractions were concentrated by ultrafiltration with a PM-10 membrane and dialyzed against 5 mM potassium phosphate buffer, pH 7.8.

The dialyzed SOD was applied to a 2.6 cm × 86 cm Sephadex G-200, which was equilibrated and eluted with 5 mM potassium phosphate buffer, pH 7.8. Elutions containing SOD activity were collected in the fractions 33–43, Fig. 3. Enzyme was eluted from the column resulting in a 52-fold increase in the specific activity.

Further purification was achieved by applying the active fractions to a second DEAE-52 1.5 cm × 25 cm column. Enzyme was eluted with a linear gradient of NaCl (0–250 mM), in 5 mM potassium phosphate buffer, pH 7.8. The SOD activity was observed in the fractions 37–47, Fig. 4. Twenty-three percent of SOD activity was recovered in pooled fractions with a 267.2-fold increase in specific activity that was determined to be 4843.07 U/mg protein.

A summary of the purification procedures is presented in Table 1. The homogeneity of the purified Cu,Zn SOD was examined by SDS-PAGE (Fig. 5A), high-performance liquid chromatography, and mass spectrometry analysis. Purified SOD was injected on a Nucleosil RPC18 column (Fig. 6), via elution with a linear gradient for 60 min using the elution

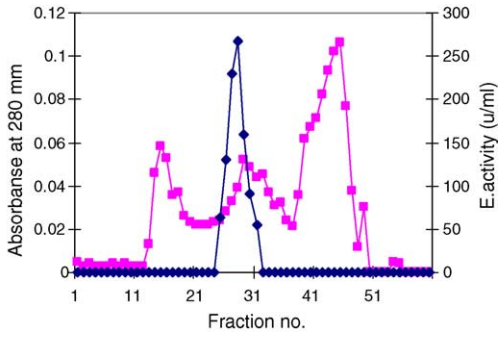

Fig. 2. Ion exchange chromatogram (DEAE-52 column, 1.5 cm × 25 cm) of the active fractions received from the ammonium sulphate precipitation. The column was equilibrated with 5 mM phosphate buffer, pH 7.8, and eluted with a linear NaCl gradient (0–0.5 M). Fractions of 3 ml were assayed for SOD activity (♦) and absorbance at 280 nm (■).

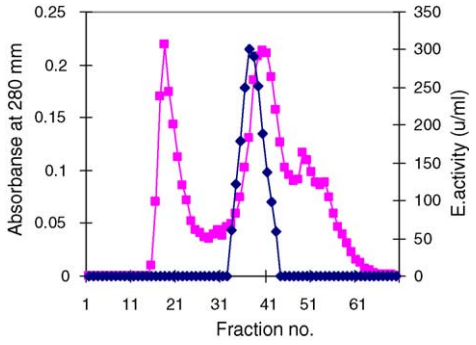

Fig. 3. Gel exclusion chromatography (G-200 column, 2.65 cm × 86 cm) of the dialyzed and concentrated fractions received from the ion exchange chromatography. The column was equilibrated and eluted with 5 mM phosphate buffer, pH 7.8. Fractions of 3 ml were assayed for SOD activity (♦) and absorbance at 280 nm (■).

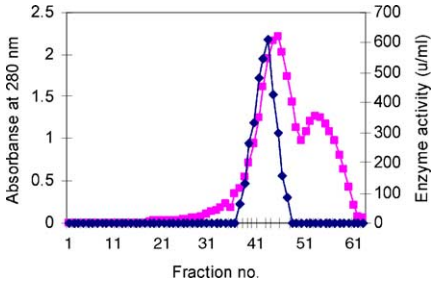

Fig. 4. Ion exchange chromatogram (DEAE-52 column, 1.5 cm × 25 cm) of the active fractions received from the gel chromatography. The column was equilibrated with 5 mM phosphate buffer, pH 7.8, and eluted with a linear NaCl gradient (0–0.25 M). Fractions of 3 ml were assayed for SOD activity (♦) and absorbance at 280 nm (■).

