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Cu, Zn Superoxide Dismutase from *Radix Lethospermi* seed: Purification and Characterization

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Abstract

The Cu,ZnSOD was purified from *Radix lethospermi* seed by ammonium sulphate precipitation followed by column chromatography using DEAE-FF, Sephadex G-100and hydroxylapatite chromatography. Before extraction lipid was removed by super critical fluid extraction (SCF). Pure copper,zinc superoxide dismutase had a specific activity of 3555.9 units per milligram protein and was purified 211.5-fold, with a yield of 19.3 %. The isozyme has a molecular weight of 33 KDa and is composed of two non-covalently joined equal subunits, having 0.93±0.02 g.atom Cu and 0.79±0.01 g.atom Zn for each. The purified enzyme was stable over a pH range of 6.0-9.0 at 25°C and a temperature range of 25-45 °C. The purified *RLS* Cu,ZnSOD was sensitive to both cyanide and hydrogen peroxide which is typical of Cu,Zn SODs but was not inhibited by DTT, NaN_3 and β-mercaptoethanol.

الخلاصة

تم تنقية وتشخيص أنزيم دايسمبوتيس فوق أوكسيد Cu, Zn من بذور Radix lethospermi بالترسيب بوساطة كبريتات الامونيوم يتبعها كروماتوغرافيا العمود باستخدام(DEAE-FF) وسيفادكس – G 100 وكروماتوغرافيا هيدروكسيد الابتيت (hydroxyl lapetic chromat). تم إزالة الدهون (lipid) قبل الاستخلاص بوساطة الاستخلاص المائع فوق الحرج Super critical fluid extraction يمتلك سوبر أوكسيد ديسميوتير النحاس، الخارصين Cu, ZnSOD النقي فعالية خاصة مقدارها 3555.9 وحدة لكل مليغرام من البروتين وتم تنقيته 211.5 مرة بمنتوج مقدار 19.3% للايسوزيم وزن جزيئي (33 KDa) وانه يتكون من وحدتين متساويتين متصلتين بطريقة غير تساهمية لها 0.93 ± 0.02 . ذرة نحاس و 0.79 ± 0.01 ذرة خارصين لكل وحدة. يكون الأنزيم النقي مستقراً عند مدى دالة هيدروجينية تتراوح بين 6.0 – 9.0 عند درجة حرارية 25 م° وعند مدى حرارة بين 25 – 45 م°. يكون RLS Cu, ZnSOD النقي حساس لكل من السيانيد وبيروكسيد الهيدروجين وهذا هو مثالي بالنسبة إلى Cu, ZnSOD لكنه لا يخمد (Inhibited) من ال DTT و NaN_3 و B–ميركبنثو ايثانول.

Introduction

Superoxide dismutase (SOD) (E C 1.15.1.1.) is a metalloenzyme that dismutates the superoxide anion (O_2^-) to H_2O_2 and O_2 as a part of the defense against oxidative damage in aerobic organisms [1-4].

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 type of SOD have been reported. Cu,Zn SOD the most studied form, is usually present in the cytosol of eukaryotes, fungi, mammalian cells and in higher plants. It was reported to be present also in, chloroplast and glyoxysomes [6-9]. Cu,Zn SODs apparently have an evolutionary origin independent from MnSOD and FeSOD, which show a close evolutionary relationship.

MnSODs are widely distributed among prokaryotes, and in eukaryotes are mainly localized in mitochondria. However, in higher plants MnSODs also occur in different types of peroxisomes [10,11]. FeSOD has been found in bacteria, blue-green algae and protozoa [12,13]. In higher plants FeSODs 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,ZnSOD [15], hydrogen peroxide irreversibly inhibits both FeSOD and Cu,ZnSOD [16,17], while azide inhibits these enzymes in the following order : Fe SOD > Mn SOD > Cu,Zn SOD [18]. SODs play an important role in surviving against the presence of $O_2^{\cdot}$, influence aging, cancer and some very important diseases such as cataract, retinal damage artheriosclrosis, amyloidosis ischemia, essential hypertension and age-dependent immune deficiency disease [19].

