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USE OF CHITINASE AND CHITOSANASE TO FORM SPHAEROPLASTS FROM *PILOBOLUS* SPORANGIOSPORES

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ABSTRACT. Extracting cell contents, such as DNA and protein, from cells with cell walls creates a special challenge because the wall has to be removed without damaging the contents of the cell itself. One method to remove cell walls is to use enzymes to break down cell wall components to release the cell's sphaeroplast. This study was undertaken to develop a technique that could provide sphaeroplasts from sporangiospores of the zygomycete, *Pilobolus*. Sphaeroplasts from some fungi have been prepared by digesting the germ tube cell wall more successfully than digesting the spore wall itself. However, sphaeroplasts were released more readily from ungerminated *Pilobolus* sporangiospores than from the germ tubes growing from spores. An enzyme suspension containing a combination of chitinase and chitosanase readily produced intact sphaeroplasts from which sufficient DNA could be extracted to do sequencing studies.

Keywords: *Pilobolus*, chitinase, chitosanase, sphaeroplasts, zygomycetes

Molecular techniques are central to understanding many aspects of biology and can be used to identify the various taxa and to study the relationships of a wide range of organisms. Cell contents, whether DNA, RNA or protein can be extracted from cells and purified. Once nucleic acids or proteins have been extracted, many molecular techniques can be employed and the literature is replete with techniques that have been used with specific organisms. However, it is particularly difficult to obtain cellular contents from cells with resistant cell walls.

Mycelia are used frequently as the source of fungal cell components because hyphal cell walls are often weaker than spore cell walls (Lalithakumari 2000). However, use of hyphal cells requires fungal growth before the cellular contents can be obtained. It has been reported that isolates of *Pilobolus* cannot be cultivated on agar (Gronvold et al. 2004). While that is often the case, some isolates can be maintained in the laboratory, but the majority of isolates of *Pilobolus* cannot be cultured on laboratory media and are lost before they can be examined. When isolates that cannot be cultured are lost, we can never know whether they were isolates of taxa studied previously, or whether they are new taxa that have never been collected. If cellular components could be obtained from spores of individual sporangia, it would not be necessary to culture the fungus. For this reason, the focus of this work was to develop a technique to use spores collected from the sporangia of *Pilobolus* growing on natural media as the source of cellular material.

Sporangiospores of *Pilobolus* are contained within sporangia, making the sporangium the repository of a sample of cells that can be a source of DNA or protein. However, these cells are covered with rigid cell walls that must be broken to obtain cellular contents. Use of enzymes to degrade the ungerminated sporangiospore cell walls, or of the cell walls of germ tubes from germinating spores, appeared to be one of the methods of choice.

The literature revealed a range of protocols using enzymes to degrade fungal cell walls (Binding & Weber 1974, 1978; Bon et al. 2000; Cheng & Belanger 2000; Conway et al. 2001; Petit & Arveiler 1994). However, most techniques describe sphaeroplast formation in basidiomycetes or ascomycetes (Lalithakumari 2000).Little work has been done with zygomycetes and an extensive search of the literature revealed no protocols for removing *Pilobolus* sporangiospore cell walls. We fo-

cused on the work of Binding & Weber (1974, 1978) since their work was with *Phycomyces*, a zygomycete, and tried several variations with *Pilobolus*.

While the chemical nature of *Pilobolus* sporangiospore cell walls has not been reported, it is widely assumed that, as with other zygomycetes, they are comprised of some combination of chitin and/or chitosan. Thus, we attempted to use chitinase and chitosanase to degrade sporangiospore cell walls.

METHODS

Isolates and culturing of *Pilobolus*.—Four isolates of *Pilobolus*, three of *P. kleinii* and one of *P. crystallinus*, were used in this study. *Pilobolus crystallinus* and two isolates of *P. kleinii* were obtained from herbivores in Yellowstone National Park; the third isolate of *P. kleinii* was from cattle from southern New Mexico. These isolates were maintained at ambient temperature of 22 °C ± 2 °C, with alternating 12 h light and dark periods of 2000 lux cool white fluorescent illumination. Isolates were cultured on a synthetic hemin medium (SHM) (Levetin & Caroselli 1976) in disposable plastic Petri dishes sealed with Parafilm®. Upon discharge, the sporangia of *Pilobolus* adhered to the lids of the Petri dishes and were collected for enzyme experiments using a sterile inoculating loop.

