SHORT COMMUNICATION

Preparation of Spheroplasts of *Schizosaccharomyces pombe*

By DOUGLAS P. DICKINSON¹* AND IRVIN ISENBERG²

¹ Department of Zoology, University of Edinburgh, Edinburgh EH9 3JT, U.K.

² Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, U.S.A.

(Received 30 July 1981)

A method is described for the conversion of *Schizosaccharomyces pombe* cells to spheroplasts using Novozym 234, a commercially available enzyme preparation. Both exponentially growing and stationary phase cells can be quantitatively converted to spheroplasts on a large scale. A means of disrupting these spheroplasts is described which gives an efficient release of organelles. The resulting homogenate is suitable for subcellular fractionation. The spheroplasts can also be regenerated to give dividing cells, thus providing an approach to genetic manipulation in *S. pombe*.

INTRODUCTION

A cell wall is generally an obstacle to the isolation of organelles from micro-organisms, and it must be removed or disrupted as a first step. In the fission yeast *Schizosaccharomyces pombe*, the shape of the cell is maintained by a tough, rigid polysaccharide wall, forming a cylindrical cell with rounded ends. This wall is composed of a β -glucan fraction (46–54%) containing (1→3)- and (1→6)-linked saccharides, and a (1→3)- α -glucan fraction (28%) (Bush *et al.*, 1974). Mechanical methods necessary to disrupt this wall, such as violent agitation with glass beads, risk seriously damaging internal structures, and gentler methods, such as enzymic digestion of the wall, are preferable.

Kopecka (1975), using a combination of snail and *Trichoderma viride* enzymes, and Schwenke & Nagy (1978), using a combination of snail enzymes and a (1→3)- α -glucanase and a (1→3)- β -glucanase, have reported a quantitative conversion to spheroplasts of exponentially growing *S. pombe* cells. However, neither reported spheroplast formation from stationary phase *S. pombe*, and both methods required the laboratory production of the appropriate enzymes.

Recently, Stephen & Nasim (1981) described the use of Mutanase, a commercial enzyme preparation (since renamed Novozym 234), to produce protoplasts in high yield from a variety of yeasts, including *S. pombe*. In this paper, we present a more detailed report on the use of Novozym 234 to prepare spheroplasts from *S. pombe* cells. Both exponentially growing and stationary phase cells can be quantitatively converted to spheroplasts. Since Novozym 234 is commercially available, our method may be easily adopted by any laboratory.

METHODS

Novozym 234 was obtained from Novo Enzyme Products Ltd, 2B Thames Avenue, Windsor, Berkshire, U.K. This enzyme preparation was previously supplied under the names 'Mutanase' and 'Cell Wall Lysing Enzymes SP 234'.

Cells of wild-type *Schizosaccharomyces pombe* strain 972h⁻ were grown with shaking at 30 °C in 100 or 200 ml batches of media containing 3% (w/v) glucose, 0.5% (w/v) peptone (Difco or Oxoid), 0.5% (w/v) yeast

extract (Difco). To obtain exponentially growing cells, cultures were incubated for 18 h to a density of $5\text{--}10 \times 10^6$ cells ml^{-1} . To obtain stationary phase cells, the cultures were incubated for a further 48 h to a final density of about 4×10^7 cells ml^{-1} . The cells were harvested by centrifugation at 2000 *g* for 5 min, and washed once with distilled water.

Spheroplasts were prepared from cells essentially as described by Schwenke & Nagy (1978). Prior to digestion with the enzyme, the cells were treated by resuspension at a density of $1\text{--}2 \times 10^7$ cells ml^{-1} in 100 mM-EDTA, 100 mM-2-(*N*-morpholino)ethanesulphonic acid (MES), pH 6.0, to which dithiothreitol had been added, just before use, to a final concentration of 5 mM. The suspensions were incubated at 30 °C with shaking. Exponential phase cells were pretreated for 20 min, and stationary phase cells for 60 min. After pretreatment, the cells were harvested by centrifugation at 5000 *g* for 1 min, and washed once with 0.6 M-sorbitol, 20 mM-MES, pH 6.0.

