Selectivity to K\(^+\) and Na\(^+\) of Protoplast Fractions Isolated from Different Regions of *Aspergillus nidulans* Hyphae

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The selectivity to K\(^+\) and Na\(^+\) of protoplast samples representing cytoplasm isolated from different regions of the hyphal filament of *Aspergillus nidulans* was investigated. Concentrations of both ions contained in successive protoplast fractions were measured. During lytic digestion, protoplasts were released first from apical regions and subsequently from progressively older regions of hyphae. A low K\(^+\)/Na\(^+\) ratio was found in protoplasts containing primarily apical cytoplasm and a high K\(^+\)/Na\(^+\) ratio was found in protoplasts originating from older regions of hyphae. The ratios were the same whether MgSO\(_4\) or mannitol was used as stabilizer. Absolute concentrations of both ions were higher in protoplasts of apical origin. Protoplasts stabilized in mannitol lost more ions than those stabilized in MgSO\(_4\) over an 8 h incubation period. Na\(^+\) losses were higher from apical protoplasts whereas K\(^+\) losses were higher from protoplasts liberated from older regions of hyphae. The addition of divalent metal cations (1.5 mM-Mn\(^{2+}\) or Mg\(^{2+}\)) reduced losses of Na\(^+\) from protoplasts but did not affect loss of K\(^+\). Data obtained using protoplast samples were related to those obtained for intact mycelium. Absolute losses of both ions from mycelium were lower than for protoplasts but when compared on a protein basis the data suggested that protoplasts possess properties similar to those of intact mycelium in terms of K\(^+\) and Na\(^+\) selectivity.

INTRODUCTION

Fungal protoplasts are recognized as being important in the preparation of cell-free extracts (Coakley et al., 1977) and also in the investigation of the biochemical anatomy of fungal hyphae (Peberdy, 1976, 1979; Isaac, 1985). Protoplasts possess properties very similar to those of intact mycelium (Isaac, 1978; Isaac & Gokhale, 1983; Gokhale & Isaac, 1984), after allowing for the degree and nature of any inevitable disruption which occurs during their formation, and subsequent effects on their biochemistry.

For the release of protoplasts, lytic techniques (Coakley et al., 1977) involving a range of different stabilizing solutions have been widely investigated as a result of the need to liberate large numbers of protoplasts for biochemical investigations (Peberdy et al., 1976; Peberdy, 1979; Isaac, 1978). Characteristic protoplast release patterns can now be identified and reproduced for individual lytic systems (Peberdy et al., 1976; Isaac, 1985). Additionally it is possible to isolate samples of protoplasts, from filamentous fungi, representing different regions of hyphal cytoplasm (Isaac et al., 1978). Apical protoplasts are released first, on dissolution of hyphal tips. Those protoplasts released subsequently arise from progressively older regions of hyphae. Temporal fractionation of protoplasts, as these are liberated from mycelium, represents a longitudinal fractionation of hyphal cytoplasm. Such protoplast preparations provide a means of biochemical analysis of the longitudinal variation within hyphae (Isaac, 1978, 1985).

Protoplast preparations are well suited to the investigation of solute transport across the plasma membrane, but although permeability properties of bacterial protoplasts have been studied there are few reports concerning fungal protoplasts. Elorza et al. (1969) studied the regeneration of *Aspergillus nidulans* protoplasts and found evidence that the permeability...
properties remain unaltered after cell wall removal. Theuvenet & Bindles (1980) confirmed the same situation for K+ and Rb+ uptake in yeast protoplasts.

This paper investigates the selectivity to K+ and Na+ of protoplasts representing cytoplasm isolated from different regions of the hyphal filament of Aspergillus nidulans. The effects of an inorganic salt (MgSO4) and a sugar alcohol (mannitol) as osmotic stabilizers are also assessed.

METHODS

Organism. Aspergillus nidulans BDUN 33 (University of Nottingham collection) was maintained on 1-0% (w/v) malt extract agar (Oxoid) at 35 °C. Conidia for inoculation into liquid culture were harvested from 3-d-old cultures. Mycelium for protoplast production was grown in 2-1 Erlenmeyer flasks containing 500 ml mineral salts medium (Vogel, 1964) supplemented with 10 g glucose l-1 and inoculated with 2 × 108 spores ml-1 final concentration. Incubation was at 30 °C for 18 h on a rotary shaker (200 r.p.m.).

