D. Chitinase

Enzyme chitinase (EC 3.2.1.14) catalyzes the cleavage of a bond between C1 and C4 of two consecutive N-Acetyl Glucosamine (NAG) monomers of chitin. The presence of chitinase (CHI) as a thermo sensitive and diffusible antifungal factor in orchid bulbs was first described in 1911. There are two kinds of chitinases: endochitinases and exochitinases. Most plant CHIs are endochitinases that randomly hydrolyze chitin polymers. Endochitinase cleaves chitin randomly at internal points within the polymer, releasing soluble, low-molecular weight multimers of NAG such as chitotetraose and chitotriose and the dimer, di-acetylchitobiose, which predominates. Some plant chitinases also show lysozyme activity, which hydrolyzes the β-1, 4-linkages between N-acetylmuramic acid and NAG residues in peptidoglycan (Roberts & Selitrennikoff, 1988). Since chitin is not a component of plant cell wall the presence of CHI in plants has been attributed to their role in protection from chitin-containing parasites like fungi and insects (Abeles et al. 1970; Bell, 1981; Boller, 1985). Members of the family Solanaceae have been preferentially used as model systems to investigate any physiological and molecular mechanisms of growth and development. In the present study also tomato has been selected for studying optimal physical parameters for production of CHI in its seedlings.

1. Materials and method

1.1 Preparation of colloidal chitin

Colloidal chitin was prepared and assayed according to the method of Berger & Reynolds (1958). In this method, ten grams of chitin was slowly dissolved in 400 ml of concentrated HCl with constant stirring in cooling conditions; which was then incubated in water bath at 37°C until viscosity decreased. To this mixture 4.0 litres of sterile D/W was added and left overnight at 4°C. The supernatant was slowly decanted and the precipitate was collected on a whattman filter...
paper and washed thoroughly with sterile D/W to attain neutral pH. The colloidal chitin thus obtained was dissolved in required amount of D/W prior to use.

1.2 Preparation of homogenate for enzyme assay, ammonium sulphate fractionation and dialysis

Homogenate for CHI enzyme extraction was prepared according to Reissig et al. (1955). The supernatant obtained on homogenizing with 0.1 M Sodium Citrate buffer (pH 5) was used for enzyme assay. Enzyme homogenates were stored in at 4°C until utilized for biochemical analysis. Chitinase crude homogenate were subjected to 0-80% (w/v) saturation with ammonium sulphate at cold conditions. The saturated solutions were left overnight at 4°C and the precipitated proteins were separated by centrifugation at 7000×g for 10 min at 4°C. The precipitate was dissolved in 33 ml of 0.1M Sodium Citrate buffer (pH 5). The concentrated sample with maximum specific activity was dialyzed for 8 h against 1 L of above buffer for further use (modified method of Lokhandwala & Bora, 2013).

1.3 Chitinase assay

CHI assay was performed according to Reissig et al. (1955). The Chitinase activity in Lycopersicon esculentum Mill seedlings was measured using colloidal chitin as a substrate for the homogenate prepared, as mentioned earlier. Absorbance was measured at 585nm on shimadzu made UV-VIS 1800 spectrophotometer. One unit of enzyme activity was expressed as 100μg NAG/ml chitin solution in 1h at 37°C.
1.4 **Kinetic studies**

1.4.1 **Optimum pH and pH stability profile**

The optimum pH value for partially purified chitinase was estimated by assaying enzyme activity at different pH levels. The assay was carried out in the presence of different buffers with different pH such as 50mM Glycine-HCl buffer (pH 1, 2, 3 and 4), 50mM Sodium Phosphate buffer (pH 5, 6, 7 and 8) and 50mM Glycine-NaOH buffer (pH 9 and 10) separately in an assay mixture. The pH stability for chitinase was assayed for pH ranging from 1 to 10 for 5 days by incubating with equal volume (0.5ml) of enzyme extract and respective buffer. Residual enzyme activity was measured by above mentioned method at every 24 hrs (modified method of Lokhandwala & Bora, 2013).

1.4.2 **Optimum temperature and temperature stability profile**

The relative enzyme activity for partially purified chitinase was measured at different temperatures in the range of 0°C to 80°C to find out the best suited temperature. In order to determine thermal stability of enzyme, 0.5ml of partially purified chitinase was assayed at regular time intervals (which varied according to the temperature). Residual activity was assayed by above mentioned method at fixed time intervals and compared to control (Ebiloma et al. 2011).