Enzyme studies.—A solution of 1% chitosanase was made by adding 16.3 ml of 0.1 M phosphate buffer at pH 5 to the tube containing 0.163 mg of lyophilized chitosanase (Sigma C-9830). A solution of 2% chitinase was made by adding 0.3 mg powdered chitinase (Sigma C-7809) to 15 ml of 0.1 M phosphate buffer at pH 6.

Solutions containing both enzymes were prepared by adding 25 µl of 1% chitosanase solution and 25 µl of 2% chitinase solution to 50 µl of 3.0 M sorbitol containing 1% Tween 20®. This resulted in a working solution of 0.25% chitosanase and 0.5% chitinase in 1.5 M sorbitol with 0.5% Tween 20®. The mixture of both enzymes together (pH 5.5, 30 °C) was used to degrade the spore walls. All treatment solutions contained 1.5 M to 3.2 M sorbitol which reduced cytolysis of sphaeroplasts, and Tween 20® to reduce sporangiospore clumping.

Multiple enzyme treatments were attempted both with germinated and ungerminated sporangiospores. Chitinase and chitosanase were used individually at their optimum pH values and temperatures. Then, both enzymes were used sequentially. Chitinase (pH 6, 24 °C) was used before chitosanase (pH 5, 37 °C), and chitosanase was used before chitinase to degrade spore walls.

Germinated sporangiospores were produced by introducing 8–10 sporangia from a Petri dish lid into a solution of 50% SHM and 50% water and incubating the spores at 37 °C for 24 h and then at room temperature for 24 h.

Ungerminated sporangiospores were used to produce sphaeroplasts by introducing 8–10 sporangia, collected from a Petri dish lid, to a 1.5 ml micro-centrifuge tube containing 100 µl of one of the enzyme solution regimens listed above. This was done for all four isolates. The tubes were vortexed for 10 sec and incubated at 30 °C for 4 h. Following incubation, 15 µl samples of spore suspension were removed and examined for sphaeroplast formation using brightfield, phase contrast, fluorescent, or Huffman modulated interference contrast microscopy. Fluorescent staining, using calcofluor white (Harrington & Hageage 1991) made empty cell walls of the