Earlier, we have reported the isolation and purification of SOD isozyme from *Radix Lethospermi* seed *(RLS)* [20]. In the presents work we describe the identification of Cu,ZnSOD from a Radix Lethospermi seed *(RLS)*, under optimized conditions and study its characterization. Thus, to shed further light on the properties of Cu,ZnSOD from (*RLS*) as a Chinese traditional medicine, and to compare it with the properties of other kinds of SODs.

Materials and Methods

Radix Lithospermi seed *(RLS)* were purchased from Dalian city, Liaoning province, P. R. of China. Commassie brilliant blue G-250, Folin-Ciocalteau, molecular weight marker kits, were from (Pharmacia, Sweden). All chemical reagents used were of analytical grade.

The used instruments were a spectrophotometer (UV-2100, UNICO, Japan), electrophoresis (BIO-RAD, Protean □, CA, USA), and atomic absorption (Varian, Spectr AA-300 Plus).

1. Super Critical Fluid Extraction (SCF)

Radix lethospermi seed *(RLS)* was grinded using electrical blender machine. Seeds were treated with super critical fluid extraction processes (SCF) [21], under, extraction pressure, 30 (MPa), separation pressure, 5.8 (MPa), extraction temperature, 37 °C and flux, 100 Kg/hr.

2. Isolation and Purification of *RLS* Cu,ZnSOD

2.1. Extraction

500 g of *Radix Lethospermi* seed *(RLS)* 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 EDTA, using mechanical stirrer for 8 hrs. The homogenate was filtered through six layers of cheesecloth and centrifuged at (12,000 × g) for 30 min.

2.2. Ammonium Sulphate Fractionation

The homogenate was brought to 50% saturation with solid ammonium sulphate. After 2 hr of stirring, the precipitate was removed by centrifugation at (13,000 × g) for 15 min. the supernatant was brought to 80% saturation with ammonium sulphate, and stirrered for 2 hr, 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 0.5 mM β-mercaptoethanol.

2.3. Ion Exchange Chromatography

The sample was dialyzed for 40 h against 5 mM potassium phosphate pH 7.8, containing 2 mM β-mercaptoethanol and 0.1mM EDTA. The dialysate(9.8 mg protein/ml) was applied to a column (1.5 cm × 25cm) of DEAE-FF equilibrated 5 times (column volume) with the same buffer, Cu,ZnSOD was eluted by washing with 500 ml of the same buffer with a gradient of (0-350 mM) NaCl, at a flow rate of 15ml h^{-1} cm^{-1}.

2.4. Chromatography on Sephadex G-100

SOD fractions from the previous step were put on a column (2.6 cm × 86 cm) of Sephadex G-100 equilibrated with 5 mM potassium phosphate buffer, pH 7.8 sample volume of 2 ml (15mg protein/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.

2.5. Chromatography on Hydroxylapatite

The SOD fractions were dialyzed against 4 liter of 20 mM potassium phosphate, pH 7.0, overnight and applied to a (2.6 × 40) cm column of hydroxylapatite previously equilibrated with dialysis buffer. After washing the column with 2 bed volume of buffer, SOD fractions were collected and concentrated by lyophilization and used for further studies.

3. Electrophoretic Procedures

Sodium dodecyl sulphate-plyacrylamide 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 hr. Samples were solubilized at 100 °C for 5 min in 10 mM tris-HCl (pH 7.6), containing 5% β-mercaptoethanol, 2.5% SDS, and 1 mM EDTA. Protein bands were visualized in gels by staining with Commassie brilliant blue G-250 according to Reisner [23].

Non-denaturing polyacrylamide gel electrophoresis was performed in the absence of SDS following the method of Beauchamp and Fridovich [24]. Isoforms of SOD were separated by anodic electrophoresis on 7.5% polyacrylamide vertical slab gels incorporating a 5% stacking gel. Following electrophoresis (50A, 4h at 4 °C), the gels were stained for SOD activity. After incubation in dark for 20 min at room temperature in a solution containing 50 mM sodium phosphate buffer (PH 7.8), 0.1 mM EDTA, 3mM riboflavine and 0.25 mM nitroblue tetrazolium chloride, gels were rinsed twice in distilled water, placed on a glass plates and illuminated for 15 min under a 40-W lamp placed 30 cm above the gel.