Protoplast isolation. Protoplasts were prepared by a method modified from Peberdy & Isaac (1976) using either Cytophaga lytic enzyme L1 (BDH) at a final concentration of 20 mg ml-1 or Aspergillus autolytic enzyme (Isaac & Gokhale, 1982) with either 0-6 M-MgSO4 or 0-8 M mannitol as stabilizer. All mixtures were buffered with 0-05 M-Tris/maleate except where otherwise indicated. Protoplasts were harvested from these mixtures at 1, 2 and 3 h after the start of lytic digestion; residual mycelium was replaced in the lytic mixture after each harvest. Harvesting was done by the method of Isaac et al. (1978). Protoplasts were counted in a haemocytometer.

Determination of ion concentration. Concentrations of K+ and Na+ were determined by atomic absorption spectrophotometry, using a Unicam SP 90A series 2 atomic absorption spectrophotometer. Standards were prepared in double-distilled water, using 'AnalaR' salts. Protoplast suspensions were lysed in water before assay. Replicate protoplast samples were stored for up to 8 h in isotonic Na+/K+-free, buffer/stabilizer solution at 25 °C, using a known protoplast concentration. Na+ and K+ concentrations were measured in the supernatant at intervals during storage. Ion concentrations in supernatants and protoplast samples were assayed in the same way.

Dry weight determination. Samples were filtered through Whatman 542 paper discs, thoroughly washed with distilled water and dried to constant weight at 80 °C.

Protein determination. Protein was determined by the Lowry method using bovine serum albumin as standard.

RESULTS

The liberation of protoplasts from Aspergillus nidulans using 0-6 M-MgSO4 as stabilizer and either 0-01 M-sodium phosphate buffer or 0-05 M-Tris/maleate buffer pH 5-8 is shown in Fig. 1. Characteristic sigmoidal release patterns were obtained during a 3 h incubation period. Final yields obtained from the two buffer systems were similar. To avoid contamination from Na+, Tris/maleate buffer was used in further experiments. Protoplasts were also obtained using mannitol as stabilizer but yields were low. However, mannitol was more successful as a stabilizer than either maltose or sucrose (data not shown).

The concentrations of K+ and Na+ contained in protoplast samples from successive fractions, isolated after 1, 2, and 3 h lytic incubation, are given in Table 1. Concentrations of both ions in protoplasts liberated in 0-6 M-MgSO4, calculated on a protoplast basis, were significantly higher after 1 h than after either 2 h or 3 h and also there were higher concentrations of Na+ than K+ in the 1 h fraction. Protoplasts liberated in 0-8 M mannitol after 1 h also contained significantly higher levels of both ions than protoplasts of subsequent fractions. Using this stabilizer system lower concentrations of both K+ and Na+ were found in the 2 h fractions. Concentrations calculated on a unit protein basis showed qualitatively similar results. In both stabilizers Na+ concentrations (mg protein)-1 were higher in protoplasts released after 1 h than in protoplasts of subsequent fractions. K+ levels in MgSO4-stabilized protoplasts, however, were higher in both the 1 h and 2 h fractions than in the 3 h fraction. K+ concentrations in mannitol-stabilized protoplasts were higher in protoplasts liberated after 3 h. The concentrations of both ions in MgSO4-stabilized protoplasts were greater than those in mannitol-stabilized preparations.

Data for the ratio of sodium to potassium in these successive protoplast fractions are also given in Table 1. In both stabilizer systems the ratio was less than 1-0 for the 1 h protoplast fractions. The ratio was higher in subsequent fractions although it was lower in the 2 h fraction than for protoplasts released after 3 h, a similar trend being observed for both stabilizer systems used.

