DISTINCTION BETWEEN LIPASE AND PHOSPHOLIPASE ACTIVITIES OF MUCOR JAVANICUS MYCELIA

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1. Introduction

The phospholipase A₁ (EC 3.1.1.32) activities of Mucor javanicus, Rhizopus arrhizus [1] and other [2] fungal mycelia have been demonstrated using an assay system in which the substrate is dissolved in diisopropyl ether and the enzyme is present as a solid phase attached to the fungal cells. Other workers have indicated that there is some phospholipase activity associated with purified extracellular lipase preparations from both R. arrhizus [3] and M. javanicus [4].

Peculiar difficulties arise in examining the specificity of insoluble enzyme fractions since they are not susceptible to the usual procedures for purification and isolation used with soluble enzymes. It has consequently been necessary to determine by indirect means whether the phospholipase A₁ activities of fungal mycelia could be attributed to lipase. To ascertain whether there was correlation between the two types of activity, the lipase and phospholipase activities of M. javanicus were examined and compared in a variety of circumstances using a reaction system described previously [1,2,5].

2. Materials and Methods

2.1. Microorganisms and substrates

Cultures of M. javanicus (CMI 25330) were obtained from the Commonwealth Mycological Institute (Surrey, England). Mycelial preparations were obtained as described previously [1] using growth media based on that of Fukomoto et al. [6].

Egg lecithin (B.D.H. Chemicals Ltd.) was used in phospholipase assays as previously described [1]. For studies on lipase activity, commercial, refined olive oil was found to be suitable. Absence of lipids other than triglyceride was confirmed by thin-layer chromatography.

2.2. Phospholipase and lipase assays

Phospholipase assays were carried out at 50°C using the diisopropyl ether solvent system previously described [1].

Assays for lipase were carried out in the same manner as those for phospholipases by substituting a solution of olive oil (usually 10% w/v) in diisopropyl ether for the lecithin solution. Lipase activity was determined by titration of liberated fatty acid.

2.3. Heat inactivation studies

250 mg of a mycelial preparation of M. javanicus were suspended in 2.5 ml McIlvaine buffer in a thin-walled test-tube. After the contents had been maintained at the appropriate temperature for 5 min, the tube and contents were cooled in a melting-ice bath and the preparation recovered by centrifugation (20 min at 1500 × g), washing and freeze-drying. The supernatant was also retained and freeze-dried. Control samples were obtained by suspending the mycelial preparation in the appropriate buffer at 0°C for 5 min and then washing and freeze-drying the centrifuged material.
2.4. Disintegration of cell walls

This was carried out using a rotary bead mill ("Dynomill" Type K.D.L., W.A. Bachofen, Basle, Switzerland) as previously described [1].

3. Results

3.1. Heat inactivation

The effects of prior heat treatment on lipase and phospholipase activities of *M. javanicus* mycelia at different temperatures and pH values are shown in Fig. 1. The effect of duration of heat treatment at 74°C is also shown in Fig. 2. It can be seen from Fig. 1, that phospholipase activity is almost entirely destroyed at 74°C and pH 7.4 whilst lipase activity undergoes a much smaller diminution. Likewise in Fig. 2 the rapid loss of phospholipase activity contrasts with the relative stability of lipase activity.

3.2. Effect of mixed substrates

If the lipase and phospholipase activities of *M. javanicus* mycelia were associated with two distinct enzymes then independent action against triglycerides and phosphatidylcholine would be anticipated. On the other hand were the same enzyme responsible for both activities it would be expected that the action of the enzyme on one substrate would be reduced by the presence of sufficient quantities of the other substrate, i.e. substrates would compete for the active centre.