4. Determination of Molecular Weight (*Mr.* and subunit size)

The native molecular weight of the purified Cu,ZnSOD was determined by gel filtration on a Sephadex G-100 column (2.6 × 86 cm) (Pharmacia). The column was calibrated with following standard proteins: bovine serum albumin(66 kDa), ovalbumin (43 kDa), trypsin inhibitor (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 II slab cell. Standard used were rabbit phosphorylase b (97.4 kDa), bovine serum albumin (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 Commassie brilliant blue G-250.

5. SOD Activity Assay

Superoxide dismutase activity was determined by the modified procedure of pyrogallol autooxidation [25]. One unit of activity is defined as the amounts of SOD required to give 50% inhibition of the autooxidation of pyrogallol. Specific activity is defined as units of activity per milligram of protein.

6. Protein Determination

Protein determination between the purification steps was estimated Colometrically using Follin-Ciocalteau reagent, following Lowry method [26]. Crystalline bovine serum albumin was used as a standard. Protein concentrations in the fractions from the column were determined

spectrophotometrically by measuring the absorbance at 280 nm.

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 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.

8. 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 5mM 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,ZnSOD was examined by performing enzyme incubation for different times interval(0.5-24h) in 5 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 samples was assayed under standard conditions.

9. Effect of Chemicals and Inhibitions on SOD Activity

The effect of some compounds known to be selective inhibitors of SOD activity, were investigated. The enzyme solution containing each compounds was incubated in 5 mM phosphate buffer, pH 7.8 and 25 °C for different time intervals. SOD control reactions were carried 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^- radicals (ΔAm^{-1}) was kept constant by adding an appropriate dilution of XOD. The following inhibitors were tested: 2 mM of DTT, NaN_3, β-mercaptoethanol, SDS, EDTA, urea, H_2O_2 and KCN.

Results

1. Purification of Enzyme

Radix Lethospermi seed *(RLS)* is one of the Chinese traditional medicine which is contained about 23% of its dry weight as a fat, so the first process was the super critical fluid extraction (SCF), to separate the fat content from its seed and to prevent the interferences to the purification processes caused by production of turbidity due to formation of the creamy fat layer [21].

The enzyme was purified from extract of *Radix lethospermi* seed by ammonium sulphate fractionation, ion exchange chromatography using DEAE-FF, Sephadex G-100 and consecutive charge transfer column chromatography using hydroxylapatite.

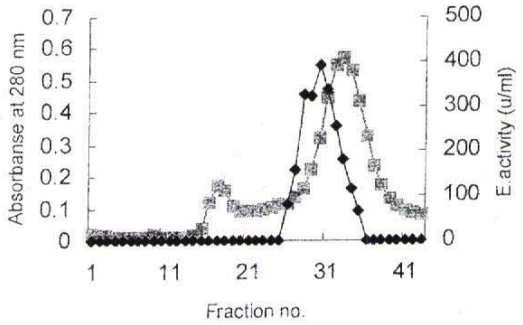
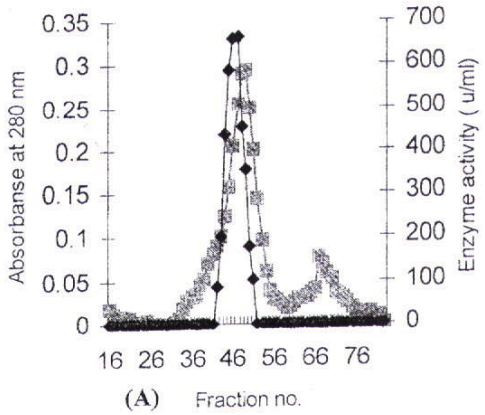


Fig.1: Ion exchange chromatogram (DEAE-FF column, 1.5 × 25 cm) of the active fractions received from the ammonium sulphate precipitation. The column was equilibrated with 5 mM phosphate buffer, pH 7.8, and eluted with a linear NaCl gradient (0-0.35M). Fractions of 3 ml were assayed for SOD activity (♦) and absorbance at 280 nm (■).

