FURTHER ENZYMATIC STUDIES

SUPEROXIDE DISMUTASE
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Superoxide dismutase (SOD, EC 1.15.1.1) is a metalloenzyme widely distributed among animals (205,206), plants (207,208) and microbes (209,210). It catalyze the conversion of superoxide radicals which are produced as a common metabolic intermediates in a variety of biological oxidations to hydrogen peroxide (211).

$$\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

Superoxide dismutase plays an important role in protecting the cells against the indirect deleterious effects of these free radicals (212). SOD protects normal cells from damage due to anticancer drugs (213) and also used to treat patients with acute paraquat poisoning (214). Based on the type of metal present at the active site of SOD three distinct classes (Cu-Zn, Fe and Mn) of SOD enzymes were reported (215). All three types of SODs were isolated and characterized extensively from different sources. The iron and manganese containing SODs are characteristic of microorganisms and show a high degree of sequence homology (216) while Cu-Zn SODs are characteristic of eukaryotic cytosol (217) and have no sequence homology with Fe or Mn SODs, which are totally unrelated to the Cu-Zn SODs except for their catalytic activity (218). Mn-SOD has been isolated and characterized from higher plants (219-221), human liver (205) and bacteria (222).
The gram-positive bacteria most frequently contain Mn-SOD (223) whereas gram-negative bacteria generally contain both Fe and Mn-SOD (224). However, gram-positive bacteria, such as staphylococcus aureus contain both Fe-SOD and Mn-SOD (224).

This chapter describes the isolation and characterization of Mn-SOD from the venom of scorpion H. fulvipes. Ours is the first report about the presence of SOD in venoms.

RESULTS

Purification

As shown in Table 13 about 100 mg of the venom was dissolved in 4 ml of saline and ammonium sulfate was added to 40% saturation with constant stirring over a period of 30 min. After standing at 4°C for 15 min the suspension was centrifuged for 20 min at 4000 X g. The precipitate containing SOD activity was dissolved in phosphate buffer (0.01 M, pH 7.6) and dialysed against the same buffer for 24 hr and subjected to gel filtration chromatography on a Sephadex G-100 column (0.6 X 50 cm). SOD activity was eluted with 50 mM phosphate buffer (pH 7.6) at a flow rate of 24 ml/hr (Fig.34). Fractions of 4 ml each containing SOD activity were pooled, dialysed and concentrated. The ion-exchange column of DEAE-cellulose (1.6 X 32 cm) was equilibrated with
Table 13: Purification of Mn-SOD activity from the venom of scorpion Heterometrus fulvipes

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (Units)</th>
<th>Specific activity (Units/mg protein)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude venom</td>
<td>100</td>
<td>270</td>
<td>2.7</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation</td>
<td>45</td>
<td>230</td>
<td>5.1</td>
<td>1.9</td>
<td>75</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>25</td>
<td>167</td>
<td>6.7</td>
<td>2.5</td>
<td>62</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>0.57</td>
<td>124</td>
<td>218</td>
<td>80.7</td>
<td>44.44</td>
</tr>
</tbody>
</table>
Fig. 34: Elution profile of SOD from Sephadex G-100 column (0.6 x 50 cm). Venom protein 45 mg in 2 ml of phosphate buffer (0.01 M, pH 7.6) was loaded on the column. Elution was carried out with the same buffer collecting 4 ml fractions at a flow rate 24 ml/hr. Protein of the fractions was monitored at 280 nm. SOD activity was determined using 0.1 ml aliquots of each fraction (--; protein, (--; SOD activity).
Fig. 34: Elution profile of SOD from Sephadex G-100 column.
0.01 M phosphate buffer (pH 7.6). The fractions eluted from the Sephadex G-100 column containing SOD activity were applied to the DEAE - cellulose column and unbound material was removed. The column was eluted using a linear salt gradient of 0.1 - 0.4 M sodium chloride in the starting buffer. The flow rate was 18 ml/hr and fractions of 3 ml were collected (Fig.35).

Properties of the Enzyme

SOD was purified to 81 fold with 44% yield. The homogeneity of the enzyme was established by PAGE (7.5% gel) as shown in Fig.36a and its activity staining in gels was shown in Fig.36b. The molecular weight of the enzyme was found to be 100,000 by 10% SDS - PAGE in presence of 2-mercaptoethanol as shown in Fig.12. The enzyme is a monomer consisting of a single polypeptide chain. The optimum reaction temperature was 45°C (Fig.37). Pre-exposure of the enzyme above 55°C led to 70% loss of activity while no enzyme activity was found at 80°C.

