Kinetic properties of cell wall bound superoxide dismutase in leaves of wheat (Triticum aestivum L.) following stripe rust (Puccinia striiformis) infection

Bavita Asthir1*, A Koundal1 and N S Bains2
Department of Biochemistry1, Department of Plant Breeding and Genetics2, Punjab Agricultural University, Ludhiana 141004, Punjab, India
Received 25 March 2011; revised 07 July 2011

Stripe rust (Puccinia striiformis f.sp. tritici) is the most devastating disease of wheat (Triticum aestivum L.) accounting huge economical losses to the industry worldwide. HD 2329 was a widely grown wheat cultivar which had become highly susceptible to stripe rust and was used to understand the biochemical aspects of the host pathogen interaction through characterization of superoxide dismutase (SOD). In the present study, two types of SOD, ionically or covalently bound to the particulate fraction were found in the stripe rust infected and uninfected wheat leaves of susceptible cultivar HD 2329. Cell walls of leaves contained a high level of SOD, of which 41-44% was extractable by 2 M NaCl and 10-13% by 0.5% EDTA in infected and uninfected leaves. The NaCl-released SOD constituted the predominant fraction. It exhibited maximum activity at pH 9.0, had a $K_m$ value of 1.82-2.51 for uninfected and 1.77-2.37 mM for infected, respectively with pyrogallol as the substrate, and a $V_{max}$ of 9.55-21.4 and 12.4-24.1 $\Delta A$ min$^{-1}$g$^{-1}$FW. A temperature optimum of 20°C was observed for SOD of both uninfected and infected leaves. SOD showed differential response to metal ions, suggesting their distinctive nature. Inhibition of wall bound SOD by iodine and its partial regeneration of activity by mercaptoethanol suggested the involvement of cysteine in active site of the enzyme. These two forms showed greater differences with respect to thermodynamic properties like energy of activation ($E_a$) and enthalpy change ($\Delta H$), while entropy change ($\Delta S$) and free energy change ($\Delta G$) were similar. The results further showed that pathogen infection of the leaves of susceptible wheat cultivar induced a decrease in the SOD activity and kinetics which might be critical during the response of plant cells to the infection.

Keywords: Puccinia striiformis, Superoxide dismutase, Thermodynamic properties, Triticum aestivum

Puccinia striiformis (f. sp.) tritici is an obligate biotrophic pathogen that causes leaf stripe rust on wheat (Triticum aestivum L.). It is a major constraint in wheat production and is a severe threat to food security worldwide. Crop losses and invariable use of fungicides has necessitated the development of more affordable control strategies against stripe rust. Many disease resistant genes and various defense-related genes are involved in conferring resistance against plant pathogens. They act at different levels in the host-pathogen interaction.

Reactive oxygen species (ROS) are produced in both unstressed and stressed cells. Plants have well developed defense systems against ROS, involving both limiting the formation of ROS as well as instituting its removal. Within a cell, the superoxide dismutases (SODs; EC 1.15.1.1) constitute the first line of defence against ROS and exist as a group of metalloenzymes that catalyze the disproportionation of superoxide radicals ($O_2^-$) generated in different cellular compartments to $H_2O_2$ and $O_2$. In the apoplast, SODs are an important class of enzymes responsible for the formation and degradation of ROS. Plants have a great diversity of SOD and these include cell wall SODs that play significant role in cell signaling events, since the product of its reaction $H_2O_2$ is an important transduction signal molecule in different physiological processes and plant stress. Moreover, many more isoforms can be generated by post-transcriptional and post-translational modifications and their activity is influenced by various stresses, including pathogen infection. The identification and study of the properties of SOD under stress conditions is essential for the possible manipulation by plant stress resistance.

The ability of plants to overcome oxidative stress only partly relies on the induction of SOD activity and other factors can regulate the availability of the substrate for SOD. It is reported that possibly different plant species and tissues involve different mechanisms in protection against oxidative stress. But, it is unclear whether this effect is on the gene...
expression or on the kinetic properties of SOD. In the present work, we demonstrate the occurrence of at least two distinct SOD activities (released by NaCl and EDTA) associated with the cell wall of leaves of susceptible cultivar HD 2329 of wheat.

Materials and Methods

Plant material
HD 2329 cultivar of wheat (Triticum aestivum L.) was grown in 12 inch diameter pots filled with clay loam soil as per standard practice for raising wheat plants. Plants were grown in 3 replication (3 plants/pot) and inoculated at boot stage with mixture of Puccinia striformis pathotype prevalent in the region using fresh ureidial suspension (300 uredia cm⁻²). Inoculated and control plants were then incubated at 13°C ± 2°C in opaque polythene chamber for 48 h to ensure saturated atmosphere for infection. Flag leaves showing 40% infection (i.e. 40% of the leaf area infected or covered with rust pustules) were selected for laboratory studies along with sample for the control treatment which were free from infection.