The loss of Na+ from successive protoplast fractions was measured during storage for 8 h (Fig.
Cation selectivity of protoplasts

Table 1. Concentrations of K⁺ and Na⁺ in successive protoplast fractions isolated from Aspergillus nidulans

<table>
<thead>
<tr>
<th>Stabilizer</th>
<th>Protoplast fraction</th>
<th>K⁺ concn</th>
<th>Na⁺ concn</th>
<th>K⁺/Na⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄</td>
<td>1 h</td>
<td>12.08 ± 4.64</td>
<td>2.33 ± 0.23</td>
<td>38.17 ± 14.45</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>2.98 ± 0.06</td>
<td>2.83 ± 0.08</td>
<td>3.40 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>3 h</td>
<td>1.49 ± 0.02</td>
<td>1.50 ± 0.13</td>
<td>1.02 ± 0.77</td>
</tr>
<tr>
<td>Mannitol</td>
<td>1 h</td>
<td>8.73 ± 0.72</td>
<td>0.39 ± 0.01</td>
<td>22.25 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>0.52 ± 0.01</td>
<td>0.46 ± 0.15</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>3 h</td>
<td>1.31 ± 0.01</td>
<td>0.98 ± 0.03</td>
<td>0.45 ± 0.08</td>
</tr>
</tbody>
</table>

Results are given ± SEM. *, 1 h significantly different from 2 h and 3 h at 5% probability level, using a t-test; **, 1 h significantly different from 3 h at 5% probability level, using a t-test; *** 1, 2 and 3 h significantly different at 5% probability level, using a t-test.

2). It was not possible to measure ion loss from 1 h or 2 h protoplast fractions stabilized with mannitol because the yields of protoplasts obtained were very low. However, yields obtained after 3 h were similar to those obtained with MgSO₄ stabilizer. Protoplasts released after 3 h lytic incubation in mannitol were very permeable to Na⁺, showing a rapid loss. MgSO₄-stabilized protoplast fractions were not all equally permeable to internal Na⁺. The 1 h and 2 h protoplast fractions were less permeable and losses were significantly lower in them.

The loss of K⁺ from protoplast fractions was also measured during storage for 8 h (Fig. 3). Protoplasts released after 3 h lytic incubation in both stabilizers appeared to be very much less permeable to K⁺ than Na⁺. Leakage of K⁺ from MgSO₄-stabilized protoplast samples was highest in 1 h fractions and lowest in those released after 3 h.

The loss of K⁺ and Na⁺ from protoplasts stabilized with mannitol supplemented with either 1.5 mM-Mn²⁺ or Mg²⁺ measured during storage for 8 h is shown in Figs 4 and 5. The addition of either of these divalent metal cations did not affect the loss of K⁺ from protoplasts. However, the permeability of protoplasts to Na⁺ was much lower in the presence of either Mn²⁺ or Mg²⁺.

The loss of K⁺ and Na⁺ from mycelium suspended in either isotonic MgSO₄ stabilizer (Fig. 6) or isotonic mannitol, with and without supplementation with 1.5 mM-Mn²⁺ (Fig. 7) was
measured. Mycelium retained K⁺ but lost Na⁺ in MgSO₄ buffer/stabilizer. Both ions were lost on storage in mannitol buffer/stabilizer although Na⁺ was lost at a faster rate than K⁺. In mannitol the accumulation of the two ions in the external medium occurred at a higher rate than in MgSO₄. Inclusion of Mn²⁺ resulted in a reduction of the loss of Na⁺ although the effect was less pronounced than for protoplasts.
Cation selectivity of protoplasts

**DISCUSSION**

Hyphal fractionation of *Aspergillus nidulans* mycelium was previously described (Isaac et al., 1978, 1979; Isaac & Gokhale, 1983; Gokhale & Isaac, 1984) using either 0.6 M-KCl or 0.6 M-MgSO₄ as stabilizers, buffered to pH 5.8 with sodium phosphate buffer. The characteristics of lytic digestion of *A. nidulans* hyphae in both Tris/maleate and sodium phosphate buffered systems were very similar in terms of release pattern, final yield and protoplast morphology. Protoplasts liberated by these systems were released as a result of similar hyphal fractionation and would be expected to arise from similar hyphal origins (Isaac et al., 1978, 1979).

