Purification and Characterization of a Lipoxygenase Isozyme from Oat Leaves Inoculated with Incompatible Race of

*Puccinia coronata avenae*

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Key words: *Puccinia coronata*, oat, lipoxygenase, resistance, purification.

Lipoxygenases (LOXs) are an oxidoreductase which catalyze the hydroperoxidation of fatty acids having cis, cis-1,4-pentadien structures. These enzymes are widely distributed in higher plants. The physiological role of plant LOX has not been elucidated, although it has been associated with ripening, abscission, senescence, and resistance to plant pathogens.

In fungal-infected leaves, the increase of LOX activity has generally been much larger in lines of a specific resistant to a particular fungus than in susceptible lines. Recently, new isozyme was found in an incompatible host but not in a compatible one, blast fungus.

In previous study, we reported that the two isozymes (LOX-1 and LOX-5) in the incompatible oat-crown rust system are de novo synthesized and their activities are causally linked with the resistance. In the further experiments, it is suggested that LOX-5 was considered to be the same with LOX-1.

In this paper, the purification of oat leaves LOX isozyme and some of its characters are examined.

Oat leaves (100 g) inoculated with race 226 were harvested 48 hr after inoculation and homogenized in 500 ml of 100 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA by Waring blender (20,000 rpm). Then, the homogenate was filtered through 2 layers gauze and centrifuged at 10,000 × g for 15 min. The first protein of supernatant precipitating 50% saturation with (NH₄)₂SO₄ was removed by centrifugation, then the second supernatant precipitating 80% saturation was collected by centrifugation (20,000 × g, 30 min), dissolved in 20 mM Tris-HCl buffer (pH 7.2) and dialyzed overnight against the same buffer.

The dialyzed solution was concentrated against Aquacide II-A in dialysis bags and was subjected to chromatography using DEAE-Sepharose CL-6B column (1.2 × 50 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.2). The column was washed with the same buffer followed by elution with a linear gradient of 0.0-0.5 M NaCl in the same buffer.

Analytical disc electrophoresis was performed in a 6.0% polyacrylamide gel according to the method of Davis. LOX isozymes on the gel was visualized by the method as described. Glycoprotein was visualized on gel by staining with periodic acid-Schiff (PAS) reagent according to Glossmann and Neville. A method for staining iron proteins on gel was carried out as described by Kuo and Fridovich. Proteins were stained by silver stain kit or Coomassie brilliant blue.

Isoelectrofocusing (IEF) in polyacrylamide gel was performed in the pH range 3.5-10 as previously described, but Nonidet P-40 and urea were omitted from gel in this experiment. LOX activity was
Table 1. Purification of LOX-1 from oat leaves inoculated with *Puccinia coronata* race 226 (100 g leaves used)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Activity (U)</th>
<th>Specific activity (U/mg protein)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2112</td>
<td>78396</td>
<td>37</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate (50-80%)</td>
<td>110</td>
<td>3740</td>
<td>34</td>
<td>5.2</td>
<td>0.9</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B (Numbers 7 and 8)</td>
<td>0.88</td>
<td>372</td>
<td>423</td>
<td>0.04</td>
<td>11.4</td>
</tr>
</tbody>
</table>

LOX activity was measured as hydroperoxides (ε = 25,000 M⁻¹·cm⁻¹) produced at 25°C by monitoring the increase in absorbance at 234 nm.

The recovered yield percentage during purification is shown in Table 1. In first step, the activity of LOX-1 was found in the precipitate of between 50 and 80 saturation with (NH₄)₂SO₄. Further purification was accomplished by ion-exchange chromatography. Activity of LOX-1 was found in the protein passed on DEAE-Sepharose CL-6B column eluting with 20 mM Tris-HCl buffer (pH 7.2). When 5 ml of the eluate was collected, fractions of number 7-9 contained LOX-1 isozyme (Fig. 1). However, number 9 showed slight contamination of LOX-2. LOX-1 in fractions No. 7 and 8 was verified to be highly purified one by disc-PAGE, because any of other proteins were not detectable (Fig. 2-A).

The enzyme fractions (numbers 7 and 8) were pooled and concentrated by adding (NH₄)₂SO₄ to 80% saturation. LOX-1 was purified by about 11-fold to crude extract.