Extraction and solubilization of SOD
Excised leaves (30 g) were homogenized with a chilled pestle and mortar in 100 ml of sodium phosphate buffer (pH 6.5). Cell walls obtained after extraction of the soluble fraction were washed thoroughly with 1 L of distilled water. The pellets were suspended in 100 ml of sodium phosphate buffer (pH 6.5) and suspension was kept for 24 h at 4°C. The extract was collected under vacuum filtration and designated as the EDTA-released fraction. Kₘ and Vₘₐₓ were determined by the double-reciprocal method of Lineweaver and Burk employing weighted least squares linear regression analysis. The formulae used to calculate energy of activation, enthalpy and entropy changes were: Eₐ = –2.303 R x slope (slope of the linear plot of log Vₘₐₓ vs 1/T, where R is Gas constant = 8.314 JK⁻¹mol⁻¹), ΔH = –slope x 2.303 R (slope of the linear plot of pKₘ vs 1/T where, pKₘ = –log Kₘ), ΔG = ΔH - TΔS, ΔS was calculated from the standard thermodynamic relation where ΔG was defined as the free energy of substrate binding. Kₘ was regarded as the dissociation constant of enzyme-substrate complex (ES ↔ E + S), the values of ΔH and ΔS represented the changes occurring during the formation of Michaelis complex.

Effect of pH and temperature on enzyme activity
The effect of pH on SOD activity was determined by estimating its activity in buffers ranging in pH from 5.0 to 10.0. For the pH range 5.0, the Na-acetate buffer was used; for the pH range 6.0 to 7.0 and 8 to 10.0 a phosphate and Tris buffer were used, respectively. Thermal stability of the enzyme was examined by first incubating 0.2 ml of enzyme preparation at –10, 0, 10, 20, 30, 40, 50 and 60°C, respectively, for 2 h, followed by assaying the enzyme activity as described above.

Inhibition of enzyme by iodine and its regeneration by β-mercaptoethanol
Inhibition of enzyme activity by iodine was examined at varying concentrations ranging from 0.05-1.0 mM and the inhibited enzyme was referred as iodine-SOD. The reaction mixture contained 0.1 ml enzyme extract and 0.1 ml of 0.1 mM iodine mixed in 100 mM Tris HCl buffer (pH 8.2). After 5 min the iodine-SOD was added with 0.6 mM pyrogallol, 1.5 ml 100 mM Tris HCl buffer (pH 8.2), 0.5 ml 6 mM EDTA and 0.1 ml of the enzyme extract. The rate of autooxidation of pyrogallol was taken from the increase in absorbance at 420 nm in a spectrophotometer after an interval of 15 s up to 2 min. One unit (U) of SOD was the amount of enzyme that inhibited pyrogallol autooxidation by 50%, monitored at 420 nm on Elico spectrophotometer (UV-SL 159). Enzyme activity was expressed as change in absorbance (ΔA₄₂₀ min⁻¹ g⁻¹FW).

Effect of metal ions on enzyme activity
For ascertaining the effects of metal ions i.e. Ca²⁺, Cd²⁺, Na⁺, K⁺, Zn²⁺, Co²⁺ and Cu²⁺ on the activity of SOD, the reaction mixtures (3.1 ml) contained 1 ml 0.6 mM pyrogallol, 1.5 ml 100 mM Tris HCl buffer...
(pH 8.2), 0.5 ml 6 mM EDTA and 0.1 ml of enzyme extract plus different metal ions were used. The activity of the enzyme was determined.

**Statistical analysis**

Results were based on at least three replicates. The values were statistically analyzed by a one-way analysis of variance. The differences were considered significant when P ≤ 0.05.

**Results and Discussion**

We performed different washing procedures to determine if SOD activity was loosely bound to the cell wall or entrapped inside the vesicles. Two types of SOD, ionically or covalently bound to the particulate fraction existed in stripe rust-infected and uninfected wheat leaves of HD 2329. The extract obtained after the cell walls was suspended in 2 M NaCl and contained 41-44% of the total SOD activity. After extraction with NaCl, the cell walls were further extracted with 0.5% EDTA. About 10-13% of the SOD could be extracted with EDTA. These treatments revealed the presence of at least three types of SOD in the cell walls of wheat leaves with two solubilized with NaCl and EDTA and the third tightly bound to the residual cell walls. Similar release of SOD by salt stress from the cell wall of moss is reported by Nakata et al. 12.

