Cellulolytic enzymes associated with the fruit rots of \textit{Citrus sinensis} caused by \textit{Aspergillus aculeatus} and \textit{Botryodiplodia theobromae}

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Abstract

\textit{Botryodiplodia theobromae} and \textit{Aspergillus aculeatus} were inoculated on carboxymethylcellulose (CMC) medium and filter papers. The hydrolysis of the CMC medium and degradation of the filter papers were observed, indicating the production of the $C_1$ and $C_x$ cellulases by the two rot pathogens. The $C_1$ and $C_x$ enzymes were also detected in filtrates of rotted orange fruits incited by the two rot pathogens. The cellulases could not induce rot development on their own. However, when they were added to pectinases in an enzyme inoculum, the incubation period for inducing rot development was shorter; thus establishing a secondary role for the cellulases in the rot development. This secondary role of the cellulases produced by the two fungi was found to be at peak at pH 7 and a temperature range of 25° - 30 °C in the two fungi.

Introduction

The role of pectinolytic enzymes in tissue maceration and cell separation during rot development has been greatly emphasised and well documented (15, 18). However, the role of other enzymes such as cellulases in rot development has not been well established. Husain (4) reported that comparatively little is known about cellulases of plant pathogens \textit{in vivo}. According to Husain (4) and Husain & Dimond (5) only a relatively small proportion of plant pathogens are able to degrade insoluble cellulose even after it has been physically or chemically modified to make it more susceptible to enzyme action. Wood (17) claimed that it was the breakdown of the insoluble cellulose that was more important in the study of rot development and that investigations based only on the degradation of soluble cellulose would have a limited impact. He also asserted that only cellulases of a few fungi have been studied in detail and even with the same organism, there appears to be a general disagreement as to the degradation of native cellulose.

Further, there are different views on the number and types of cellulases that degrade cellulose. There could be only one type (14), two types (6), or a number of enzymes (3, 10). However, the most recent reviews are those of Mandels & Reese (7) and Mandels & Steinberg (8) that cellulose utilization by fungi depends on their capability to produce the $C_1$ and $C_x$ enzymes.

The production \textit{in vitro} and \textit{in vivo} of cellulases by \textit{Botryodiplodia theobromae} and \textit{Aspergillus aculeatus} as well as the role of the cellulases in the development of the post-harvest rots of \textit{Citrus sinensis} are reported in this paper.

Materials and methods

\textit{Production and quantitative estimation of cellulases in culture, healthy and diseased orange fruits}

The production of cellulases by the two rot organisms in culture as well as in rotted and healthy orange tissues were investigated. The utilization of
an insoluble (native) form of cellulose by the test fungi was tested by following the modified method of Garrett (2). Wads of 10 filter papers (Whatman No. 19.0 cm) were oven dried, weighed and placed in each of eight sterile Petri dishes. Fifty millilitres of a basal medium containing the following (in mg/liter) were added to each Petri dish: K$_2$HPO$_4$, 1000; MgSO$_4$·7H$_2$O; 300, peptone, 1000; yeast extract, 100 and 1 ml of microelement solution (1). The pH was adjusted to 7.0 with 0.2 N NaOH after sterilization.

The cellulolytic activity of the rot organisms was tested in liquid cellulose culture, and rotted orange fruits using a modified method of Reese & Mandels (11). Two liquid media, carboxymethyl cellulose BDH of Na salt (CMCM) and basal medium lacking carboxymethyl cellulose (NCMC) were employed. Orange fruits were inoculated with test fungi. An average volume of 50 ml of fruit juice from each of the healthy and rotted fruits was taken for the enzyme estimation. The determination of reducing sugars (R.S.) in CMCM, NCMC and fruit juices was done. One gram of 3, 5, dinitrosalicylic acid, 20 ml of 2 N NaOH, 30 gram of potassium sodium tatarate were dissolved in 100 ml of water, heated for 5 mins. in boiling water. Three ml of this reagent was added to 1 ml of each enzyme filtrate (from NCMC, CMCM and fruit juice) and then diluted with 10 ml of H$_2$O. The amount of the reducing sugars produced was estimated by determining the optical density (absorption spectrum) at 550 nm wavelength with a Zeiss Spektrophotometer PM 2A. The values of the reducing sugars produced were read off from a calibration curve earlier constructed from the optical densities of varying concentrations (0, 0.125, 0.25, 0.5, 1.0 and 2.0 mg/ml) of standard solutions of D-glucose.