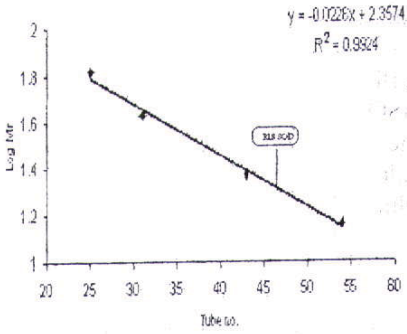
Table 1. Purification steps of *RLS* SOD

Steps	Activity (u/ml)	Total activity (u)	Protein content (mg/ml)	Specific activity (u/mg)	Volume (ml)	Yield %	Purification fold
Crude	173.5	52050	10.32	16.81	300	100	1
Ammonium Sulphate (50~80)%	1412.78	49447.5	9.8	144.16	35	95	8.58
DEAE-FF	2665.02	33312.78	5.28	504.7	12.5	64.0	30.04
Sephadex G-100 + concentrate	2823.88	21179.14	2.98	947.6	7.5	40.69	56.37
Hydroxylapatite	401.82	10045.65	0.113	3555.92	25	19.3	211.5

The specific activity of the purified enzyme was 3555.92 units/mg protein. The yield was 19.3% at the end of purification with a purification fold of 211.5- fold. The summary of the purification procedure is presented in Table 1. The enzyme was precipitated at 80% saturation with solid ammonium sulphate. The enzyme bound to the ion-exchanger DEAE-FF result in the removal of the contaminating proteins. Fig.1 gives the elution profile of SOD on the ion exchange chromatography column revealing a single peak. The SOD activity was found in the fractions 26-35 (Fig. 1). Gel- filtration of the enzyme on Sephadex G-100 showed a single peak. The enzyme was eluted in the fractions 42-51 (Fig. 2,A). The fractions eluted from the gel-filtration column resulted in a 40-fold of purification.



(A)



(B)

Fig. 2. (A) Gel exclusion chromatography (G-100 column,2.6 × 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 (■). (B) Determination of relative molecular mass of *RLS* Cu,ZnSOD using Molecular exclusion chromatography on a column of Sephadex G-100.

Subsequent step of purification occurred by a kind of charge transfer chromatography, hydroxylapatite chromatography. Cu,ZnSOD was polished because it was not adsorbed although other proteins were. Nineteen percent of SOD activity was recovered in pooled fractions with a 211.5-fold increase in specific activity, that was determined to be 3555.92 units per milligram protein. The electrophoretic patterns of the native and SDS-PAGE are depicted in fig. 3. The purity of the SOD was confirmed by polyacrylamide gel electrophoresis. Non-denatured polyacrylamide gel stained with substrate for SOD revealed a single band corresponding to the major protein band hence confirming the presence of the enzyme (Fig. 3). The native molecular weight of the purified enzyme was determined by gel exclusion chromatography on Sephadex G-100 column. The value obtained from three determinations was 33 kD, (Fig 2 B). The subunit molecular weight of RLS Cu,ZnSOD was estimated to be 16.5 kDa by SDS/PAGE electrophoresis (Fig. 4). These results indicates that the enzyme is composed of two non-covalently joined subunits of equal size.

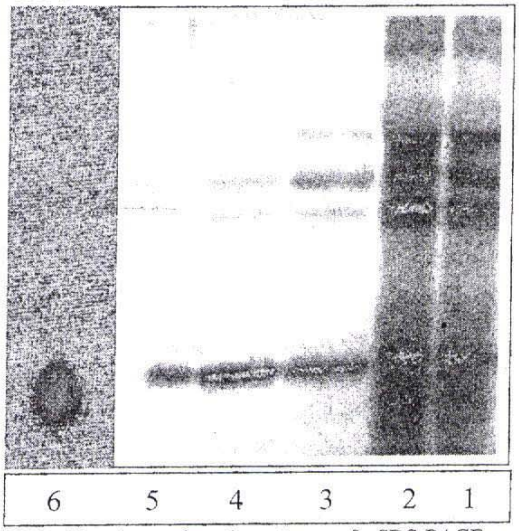
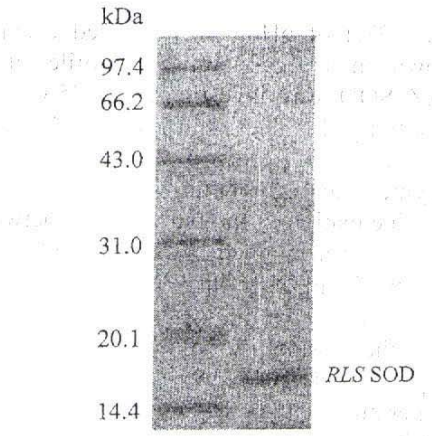
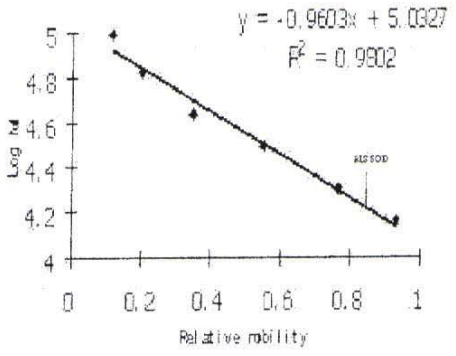