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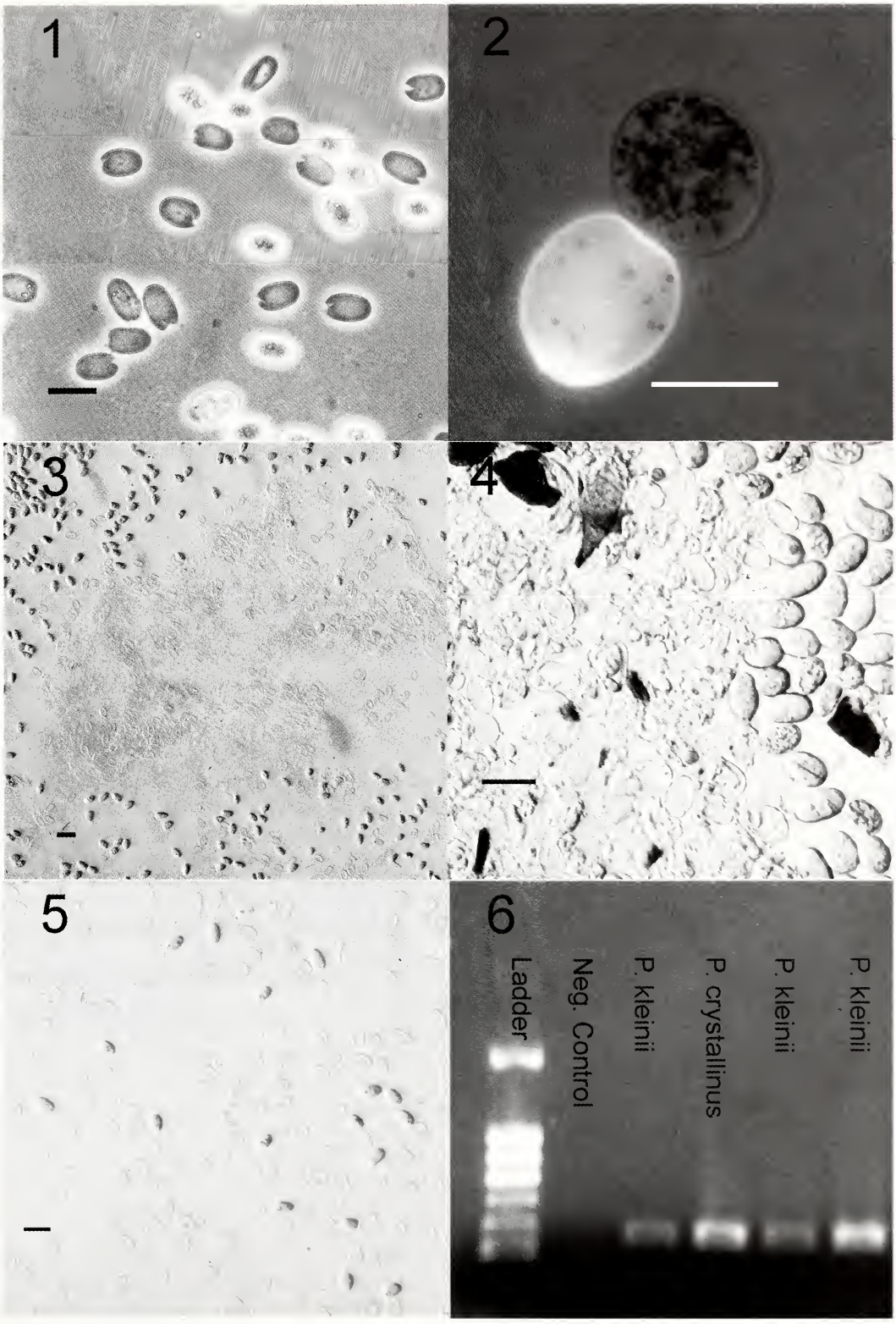
Figures 1–6.—1. *Pilobolus crystallinus*, 200×. Phase contrast microscopy shows empty sporangiospore cell walls and intact sporangiospores. Note the fissures near the ends of the spores when sphaeroplasts were released. Scale = 10 µm; 2. *Pilobolus kleinii*, 500×. Fluorescent microscopy shows a single sphaeroplast emerging from sporangiospore stained with 1% calcofluor white. Scale = 10 µm; 3. *Pilobolus kleinii*, 50×. Huffman modulated interference contrast microscopy shows degraded spore walls surrounded by intact spores. Scale = 10 µm; 4. *Pilobolus kleinii*, 200×. Huffman modulated interference contrast microscopy shows boundary between sporangiospores cell walls (left) and intact sporangiospores (right). Scale = 10 µm; 5. *Pilobolus kleinii*, 100×. Huffman modulated interference contrast microscopy shows a sample of spores after enzyme treatment. Over 70% of the spores in this field of view were lysed. Scale = 10 µm; 6. Agarose gel electrophoresis of amplified DNA from *Pilobolus* sporangiospores shows bands of approximately 520 bp fragments of DNA.


1
2
3
4
5
6
Ladder
Neg. Control
P. kleinii
P. crystallinus
P. kleinii
P. kleinii

spores show up particularly well. Images were captured on 35 mm Kodachrome® 64 film and digitized using a Nikon Coolscan® V-ED 35mm Film Scanner. Adobe Photoshop Elements® was used to crop, insert scale, remove color and sharpen images.

DNA isolation and study.—A suspension of sporangiospores that had been lysed using the combined enzyme solution was washed twice with pH 5.5 phosphate buffer, centrifuged for 5 min at 3000 rpm, and added to tubes of a MoBio UltraClean® Soil DNA kit. The initial steps in the protocol requiring mechanical cell disruption were omitted, and the DNA purification steps of the protocol were followed. DNA was stored in water. Samples of DNA were amplified using AmpliTaq® Gold DNA polymerase (Applied Biosystems, Foster City, California) and a dNTP mix (Promega Corporation, Fitchburg, Wisconsin). Thermal cycling was conducted in an Elmer Perkin GeneAmp® PCR System 2400. Primer sets NS1 and NS2 used in these experiments were from White et al. (1990). Reaction conditions for thermal cycling were 95 °C for 5 min, followed by 36 cycles of 95 °C for 1 min, 55 °C for 1 min 30 sec, 72 °C for 1 min 30 sec, followed by an extension at 72 °C for 10 min. PCR products were subsequently purified using the QIAquick® PCR Purification Kit (Qiagen, Inc., Valencia, California).