To test the capacity of Novozym 234 to produce spheroplasts, the washed, pretreated cells were resuspended at a density of $1\text{--}2 \times 10^7$ cells ml^{-1} in solutions containing different concentrations of the enzyme. The enzyme was dissolved in a buffer of 0.6 M-KCl, 0.01 M-citrate/phosphate, pH 5.85, to which dithiothreitol had been added, just before use, to a final concentration of 5 mM. The suspensions were incubated at 30 °C with shaking.

The formation of osmotically fragile cells was monitored by taking 50 μl samples, diluting them 20-fold with distilled water, and viewing under a phase-contrast microscope. Although they retained much of their structure when lysed, cells and spheroplasts became phase-dark and could thus be distinguished from the phase-bright intact cells. The efficiency of conversion of exponential phase cells was further tested by taking samples of cells before and after digestion, diluting them 100-fold with 0.6 M-sorbitol, 0.2 M-KCl, 20 mM-MES, pH 5.0 (SKM buffer), and counting them on opposite sides of a haemocytometer. Cells with visible cell plates were counted as one cell.

When spheroplast formation was judged to be complete, 5–10 ml of the suspension was layered on to 20 ml of 0.8 M-sucrose, 0.2 M-KCl in a 50 ml tube and centrifuged in a swing-out rotor for 5 min at 5000 *g*. The supernatant was carefully removed by pipette, and the spheroplasts were either used immediately or stored in SKM buffer at 4 °C.

RESULTS AND DISCUSSION

The Schwenke & Nagy (1978) protocol for preparing spheroplasts of *S. pombe* was based upon solubilizing the three main components of the wall: snail gut juice was used as a source of a (1 $\rightarrow$ 6)- β -glucanase, and (1 $\rightarrow$ 3)- α -glucanase and (1 $\rightarrow$ 3)- β -glucanase were prepared by Dr E. T. Reese of the Natick Laboratories, Natick, Mass., U.S.A. As the principal activity in Novozym 234 is a (1 $\rightarrow$ 3)- α -glucanase, we tested this enzyme, using the Schwenke & Nagy (1978) protocol, for its effect on wild-type *S. pombe* strain 972h⁺. Initially, we monitored the formation of osmotically fragile cells. Used alone on exponentially growing cells, Novozym was very potent: as little as 1 mg ml^{-1} produced 100% fragility in about 100 min, and 3 mg ml^{-1} produced 100% fragility in 20 min.

The development of osmotic fragility was accompanied by changes in the shape of the cells. After 30–40 min incubation with 3 mg Novozym 234 ml^{-1} , many cells were dumbbell-shaped, or showed prominent bulges in the wall, either at one end or in the middle of the cell (Fig. 1*a*). These cells continued to round up and eventually formed spherical cells. Also, some spheroplasts were formed by extrusion from an *S. pombe*-shaped wall 'ghost'. After 210 min, all the cells had been converted to spheroplasts either by extrusion, or by rounding-up, and no 'ghosts' or cell wall debris could be seen (Fig. 1*b*). However, some of the cells and spheroplasts lysed during the digestion (phase-dark particles in Fig. 1*b*).

With stationary phase cells, 3 mg Novozym 234 ml^{-1} gave a 100% yield of osmotically fragile cells after 190 min. However, although no *S. pombe*-shaped cells or cell walls were visible after 5 h of digestion, only 52% of the original cells were present as intact spheroplasts, the rest having apparently lysed.

During the studies described above, it was necessary to use concentrations of enzyme that gave rates of digestion sufficiently low to enable the course of the digestion to be followed. As a result of the slow rates of digestion, a fraction of the cells lysed before they could form spheroplasts. At Novozym 234 concentrations of 30–45 mg ml^{-1} , the proportion of lysed cells was found to be negligible (typically 0–5%). Digestions of mid-exponential or stationary phase cells suspended at a density of $2\text{--}8 \times 10^9$ cells ml^{-1} with 30–45 mg Novozym 234 ml^{-1} gave a total conversion to spheroplasts, as judged by microscopic observation. This occurred within 10–20 min for exponential phase cells, and within 45 min for stationary phase cells.

Fig. 1. Exponentially growing cells of *S. pombe* 972h⁻ were pretreated for 20 min and then digested with Novozym 234 (3 mg ml⁻¹) at 30 °C for 40 min (a) and 210 min (b). The bar markers represent 20 µm.