A (100% H_2O, 0.1% TFA) and B (80% CH_3CN, 0.058% TFA) at a flow rate of 1 ml min^{-1}. One peak is appeared in the chromatogram (Fig. 6), indicating that Cu,Zn SOD obtained from the last step of purification was homogeneous. The purity was above 95% as estimated from gel scanning and display as a single peak on mass spectrum (Fig. 7).

The molecular weight of RLS Cu,Zn SOD was determined through several methods. The rough size of the subunit determined by SDS-PAGE was 16 kDa (Fig. 5A and B). The exact molecular mass as measured by means of matrix-assisted laser desorption ionization mass spectrometry [MALDI-TOF], was determined to be 15.166 kDa for one subunit (Fig. 7). On gel filtration a single peak was obtained with an M_r of 32 kDa for a whole enzyme. These results indicate that the enzyme is composed of two non-covalently joined subunits of equal size.

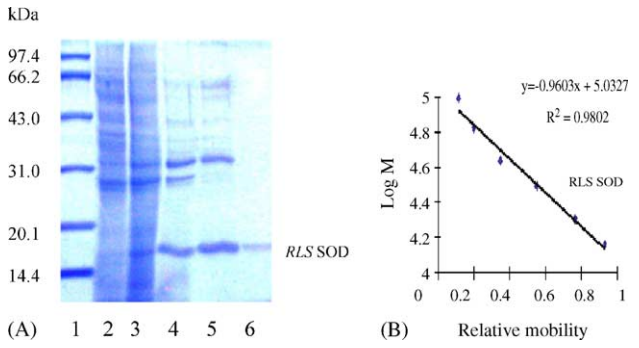

Fig. 5. (A) Polyacrylamide gel electrophoresis (12% gel) of purified RLS Cu,Zn SOD. Lane 1, standard mixture: (a) rabbit phosphorylase b (97.4 kDa); (b) bovine serum albumine (66.2 kDa); (c) rabbit actin (43 kDa); (d) bovine cabonic anhydrase-(31 kDa); (e) trypsin inhibitor (20.1 kDa); (f) hen white lysozyme (14.4 kDa). Lane 2, crude enzyme. Lane 3, ammonium sulphate precipitation step. Lane 4, DEAE-52 (first). Lane 5, Sephadex G-200. Lane 6. Purified RLS Cu,Zn SOD, from the second step of DEAE-52. (B) Determination of relative molecular weight of RLS Cu,Zn SOD by SDS-PAGE.

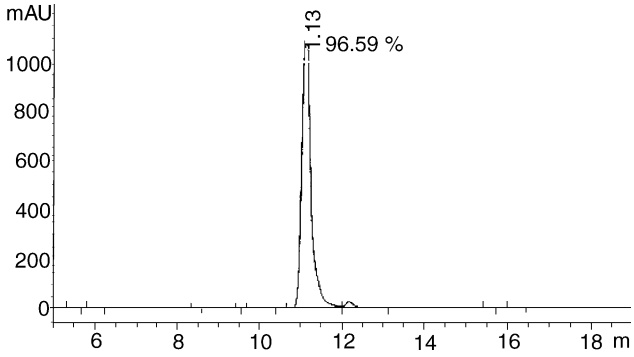

Fig. 6. HPLC chromatogram of the purified RLS Cu,Zn SOD.

3.2. Characterization of enzyme

3.2.1. Ultraviolet absorption spectrum

The ultraviolet absorption spectra of the purified Cu,Zn SOD is shown in Fig. 8. It has an absorption maximum at 269 nm, which is a characteristic peak due to phenylalanine. No absorption maximum was observed around 280 nm indicating a low content or lack of tyrosine and tryptophan. The spectra is typical of most Cu,Zn SOD (250–270 nm), and remarkably similar to those of rice, wheat,