In NaCl-released fraction of infected and uninfected leaves, SOD showed maximum activity at pH 9.0 (Table 1). MnSOD from wheat seedlings is found to be stable over pH 7.0-9.0 with an optimum pH of 8.0, but is sensitive to extreme pH, particularly to acidic pH13. By using 1.2-2.0 mM pyrogallol and pH range from 7.5 to 10.0, $K_m$ and $V_{max}$ were determined at each pH (Table 2). At pH 8.5-9.0, the enzyme exhibited strong affinity to pyrogallol as revealed by $K_m$ values, which ranged from 1.82–1.97 (mM) for uninfected and 1.77–1.88 (mM) for infected leaves. The results indicated that at pH 9.0, which was nearly the optimum pH, the $K_m$ was lowest. Plotting the values of log$V_{max}/K_m$ and log$V_{max}$ against pH, the curves were obtained (Fig. 1). The application of Dixon’s rule showed that (i) the pK value of ionizing groups existing in the free enzyme was 9.0-9.7 in uninfected and 9.5-9.7 in infected leaves, and (ii) in the ES complex, there were ionizing groups with pK values of 9.4-9.6 in uninfected and 9.6-9.9 in infected leaves. A comparison of the pK values of ionizing groups in the enzyme with the ones listed by Dixon and Webb14 indicated the possibility of participation of amino groups during enzyme catalysis.

The enzyme exhibited temperature optima at 20°C (Table 1). With increase in temperature from –10 to

<table>
<thead>
<tr>
<th>pH</th>
<th>% of Maximum SOD activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>2.1 ± 0.03</td>
</tr>
<tr>
<td>6.0</td>
<td>7.0 ± 0.04</td>
</tr>
<tr>
<td>7.0</td>
<td>13.3 ± 0.80</td>
</tr>
<tr>
<td>8.0</td>
<td>80.0 ± 2.7</td>
</tr>
<tr>
<td>9.0</td>
<td>100</td>
</tr>
<tr>
<td>10.0</td>
<td>65.3 ± 2.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>% of Maximum SOD activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>-10</td>
<td>50 ± 3</td>
</tr>
<tr>
<td>0</td>
<td>68 ± 4</td>
</tr>
<tr>
<td>10</td>
<td>85 ± 6</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>40</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>50</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>60</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

Table 1—Effect of pH and temperature on activity of wall-bound SOD released by NaCl from uninfected and infected leaves of HD 2329

![Fig. 1—Effect of pH on $K_m$ and $V_{max}$ of wall bound SOD [(A): Relationship between pH and log $V_{max}/K_m$; and (B): Relationship between pH and log $V_{max}$ in uninfected (-●-) and infected (-▲-) leaves of cultivar HD 2329]
20°C, there was a gradual increase in the enzyme activity. It exhibited lowest $K_m$ at temperature 20°C (Table 2). Most SOD from plants and animals seemed to have low temperature optima. Apoplastic, cytosolic and soluble SOD of several plant tissues has shown temperature optima between 20 and 35°C. The thermal stability of SOD was also observed in the present study, wherein enzyme stability was demonstrated from −10 to 60°C with an optimum temperature of 20°C. SOD showed significant thermostability with an increase in SOD activity up to 45°C. However, beyond this temperature, activity fell as reported for other higher terrestrial plants and lower plants, possibly because the heat denaturation of the enzyme being dependent on its sub-unit structure.

SOD was present in the cell walls (data not shown), which is characteristic of pathogen-induced SOD. Similar accumulation of Mn-SOD bound to peroxisomal membranes has been observed in watermelon cotyledons. Linear plots were obtained between log $V_{max}$ and $pK_m$ versus 1/T (Fig. 2), suggesting that $K_m$ and $V_{max}$ were simple constants rather than complex functions of several velocity constants. The energy of activation ($E_a$) calculated from Fig. 2A were 20.62 and 16.87 kJmol$^{-1}$ for the uninfected and infected NaCl–released fractions, respectively. The corresponding values of enthalpy change ($\Delta H$) calculated from Fig. 2B were −32.81 and −20.19 kJmol$^{-1}$ for the uninfected and infected NaCl-released fractions, respectively. The entropy change value ($\Delta S$) for the enzyme at 30°C was, therefore, −5.78 and −5.88 JK$^{-1}$mol$^{-1}$ for uninfected and infected NaCl-released fraction (Table 3), respectively.

