The Pectic Enzymes of the Fungus Byssochlamys fulva

By W. W. Reid

Research Department, H. W. Carter and Co. Ltd., Coleford, Gloucestershire

(Received 17 April 1951)

It was reported by Beavan & Brown (1949) that cultures of Byssochlamys fulva Olliver & Smith produced proteocellulase, but neither polygalacturonase nor pectin esterase; they concluded that B. fulva produced a disaggregating enzyme, which lowered the viscosity of pectin solutions, without the formation of free reducing groups. A re-investigation of the problem has been made, and in a preliminary note (Reid, 1950a), it was suggested that B. fulva produced both polygalacturonase and pectin esterase.

EXPERIMENTAL

Enzyme preparations

Two strains of Byssochlamys fulva were used; one was received from Mr L. D. Galloway (I) and the other from Miss M. Olliver (II). B. fulva is considered by Thom & Raper (1945) to be an ascoporic phase of Paecilomyces variotii and isolates of the non-ascoporic phase have been found to produce small amounts of pectic enzymes (unpublished data).

Previous work with other microfungi had shown that several cultures produced good yields of enzymes on bran. In some fungi, e.g. P. chrysogenum (Phaff, 1947), the enzyme was adaptive, in others (e.g. Aspergillus foetidus Thom & Raper), the nitrogen source was all important. In the present work, cultures were grown on bran supplemented with organic nitrogen, e.g. peptone or protein hydrolysates. When the enzyme formation was optimal, the culture was extracted with chloroform water and the extract was either dialysed against running tap water, followed by distilled water, or precipitated with acetone (70 % v/v) at 4 °C and vacuum dried.

Estimation of enzyme activity

Viscometric method. A ‘four-point’ viscometric assay was used (Reid, 1949; Ayres, Hooper & Reid, 1951) with sodium pectate at pH 5-0 for polygalacturonase and citrus pectin at pH 3-5 as an approximate estimate of combined pectin esterase and polygalacturonase activity. A preparation of Pectinol 10 M (Rohm and Haas Co., Philadelphia) was preserved over silica gel at 4 °C as a standard.

Glycosidic hydrolysis. (a) The enzyme was added to a 1 % solution of sodium pectate at pH 5-0 and 25 °C. Samples were withdrawn at intervals and titrated by the hypodiode method of Wiltstättel & Schüdel (cf. Hinton, 1939) against d-galacturonic acid as a standard.

(b) The colorimetric method of Sumner (1924-5), as modified by Smith & Stocker (1949), was used. The sample to be analysed was brought to a total volume of 5 ml., 1 ml. of the dinitrosalicylic acid reagent added, and the tubes heated for 15 min. in a boiling-water bath and cooled rapidly in running water. The red-brown colour which developed was read in a Hilger Biochem Absorptiometer with a green OGI filter. A standard curve was constructed using d-galacturonic acid, and the linear portion (0-7-2-0 mg. polygalacturonic acid) was used.

Pectin esterase assay. (a) The titrimetric method of Kertesz (1937) was used, and the enzyme solution was well dialysed to remove buffering substances. Methyl red was used as an indicator, the solution was made 0-1 M with NaCl, and 0-2 N NaOH added continuously from a micro-burette to maintain the pH at 6-0.

(b) The enzyme solution was added to a 2 % solution of citrus pectin at pH 6-0. Samples were removed and distilled at intervals and the methanol in the distillate estimated by the Denigès method (modified by the A.O.A.C., 1945). Methanol (British Drug Houses Ltd., Analar grade) was used as a standard.

Substrates

Pectin. 100 grade pectin (British Drug Houses Ltd.) was well washed with 70 % (v/v) ethanol to remove soluble carbohydrate, then with absolute ethanol and dried in vacuo. For the Kertesz (1937) method for estimating pectin-esterase activity, the buffer substances present were removed, either by acidifying a 1 % solution with HCl followed by dialysis against distilled water or by shaking a 1 % solution with Bio-Deminrolit (Permutit Co. Ltd.).

Pectic acid. To a 1 % solution of 100 grade pectin, NaOH solution (33 %, w/v) was added to pH 10-0 while stirring in a Waring blender. After 1 hr. the solution was adjusted to pH 1-0 with HCl (10 %, w/v). The pectic acid gel was pressed in a small screw press, washed with 70 % ethanol containing 1 % HCl followed by 70 % ethanol until free from HCl, left under absolute ethanol for 24 hr., the excess ethanol pressed out, and the fibrous mass dried in vacuo at 50-70 °C for several hours.