Table 1
Purification steps of RLS SOD

Steps	Activity (U/ml)	Total activity (U)	Protein content (mg/ml)	Specific activity (U/mg)	Volume (ml)	Yield (%)	Purification fold
Crude	173.1	54,526.5	9.55	18.12	315	100	1
Ammonium sulphate (50–75%)	2,175.2	54,380	11.51	188.98	25	99.73	10.42
DEAE-52 + concentrate	6,239.25	38,995.31	8.98	694.79	6.25	71.51	38.34
Sephadex G-200 + concentrate	2,835.1	24,807.12	2.97	954.57	8.75	45.495	52.68
DEAE-52	125.92	12,843.84	0.026	4,843.07	102	23.55	267.27

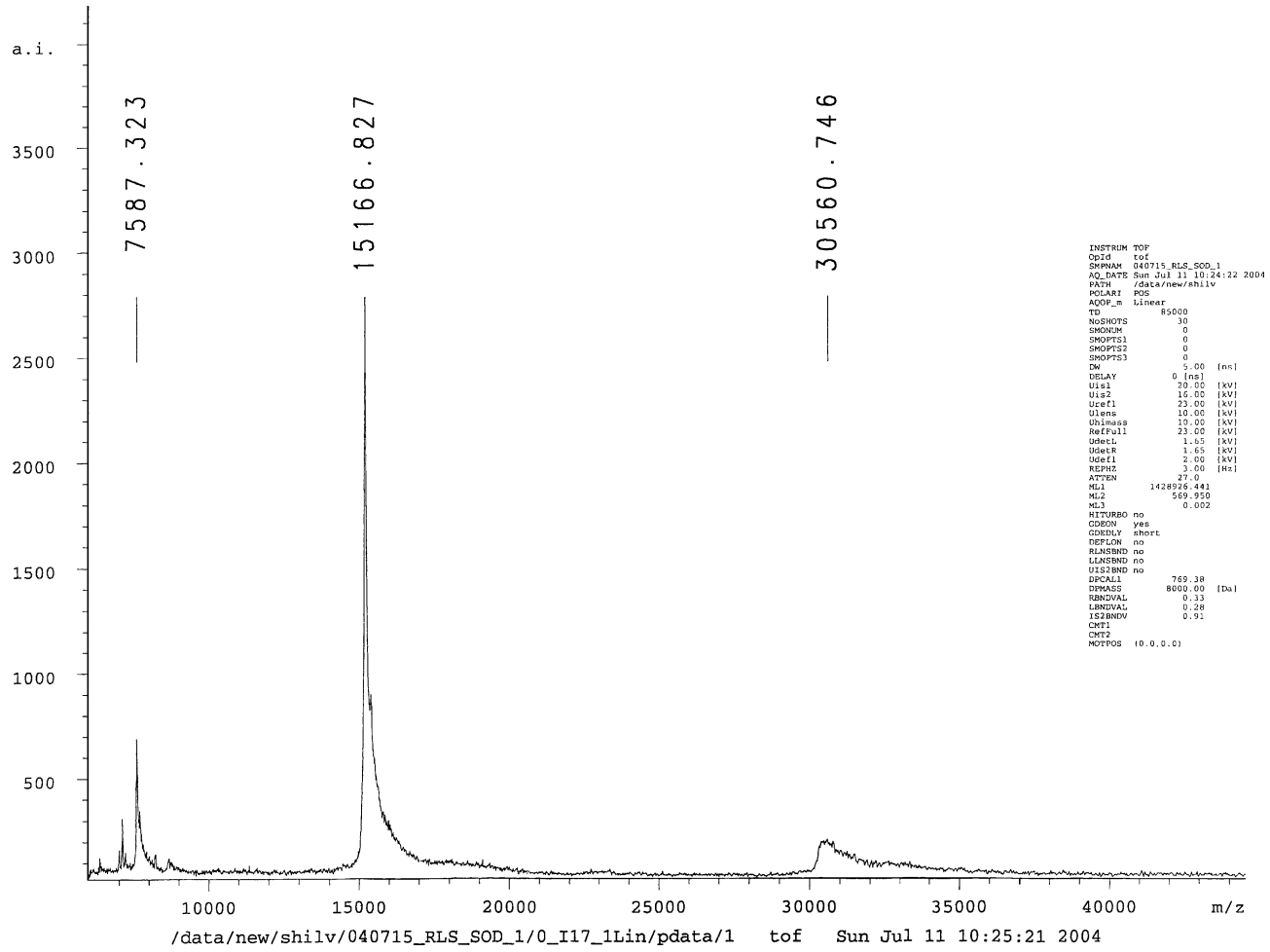

Fig. 7. Matrix-assisted laser desorption mass spectroscopy (MALDI-TOF) of the purified RLS Cu,Zn SOD. The sample (10 pm) was dissolved in 0.1% (v/v) TFA and applied on to a target. Analysis was carried out in α-cyano-4-hydroxy-cinnamic acid. Solution of trypsinogen (M_r = 23,982 Da) and bovine serum albumin (M_r = 66,431 Da) were used to calibrate the mass scale.

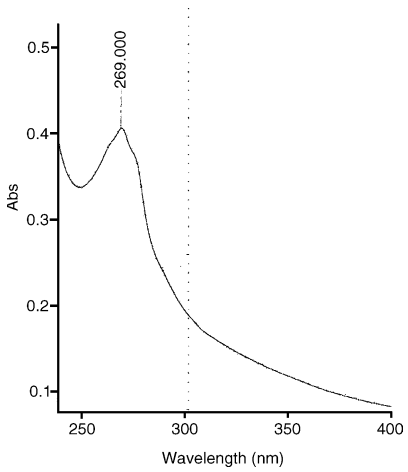

Fig. 8. Ultraviolet absorption spectrum of RLS Cu,Zn SOD. The enzyme, at a concentration of 250 μg/ml in 5 mM potassium phosphate, pH 7.8 was examined.

pea, spinach and maize cupro-zinc superoxide dismutases [26–30].

3.2.2. Metal content

The enzyme was assayed for copper, zinc, iron and manganese by atomic absorption spectroscopy after exhaustive dialysis to remove traces of contaminating metals. The purified SOD contained 0.93 ± 0.02 g atom Cu and 0.79 ± 0.01 g atom Zn per subunit. Fe and Mn concentration were below the detection level.

3.2.3. Amino acids analysis