Substrate specificity of LOX-1 was determined on linoleic acid, linolenic acid, arachidonic acid and their esters. Among the common substrates tested, linoleic acid was the best substrate for LOX-1 (Table 2), but the activity reduced in the various acid esters as substrates. LOX-1 activity was highest at pH 5.0, similar to rice leaf LOX-3⁴⁹.

The Michaelis-Menten constants were determined with the spectrophotometric assay at optimum conditions (25°C, sodium acetate buffer, pH 5.0). Values for the apparent Km and Vmax of the LOX-1 isozyme calculated from a Linewaver-Burk plot using linoleic acid as substrate were 23 μM and 0.49 μM/min, respectively.

The stimulatory and inhibitory effect of metal ions on LOX-1 is shown in Table 3. Among ions tested, LOX-1 was stimulated by 0.1-5.0 mM Fe²⁺. α, α'-Dipyridyl of metal chelator reduced activity by 60% at 0.1 mM. At 5 mM, Mg²⁺, Ca²⁺, Mn²⁺ and Co²⁺ inhibited 30-50% of enzyme activity.

The effect of temperature on enzyme stability was examined by measuring the residual activity...
Fig. 2. Diagrammatic illustration of preparations of LOX-1. A, polyacrylamide gel electrophoresis (PAGE, 6% gel); B, isoelectrofocusing (IEF, pH range 3.5-10). Purified LOX from 50-80% (NH₄)₂SO₄ saturation (lane 1) and DEAE-Sepharose CL-6B chromatography (lane 2). LOX was visualized by o-dianisidine staining. LOX-1 was indicated by star. pI: pI-value.

Table 2. Substrate specificity on oat LOX-1 isozyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic acid</td>
<td>100</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>55</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>2</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>14</td>
</tr>
<tr>
<td>Methyl linolenate</td>
<td>22</td>
</tr>
</tbody>
</table>

LOX activity is defined as in Table 1.

Table 3. Effect of different ions on the activity of oat LOX-1 isozyme

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
<th>Ion</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td>Ca²⁺</td>
<td>0.1</td>
<td>87</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>0.1</td>
<td>274</td>
<td>Fe³⁺</td>
<td>1.0</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>128</td>
<td></td>
<td>5.0</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>118</td>
<td></td>
<td>0.1</td>
<td>64</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.1</td>
<td>87</td>
<td>Mn²⁺</td>
<td>1.0</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>98</td>
<td></td>
<td>5.0</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>68</td>
<td>Co²⁺</td>
<td>0.1</td>
<td>71</td>
</tr>
</tbody>
</table>

LOX activity is defined as in Table 1.

after the treatment at 40-80°C for 15 min. LOX-1 activity remains about 80% of its original one after treatment at 60°C. At 70°C, the activity of LOX-1 lost more than 80%.

Inhibition of the activity by SH-reducing reagents such as reduced glutathion, dithiothreitol and 2-mercaptoethanol inhibited 40-50% activity.

The isoelectric points of the purified isozyme were determined by IEF (Fig. 2-B). LOX-1 exhibited a pI-value of 5.14. Other proteins were not detectable. LOX-1 on polyacrylamide gel was shown to be
iron-containing glycoprotein\(^2,5\)).
These findings differ from those reported on oat LOX by Gardner\(^3\). Immunohistochemical assay by the use of purified LOX-1 enzyme will be provided in the further study of clarify the localization of resistant specific LOX in oat leaf tissues.

**Literature cited**


和文摘要

田中明美・山本弘幸・前家正起・丸山安恒・谷利一：不親和性冠さび面感染エンパク症からのリポキシゲナーゼのイソザイムの単離とその性質

エンパク冠さび病の抵抗性発現時に *de novo* 合成されるリポキシゲナーゼ（LOX-1）を50〜80%鈉和の硫酸アンモニウム沈殿、DEAE-Sepharose CL-6 B カラムクロマトクロム法によって单一蛋白質として単離した。LOX-1の最適pHは5.0で、基質特異性はリノール酸で高く、同基質に対する *Km* 値、*Vmax* はそれぞれ23μM、0.45μM/minであった。pH値は5.14であり、鈉を含んだ糖酸蛋白であった。LOX-1の活性はFe**イオンで促進され、Mg**, Ca**, Mn**, Co**イオンおよび還元剤のDTT、2-MEなどによって阻害された。

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