Fig.3. The electrophoretic patterns of SDS-PAGE and Native-PAGE of purified SOD from *Radix Lethospermi* seed. The purified enzyme was electrophoresed on 12 % polyacrylamide gel under denaturing conditions. The protein bands of the gel were stained with Coomassie blue. (1) Crude enzyme; (2) 50-80% ammonium sulphate precipitation; (3) Ion-exchange chromatography; (4) Gel-filtration chromatography; (5) Hydroxylapatite chromatography. The purified enzyme was run on 7.5% non-denaturing polacrylamide gel and stained for SOD activity. (6) Activity staining of purified SOD.



(A)



(B)

Fig.4. (A).Polyacrylamide gel electrophoresis (12% gel) of purified *RLS* Cu,ZnSOD. 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. Purified *RLS* Cu,ZnSOD. (B). Determination of relative molecular weight of RLS Cu,ZnSOD by sodium dodesyl sulphate/ PAGE.

3.2. Characterization of Enzyme

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.

The effect of pH on the purified enzyme is shown in Fig. 5. Activity profile of *RLS* Cu,ZnSOD was investigated at 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 it's activity after incubation in the buffer at pH between 6 and 9, and it retained more than 60 % of it's activity till pH 12, but it was inactivated by nearly 50 % at pH 5

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 20~45 °C (Fig.5). However, the activity was peaked at temperature 25 °C. The enzyme was inactivated rapidly at temperature above this range. It retained about 53.5 % of it's activity at 60°C, while only 15 % of it's activity retained after incubation at 85 °C for 30 min.

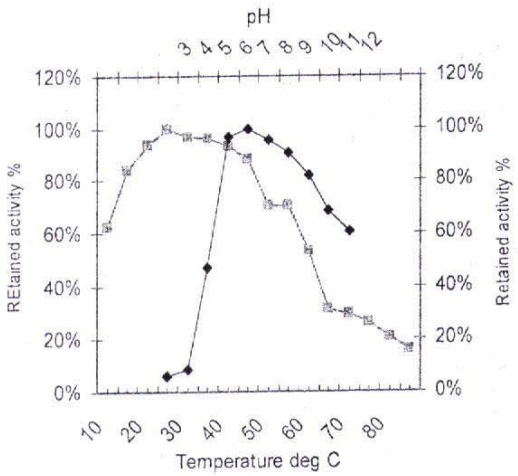


Fig.5. Effect of temperature (■) at pH 7.8 and pH (♦) on SOD enzyme stability at 25 °C.

Influence of various chemicals and inhibitors on SOD activity revealed that it was inhibited 16% and 10 % when incubated with 2 mM of EDTA and SDS respectively (Fig. 6), while enzyme inhibition was approximately 76.4% and 89.5% on the presence of 2 mM H_2O_2 and KCN respectively, as described previously, the Cu,ZnSODs are sensitive to both H_2O_2 and KCN [27]. *RLS* Cu,ZnSOD was not sensitive to other inhibitors.