Table 2 shows the results of RLS Cu,Zn SOD amino acids analysis with a comparison of amino acid analysis of rice and pea Cu,Zn SODs [26,28]. As expected from the absorption spectrum in ultraviolet region, low content of tyrosine and lack of tryptophan were observed, and phenylalanine was the only amino acid having absorption in the ultraviolet region. RLS Cu,Zn SOD consists of 330 amino acid residues. The

Table 2
Amino acid residues mol^{-1} Cu,Zn SOD

Amino acid	RLS Cu,Zn SOD	Rice Cu,Zn SOD [26]	Pea Cu,Zn SOD [28]
Aspartic	28	30	45
Threonine	20	26	30
Serine	18	24	14
Glutamic	36	32	19
Proline	10	18	14
Glycine	74	56	56
Alanine	44	24	21
Half-cystine	2	4	6
Valine	22	32	21
Methionine	2	2	0
Isoleucine	18	8	20
Leucine	20	26	21
Tyrosine	2	0	0
Phenylalanine	6	6	9
Lysine	12	12	10
Histidine	8	12	18
Arginine	8	8	6
Tryptophan	0	0	0
Total	330	320	310

amino acid analysis was different from the value given for the respective Cu,Zn SODs of different origin. RLS Cu,Zn SOD showed a lower content of threonine and proline than that of rice and pea Cu,Zn SODs, which were almost compensated by a higher content of histidine. RLS Cu,Zn SOD also was characterized by a higher content of glutamic, glycine and alanine, as compared to levels of these amino acids in rice and pea Cu,Zn SODs. Another view comparing amino acid composition reported the most dominant amino acid of RLS Cu,Zn SOD was glycine, followed by alanine, aspartic and valine. On the other hand, the most predominant amino acids of Cu,Zn SODs from other sources were glycine, followed by aspartic, valine and glutamic. RLS Cu,Zn SOD was in a good agreement with other sources in a low content of aromatic amino acids.