The substrates were dispersed to the appropriate concentrations in either distilled water or buffer (Moll ivaine) in the cold.

RESULTS

In a preliminary investigation it was shown (Table 1) that, compared with other cultures, Byssochlamys fulva (II) was active in the production of pectic enzymes, whereas (I) was very poor. The ‘high level’ enzyme-substrate mixtures from the assay of the two B. fulva cultures and the Pectinol standard were maintained at 25 °C for 18 hr. Sodium hydroxide was added to pH 10-0, the mixture left for 30 min. to
demethylate the uronides present, and a calcium pectate determination carried out (cf. Hinton, 1939). From the weight and appearance of the

Table 1. The pectic enzyme activity of 5-day cultures (dry basis) of microfungi, by a viscometric 'four point' assay on 0-4 % pectin at pH 3-5
(Activity of culture compared with that of Pectinol 10 M used as standard M indicator, arbitrary enzyme unit.)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Temp. of incubation (°)</th>
<th>Assay value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. chrysogenum</td>
<td>25</td>
<td>1.2 M</td>
</tr>
<tr>
<td>Stachybotrys spp.</td>
<td>25</td>
<td>1.2 M</td>
</tr>
<tr>
<td>Monilia spp.</td>
<td>25</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Aspergillus foetidus, n.sp.</td>
<td>37</td>
<td>3.0 M</td>
</tr>
<tr>
<td>Thom &amp; Raper</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. fulva (I)</td>
<td>37</td>
<td>0.04 M</td>
</tr>
<tr>
<td>B. fulva (II)</td>
<td>25</td>
<td>0.9 M</td>
</tr>
<tr>
<td>B. fulva (II)</td>
<td>37</td>
<td>0.7 M</td>
</tr>
</tbody>
</table>

resulting calcium pectate precipitates (Table 2) the enzyme action had markedly changed the colloidal properties of the pectin. A similar result was obtained by precipitating reaction mixtures with acetone (70%, v/v); in one such experiment the resulting precipitate from the original substrate was 370 mg., whereas two other samples treated for 20 hr. with different extracts of B. fulva (II) gave precipitates of 221 and 150 mg.

Table 2. The action of Byssochlamys fulva extracts and Pectinol 10 M on 0-4 % pectin at pH 3-5 for 18 hr. as measured by the calcium pectate value

<table>
<thead>
<tr>
<th>Character of calcium pectate ppt.</th>
<th>Calcium pectate remaining (%)</th>
<th>Calcium pectate remaining (mg./20 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectin substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pectin substrate + B. fulva (I)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pectin substrate + B. fulva (II)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pectin substrate + Pectinol 10 M</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Additions</th>
<th>Reducing material formed (as mg. D-galacturonic acid/2 ml.)</th>
<th>Methanol liberated (mg./2 ml.)</th>
<th>Methanol liberated (% total methoxyl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4 ml. Pectinol +1 ml. water</td>
<td>22.23</td>
<td>2.2</td>
<td>61.1</td>
</tr>
<tr>
<td>2</td>
<td>As 1, but enzyme heat-inactivated</td>
<td>0.0</td>
<td>0.0</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>5 ml. solution of acetone-precipitated B. fulva preparation</td>
<td>7.98</td>
<td>2.0</td>
<td>55.5</td>
</tr>
<tr>
<td>4</td>
<td>As 3, but enzyme heat-inactivated</td>
<td>0.0</td>
<td>0.0</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>50 ml. 2 pectin in pH 5-0 buffer +50 ml. dialysed B. fulva extract</td>
<td>12.54</td>
<td>1.1</td>
<td>61.1</td>
</tr>
<tr>
<td>6</td>
<td>As 5, but enzyme heat-inactivated</td>
<td>0.0</td>
<td>0.0</td>
<td>—</td>
</tr>
</tbody>
</table>

The marked activity of the B. fulva preparations suggested that polygalacturonase was present, and preliminary experiments indicated the formation of reducing material during enzyme action. In a later experiment, 50 ml. of culture extracts of B. fulva (II) were precipitated with acetone at 4°. The white precipitate obtained was redissolved, centrifuged from a trace of residue and the clear extract (5 ml.) added to 50 ml. of a 1% solution of a pectic acid in McIlvaine buffer at pH 5-0. The reducing value after 48 hr. (hypiodide method) corresponded to a D-galacturonic acid content of 63-3-65-5% of the theoretical. Paper chromatography from this experiment showed no soluble sugars in the enzyme and substrate blanks, but D-galacturonic acid and some higher uronides in the enzyme substrate mixtures. These results confirmed that polygalacturonase was present (see also Table 3).