However, the selectivity to K⁺ and Na⁺ of protoplasts liberated in the two stabilizer systems differed. In those experiments concerning ion loss, low yields of protoplasts were obtained early during lytic incubation in mannitol stabilizer; therefore the 3 h fraction probably represented protoplasts arising from apical, sub-apical and older regions of hyphae together. Protoplasts isolated in mannitol lost more Na⁺ during storage than those isolated in MgSO₄ even though internal concentrations of Na⁺ in mannitol-stabilized protoplasts were lower. Protoplasts stabilized with mannitol also lost more K⁺ than those isolated in MgSO₄. In both stabilizer systems K⁺ loss tended towards an equilibrium during the storage period.

Tabata et al. (1965) reported that the addition of divalent cations to *Saccharomyces cerevisiae* protoplasts increased their stability. Magnesium is important in maintaining the integrity of bacterial spheroplast membranes (Zinder & Arndt, 1956; Lederberg, 1956) and osmotic shock studies with *Escherichia coli* indicate the importance of magnesium in the maintenance of the cellular permeability barrier (Leive, 1965; Neu & Heppel, 1965). The inclusion of Mg²⁺ or Mn²⁺ with mannitol-stabilized *A. nidulans* protoplasts did not significantly increase overall protoplast yields but did decrease the rate of Na⁺ leakage.

The washing and resuspension of protoplasts in a medium free from sodium and potassium created a concentration gradient for these ions, tending to drive them out of the protoplasts. In MgSO₄ stabilizer, loss of Na⁺ and K⁺ virtually ceased after 4–5 h and may have been attributable to passive leakage down an electrochemical potential gradient. In mannitol, protoplasts released after 3 h lytic incubation lost Na⁺ rapidly; but the loss was reduced by the inclusion of Mg²⁺ or Mn²⁺. Cell membranes of both *S. cerevisiae* and *Neurospora crassa* possess an H⁺ extrusion ATPase which requires activation by Mg²⁺ (Goffeau & Slayman, 1981) and it seems likely that this is also the case in *A. nidulans*. Scarborough (1977) showed that Mn²⁺ can replace Mg²⁺ as a pump activator in *N. crassa* at a concentration of 1.5-3.0 mM. This would...
suggest that ATPase comes into operation on addition of Mg$^{2+}$, with protons being extruded instead of Na$^+$. This requires further investigation.

Care must be taken in comparing the loss of sodium and potassium from intact hyphae with that occurring from protoplasts. Some membrane transport capabilities may be damaged or lost, at least temporarily, during the process of formation and liberation of protoplasts. Elorza et al. (1969) have shown that some transport pathways do remain functional in isolated protoplasts from \textit{A. nidulans}. Experiments described here using intact mycelium in mannitol stabilizer as incubation medium showed a higher rate of K$^+$ loss than when MgSO$_4$ was used; Mn$^{2+}$ had no effect on K$^+$ loss. Loss of Na$^+$ was similar in both stabilizer solutions; Mn$^{2+}$ had no effect on Na$^+$ loss.

Losses of both K$^+$ and Na$^+$ from intact mycelium were lower than losses from protoplast samples when compared directly on a protein basis. Protoplast samples contained cytoplasmic protein alone whereas mycelial samples also included the protein component attributable to the cell wall. In \textit{A. nidulans}, cell wall protein accounts for about one-third of the total protein of whole mycelium (Isaac, 1978). Ion losses from protoplasts and intact mycelium were of a similar order of magnitude taking wall protein into account. This suggests that, in terms of permeability to K$^+$ and Na$^+$, protoplasts possess properties very similar to those of intact mycelium.

The K$^+/Na^+$ ratios obtained using protoplast samples isolated during lytic digestion showed significant differences between protoplast fractions. Protoplasts digested for 1 h contained primarily apical cytoplasm and showed a low K$^+/Na^+$ ratio; protoplasts digested for 2 h and 3 h originated from progressively older hyphal regions and exhibited higher K$^+/Na^+$ ratios. Absolute concentrations of both K$^+$ and Na$^+$ also varied between fractions; higher levels were found in protoplasts of apical origin. These data suggest that the plasma membrane exhibits differential selectivity to these ions longitudinally, between apical and older regions. This is in keeping with the results of Galpin et al. (1978) who, by X-ray microanalysis together with chemical analysis of growing mycelium of \textit{Dendrphyella salina}, suggested that the K$^+/Na^+$ ratio varied with intrahyphal location, with a low ratio at hyphal tips.

REFERENCES


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