The use of beta-D-glucanase as a substitute for Novozym 234 in immunofluorescence and protoplasting

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Novozym 234 has been used for many years to prepare protoplasts of *Aspergillus nidulans* and other fungi for transformation. It has also been very useful in immunofluorescence studies for partially digesting walls of fixed hyphae or germlings to allow antibodies to penetrate into the cytoplasm. In recent years, the availability of Novozym 234 has become problematic, and we have searched for combinations of available enzymes that are suitable for protoplasting and immunofluorescence studies in *A. nidulans*.

For immunofluorescence microscopy we have found that a mixture of beta-D-glucanase (16 mg/ml) (Catalog # 0439-1, InterSpex Products, Inc., Foster City CA), Driselase (10 mg/ml) (Catalog # 0465-1, InterSpex) and yeast lytic enzyme from *Arthobacter luteus* (81.4 U/ml) (Catalog # 369941, ICN Biomedicals, Costa Mesa, CA) is an excellent combination for rendering walls of germlings permeable to antibodies. It is better, in fact, than various mixtures we have used that include Novozym 234, particularly if the beta-D-glucanase is heat treated to reduce protease activity (Roncal *et al.*, 1991 J. Gen. Microbiol. 137:1647-1651). Details of a procedure using this combination have been published (Ovechkina *et al.*, 1999 Antimicrob. Agents Chemother. 43:1993-1999). We mention it here because the journal in which the manuscript was published may not be commonly read by fungal geneticists and because we did not call attention to the novel immunofluorescence procedure in the manuscript.

Producing *A. nidulans* protoplasts for transformation is often problematic with Novozym 234. There is enormous batch to batch (and even bottle to bottle) variation in potency and toxicity and, unfortunately, we have found that this is the case with other protoplasting enzymes as well. We have tried a large number of combinations of beta-D-glucanase (InterSpex), Driselase (InterSpex and Karlan Research Products Corporation, Santa Rosa, CA), yeast lytic enzyme (lyticase) (ICN and Sigma), chitinase (various manufacturers), Funcelase (Karlan), and beta glucuronidase (Sigma) for protoplast production in *A. nidulans*. All experiments were carried out at 30°C in 0.55 M KCl, 0.05 M citric acid, pH 5.8 (Oakley *et al.*, 1987 Gene **61**:385-399).

While it is beyond the scope of this note to discuss these experiments in detail, we have made several general observations. First, it is critical to monitor protoplast formation by phase contrast microscopy. Some strains form protoplasts much more easily than others (see below) and, as mentioned, batches of enzymes differ in their potency and toxicity. One may need to use different enzyme concentrations with different strains, and making appropriate adjustments requires that one monitors protoplast formation.

Second, swollen conidia form protoplasts much more easily than mature hyphae. We obtain most efficient protoplasting if conidia are inoculated in growth medium and incubated to the point at which conidia are swollen, but germ tubes have not yet extended. In our experience, ease of protoplasting generally correlates with conidial pigments. Swollen white or fawn conidia protoplast most easily (i.e. at lower protoplasting enzyme concentrations) while yellow conidia protoplast less readily and green conidia are most difficult to protoplast. We have not yet attempted to make protoplasts from chartreuse conidia.

Third, many enzymes or combinations of enzymes are highly toxic. They kill forming protoplasts and even swollen conidia. Death of protoplasts and conidia can be monitored by phase contrast microscopy. Toxicity does not correlate with protoplasting potency or protease activity and may vary dramatically from batch to batch of enzymes. We suspect that some of the toxicity may be due to antifoaming agents that are often added during batch fermentations to produce enzymes.

We have found two procedures that produce acceptable yields of highly transformation-competent protoplasts. A combination of 8 mg/ml beta-D-glucanase (InterSpex), 5 mg/ml Driselase (InterSpex or Karlan), and 81.25 U/ml lyticase (Sigma) is reasonably potent in producing protoplasts but causes significant protoplast death. Protoplasting efficiency varies among strains, but in a typical experiment, after 2.5 hr of protoplasting, 57% of swollen conidia were converted to protoplasts, 25% were killed and 18% were unprotoplasted.

Driselase contains a starch carrier which should be removed. We generally prepare double strength protoplasting solution (1.1 M KCl, 0.1 M citric acid, pH 5.8) and dissolve the Driselase in the double strength protoplasting solution on ice before removing the starch carrier by centrifugation (Oakley *et al.*, 1987 Gene 61:385-399). We then add the other enzymes and filter sterilize. Finally, an equal volume of growth medium containing swollen spores is added to the double strength protoplasting solution and incubation to produce protoplasts is at 30°C.

An alternative that works for some strains (particularly strains with fawn or white conidia) is to substitue Driselase alone (InterSpex or Karlan) at a final concentration of 20 mg/ml for the three enzyme mixture. Driselase is relatively non-toxic on its own, and in some strains (e.g. FGSC A722) up to 80% of swollen conidia are converted to protoplasts. Other strains we have created in our lab, however, protoplast at low frequencies when only Driselase is used. Since Driselase alone is less toxic than the three enzyme mixture, we find it useful to determine if Driselase alone works for a particular strain before trying the three enzyme mixture.

A. nidulans protoplasts are surprisingly resistant to physical shearing by vortexing or pipeting, but they are very sensitive to osmotic shock and exquisitely sensitive to detergents. In our hands, unprotoplasted material tends to lower transformation frequencies. We often separate protoplasts from unprotoplasted material by layering the protoplast suspension on to 1.0 M sucrose and sedimenting at _ 1000 X g (R_{min}) for 10 minutes. The protoplasts collect at the interface and can be harvested with a pipet, mixed with 0.6 M KCl, sedimented and carried through transformation procedures.