1.4.3 **Effect of ionic strength and incubation time**

The effect of ionic strength (0.05 to 1 M of 1.0 M Sodium citrate buffer, pH 5) and incubation time (1-8 hrs) was assayed for production of chitinase (Şişecioğlu et al. 2010).
1.4.4 Effect of metal ions and reducing agents

Stimulatory or inhibitory effect of eight metal ions (HgCl$_2$, AgNO$_3$, CaCl$_2$, MgSO$_4$, FeCl$_3$, MnCl$_2$, CuSO$_4$ and CoCl$_2$) and three reducing agents (EDTA, SDS and β- mercaptoethanol) was studied on chitinase production (Kim et al. 2003).

1.4.5 Substrate specificity

Efficiency and specificity of chitinase enzyme with four different substrates (Colloidal chitin, Carboxy Methyl Cellulose, Laminarin and Amylose) was checked. Substrates were taken in range of 1%-10% and efficacy and rate of reaction was studied using Lineweaver Burk plot.

2. Results and discussion

2.1 Ammonium sulphate fractionation and dialysis

The fraction containing 75% ammonium sulphate concentration showed maximum chitinase specific activity. This primary purification step resulted in 1.73 fold purification with 57.61% yield respectively (Table 2A). Chitinase precipitation at higher ammonium sulphate concentration justifies that chitinase is a low molecular weight enzyme. It has been reported that tomato chitinase are in range of 27-31KDa (Pegg & Brady, 2002). Similar results were obtained in study made by García-Breijo et al. (1990) wherein chitinase from tomato leaves showed maximum activity with 75% ammonium sulphate precipitation, 54.2 % yield and 1.1 purification fold.
Table 2A: Partial Purification of chitinase

<table>
<thead>
<tr>
<th>Sample used</th>
<th>Volume (ml)</th>
<th>Enzyme (µmoles/hr/ml)</th>
<th>Total Protein (mg)</th>
<th>Specific activity (µmoles/hr/ml/mg)</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>90</td>
<td>189.2</td>
<td>3.015</td>
<td>62.7</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate fraction</td>
<td>35</td>
<td>109</td>
<td>1.0</td>
<td>109</td>
<td>57.61</td>
<td>1.73</td>
</tr>
</tbody>
</table>

2.2 Kinetic studies

2.2.1 Optimum pH and pH stability profile

The optimum pH found by assaying enzyme activity at different pH levels was 5.0 in 0.1 M Sodium Citrate buffer (Figure 2A & Table 2B). The chitinase activity measured at this pH was 286.6 µmoles/hr/ml. There was a sharp decrease in chitinase activity at alkaline pH and it was almost negligible on day 5 in alkaline pH range. Therefore, chitinase seems to be more stable in acidic (pH 3 to 6) range (Figure 2B, Figure 2C & Table 2B). These results corroborates with the earlier reports on sweet potato (pH 5.0 in 0.1M acetate buffer) by Hou et al. (1998) and in tea plants (pH5-5.8) by Nisha et al. (2011). Acidic environment in plant is maintained in organelles like vacuole and apoplast where apoplast is the first site of contact with pathogens from wherein different defence responses are initiated (Bolwell et al. 2001) while vacuole has been reported as production site for synthesis of chitinase as a response against fungal pathogens (Taiz, 1992).
These reports strongly support our observations for the maximal production and stability of chitinase at acidic pH.

2.2.2 Optimum temperature and temperature stability profile

The optimum temperature for chitinase production was found to be 20°C (Figure 2D & Table 2B) where the chitinase activity was 455.6µmoles/hr/ml; beyond this temperature there was a continuous decrease in chitinase activity. Chitinase showed more stability at lower temperatures. (Figure 2E, Figure 2F, Figure 2G & Table 2B). Chang et al. (1996) reported that temperature optima for chitinase in cabbage are between 30°C- 60°C and enzyme activity decreased above 60°C. Similarly, Hou et al. (1998) also reported the temperature optima for chitinase in sweet potato as 25°C. It can be thus suggested that chitinase production is high at lower temperatures. This property of chitinase can be exploited in preventing plant from pathogen attack in cold environments and also can be used in prevention of spoilage caused to refrigerated food.

