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Original Research

Purification and properties of Manganese Superoxide Dismutase (MnSOD) from *Tamarix aphylla* L.

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Abstract: Superoxide dismutase (SOD) of the *Tamarix aphylla* leaves were detected at optimum conditions that collected in April, May and June. Results indicated the specific activity in the crude extract reaching to 36.76 unit/ mg protein. Crude SOD was purified by several techniques, precipitation with ammonium sulfate (50–75) %, Ion exchange chromatography using DEAE-cellulose and two steps of size exclusion chromatography on sephacryl S-200 column. The obtained specific activity (310 unit/mg protein) and purification fold 7.91. The purified enzyme revealed one band by SDS-polyacrylamide gel electrophoresis with molecular mass 85.703 kDa. while 89.125 kDa by Sephacryl S-200. The optimal pH and temperature for enzyme activity were 7.5, and 50ºC respectively. EDTA, SDS and NaN_3 reduced activity, contrariwise of H_2O_2 and KCN, pointed to the studied SOD is MnSOD. Michalis constant K_m and maximum velocity V_{max} values were 0.016 mM and 55.86 mM/min, respectively by using Pyrogallol as substrate. According to the results, we conclude *Tamarix aphylla* produce MnSOD which can have purified by serial purification techniques for better activity and characterized for further studies.

Key words: *Tamarix aphylla*, Superoxide dismutase, Purification, SDS- PAGE.

Introduction

Superoxide dismutase belong to Oxidoreductase Enzymes (EC 1.15.1.1), SODs have ability to transform (O_2^-) to O_2 and H_2O_2 (1). All types of these enzyme play significant role against cells damage by oxidative agents (2). SODs are metalloenzyme consists of four isozymes, Cu/ZnSOD, MnSOD, FeSOD and NiSOD. In plants, Cu/ZnSOD found in cytoplasm, chloroplast and peroxisomes, FeSOD found in chloroplast (3, 4). MnSOD present in mitochondria, is essential to a biotic life (5,4). MnSOD also had been detected in in the peroxisomal matrix (6,7) and the external side of the glycosomal membrane of higher plants (8). NiSOD not found in plants (8). In plants SODs varied in activity according to stresses conditions (9,10); MnSOD activity has been proposed to correlate with the severity of stress (11). Some detail immunological studies regarding the cellular localization of MnSOD in animal systems were reported (12,13), but not similar immunological study to locate MnSOD in plant cell was reported, in ordered to identify and locate MnSOD protein in plant during the development or in response to various stresses. SOD widely used in different fields such as medical, cosmetic, food, agricultural and chemical industries (14,15). Recently, SOD showed efficient ability to prevent or decrease of the circulation system diseases, malignant tumors, autoimmune diseases, diabetes, ageing, neurological disorders, transplantation, rheumatoid arthritis, asthma, and sterility (16). The aim of study is to found novel source of SOD may be useful in medicine fields that required purified and characterized enzyme.

Materials and Methods

Plants and Chemical Materials

Leaves of previously classified as *Tamarix aphylla* by specialist in taxonomy were collected from the farmland surrounding the University of Al-Qadisiyah, Al-Diwaniyah province during the period between April to June.

The source of chemicals was as follows: Potassium phosphate Pyrogallol, Bovine serum albumin, Acrylamide, Bis acrylamide, Ammonium sulphate, PVP, EDTA-Na_2, Casein, Hydrogen peroxide, Ovalbumin, Alkaline phosphatase, Acrylamide, Bis-acrylamide, Aldolase, Sodium azide, HCl, TEMED, Bromophenol blue, Silver nitrate, Formaldehyde, Sodium carbonate, Sodium thiosulfate, Acetic acid, Hydrogen peroxide (BDH, England). Diethylaminoethyl cellulose (DEAE). Sephacryl S-200, Blue dextran 2000 (Pharamacia, Sweden). Ammonium persulfate, Tris-base, EDTA-Na_2, KCN, EDTA, SDS (Fluka, Switzerland). Dialysis Sacs (Spectrum(USA)). Tris-HCl (Oxoid, England).

Enzyme Purification

Crude extract

SOD extracted according to (17). Fresh leaves of selected species (5g) were crushed for 20 min in 15ml of 0.1M potassium phosphate buffer (7.8) containing 1mM EDTA-Na_2 and 2% (w/v) insoluble PVP by pre-chilled mortar and pestle. The crude extract was filtered through four layers of miracloth then centrifuged at 10,000 rpm for 20 min at 4º C. and supernatant used as

source of crude enzyme.

