POLYGALACTURONASE ACTIVITY OF ASPERGILLI

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Received August 17, 1950

INTRODUCTION

POLYGALACTURONASE (PG) (Pectinase) catalyzes the hydrolysis of 1, 4-α-glycosidic linkage of anhydrogalacturonic acid units in pectic acid. In fungi PG is always associated with pectinesterase (PE)\(^1,\)\(^2\) and the separation of these two enzymes has been effected recently by use of cation exchange resin\(^3\) or by acid treatments followed by precipitation with ammonium sulphate between treatments.\(^4\) Although qualitatively the occurrence of PG in plant materials is known,\(^1\) its elaboration by phytopathological microorganisms as well as non-parasitic fungi has been studied more extensively.\(^5\)\(^--\)\(^11\) Reports are however not always in agreement regarding the optimum cultural conditions for PG secretion.

This study relates to PG elaboration by three strains of Aspergilli isolated in the laboratory and the preparation and properties of an active PG product.

EXPERIMENTAL

Measurement of Activity

PG activity was followed by determining increase in reducing power of pectin solution.\(^12\) 100 c.c. of 1 per cent. solution of citrus pectin (analysing to 1·15 per cent. ash, 75·20 per cent. uronic acid\(^13\) and 4·18 per cent. CH\(_5\)O\(^14\) on dry basis) was adjusted to pH 3·6-3·7, reported optimum for the enzyme\(^15\)\(^,\)\(^16\) and 10 c.c. of the enzyme extract was added and incubated at 37° C. for 48 hours after addition of a few drops of toluene. The reducing values were determined by Willstatter and Schudel’s hypoiodite method.\(^17\) Blank determinations were carried out using 10 c.c. of the inactivated enzyme extract. The amount of the galacturonic acid corresponding to the thiosulphate values were read off directly from Kertesz’ data\(^18\) which were suitably standardized. The activity of PG is expressed as the percentage of galacturonic acid liberated on its yield as obtained by decarboxylation with 12 per cent. hydrochloric acid according to Dickson, et al.\(^13\)

Although pectic acid is the ideal substrate for PG action, the method as employed, using a low ester pectin, was found quite satisfactory in
practice. The hydrolysis of pectinic acid to galacturonic acid by PG alone is not complete, but the presence invariably of associated PE with PG would release the esterified portion for PG action. The rate of liberation of galacturonic acid would be affected by the relative proportions of PG and PE in different fungi, but it was observed that, under the conditions of hydrolysis, the reducing value was maximum after 24–48 hours, a further 8–10 days period resulting only in 3–5 per cent. increase in the different cases.

Isolation and Culture of Organisms

A dilute glucose-Czapek-Dox medium containing 1 per cent. pectin was exposed to air. At the end of 4-5 days, eight different strains of moulds were obtained; and these were isolated in pure forms by "agar-dilution method". Out of eight strains (Aspergilli, Penicillia and Rhizopus) only three of Aspergilli designated here as Nos. I, III and V were pectolytic as observed by partial liquefaction in sodium pectate-containing solid media. The stock cultures were made and maintained on Czapek-Dox agar slants. They were subcultured thrice before transferring to liquid media.

Fifty c.c. of Czapek-Dox medium were inoculated after sterilization with 0.5 c.c. of a heavy suspension of spores of the selected strain of fungus and incubated at room temperature. At the end of 4-5 days, 2 c.c. of toluene were added to each flask and the fungal mycelia were collected on a tared filtered paper and dried to constant weight at 100° C. PG activity was determined in the culture filtrate (Table I).

<table>
<thead>
<tr>
<th>Moulds</th>
<th>Aspergillus</th>
<th>Penicillium</th>
<th>Rhizopus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Dry weight of mycelium (mg.)</td>
<td>387</td>
<td>342</td>
<td>258</td>
</tr>
<tr>
<td>PG activity</td>
<td>...</td>
<td>7·4</td>
<td>4·5</td>
</tr>
</tbody>
</table>

Only Aspergilli I, II, III and V were used for further studies. These have been identified as A. luchuensis Inui, A. flavus Link and A. niger van Tiegh respectively. A. luchuensis Inui was a strain with longer conidiophores and larger vesicles than is typical for the species. The use of pectin has been
advocated for stimulating PG formation. Supplementation of culture solution with 1 per cent. pectin stimulated only the growth of the organism without significant enzyme secretion. A time study of the growth of mould, PG activity and changes in pH and reducing values with two of the strains in pectin-enriched media is given in Figs. 1 and 2.