The cellulases were assayed by inoculating 1 ml of enzyme filtrate (from cultures and fruit juices) into a 9 ml of substrate containing 0.55% of CMC (BDH sodium salt) in 0.055 M citrate buffer of pH 4.5. The incubation was at 37 °C for 1 h. One enzyme unit produces 4 mg of reducing sugar in a 10 ml of the reaction mixture. The sugars are expressed as total reducing sugars (T.R.S.).

The effect of temperature on the activity of cellulases was investigated by incubating each of the inoculated enzyme source at each of the temperatures 10°, 15°, 20°, 25°, 30°, 40°, 50°, 60° and 70° C. After one hour, all reaction mixtures were cooled and stored at 0 °C until the determination of the total reducing sugars is required. The pH was adjusted by following the method of Nolan (9).

_Effects of cellulolytic enzymes in rot development_

Cellulases in culture (obtained as described above) and pectinases from 3 day old inoculated cultures of _Aspergillus aculeatus_ and _Botryodiplodia theobromae_ at 25 °C.

<table>
<thead>
<tr>
<th>Rot organism</th>
<th>Initial wt. of filter papers (in mg)</th>
<th>Net Loss in wt. of filter papers (in mg)</th>
<th>% wt. Loss</th>
<th>Visual estimation of growth</th>
<th>Medium</th>
<th>Mg. dry wt. mycelium on medium (in mg.)</th>
<th>R.S. (mg/ml)</th>
<th>T.R.S. (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Botryodiplodia theobromae</strong></td>
<td>660.0 ± 1.1</td>
<td>385.0 ± 1.6*</td>
<td>58.3 ± 1.2</td>
<td>***</td>
<td>CMCM</td>
<td>80.4 ± 0.4</td>
<td>0.9 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Control</td>
<td>510.0 ± 0.2</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>CMCM</td>
<td>0</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td><strong>B. theobromae</strong></td>
<td>564.0 ± 0.8</td>
<td>340.0 ± 1.7</td>
<td>60.2 ± 1.0</td>
<td>***</td>
<td>NCMC</td>
<td>85.6 ± 0.5</td>
<td>1.7 ± 0.3</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>Control</td>
<td>460.0 ± 0.4</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>NCMC</td>
<td>0</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td><strong>Aspergillus aculeatus</strong></td>
<td>621.0 ± 1.4</td>
<td>394.0 ± 1.7</td>
<td>63.4 ± 0.8</td>
<td>**</td>
<td>CMCM</td>
<td>64.8 ± 0.6</td>
<td>1.1 ± 0.1</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Control</td>
<td>464.0 ± 0.6</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>CMCM</td>
<td>0</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td><strong>A. aculeatus</strong></td>
<td>610.0 ± 1.8</td>
<td>390.0 ± 1.4</td>
<td>63.9 ± 1.5</td>
<td>**</td>
<td>NCMC</td>
<td>61.2 ± 1.7</td>
<td>0.6 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Control</td>
<td>446.0 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>–</td>
<td>NCMC</td>
<td>0</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

1 Growth – *** very good, ** good, – no growth
2 R.S. – Reducing sugars of culture filtrate
3 T.R.S. – total reducing sugars of cellulase assay
4 CMCM – carboxymethylcellulose liquid medium
5 NCMC – Liquid medium lacking carboxymethylcellulose (Basal medium)
6 Data are means of 5 replicates
7 * Standard error

Table 1. Degradation of filter papers (after 21 days) and carboxymethylcellulose (in 5 days) by cellulolytic enzymes produced by _Aspergillus aculeatus_ and _Botryodiplodia theobromae_ at 25 °C.