Precipitation by $(NH_4)_2SO_4$

The crude extracts were precipitated gradually by adding of solid $(NH_4)_2SO_4$ in cooled water bath, after centrifugation (10000 rpm for 30 min) supernatant was obtained which represent initial saturation ratios (0, 25, 50 and 75%). Solid $(NH_4)_2SO_4$ was added as above for final concentration (0-25, 25-50, 50-75 and 75-100 %). centrifugation was carried out at10000 rpm for 30 min to separate the precipitated proteins. Precipitates were suspended with 5 ml of 0.1 M potassium phosphate buffer pH7.8. The active precipitate was dialyzed against the same buffer to eliminate solid $(NH_4)_2SO_4$.

Ion exchange chromatography

The enzyme applied to DEAE-cellulose column (3.5 × 20) cm pre-equilibrated by 0.1M potassium phosphate pH 7.8. Column washed by same buffer and fractions were collected, the bound SOD was eluted by linear gradient NaCl (0-1M) dissolved in previous buffer as above for protein and enzyme activity analyzation.

Size exclusion chromatography

Sephacryl S-200 (1.5 × 80) cm column pre-equilibrated with 0.2M potassium phosphate pH 7.8 was used to increase the purity of the second peak obtained from DEAE-cellulose column step. Enzyme was eluted with the same buffer; the fractions of activity peak were collected and SOD activity was determined.

SOD assay

Pyrogallol auto-oxidation used to measure SOD activity. One unit of activity is defined as the amount of SOD required to inhibit the 50% of pyrogallol (18).

Concentration of Protein

Bradford method (19) used to estimate concentration of protein in enzyme solutions.

SOD properties

Electrophoresis

SOD purity and molecular mass were detected by polyacrylamide gel electrophoresis (SDS-PAGE) post final step of purification. It was carried out in 7.5% and 4% of stacking and resolving gel respectively each containing 10 % SDS [20]. Protein sample was incubated at 95°C for 5 min with sample buffer (0.06 M Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromphenol blue) and loading (100µl) after cooling to the gel. The gel was run in 1X SDS running buffer, after running the gel was shacked and rinsed separately with 50% methanol for 60 min and distilled water for 15 min. Protein bands was detected by silver nitrate. After staining gel was placed in developer and shake as required then stain was fixed by 50% methanol for photograph on light box. Aldolase (158 kDa), Alkaline phosphatase (80 kDa), Bovine Serum albumin (67 kDa), Ovalbumin (43 kDa) and Casein (31 kDa) were used as markers.

Molecular mass calculated by size exclusion chromatography

Sephacryl S-200 column (1.5×80) cm was equilibrated with 0.2M potassium phosphate pH7.8. Void volume (V_o) determined by blue dextran. Elution volume (V_e) of studied SOD and different standard protein Trypsin (24 kDa), Casein (31 kDa), Bovine Serum albumin (67 kDa), Alkaline phosphatase (80 kDa) and Aldolase (158 kDa) were estimated. The molecular weight of purified SOD was calculated according to relationship between V_e/V_o vs log MW of standard proteins.

Influence of pH on enzyme activity

Examined by incubations of the enzyme with 0.1M potassium phosphate at varied range of pH values (4-9) at 25ºC for 30 min.

Influence of heat on enzyme activity

Determined by incubating of enzyme and 0.1 M potassium phosphate pH 7.5 in different range of temperature (20-80) ºC for 25 min.

Influence of inhibitors, chelating factors and detergent

Inhibitors influence were investigated on SOD activity, 5 and 10 mM of KCN, NaN_3, EDTA, SDS and H_2O_2 were selected. Aliquot of SOD incubated in 0.1 M potassium phosphate pH7.5 containing inhibitors at 50 °C for 30 min. and remaining activity was measured.

Kinetics

Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) measured according to Lineweaver-Burk by plot 1/V versus 1/[S].

Influence of metal ions

Aliquot of enzyme were incubated (50°C for 30min) with 0.1 M potassium phosphate pH7.5 contain 5 and 10Mm of different ions included $MgCl_2$, KCl, $ZnCl_2$, $HgCl_2$, $CoCl_2$, $MnCl_2$, $CaCl_2$ and $CuCl_2$.