It can be seen from Fig. 3 that the presence of lecithin, in a quantity almost sufficient to produce saturation of the phospholipase does not diminish rates of triglyceride hydrolysis over the range of concentrations examined. On the contrary the amount of fatty acid produced can be best explained by the assumption that each enzyme acts on its substrate independently. Similarly Table 1 shows that the hydrolysis...
TABLE 1

EFFECT OF THE PRESENCE OF OLIVE OIL ON LPC LIBERATION

<table>
<thead>
<tr>
<th>Olive oil - mM</th>
<th>LPC formed (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>56</td>
</tr>
<tr>
<td>22.5</td>
<td>47</td>
</tr>
<tr>
<td>68.0</td>
<td>55</td>
</tr>
<tr>
<td>113.0</td>
<td>52</td>
</tr>
<tr>
<td>169.5</td>
<td>54</td>
</tr>
<tr>
<td>226.0</td>
<td>57</td>
</tr>
</tbody>
</table>

a Reaction conditions were as described in Fig. 3.
b In presence of 63.5 mM lecithin.

With the insoluble material obtained after disintegration of the mycelia. In a number of later experiments however, substantial activity was also found in the freeze-dried soluble fraction. The difference may be due to use of cornsteep liquor from a different source in the growth media, since this has been known to affect reproducibility in mycological studies [8,9]. It became possible, in consequence, to compare distribution of lipase activity with that of phospholipase A activity between insoluble cell fragments and the soluble fractions. Defatted mycelia of M. javanicus were disintegrated and separated into soluble and insoluble fractions. After centrifugation and freeze-drying of the fractions, their activities were compared with those of untreated mycelial samples.

Results are shown in Table 2. It is evident that the soluble fractions from M. javanicus possess phospholipase activity and lack lipase activity.

4. Discussion

The work described here confirms the value of solvent systems as assay media in studies on insoluble fungal phospholipases and lipases. These allow comparative studies to be undertaken on both types of enzymes using a system suitable for the solution of either substrate.

TABLE 2

COMPARISON OF LIPASE (L) AND PHOSPHOLIPASE (P) ACTIVITIES OF MYCELIAL FRACTIONS FOLLOWING CELL DISINTEGRATION

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth period (days)</th>
<th>Fraction</th>
<th>L</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. javanicus</td>
<td>4</td>
<td>unmilled</td>
<td>194</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td></td>
<td>insoluble</td>
<td>54</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soluble</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>unmilled</td>
<td>116</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>insoluble</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soluble</td>
<td>0</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>unmilled</td>
<td>194</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td></td>
<td>insoluble</td>
<td>132</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soluble</td>
<td>0</td>
<td>122</td>
</tr>
</tbody>
</table>

a The reaction mixture consisted of 2.0 ml 10% (w/v) lecithin or olive oil in diisopropyl ether, 60 mg mycelial fraction and 80 μl water. Activity is expressed as μmol fatty acid released following a 2 h incubation period.
It can be assumed that were the phospholipase A₁ activity of the mycelia studied due to the lipase known to be present then correlation of lipase activity with that of phospholipase could be anticipated irrespective of changes in conditions of growth or treatment. On the contrary it has been established that the two activities can be differentiated by prior heat treatment. Further evidence supporting the separate identities of the two activities is provided by comparative studies of their solubilisation following disintegration as it is clear that solubilisation to different degrees occurred, resulting in some instances in a lipase-free phospholipase A fraction. The results obtained from the experiments with mixed substrates also indicate that lipase is not responsible for phospholipid hydrolysis and that a discrete phospholipase A is present.

In work recently carried out [2] it was found that the ratio of lipase activity to phospholipase activity for a number of samples of mycelia from different organisms, or from organisms grown under different conditions, varies widely. This would not be expected were the two activities manifestations of the one enzyme.

While it has been accepted that phospholipase A₁ exists in prokaryotic cells such as those of *E. coli* [10], *B. subtilis* [11] and *B. megaterium* [12], its existence in filamentous fungi has been open to doubt because of the incidental phospholipase activity of the type reported in samples of purified lipase from *R. arrhizus* [13,3] and *M. javanicus* [4]. The studies reported here on the mycelia of *M. javanicus* establish the presence of true phospholipase A in filamentous fungi.

Acknowledgment

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References