EXTRACTION, PURIFICATION AND CHARACTERIZATION OF EXO-POLYGALACTURONASE FROM CUCURBITA PEPO A PUMPKIN VARIETY COLA LAMBGOL SAMRAT 2

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ABSTRACT
Polygalacturonase (PG) is the most studied cell wall hydrolase. Germinating seeds of Cucurbita pepo, the pumpkin variety cola lambgol samrat 2 exhibited polygalacturonase (EC.3.2.1.67) activity. The maximal polygalacturonase activity was observed on the fourth day of germination. Enzyme was found to be in multiple forms, which was partially purified using the techniques of (NH4)2SO4 fractionation, DEAE-cellulose chromatography and sephadex G-200 gel filtration chromatography. Paper chromatographic results shows that the enzyme is an Exo-polygalacturonase. The maximum activity of the enzyme was found at pH 4.5. The enzyme exhibited optimum temperature of 64 °C.

KEYWORDS: Exo-polygalacturonase, Cucurbita pepo, (NH4)2SO4 fractionation, DEAE-cellulose chromatography, sephadex G-200 gel filtration chromatography. Paper chromatography.

INTRODUCTION
Exo-polygalacturonase (Galacturonase 1,4-a-galacturonidase, EC.3.2.1.67) catalyses removal of D-galacturonic acid monomer from non-reducing end. It plays an important role in plant cell wall dissolution by hydrolyzing the pectin methylestrase de-esterified pectin. Exopolygalacturonase has been purified from many plant sources, especially fruits,1-5 seeds,6 and fungal sources.7-8 Pectic enzymes are widespread in the plant kingdom and their complexity is becoming more obvious. Pectolytic enzymes in higher plants are usually
associated with conversion of protopectin to soluble pectin accompanying fruit ripening. They are assumed to play an intimate part in fruit ripening and are essentially hydrolases.\[9\]

After the solubilization of pectin, de-esterification is thought to take place by the action of pectinesterase, which then allows polygalacturonase (polygalacturonide glycano-hydrolase, EC.3.2.1.15) to rupture the glycosidic linkage. The types of polygalacturonases that attack polygalacturonic acid of high molecular weight and low degree of esterification have been divided into two groups; exopolygalacturonase removes single galacturonic acid, ultimately resulting into complete hydrolysis to galacturonic acid. Endopolygalacturonase hydrolyses the substrate at random, eventually converting it into a mixture of the monomer and the dimer.\[10-12\]

The substrate of enzyme action is pectin, a polymer of unhydro galacturonic units with α-1,4-linkage and in which a large number of carboxyl groups are esterified to form methyl ester. Pectins are large polysaccharide molecules, made up (mainly) of chains of several hundred galacturonic acid residues. Enzymes in this pectinase group include polyagalacturonases, pectin methylesterase and pectin lyases. These pectinase enzymes act in different ways on the pectins, which are found in the primary cell walls and in the middle lamella. Pectins are well known also for their ability to form gels. Pectin is prominent carbohydrate in the middle lamella of plant cell wall. It is a complex of polysaccharides forming about one third of the cell wall dry substances of dicotyledonous plants. The pectic substances contribute both the adhesion between cells and the mechanical strength of the cell wall behaving in the form of stabilized gels. Pectic substances are acidic heteropolysaccharides of relative molecular masses ranging from 23,000 to 3,60,00 and occurring in varying amount in all higher plants tissues. Pectin is also present in fruits and vegetables.\[13-16\]

MATERIALS AND METHODS

Chemicals
Polygalacturonic acid, pectin and galacturonic acid were purchased from CDH laboratories. All other chemicals used were of analytical grade.

Tissue Sterilization
Seeds of pumpkin variety cola lambbol samrat-2 were surface sterilized in 0.1% HgCl\textsubscript{2} for 1 min. and thoroughly washed with distilled water. The healthy seeds were placed over water
soaked filter paper covering an approximately 0.5 cm layer of moist cotton wool in petri dishes at 25\textdegree{}C.

**Enzyme extraction**

**Preparation of homogenate**

All the steps of enzyme extraction and purification were done at 4\textdegree{} C in a cold room. Homogenizing media consisted of 1M NaCl and Sodium Metabisulphite (0.2\%).\[17\]

**Initial extraction**

A 20g quantity of the (0-8\textsuperscript{th} day) germinated seeds were homogenized with 100 mL of homogenizing medium in a chilled stainless steel jar of a waring blender for 1 min. The homogenate was left over a magnetic stirrer with slow stirring for 30 min and thereafter, filtered through four layers of muslin cloth and the filtrate was centrifuged at 10,000 rpm for 30 min in a Sorvel 5B super-speed refrigerated centrifuge using the SS34 rotor. The supernatant was treated as the enzyme extract.

**Enzyme assay**

The reaction mixture consisted of 100\mu{}L of the polygalacturonic acid (PGA) (0.1\% w/v), 125\mu{}L of sodium acetate buffer (0.15 M, pH 4.4) and 225\mu{}L of distilled water. The reaction mixture was pre-incubated for 1 min. at 60\textdegree{} C and the reaction was started by the addition of 50\mu{}L of the enzyme preparation and continued at 60\textdegree{} C for 1 h. After which the reaction was stopped by the addition of 1.5mL of copper reagent and subsequent boiling for 10 min in a boiling water bath followed by cooling in an ice bath. The controls were prepared by adding polygalacturonic acid after the addition of reagent A (copper reagent). The reducing sugars produced were estimated by measuring O.D. at 540 nm of the Schiff’s green colour produced by the addition of 1mL of reagent B (arsenomolybdate reagent) by Nelson-Somogyi method.\[10-11\]

**Polygalacturonase standard curve**

0.18mM polygalacturonic acid was used as standard solution. Absorbancies were taken at 540nm.