Results and Discussion

Extraction and Purification

SOD extracted from leaves of *T. aphylla* showed specific activity (36.76 unit/ mg). Selected plant adopted to tolerance high level of stress by sodium chloride (21) may be reflect presence of anti-stress enzymes such as SOD. Precipitation by 50-75 % ammonium sulphate (Fig 1) appeared increase in specific activity (60.36 unit/mg protein) in other hand the lowest located in 75-100%. SOD extract from *Picea abies* L. precipitate with saturation ratio 55-75 % (22) whereas from *Ribes nigrum* was 0-40 % followed by 40-80 % (23).

Next steps of purification by DEAE-Cellulose column reveals two peak of SOD isoform with specific activity 74.14. and 95.41 unit/mg protein respectively (table 1 and Fig 2). MnSOD purified from *Citrullus*

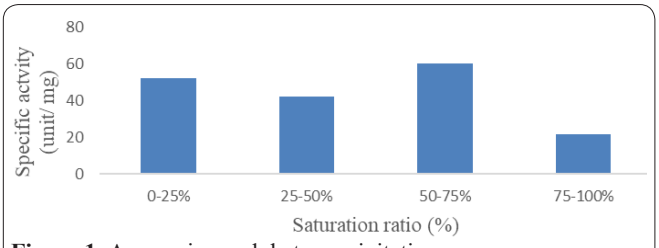

Figure 1. Ammonium sulphate precipitation.

Table 1. Purification steps of SOD from *T. aphylla* L. leaves.

Step	Volume (ml)	Enzyme activity (unit/ml)	Protein (mg/ml)	Specific activity (unit/mg protein)	Total activity (unit)	Purification fold	Recovery (%)
Crude SOD	40	5.15	0.132	39.015	206	1	100
Ammonium sulphate	10	10.3	0.166	62.04	103	1.59	50
DEAE-cellulose (first peak)	30	3.15	0.0424	74.29	94.5	1.90	45.87
DEAE-cellulose (Second peak)	25	3.5	0.0366	95.62	87.5	2.45	42.47
1st sephacryl S-200	20	3.25	0.0211	154.02	65	3.94	31.55
2nd sephacryl S-200	15	3.1	0.01	310	46.5	7.94	22.57

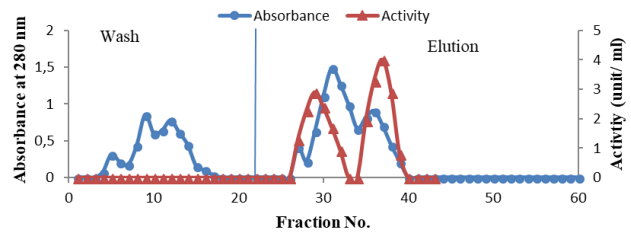

Figure 2. Ion exchange chromatography on DEAE cellulose column.

lanatus Schrad by using DEAE-Cellulose with specific activity 30.71 unit/mg protein (24), also clarify from *Agave attenuatta* (306.43 unit/mg protein) (25).

The major active peak (second peak) of highest activity and purification fold eluted on sephacryl S-200 column. First and second size exclusion were applied to increase purity (table 1, Fig 3 and 4), separate single peak of enzyme activity contains purification fold 3.92 and 7.94 respectively. Different gel columns were used for isolation of SOD from *Camellia sineses* L. and *Curcuma aeruginosa* solutions have clarification fold 34.7 and 158.7 respectively (26) (27).

SOD properties

Purity and molecular mass

One band of enzyme appeared in SDS-PAGE has

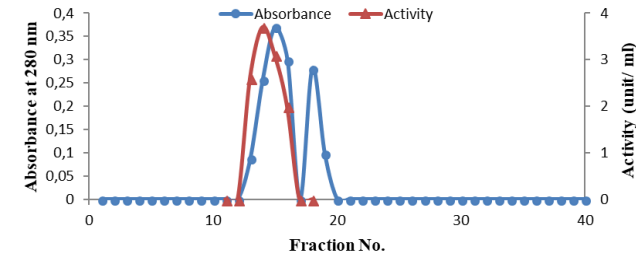

Figure 3. 1st Size exclusion chromatography by sephacryl S-200 column.