Examination of the activity of several extracts from B. fulva (I) and (II) for polygalacturonase by the 'cup-plate' assay, Reid (1950b) showed that small but definite amounts of the enzyme were present, as was also the case with approximately a hundred other cultures of microfungi from different genera, indicating that polygalacturonase formation is a common feature among microfungi (Solomons, 1951).

Table 3. Reducing substances formed and methanol liberated by the action of Byssochlamys fulva preparations and Pectinol 10 M, on pectin at pH 5-0 for 120 hr.

(Reducing substances determined by the hypiodide method on 2 ml. enzyme-substrate mixture, and methanol determined in the distillate from 2 ml. enzyme-substrate mixture, nos. 1-4, 50 ml. 2% pectin (9-0% MeO) + enzyme + water to 55 ml.)
The relationship between reducing groups liberated and reduction in viscosity with a pectic substrate

As polygalacturonase was present in the enzyme preparations, it seemed likely that the failure of Beavan & Brown (1949) to detect any liberation of reducing groups was due to the very low activity of their enzyme preparations. Examination of their data indicated that to reduce the specific viscosity $\eta_{sp}$ of a substrate to 50% of the initial value, a reaction time of approximately 12 hr. was necessary, whereas the majority of the preparations in the present paper required 20–25 min.

Preparations of *B. fulva* (II) were compared with Pectinol 10M, the latter being taken as typical of a preparation of high polygalacturonase content. An attempt was made to examine the liberation of reducing groups using amounts of each preparation that would give a rate of reduction in viscosity comparable to that given in Fig. 1 (in Beavan & Brown, 1949). The amount of reducing material was too small to be detected by the hypoiotide method, but just detectable by the colorimetric method of Sumner (1924–5). The latter method was more sensitive than the hypoiotide method, and gave good recoveries for various dilutions of pectin, and pectin-enzyme solutions. Although it was possible to demonstrate small amounts of reducing material by the colorimetric method in the early stages of enzyme action, the results were subject to considerable error. However, by taking larger amounts of *B. fulva* preparations and Pectinol, estimating the glycosidic hydrolysis, and extrapolating the curve back to the zero time, it was found that for a 50% drop in viscosity the accompanying glycosidic hydrolysis was 0.5–1.0% of the theoretical.

**Pectin-esterase activity of Byssochlamys fulva**

Using the Kertesz (1937) titrimetric method it was found essential to free both substrates and enzyme solutions from buffer substances. In typical experiments 40 ml of dialysed culture extracts were added to 40 ml of 5% pectin solution containing methyl red and sodium chloride to 0.2M. The pH of the mixture was adjusted to 6.0 with 0.2N-sodium hydroxide, and with the majority of the preparations there was marked acid production in 1–2 hr. at 25°. With concentrated enzyme preparations prepared by dialysis and solvent precipitation, the acid produced could be titrated continuously. One such preparation produced acid at an initial rate equal to 2.4 ml 0.2N-sodium hydroxide/hr. In the nomenclature of Kertesz (1937) this was equivalent to 0.0068 F.M.U. (pectin methyl esterase units) in 0.1M-sodium chloride, at pH 6.0 and 25° per ml of enzyme solution.

To confirm that the acid production was in fact due to demethylation of the pectin, samples of a *B. fulva* preparation and heated control samples were added to a pectin solution at pH 6.0, and samples distilled at intervals and the methanol in the distillate determined. From Table 3 it may be seen that methanol was liberated during the enzyme action.

**Arabanase and galactanase of Byssochlamys fulva**

An enzyme preparation was added to a solution of apple pectic acid, buffered to pH 5.0, which had been freed from soluble sugars, but contained considerable araban and galactan. Paper chromatograms after 24 hr. reaction showed no soluble sugars in the enzyme and substrate solutions alone, but with the enzyme-substrate mixture, in addition to uronides, well defined spots of arabinose and galactose were obtained, indicating arabanase and galactanase in the enzyme solution.