**Protein determination**

Protein determination was carried out according to the procedure of Lowry using bovine serum albumin as standard.\[18-19\]
Enzyme activity at different days of germination

Enzyme extracts at different days of germination were estimated for polygalacturonase activity by Nelson-Somogyi method.

Effect of physiological factors on polygalacturonase production

Effect of temperature

For the temperature optimization of polygalacturonase was diluted with ammonium sulphate fractionated enzyme 10 times, experimental and control tubes were prepared, incubated at different temperature (30° C - 80° C) and assayed for enzyme activity.

Effect of pH

For pH optimization of polygalacturonase was diluted with ammonium sulphate fractionated enzyme 10 times, experimental and control tubes were prepared, incubated at different pH (1.0 – 6.0) and assayed for enzyme activity.

Enzyme purification

All the steps of enzyme purification were carried at 4° C.

Briefly, seeds of different day germination were homogenized in a homogenizing medium followed by centrifugation at 15,000 for 30 min. The supernatant was used as enzyme source for which enzyme activity was measured at 540 nm. To 100mL of the enzyme extract, (NH₄)₂SO₄ was added with constant stirring to achieve 30% saturation. After storage for 3 h, it was centrifuged at 10,000 rpm for 30 min and the supernatant was brought to 70% saturation again with (NH₄)₂SO₄. The pH was maintained at 7.5 by the addition of 1% (v/v) ammonium hydroxide. After overnight storage, the mixture was centrifuged at 10,000 rpm for 30 min. The pellet thus obtained was dissolved in 10 mL of distilled water containing 0.1M NaCl and 0.02% Sodium Metabisulphite (solution A) and dialyzed against the same solution for 6 h with two changes of solution A. The dialyzed fraction was centrifuged at 10,000 rpm for 10 min to obtain a clear supernatant.

Supernatant obtained was loaded onto a DEAE cellulose column (2 x 10cm), pre-equilibrated with solution A. The enzyme from the column was eluted stepwise (by passing 2 to 3 bed volumes of the medium containing 0.1-1.0 M NaCl serially and fractions were collected. The fraction of enzyme having high specific activity was applied onto a sephadex G-200 column (1.5 x 55cm) equilibrated with solution A. Fractions of 3 mL each were collected at a flow rate of 10 mL/h.
Determination of exo or endo-polygalacturonase

The paper chromatography of the reaction products was performed according to the procedure of Rexova-Benkova.[20] The sample spots were applied onto a chromatography paper at 3cm from the bottom. The paper was subjected to ascending chromatography using ethyl acetate: acetic acid and water (18:7:8). The reducing sugar spots were stained using Aniline diphenylamine.

RESULTS AND DISCUSSION

Galacturonases releases uronides from the cell wall and it is observed that the enzymatic release and metabolism is increased during growth responses.[21-22] The experimental setup is shown in figure 1 and figure 2. The absorbance showing multiple peaks confirms that enzyme polygalacturonase found in multiple forms in this pumpkin variety.

The change in pumpkin polygalacturonase with days of germination has been shown in figure 3. The maximal polygalacturonase activity was observed on the 4th day of germination. The enzyme was thus partially purified from the 4th day germinated pumpkin seeds. The summary of the enzyme purification is given in table 1 and figure 3.

Fig 3: The change in Polygalacturonase enzyme activity during germination of Cola lambol samrat 2
Table No.1: Enzyme activity at different days of germination

<table>
<thead>
<tr>
<th>No of days</th>
<th>Test sample</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.22</td>
<td>0.032</td>
</tr>
<tr>
<td>1</td>
<td>0.100</td>
<td>0.031</td>
</tr>
<tr>
<td>2</td>
<td>0.080</td>
<td>0.029</td>
</tr>
<tr>
<td>3</td>
<td>0.315</td>
<td>0.035</td>
</tr>
<tr>
<td>4</td>
<td>0.54</td>
<td>0.033</td>
</tr>
<tr>
<td>5</td>
<td>0.417</td>
<td>0.032</td>
</tr>
<tr>
<td>6</td>
<td>0.395</td>
<td>0.034</td>
</tr>
<tr>
<td>7</td>
<td>0.361</td>
<td>0.031</td>
</tr>
<tr>
<td>8</td>
<td>0.287</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Temperature optimization

The temperature optimization of polygalacturonase was done after diluting 10 times ammonium sulphate fractionated enzyme. Experimental and control tubes were prepared, incubated at different temperature (30°C - 80°C) and assayed for enzyme activity. The maximum activity was found at 64°C with half maximum activity at 60°C and 68°C.

pH optimization

For the pH optimization of polygalacturonase, ammonium sulphate fractionated enzyme was diluted 10 times, experimental and control tubes were made, incubated at different pH (1.0 – 6.0) and assayed for enzyme activity. The maximum activity was found to be at 4.5.

Determination of exo or endo-polygalacturonase by Paper Chromatography

After spraying the developed chromatographic paper with aniline diphynelamine reagent, two bands were observed; one for test sample and other for marker galacturonic acid. There was no band for marker digalacturonic acid. It confirms that it is an exo-polygalacturonase enzyme.

![Fig 4: Elution profile of Cola lambol samrat 2 exo polygalacturonase from DEAE Cellulose Column](image)
Fig 5: Elution profile of *Cola lambol samrat 2* exo polygalacturonase from Sephadex G-200 Column

REFERENCES