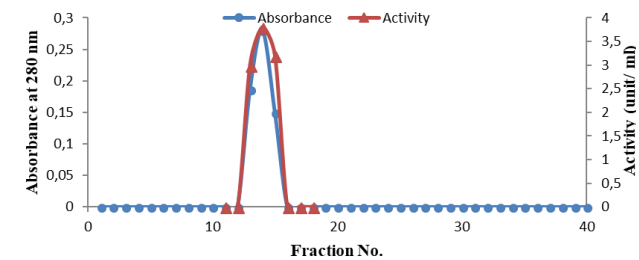

Figure 4. 2nd Size exclusion chromatography by sephacryl S-200 column.

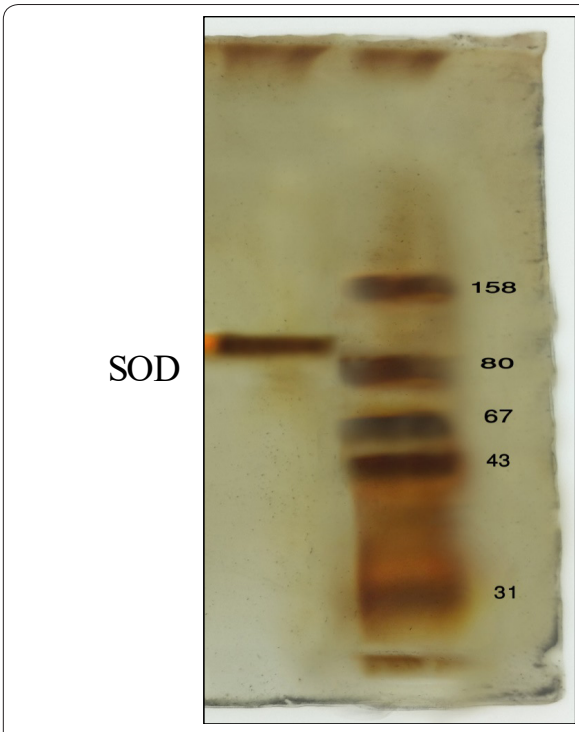

Figure 5. SOD from *T. aphylla* L. leaves on SDS-PAGE using different molecular weight protein markers (M) ranged from 31-158 kDa

molecular mass 85.703 kDa (Fig 5 and 6) whereas by sephacryl S-200 column was 89.125 kDa (Fig 7). The molecular mass of one band MnSOD purified from the Nicotiana sp. was 17.5 kDa determined by SDS-PAGE (28), While same enzyme purified from *Citrullus lanatus* Schrad showed four bands each one has molecular mass 27 kDa (24). Mn, Fe and Cu/Zn SOD separated from *Mesembryanthemum crystllinum* have molecular mass 82, 48 and 34 respectively estimated by gel filtration (29).

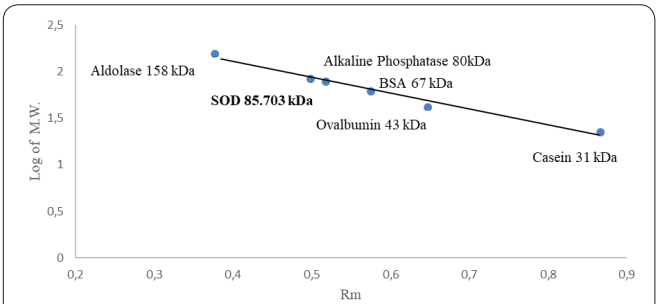

Figure 6. Molecular mass of SOD by SDS-PAGE purified from *T. aphylla* L.

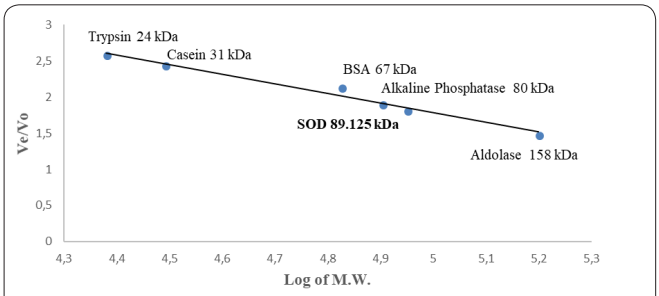

Figure 7. Molecular mass of SOD by sephacryl S-200.

Influence of pH on enzyme activity

SOD activity showed optimum activity at pH 7.5 (Fig 8). Different result was recorded of SOD purified from the seeds of *Amaranathus spinosus* which has typical activity at pH 5.0 (30), in other hand MnSOD from *Camellia sineses* has optimum pH 8.0 (31).