Intact cells were very rarely seen after complete digestion. Haemocytometer counts of exponential phase cells before and after digestion gave $54.1 (\pm 6.0) \times 10^4$ cells ml⁻¹ and $62.8 (\pm 3.1) \times 10^4$ spheroplasts ml⁻¹, respectively, thus confirming qualitative microscopic observations.

To test their viability, spheroplasts were thinly spread on an iso-osmotic agar plate (0.2 M-KCl, 0.6 M-sorbitol, EMM 3 minimal medium, 2% agar) and incubated at 25 °C for 24 h. At this time, small colonies had formed around each individual spheroplast. The colonies consisted of a central region of irregularly-shaped cells, surrounded by a layer of cells having a typical *S. pombe* shape. The spheroplasts therefore form *S. pombe*-shaped cells after several divisions of an intermediate form of irregular shape.

The spheroplasts formed by Novozym 234 were osmotically sensitive and ruptured when the osmotic strength of the buffer was reduced too far, releasing some of the internal organelles. However, the lysed spheroplasts retained much of their spherical shape, and a considerable amount of internal structure. When lysed in 18% (w/v) Ficoll (which has a high refractive index), nuclei and vacuoles could be seen trapped within the lysed spheroplasts.

Detergent extraction of lysed spheroplasts with 0.1% (w/v) Triton X-100, 0.1% (w/v) deoxycholate revealed an almost spherical wall-remnant ghost. This ghost was resistant to Pronase and was presumably a residual polysaccharide component of the wall.

To release the cell organelles efficiently, a suspension of spheroplasts was homogenized in 18% Ficoll using a motor-driven polyethylene homogenizer with a 1 mm diameter clearance. This was found to give excellent disruption of spheroplasts and release of organelles.

The inclusion of snail juice and Zymolyase [which contains a (1→3)- β -glucanase as its principal component] in the incubation mixture had no significant effect on the course of spheroplast formation, or on the remaining structure surrounding the spheroplast. As Novozym 234 contains a variety of other enzyme activities besides (1→3)- α -glucanase, it seems likely that these include sufficient β -glucanase activities to complete the wall digestion.

The quantitative conversion by Novozym 234 of cells to osmotically fragile spheroplasts, and their subsequent capacity to revert to dividing cells having a pombe shape, provides an approach to genetic manipulation in *S. pombe*. Recently, Beach & Nurse (1981) have used

Novozym 234 (described as Mutanase in their paper) to develop a transformation system in *S. pombe*. Novozym 234 also provides material that can be used to obtain nuclei or other organelles. Up to now, obtaining large quantities of spheroplasts of *S. pombe* has been difficult. Our procedures provide a simple and effective method.

The work of D.P.D. was supported by the Royal Commission for the Exhibition of 1851. The work of I.I. was supported by Public Health Service grant GM25794.

The authors thank Professor J. M. Mitchison for discussions, and I.I. thanks him for his hospitality during a sabbatical leave in Edinburgh. They also thank other members of the laboratory for conversations. They thank Dr W. D. Cowan of Novo Enzyme Products Ltd for samples of Novozym 234.

REFERENCES

- BEACH, D. & NURSE, P. (1981). High-frequency transformation of the fission yeast *Schizosaccharomyces pombe*. *Nature, London* **290**, 140–142.
- BUSH, D. A., HORISBERGER, M., HORMAN, L. & WURSCHE, P. (1974). The wall structure of *Schizosaccharomyces pombe*. *Journal of General Microbiology* **81**, 199–206.
- KOPECKA, M. (1975). The isolation of protoplasts of the fission yeast *Schizosaccharomyces* by *Trichoderma viride* and snail enzymes. *Folia microbiologica* **20**, 273–276.
- SCHWENKE, J. & NAGY, M. (1978). Preparation of protoplasts of *Schizosaccharomyces pombe*. *Methods in Cell Biology* **20**, 101–105.
- STEPHEN, E. R. & NASIM, A. (1981). Production of protoplasts in different yeasts by Mutanase. *Canadian Journal of Microbiology* **27**, 550–553.