The enzyme (20 µg/ml) was incubated separately with 20 mM H₂O₂ or chloroform and ethanol mixture (3:5 v/v) at room temperature (28°C ± 2) for an hour with proper controls and subjected to polyacrylamide gel electrophoresis followed by activity staining. One of the control gels was stained for activity in the presence of 2 mM cyanide and all
Fig.35: DEAE-cellulose chromatography of SOD fractions from Sephadex G-100 (Fig.34). The pooled fractions from Sephadex G-100 column were dialysed, concentrated and applied to a DEAE-cellulose column (1.6 x 32 cm) equilibrated with phosphate buffer (0.01 M, pH 7.6). The column was eluted with a linear gradient of 0-0.4 M NaCl at a flow rate of 18 ml/hr and the effluent was collected in 3 ml fractions (o--o, protein; o--------o, SOD activity).
Fig. 35: DEAE-cellulose chromatography of SOD fractions from Sephadex G-100 column.
Fig. 36a: Polyacrylamide gel (7.5%) electrophoresis of the purified SOD from H. fulvipes venom. The purified enzyme preparation (35µg) was subjected to electrophoresis at pH 8.3 in 7.5% gel. The gel was stained with Coomassie brilliant blue R-250.

Fig. 36b: Activity staining for Mn-SOD (10 µg) purified from H. fulvipes venom. The gels after PAGE were immersed in solution A for 20 min and 20 min in solution B. Then the gels were exposed to fluorescent light for 10 min.
Fig. 37: Temperature profile of SOD activity purified from H. fulvipes venom.
others were stained to localize the enzyme in polyacrylamide gels. These experiments showed that the enzyme was sensitive to chloroform-methanol mixture and insensitive to either cyanide or hydrogen peroxide. The results indicate that the enzyme contained manganese.

The purified enzyme was subjected to Atomic absorption spectrophotometry and the metal content was determined. The enzyme contained approximately one atom (atoms Mn/Mol 1.04) of manganese per molecule and thus the metal protein stoichiometry was 1:1 with Mn-SOD.

The results of different inhibitors on Mn-SOD from H. fulvipes venom are shown in Table 14. Complete inhibition of enzyme activity was found with 1% SDS, 70% and 90% of enzyme activity was inhibited in presence of 1 mM guanidinium chloride and 6 M urea respectively. The metal chelators showed various degrees of inhibition. About 70% of activity was inhibited with EDTA, while 17% of activity with diethyldithiocarbamate and o-phenanthroline. p-Hydroxymercuribenzoate a sulfhydryl reagent, inhibited the enzyme activity completely at 1 mM concentration while azide and nitroprusside inhibited upto 88% and 78% respectively. Inhibition of H. fulvipes SOD activity by the antiserum raised against whole venom is shown in Fig.(38) and complete inhibition was found with 0.1 ml antiserum of 1:50 dilution.
Table 14: Effect of different concentrations of inhibitors on Mn-SOD activity from H. fulvipes venom

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Percent activity *</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.25 mM</td>
</tr>
<tr>
<td>PHMB</td>
<td>85</td>
</tr>
<tr>
<td>SDS</td>
<td>71</td>
</tr>
<tr>
<td>DDC</td>
<td>93</td>
</tr>
<tr>
<td>GdmCl</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Azide</td>
<td>75</td>
</tr>
<tr>
<td>Nitroprusside</td>
<td>57</td>
</tr>
<tr>
<td>EDTA</td>
<td>50</td>
</tr>
<tr>
<td>o-Phenanthriolne</td>
<td>83</td>
</tr>
</tbody>
</table>

* Enzyme activity in the absence of inhibitor in the assay mixture is taken as 100%. All assays were carried out in triplicate and the results are the average of three values.
Fig. 38: Inhibition of SOD activity by antiserum raised against H. fulvipes venom. The purified enzyme (20 μg/ml) was incubated for 1 hr at 37°C with the indicated concentrations of antiserum (1:50 dilution). The residual SOD activity was measured.
DISCUSSION

Manganese containing superoxide dismutase was characterized from diverse sources including bacteria, yeast, algae, fungi, higher plants and animals but not from animal venoms. An attempt was made to compare the properties of Mn-SOD purified from the venom of scorpion H. fulvipes with Mn-SODs purified from other sources. The enzyme was isolated to homogeneity by three step procedure involving ammonium sulfate fractionation, gel filtration and ion-exchange chromatography.

The molecular weight of H.fulvipes Mn-SOD was found to be 100,000. It is closely related to the mol. wt. of Mn-SOD reported from pisum sativum leaves (225) and differ from Mn-SODs reported from mung bean (220), bajra (221) and ground nut (226).

The venom Mn-SOD is a single polypeptide chain in contrast to the dimeric Mn-SODs reported from photosynthetic organism (227) and red alga (228). Mn-SODs containing four subunits were also reported from bovine heart mitochondria (229), human liver (205). The presence of manganese in H.fulvipes venom SOD is confirmed by Atomic absorption analysis of the metal. The results indicate that the enzyme contains one atom of Mn/molecule similar to Mn-SODs reported by Sevilla et al (225). However, Mn-SODs
containing 2 and 4 atoms of Mn/molecule were also reported (230,231).