Growth was rapid initially but slowed down after 5-7 days. PG activity was maximum after 4th day of incubation and diminished gradually thereafter. There was parallelism, especially with *A. flavus*, in reducing power by glycosidic hydrolysis of the pectin molecule; its subsequent decrease is due possibly to utilization of galacturonic acid by the mould for its growth. The comparatively slow increase in reducing value with

Fig. 1. Changes in Mycelial Weight, PG Activity, Reducing Value and pH during Growth of *A. flavus*
A. niger would suggest that the organism utilized galacturonic acid more rapidly, its accumulation in the medium therefore not being well marked. The initial small drop in reducing value is due to the utilization of glucose of the medium by the mould. After an initial drop in pH, the value rises gradually in case of A. niger, while with A. flavus a more or less steady pH is maintained for a prolonged period. The liberation of galacturonic acid may account in part for the initial drop in pH in both cases.

Maximum elaboration of enzyme took place, when the pH of the medium was between 3.0 and 4.5. Higher pH values were detrimental to formation of PG but mycelial weight was comparatively less affected (Fig. 3). It should be stated here that formation of PG was mostly exocellular with all
the strains, the mycelial extracts (aqueous or 5 per cent. glycerine) showing negligible activity.

**Use of Solid Media**

Wheat bran is a complete habitat for growth of moulds and proved very satisfactory as medium for production of mould amylases. In the following experiments 10 g. lots of wheat bran moistened with 15 c.c. of water were sterilized (at 20 lbs. pressure for 30 minutes) and inoculated after cooling with 0·5 c.c. of the spore suspensions. At the end of every 24 hours, one flask was taken for extraction of the enzyme. Thirty five c.c. of water were added to the flask and kept overnight for autolysis after addition of toluene. The extract was squeezed out, made up to volume, centrifuged and used for determination of PG activity (Table II).
TABLE II. Use of Wheat Bran as Medium for Growth

<table>
<thead>
<tr>
<th>Age of culture (days)</th>
<th>PG activity</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. luchuensis</td>
<td>A. flavus</td>
<td>A. niger</td>
</tr>
<tr>
<td>2</td>
<td>2.38</td>
<td>7.00</td>
<td>12.8</td>
</tr>
<tr>
<td>3</td>
<td>7.92</td>
<td>10.42</td>
<td>17.11</td>
</tr>
<tr>
<td>4</td>
<td>10.76</td>
<td>13.13</td>
<td>22.11</td>
</tr>
<tr>
<td>5</td>
<td>8.74</td>
<td>11.68</td>
<td>16.77</td>
</tr>
<tr>
<td>6</td>
<td>7.14</td>
<td>9.40</td>
<td>15.97</td>
</tr>
</tbody>
</table>

PG activity was better with wheat bran than when synthetic liquid medium was employed. The enzyme content was maximum after 4 days which was the period of harvesting in subsequent experiments. The use of 5 per cent. glycerine for extraction of the mouldy bran resulted in 90–180 per cent. greater recovery of the enzyme (Table III).

TABLE III. Extraction of Enzyme by Aqueous Glycerine

<table>
<thead>
<tr>
<th></th>
<th>PG activity</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. luchuensis</td>
<td>A. flavus</td>
<td>A. niger</td>
</tr>
<tr>
<td>Water extract</td>
<td>10.76</td>
<td>13.13</td>
<td>22.11</td>
</tr>
<tr>
<td>Glycerine extract</td>
<td>10.83</td>
<td>37.60</td>
<td>43.84</td>
</tr>
</tbody>
</table>

Addition of pectin had practically no effect on production of PG but supplementation with hydrolyzed casein resulted in definite stimulation (Table IV).

TABLE IV. Effect of Addition of Pectin and Tryptic Digest of Casein Hydrolysate* to Wheat Bran

<table>
<thead>
<tr>
<th>Supplement</th>
<th>PG activity</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. luchuensis</td>
<td>A. flavus</td>
<td>A. niger</td>
</tr>
<tr>
<td>None</td>
<td>19.0</td>
<td>37.9</td>
<td>42.6</td>
</tr>
<tr>
<td>1% Pectin</td>
<td>18.9</td>
<td>38.3</td>
<td>41.0</td>
</tr>
<tr>
<td>0.1% Casein hydrolysate</td>
<td>34.0</td>
<td>63.9</td>
<td>67.1</td>
</tr>
</tbody>
</table>

* Aminoxyl: Raptakos, Brett and Co. Ltd., Bombay.
Preparation and Partial Purification of PG Enzyme

Wheat bran containing 0.1 per cent. enzymic casein hydrolysate was inoculated with spore suspensions of each of three *Aspergilli* organisms. At end of 4 days, the material was extracted with 5 per cent. glycerine, strained and centrifuged at 3,000 r.p.m. for 5 minutes. Alcohol was added to the centrifugate to a concentration of 80 per cent. The precipitate obtained after centrifuging was dried in a vacuum oven at 40°C. The activities of the alcohol precipitates were 9.2, 15.9 and 24.0 per cent. (expressed as per cent. on maximum yield of galacturonic acid) for *A. luchuensis*, *A. flavus* and *A. niger* respectively.