3.2.4. *The effect of pH and temperature on SOD activity*

The effect of pH on the purified enzyme is shown in Fig. 9. An activity profile of RLS Cu,Zn SOD was performed at

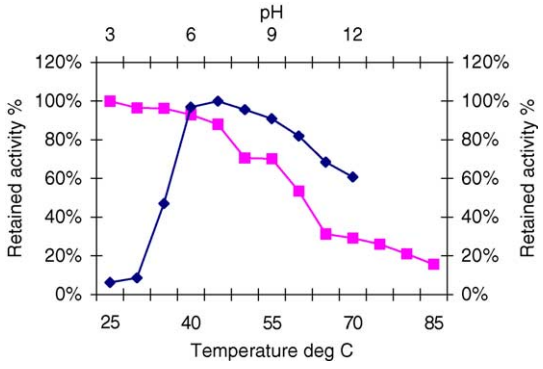

Fig. 9. Effect of temperature (■) at pH 7.8 and pH (♦) on SOD enzyme stability at 25 °C.

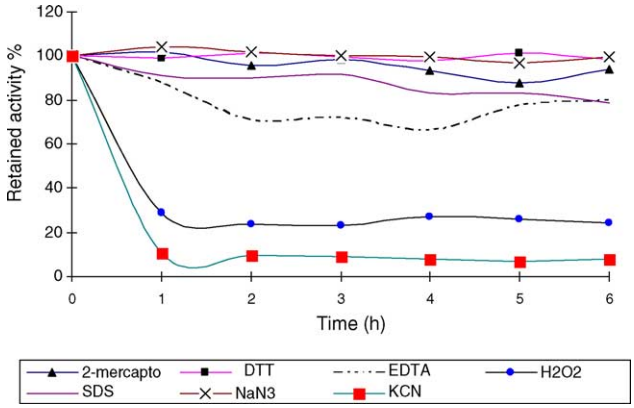

Fig. 10. Effect of 2 mM of some substances, as referred above on the RLS SOD activity, depend on incubation time (h), at pH 7.8.

25 °C and at different pH values. Optimum SOD activity was obtained in the buffer at pH 7.0. The enzyme retained more than 90% of its activity after incubation in the buffer at pH between 6 and 9, but it was inactivated by nearly 50% at pH 5 and it retained more than 60% of its activity till pH 12.

The thermostability of the enzyme was examined at pH 7.8. The enzyme had temperature optima with not much change in activity over the range from 25 to 45 °C (Fig. 9), but was inactivated rapidly at temperatures above this. The enzyme retained about 53.5% of its activity at 60 °C, while only 15% of its activity retained after incubation at 85 °C for 30 min.

3.2.5. *The effect of some substances on SOD activity*

Fig. 10 shows that DTT, β-mercaptoethanol and NaN_3 have no effect on the SOD stability. While, enzyme inhibition was approximately 34 and 18% with 2 mM of ethylendiamine tetra acetic acid and sodium dodecyl sulphate over 4 h. RLS Cu,Zn SOD was inhibited by 2 mM H_2O_2 to more than 70% after 1 h, and it was inhibited to more than 90% in the presence of 2 mM KCN for 1 h incubation time, as described previously, the Cu,Zn SODs are sensitive to both H_2O_2 and KCN [31].

4. Discussion

Super critical fluid extraction, was the first step in the isolation and purification of Cu,Zn SOD from *R. lethospermi* seed, to remove lipid content and other pigments, which was about 23% of dry weight. Protein separation was performed by precipitation with 50–75% ammonium sulphate, which obtained the maximum activity yield of SOD, then DEAE-52 ion exchange chromatography, Sephadex G-200 gel exclusion chromatography and DEAE-52 ion exchange. According to this procedure, the specific activity was found to be 4843 U/mg protein, corresponding to a 267.3-fold of purification with a yield of 23.55%. The homogeneity of the purified RLS Cu,Zn

SOD was confirmed by SDS-PAGE, HPLC and mass spectroscopy.