2.2.3 Effect of ionic strength and incubation time

Sodium citrate buffer (pH 5.0) with ionic strength of 0.1M was found to be optimum for chitinase production (Figure 2H and Table 2B). In present study, chitinase activity is maximum at 0.1M (248.35µmoles/hr/ml) and it reduces with increasing molarity. Chitinase possesses antifungal activity (Roberts & Selitrennikoff, 1988) and that depends on ionic strength. With increasing ionic strength antifungal activity reduces (Broekaert et al. 1995). So it becomes one of the important parameter to be studied as knowing optimum molarity may indirectly help in obtaining maximum antifungal activity from chitinase in plants. In present study, chitinase activity was highest (230.84µmoles/hr/ml) after 3 hrs of incubation period (Figure 2I and Table 2B). Since chitin is a high molecular weight compound so enzymes may require more incubation
time to digest it; which is also evident in present study. Microorganisms take very long time to
digest the colloidal chitin hence their optimum incubation time varies greatly from plants.
Brzezinska and Donderski (2001) reported 192 hrs of incubation time in *Aeromonas* Genus. In
present study chitinase activity reduces rapidly after 5 hrs of incubation which might be due to
over accumulation of its product (NAG).

2.2.4 Effect of metal ions and reducing agents

All heavy metals selected for present study showed reduction in chitinase activity among which
HgCl$_2$ being the most potent inhibitor; while amongst the reducing agents β-ME showed
enhancement in chitinase activity while EDTA and SDS showed mild inhibition in chitinase
activity (Figure 2J). Ueno et al. (1990) also reported similar metal inhibition and
mercaptoethanol enhancement. Similar results were reported by Kim et al. (2003) in which
chitinase activity is completely inhibited by HNO$_3$, in *Streptomyces* sp. M-20 in which authors
suggested that inhibition of chitinase by Hg$^2+$ is due to presence of sulfhydryl groups on the
active site of the enzyme. In present study also it can be speculated that inhibition of chitinase
activity may be due to presence of sulfhydryl group.

2.2.5 Substrate specificity

Partially purified enzyme showed activity only with colloidal chitin and not with laminarin,
amylose or carboxymethyl cellulose (Figure 2G). This shows absence of other enzymes like
glucanases, amylases or cellulases. Lineweaver-Burk plot obtained for varying concentration of
chitin showed Km and Vmax values as 11.5mM and 166.6µmoles/hr respectively. Hollis et al.
(1997) reported Km and Vmax values for Barley chitinase to be 3µM and 1.26µmoles/min/ml
respectively. Chitin and its derivatives are widely used as biomedical materials because of their
versatility and biocompatibility. Assay of chitinase had its own setbacks because of its expensive substrates. In present study partially purified chitinase from tomato shows significant activity with colloidal chitin which comes out to be a low cost assay for chitinase.

Table 2B: Physico-chemical and kinetic properties of partially purified chitinase

<table>
<thead>
<tr>
<th>Physico-chemical and kinetic properties</th>
<th>CHI</th>
<th>Enzyme activity (μmoles/hr/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>5.0</td>
<td>28655.92</td>
</tr>
<tr>
<td>Stable pH range</td>
<td>3-6</td>
<td>-</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>20°C</td>
<td>48555.5</td>
</tr>
<tr>
<td>Stable temperature range</td>
<td>10°C to 30°C</td>
<td>-</td>
</tr>
<tr>
<td>Optimum Molarity</td>
<td>0.1M sodium citrate buffer; pH 5.0</td>
<td>24835.1</td>
</tr>
<tr>
<td>Optimum incubation time</td>
<td>3 hrs</td>
<td>23083.9</td>
</tr>
<tr>
<td>Inhibitory agent/s</td>
<td>HgCl2</td>
<td>0</td>
</tr>
<tr>
<td>Enhancing agent/s</td>
<td>β- Mercaptoethanol</td>
<td>43605</td>
</tr>
<tr>
<td>Km (mM)</td>
<td>11.5</td>
<td>-</td>
</tr>
<tr>
<td>Vmax (μmoles/hr)</td>
<td>166.6</td>
<td>-</td>
</tr>
<tr>
<td>Vmax/Km</td>
<td>14.48</td>
<td>-</td>
</tr>
</tbody>
</table>

3. Conclusion

Anti-oxidant enzymes like Peroxidase, polyphenol oxidase, catalase and hydrolase enzyme like Chitinase are important yardsticks to analyze the defense enhancement ability of mycorrhizal colonized plants. In present study all of these four enzymes were partially purified and their optimum kinetic parameters were known. These results aided the researcher for optimal production of them in further experiments.