**DISCUSSION**

It is clear from the above results that *B. fulva* produces the pectin-esterase, polygalacturonase, arabanase and galactanase necessary to degrade the pectin complex. The action of these enzymes in pectin results in a drop in viscosity and the formation of reducing groups, but for reasons previously discussed (Jermyn & Tompkins, 1950; Reid, 1950c), the exact relationship between these two effects is difficult to assess. In the present work, a 50% drop in viscosity was accompanied by a glycosidic hydrolysis of 0.5–1.0% theoretical. As the preparations of Beavan & Brown reduced the viscosity of pectin slowly, it follows that the amount of glycosidic hydrolysis was very small; for example in Table 2 of Beavan & Brown's paper an increase in reducing value during enzyme action did occur, equivalent to 0.02–0.05 ml 0.1N-Iodine, which may in fact have been a real increase, although it was of the same magnitude as the error of the determination. It would appear that Beavan & Brown were unable to detect pectin-esterase and polygalacturonase activity due to the low activity of their preparations; this low activity may have been in part due to the fact that they grew their cultures on Czapek solution, a medium which often produces weak extracts, and precipitated the extracts with ethanol at room temperature, a procedure which markedly inactivates pectic enzyme preparations.

In the light of these findings there is no evidence for the presence in *B. fulva* of a special 'disaggregating enzyme' as postulated by Beavan & Brown, although as suggested previously Reid (1950c) arabanase and galactanase may contribute to the degradation brought about by polygalacturonase.
SUMMARY

1. It has been shown, contrary to the results of Beavan & Brown (1949), that Byss hologlumus fulva produces pectin esterase and polygalacturonase.

2. It is suggested that the results of Beavan & Brown were due to the low activity of their enzyme preparations, which made the detection of pectin esterase and polygalacturonase very difficult by the methods they used.

Thanks are due to Miss M. Olliver and Mr L. D. Galloway for cultures of B. fulva, to Mr G. L. Solomons and Mr A. Phipps for experimental assistance and the Directors of H. W. Carter and Co. Ltd., for permission to publish this paper.

REFERENCES


The Fermentation Process in Tea Manufacture

12. THE ORIGIN OF CARBON DIOXIDE

BY E. A. H. ROBERTS AND D. J. WOOD

Tocklai Experimental Station, Indian Tea Association, Cinnamara, Assam, India

(Received 3 March 1951)

There are considerable differences of opinion on record regarding the origin of the carbon dioxide produced in fermentation, and the extent to which rolling and fine mincing destroy respiratory activity in the tea leaf.

Sreerangachar (1941, 1949) is of the opinion that there is much virtually undamaged tissue both in the fine minces used in manometric work, and in factory-rolled leaf, and that the carbon dioxide evolved during fermentation arises from normal respiration in such undamaged leaf. He produced experimental evidence which showed that the minces used in his manometric experiments contained appreciable amounts of intact tissue, but, as will be shown below, the same criteria when applied to our minces indicate a much greater degree of homogenization.

Roberts (1941c), on the other hand, concluded that fine mincing results in almost complete suppression of respiratory activity. This was indicated by the absence of anaerobic fermentation in a fine mince (Deb & Roberts, 1940), and by the low rates of oxygen uptake and carbon dioxide output observed in minces after polyphenol oxidation was complete (Roberts, 1939). It was pointed out that carbon dioxide output in a mince runs parallel with oxygen uptake and that ascorbic acid, which reduces o-quinones, completely stops carbon dioxide output (Roberts, 1939). This output was believed, therefore, to depend upon oxidation of the polyphenols.

In the fermentation of factory-rolled leaf the Löwenthal non-tan titre was observed to decrease (Roberts & Sarma, 1938). The non-tans were then thought to include various respiratory substrates. A fall in reducing sugar content on fermentation was also recorded (Roberts, 1939) and it was suggested that reducing sugars and other respiratory substrates in the non-tan fraction were oxidized to carbon dioxide by the o-quinones produced in fermentation, the o-quinones functioning as H-acceptors in the dehydrogenations involved. Depletion of carbohydrates in the tea leaf by water culture in the dark for 3 days would therefore be expected to reduce carbon dioxide output in the fermentation of a leaf mince. As Sreerangachar (1941) has pointed out, the failure of Deb & Roberts (1940) to observe such an effect is inconsistent with the view that carbon dioxide is formed as a result of the oxidation by o-quinones of glucose and other respiratory substrates.