The temperature tolerance of venom Mn-SOD revealed that it is stable up to 50°C which resembled maize Mn-SOD (232). H.fulvipes Mn-SOD completely lost its activity at 80°C in contrast to Mn-SODs from mung bean (220), bajra (221) and ground nut (226) which are completely stable even at 70°C. However venom enzyme is more stable than pea leaf enzyme (225).

Inhibition of venom Mn-SOD activity by PHMB may be due to the exposure of greater number of half cystine residues present in the enzyme which is in agreement with the pea leaf enzyme (233).

Mn-SODs are sensitive to SDS than Cu-Zn SODs. Mn-SOD isolated from eukaryotic sources (220,226) are inactivated by SDS, accordingly venom enzyme also inhibited.

Cyanide has no effect on venom Mn-SOD as reported for other Mn-SODs in literature. Inhibition of venom enzyme activity by DDC, a specific inhibitor of Cu-Zn SODs is insignificant. This property was used to identify the metal ion present in SODs in crude samples.

The denaturation of Mn-SOD by guanidinium chloride is similar to that of ground nut Mn-SOD (226).
Azide and nitroprusside inhibit all forms of SODs irrespective of metal present and regardless of the source from which the SOD isolated (234, 235). Accordingly H. fulvipes Mn-SOD was inhibited to 85% and 70% by azide and nitroprusside respectively.

Inhibition of H. fulvipes venom SOD by antiserum raised against whole venom indicate that the enzyme has antigenic property.
SUMMARY AND CONCLUSIONS
The venom from *H. fulvipes* scorpion was analyzed for its toxicity and enzymatic principles. An antiserum was prepared against the venom and its efficiency was tested.

$LD_{50}$ value (i.p) determined for *H. fulvipes* venom was 100 µg per gram body weight in mice. Histopathological examination of liver, lung, heart, kidney and brain tissues showed acute congestion, wide spread haemorrhages, edema and degenerative changes in cells. Brain showed meningial blood vessels while areas of emphysema and collapse were noticed in lungs. In antivenom + venom treated animals no such pathological changes were observed.

Four enzymatic principles viz. phospholipase $A_2$, hyaluronidase, phosphodiesterase and superoxide dismutase were isolated from the venom using ammonium sulphate fractionation, gel filtration and ion-exchange chromatography.

The homogeneity and native molecular weight of the purified enzymes were determined by PAGE and SDS-PAGE.

$PLA_2$ showed a single band on PAGE. The final preparation was purified 78 fold with specific activity of 3525. The molecular weight of the enzyme was found to be 16,000 and it was optimally active at pH 7.4 and temperature at 50°C. Substrate specificity studies revealed that the enzyme is more specific for phosphatidylcholine. Divalent metal ions like calcium, magnesium and zinc stimulated the
enzyme activity while mercury ion and EDTA inhibited. The enzyme exhibited a fluorescence emission maximum between 310-320 nm.

The purified PLA$_2$ showed hemolytic activity on sheep erythrocytes. Lecithin enhanced the hemolytic activity of the enzyme.

Hyaluronidase has a molecular weight of 82,000. The final preparation was purified 27 fold with 42% yield. The optimum pH for enzyme activity was 4.0. Heparin inhibited the enzyme activity.

Phosphodiesterase was purified 109 fold. The enzyme had a molecular weight of 115,000 and it was optimally active at pH 7.0 and temperature at 65$^\circ$C. Dithiothreitol, glutathione, cysteine and EDTA inhibited the enzyme activity.

The purified SOD has a molecular weight of 100,000. Optimum pH for enzyme activity was 8.5 and optimum temperature was 45$^\circ$C. The enzyme was not sensitive to either cyanide or hydrogen peroxide but was inhibited by chloroform-ethanol mixture and p-hydroxymercuribenzoate. Metal chelators, EDTA, o-phenanthroline and diethyldithiocarbamate inhibited the enzyme activity in decreasing order. Denaturing agents like sodium dodecylsulfate, guanidinium chloride and nitroprusside inhibited the enzyme activity to
various extent.

An antiserum raised against the scorpion venom neutralized the hemolytic activity of PLA₂ on erythrocytes and hydrolytic effect of phosphodiesterase on nucleic acids.

Further, the antivenom inhibited the catalytic activity of all the four enzymes.

Among the four enzymes, three enzymes phospholipase A₂, hyaluronidase and phosphodiesterase are known to be toxic components of the venom but not the SOD.

In crude venom, it is possible that the absolute SOD activity may be suppressed by other dominating enzymes such as phospholipase A₂, hyaluronidase and phosphodiesterase. This may be the reason for the total toxicity of the venom inspite of the presence of SOD, a free radical scavenger.

It is concluded that this specific potent anti-scorpion venom may be used as an antidote immediately after scorpion sting.