The formulas used for calculating $E_a$ and $\Delta H$ were identical, but these values were derived from different curves (Fig. 2). Because $K_m$ was affected more than $V_{max}$ and also the values of $K_m$ were far greater than $V_{max}$ (Table 2), so the values obtained were related to the formation of product rather than to the formation of ES complex or to overall reaction. $E_a$ corresponds to energy of activation and its value was comparatively less in the infected leaves of NaCl-released fraction than uninfected leaves, indicating probably that infected leaves cannot tolerate high stress and the rate of the reaction will be slower under such conditions. This was further reflected in a low temperature tolerance in infected leaves over uninfected ones (Table 1). Infected leaves retained

![Fig. 2—Effect of temperature on $K_m$ and $V_{max}$ of wall bound SOD in NaCl-released fractions of infected and uninfected leaves of HD 2329](image)

Table 2—Effect of pH and substrate concentration on $K_m$ (mM) and $V_{max}$ (ΔA min$^{-1}$g$^{-1}$FW) of wall bound SOD in NaCl-released fractions of infected and uninfected leaves of HD 2329

<table>
<thead>
<tr>
<th>pH</th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>7.5</td>
<td>2.51</td>
<td>12.0</td>
</tr>
<tr>
<td>8.0</td>
<td>2.19</td>
<td>13.8</td>
</tr>
<tr>
<td>8.5</td>
<td>1.97</td>
<td>19.6</td>
</tr>
<tr>
<td>9.0</td>
<td>1.82</td>
<td>21.4</td>
</tr>
<tr>
<td>9.5</td>
<td>2.00</td>
<td>17.4</td>
</tr>
<tr>
<td>10.0</td>
<td>2.28</td>
<td>9.55</td>
</tr>
<tr>
<td>Temp (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3.13</td>
<td>6.6</td>
</tr>
<tr>
<td>30</td>
<td>3.18</td>
<td>4.5</td>
</tr>
<tr>
<td>40</td>
<td>3.28</td>
<td>3.8</td>
</tr>
<tr>
<td>50</td>
<td>3.32</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Table 3—Determination of kinetic constants of wall bound SOD in NaCl-released fractions of infected and uninfected leaves of HD 2329

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_a$ (kJmol$^{-1}$)</td>
<td>20.62</td>
<td>16.87</td>
</tr>
<tr>
<td>$\Delta H$ (kJmol$^{-1}$)</td>
<td>-32.81</td>
<td>-20.19</td>
</tr>
<tr>
<td>$\Delta G$ (kJ)</td>
<td>-1.786</td>
<td>-1.803</td>
</tr>
<tr>
<td>$\Delta S$ (JK$^{-1}$mol$^{-1}$)</td>
<td>-5.78</td>
<td>-5.88</td>
</tr>
</tbody>
</table>
higher activity of SOD at all studied temperatures over uninfected leaves. Values of ∆H, a measure of enthalpy change or heat evolved during the reaction also differed under uninfected and infected conditions, being more negative under uninfected conditions, indicating change in conformation of the enzyme during infection. However, ∆G and ∆S did not vary much during infection, suggesting minimal change in ΔG and ΔS of the reaction.

The enzyme was completely inhibited by I₂ and partly regenerated with mercaptoethanol (70%), suggesting the involvement of cysteine in the enzyme active site. The disulfide bond plays an important role in stabilizing the dimer interface by anchoring the loop in the dimer interface. Ca²⁺, Cu²⁺, Zn²⁺, Cu⁺, Co²⁺ were found to be stimulators for SOD activity in both uninfected and infected leaves, while Na⁺ and K⁺ had inhibitory effect (Table 4), showing the differential effect of metal ions on the enzyme activity. The oxidation states of Cu⁺ or Cu²⁺ in vivo did not affect enzyme activity as the reaction velocity attained its maximum value instantaneously in both cases. Therefore, the use of Cu⁺ ion was expected to yield similar results. Probable mechanism of increased activity of SOD with Cu²⁺ had been correlated to increased level of glutathione content. The binding of metal ions has profound stabilizing effects on SOD. The coordination of metal ions increases SOD stability by reducing the structural fluctuations of the native interface. It has been shown that Ca²⁺ and Zn²⁺ ions can increase activity and thermostabilize different SOD isoforms.

In conclusion, the results above showed that pathogen infection of the leaves of susceptible wheat cultivar led to a reduction in SOD activity, some of which was further extractable, following application of high concentration of salt and EDTA. Experiments on the study of pH and temperature dependence of SOD, their thermodynamic properties and sensitivity to inhibitors and metal ions demonstrated that the pathogen caused the changes in the kinetics of SOD activity that might be critical during the response of plant cells to infection.

References