Influence of heat on enzyme activity

The optimum temperature of SOD activity was 50°C (Fig 9). SOD of *Cicer arietinum* have optimum temperature 30º C (32), The optimum temperature of Cu, Zn SOD purified from black soybean was 50ºC (33).

Influence of inhibitors

EDTA, NaN_3 and SDS inhibit SOD at 5, 10 mM concentration (Table 2). Inhibition of enzyme by EDTA refer to the SOD metal ion in active site (metaloenzymes) (3,4). Potassium cyanide and hydrogen peroxide dose not decrease SOD activity, suggest the SOD is MnSOD isoform. Sugercane's MnSOD not inhibit by 5mM of H_2O_2 and 3mM of KCN (34), while Cu/ZnSOD purified from the leaves of *Jatropha curcas* show decreasing in activity by increase the concentration of imidazole, EDTA and H_2O_2 (35).

Influence of metal ions

Data presented in Table 3 showed different effect on SOD activity, full activity or slightly increased when incubated with the sodium, potassium and calcium chloride at concentration 5 and 10 mM. The metals ion of mercury, copper, zinc and iron decreased SOD activity at same concentrations, in other hand the enzyme very little influenced by used manganese ion. Similar results were reported for MnSOD purified from tobacco (28).

Kinetics of SOD

Km and *Vmax* of SOD obtained from *T. aphylla* were

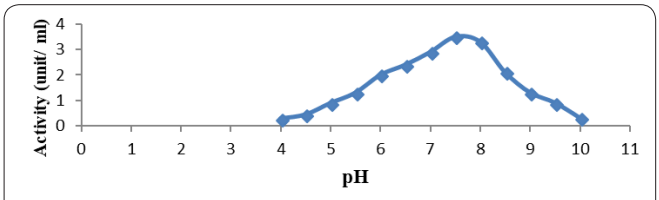

Figure 8. Optimum pH of SOD activity.

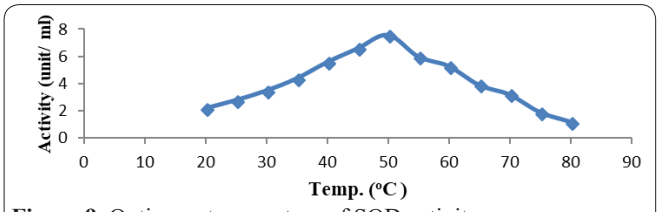

Figure 9. Optimum temperature of SOD activity.

Table 2. Influence of chelating factors, detergent and inhibitors on SOD activity.

Inhibitor	Conc. (mM)	Remaining activity (%)
Control	-	100
KCN	5	100
	10	95
H_2O_2	5	100
	10	99
NaN_3	5	32
	10	21
SDS	5	11
	10	7
EDTA	5	10
	10	4

Table 3. Influence of metal ions on SOD activity.

Metal	Conc. (mM)	Remaining activity (%)
Control	-	100
$FeSO_4$	5	44.67
	10	31.04
$HgCl_2$	5	7.21
	10	2.34
$CuCl_2$	5	84.54
	10	50.99
$CaCl_2$	5	100
	10	111
$ZnCl_2$	5	55.03
	10	28.68
$MnSO_4$	5	98
	10	102
NaCl	5	100
	10	105
KCl	5	100
	10	103

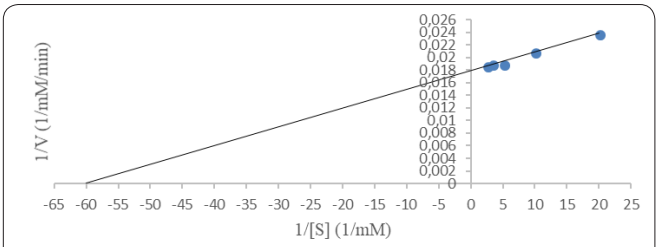

Figure 10. Line Weaver-Burk Plot to determine the SOD kinetics.

0.016 mM and 55.86 mM/min respectively (Fig 10). *Km* of *Cicer arietinum* was 10.16±2.5 µM used riboflavin as substrate (32). When nitroblue tetrazolium used as substrate *Km* and *Vmax* of *Stemona tuberosa* SOD were 62.414±0.015M and 101.0101±0.022 µmol/min/mg protein while using riboflavin as substrate, the values were 27.389±0.032M and 38.167±0.021 µmol/min/mg protein respectively (36).

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