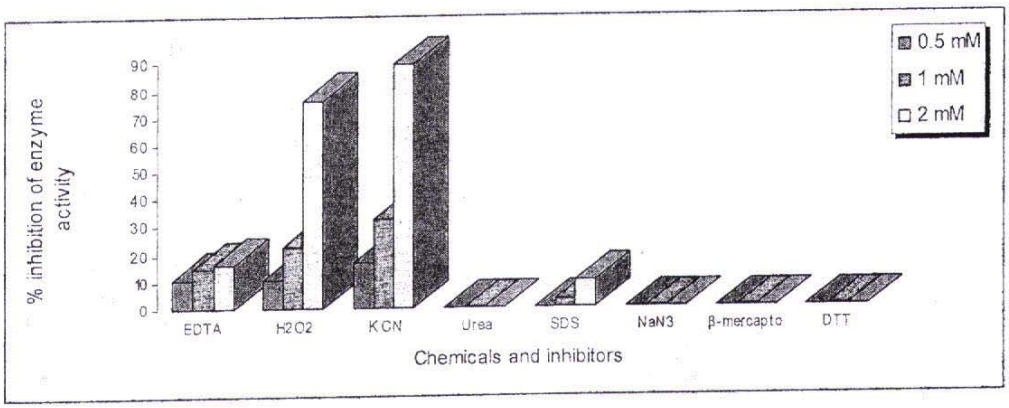


Fig.6. Effect of 2 mM of some substances, as referred above on the *RLS* SOD activity, depend on incubation time(hr), at pH 7.8.

4. Discussion

The isolation and purification of Cu,ZnSOD from *Radix Lethospermi* seed *(RLS)* was performed by super critical fluid extraction (SCF), to remove lipid content and other pigments which was a bout 23% from its dry weight. Protein purification was optimized at (50-80) % with ammonium sulphate, then DEAE-FF ion exchange chromatography, Sephadex G-100 gel exclusion chromatography and hydroxylapatite column

chromatography. According to this procedure, the specific activity was found to be 3555.92 units per milligram protein, corresponding to a 211.5 - fold of purification with a yield of 19.3%. Our purification methods were original and resulted in a higher – fold of purification than previous method reported for RLS SOD purification [20].

The apparent molecular weight of the *Radix Lethospermi* seed superoxide dismutase was estimated by SDS-PAGE electrophoresis to be 16,5 kD. The native molecular weight of the enzyme was determined by gel exclusion chromatography on Sephadex G-100. By comparison with markers of known mol. wt. *RLS* Cu,ZnSOD appeared at a position corresponding to a molecular weight of 33,0 kD. This indicates that the enzyme is composed of dimers of equal size, whose association depends upon non-covalent interactions.

The enzyme has 0.93±0.02 g.atom Cu and 0.79±0.01 g.atom Zn per subunit. Cu atom is important for SOD activity. The negative charge on the protein surface reinforces the attraction by the positive channel around the copper. The enzyme-catalyzed dismutation by Cu,ZnSOD is believed to proceed by subsequent reduction and oxidation of a Cu ion acting as an electron carrier. The Zn atom is wholly buried within the protein structure and is thought to play a structural role only, aiding protein stability [28].

Radix lethospermi seed Cu,ZnSOD was inhibited by 2mM KCN and H_2O_2. This result was reasonable, since Cu,ZnSODs are generally known to be cyanide and hydrogen peroxide sensitive enzyme [27]. It was inhibited to approximately 90 % and 70 % after incubation with 2 mM KCN and H_2O_2 for 1 hr respectively.

The inactivation of the Cu,ZnSOD has been attributed to the reduction of the enzyme-bound Cu^{2+} to Cu^{1+} by H_2O_2, followed by a fenton-type reaction of Cu^{1+} with additional H_2O_2 to form Cu^{2+}-OH. This could oxidatively attack an adjacent histidine amino acid residue of the enzyme [29]. *RLS* SOD activity was unaffected by 2mM of DTT and β-mercaptoethanol indicated that cysteine, serine and threonine residues have no important role in the SOD activity.

The purified *RLS* SOD showed optimum activity at pH of 7.0 and at temperature of 25°C. The optimum pH for SOD activity of mung bean and corn was 7.8 [30,31]. Thus, 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 [27,32]. The positive charge on the surface, in combination with electrostatic repulsion by negatively charge areas on the surface, serves to guide ($O_2^{\cdot-}$) radicals to the active-site channel.

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