The native molecular weight of the enzyme was determined by gel exclusion chromatography on Sephadex G-100. By comparison with markers of known molecular weight, RLS Cu,Zn SOD appeared at a position corresponding to a molecular weight of 32,000 ± 200 Da. SDS-PAGE of Cu,Zn SOD showed that the enzyme, in the presence and absence of β-mercaptoethanol dissociated into a single band (data not shown), that by comparison with standards, had an M_r value of 16,000 ± 100 Da (Fig. 5). Subunit molecular weight measured by mass spectra analysis (MALDI-TOF), appeared to be 15,166 Da. Thus, from these results it is suggested that the investigated enzyme is a dimmer made of identical subunits, bound by non-covalent forces. In its native and subunit molecular weight, the RLS Cu,Zn SOD is characteristic of most Cu,Zn SODs [32], and resembles many plant Cu,Zn SODs that have been characterized thus far [30,33–37].

The enzyme has 0.93 ± 0.02 g atom Cu and 0.79 ± 0.01 g atom Zn per subunit. The Cu atom is important in SOD activity due to the positive channel that exists around it, which attracts the negative protein surface charge. The enzyme-catalyzed dismutation by Cu,Zn SOD appears to proceed by subsequent reduction and oxidation of a Cu ion acting as an electron carrier, while the Zn atom, which is burried through the protein structure, plays a structure role only for the stability of protein [38].

Amino acid composition showed some variation depending on the enzyme sources, the absence of or very low tyrosine, tryptophan and methionine content was observed and was reflected in the absorption spectrum in the ultraviolet region. The RLS Cu,Zn SOD lacked tryptophan, but contained tyrosine and methionine.

R. lethospermi seed Cu,Zn SOD was inhibited by 2 mM KCN and H_2O_2. This result was reasonable, since Cu,Zn SODs are generally known to be cyanide and hydrogen peroxide sensitive enzyme [31]. It was inhibited to approximately 90 and 70% after incubated in 2 mM KCN and H_2O_2, for 1 h respectively.

The reduction of the enzyme-bound Cu^{+2} to Cu^{+1} by H_2O_2, followed by a fenton reaction of Cu^{+1} with additional H_2O_2 to form Cu^{+2}–OH could inactivate the Cu,Zn SOD. This might attack an adjacent histidine amino acid residue of the enzyme [39]. Although RLS SOD activity was unaffected by 2 mM of DTT and β-mercaptoethanol indicated that cysteine, serine and threonine residues have no important role in the SOD activity.

Generally, Cu,Zn SODs are known to be very stable three-dimensional structure and are known to show a high thermal stability [40,41]. It is suggested that hydrophobic regions of the protein might play an especially important role in its thermal stability [42]. However, at 60 °C RLS Cu,Zn SOD lost 50% of its activity and the process was not reversible. It is worth noting that RLS Cu,Zn SOD has highly unstable thermal properties, unlike cupro-zinc superoxide dismutases which have been widely reported to be resistant to thermal inactivation [40,41], except for the Cu,Zn SOD discovered in *Escherichia coli* [43].

The optimum pH value for RLS Cu,Zn SOD was 7.0, and the enzyme retained most of its activity in the range of 6.0–10.0. Thus, the dismutation rate for copper- and zinc-containing enzyme from bovine erythrocytes was essentially independent of pH in the wide range of 6.0–10.2. It can be said that a positive charge area in the active site region of the enzyme is important for the electrostatic facilitation of the catalyzed dismutation reaction [31,39,43,44]. The positive charge on the surface, in combination with electrostatic repulsion by negatively charge areas on the surface, serves to guide $O_2^{\bullet -}$ radicals to the active-site channel.

In conclusion, the above results show that the *R. lethospermi* seed Cu,Zn superoxide dismutase enzyme is a novel thermostable protein, which has a highly unstable thermal property, unlike cupro-zinc superoxide dismutases which have been widely reported to be resistant to thermal inactivation. Other properties, such as molecular weight, subunit size, metal content and effect of some compounds on SOD activity are more similar to plant Cu,Zn SODs. According to our experience, *R. lethospermi* seed seems to be a profitable and easily accessible source of this enzyme and will provide a large quantity of purified enzyme for kinetics study, correlation to tumor and other biological studies. These results will be published elsewhere.

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