During precipitation with alcohol, therefore, there was destruction of enzyme to the extent of 60–70 per cent. Fractional precipitation with increasing concentrations of ammonium sulphate resulted in comparatively smaller loss (Fig. 4); the enzyme is precipitated mostly between 40–60 per cent. concentration of ammonium sulphate; precipitates obtained at lower concentrations of the salt consisted mainly of non-enzymic matter.

![Graph](image-url)

**Fig. 4** Fractional Precipitation with Ammonium Sulphate
Using *A. niger*, a partially purified PG preparation was obtained by precipitating the enzyme extract from 500 g. of wheat bran with 60 per cent. concentration of ammonium sulphate after discarding the precipitate obtained at 20 per cent. salt concentration. The yield was about 19 g. and it had approximately 55-60 per cent. of the original activity. More or less similar results were obtained with *A. luchuensis* and *A. flavus*. The preparation was dark brown in colour and was further purified by a second salting out with ammonium sulphate in the same manner as above when a light brownish coloured product was obtained retaining about 40 per cent. of the original activity. The product was dried in *vacuo* without loss in activity. The yield was 12 g.

The activity of this preparation (10 c.c. of 0.5 per cent.) was studied with regard to time and pH using 100 c.c. of 1 per cent. citrus pectin as substrate (Fig. 5). At pH 3.7 the activity increased with time, at first

![Variation of PG Activity with Time and pH](image-url)
Polygalacturonase Activity of Aspergilli

rapidly, during 12–24 hours: further hydrolysis was slow and continued for 8–10 days. The activity at pH 3.7 was considerable; it increased rapidly with a maximum between 4 and 5; subsequent drop was also rapid.8

DISCUSSION

PG formation has been studied in case of a few Aspergilli, especially A. niger and A. oryzae. In the studies reported here, all the five different Aspergilli isolated in this laboratory were pectolytic to varying extents. Of these, A. luchuensis Inui, A. flavus Link and A. niger van Tiegh were rich in PG activity, the enzyme being mostly exocellular. The production efficiencies of A. flavus and A. niger compared very favourably with those reported for other organisms,9,10 while that of A. luchuensis was comparatively poor. The time of maximum elaboration of enzyme with all these organisms was considerably shorter than has been obtained hitherto.

Wheat bran was a better substrate than the synthetic medium for PG elaboration by the organisms. While this may be due to its better texture, promoting greater aeration, it would seem that its nutrient status has also contributed to its superiority. It is significant that supplementation with casein hydrolysate has resulted in greater PG activity thus suggesting that nitrogen source is important.

PG is shown as an adaptive enzyme by many workers,6,9 its formation being stimulated by specific substrate additions. The addition of pectin did not however stimulate the formation of PG in the Aspergilli studied here and would therefore emphasise the non-adaptive nature of PG as observed by Gaumann and Bohni10; evidently this property varies with different strains.

SUMMARY

1. Three strains of Aspergilli having good PG activity were isolated.

2. The conditions for maximum elaboration of PG were studied using synthetic liquid media as well as wheat bran.

   (i) The enzyme content was maximum after 4 days of growth; pH below 5 favoured the formation of enzyme.

   (ii) Wheat bran was a better substrate than synthetic liquid media; aqueous glycerine was very satisfactory for extraction of the enzyme from wheat bran medium.

3. Partially purified PG preparations were obtained by precipitating enzyme at 60 per cent, concentration of ammonium sulphate after discarding
the precipitate at 20 per cent. salt concentration; alcohol inactivated the enzyme to a great extent.

4. The optimum pH for activity with citrus pectin as substrate was about 4–5; hydrolysis was rapid during first 12–24 hours and decreased thereafter during a further 8–10 days period.

ACKNOWLEDGMENT

Acknowledgment is made to the Provincial Industrial Research Committee of the Bombay Government Department of Industries for a research grant in support of this investigation. Our thanks are due to Dr. R. S. Vasudeva, Head of the Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi, for identification of the organisms.

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