PCR-amplified DNA fragments were electrophoresed, prior to and following the clean-up process, on 1% agarose gels in 1× TBE buffer (50 mM Tris-HCl, 50 mM boric acid, and 1 mM EDTA) containing ethidium bromide, and visualized using a ChemiImager® 4400 Imaging System (Alpha Innotech, San Leandro, California). A 100 bp DNA ladder (Takara Mirus Bio, Madison, Wisconsin) was used as a size marker.

The PCR products were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and a sequence reaction mix comprised of 2 μl H_2O, 3 μl 5× buffer, 1 μl BigDye, 2 μl 10 mM primer, and 2 μl DNA. Thermal cycling conditions for sequencing were 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. Sequences were analyzed using an Applied Biosystems 3700 automated fluorescence system.

RESULTS

Initial studies compared the use of germ tubes of germinating spores, and ungerminated spores, as sources of sphaeroplasts. The germinated spores were exposed to several different enzyme regimens. They were introduced into (1) chitosanase alone, (2) chitinase alone, (3) chitinase followed by chitosanase, (4) chitosanase followed by chitinase, and (5) a mixture of chitinase and chitosanase. Length of treatment varied from 2–34 h.

All five enzyme treatments of germinated sporangiospores resulted in production of sphaeroplasts. However, microscopic examination of spores treated by all of these techniques showed that most spores had not ruptured. However, the mixture of chitinase and chitosanase worked as well as, but generally better, than the other treatments.

The ungerminated spores were exposed to the same enzyme regimens. Treatments with a single enzyme at the enzyme optimum temperatures and pH caused cell lysis with the release of many sphaeroplasts; however, large numbers of intact spores remained. Likewise, treatments using the two enzymes, sequentially, at their optimum temperatures and pH values, in either order, produced some sphaeroplasts, but left many intact spores. However, treatments using a mixture of chitinase and chitosanase together at 30 °C and pH 5.5 produced a higher proportion of sphaeroplasts than any other regimen of enzymes.

Microscopic techniques were used to determine whether or not sporangiospore cell walls were being degraded with the release of sphaeroplasts as a result of enzyme treatments. Brightfield microscopic views provided very little contrast between sporangiospores that were intact and those that had been degraded by the enzyme regimens. Phase contrast images of sporangiospore cell walls devoid of contents can readily be distinguished from intact sporangiospores. Many spore walls exhibit fissures near the ends of the spores (Fig. 1). Calcofluor white staining caused spore cell walls to fluoresce. Figure 2 shows a single sphaeroplast emerging from its fluorescing spore wall stained with calcofluor white. The sphaeroplast is readily visible, but much darker that the fluorescing spore wall.

Distribution of sporangiospores within the enzyme solution can be seen by preparing wet

mount slides of this material. Figure 3 shows such a wet mount demonstrating enzyme action on the cells near the center of the field. Spores near the periphery of the field of view remain intact.

Because *Pilobolus* sporangiospores are embedded in a common matrix, they tend to clump or stick together. Within a cluster of spores, the spore walls are degraded when acted upon by enzymes. These spores are the ones near the periphery of the clump. Where the enzymes do not penetrate the clumps, spores remain intact. Figure 4 shows an area in which enzymes acted upon many spores, whereas other spores remain intact. There is a distinct border between spores with enzyme degraded walls (left) and those remaining intact (right).

As clumps of spores are broken apart, those with intact cell walls can be distinguished from the empty cell walls remaining from the disrupted spores. Huffman modulated interference contrast microscopy shows the outlines of empty sporangiospore cell walls as distinct from intact spores (Fig. 5).

After determining microscopically that sphaeroplasts were being formed, it was necessary to determine whether or not DNA from the sphaeroplasts could be used for molecular studies. Gel electrophoresis of DNA prior to amplification showed no bands, however after PCR amplification DNA from these sphaeroplasts showed strong bands of 520 bp fragments of DNA from each of the isolates (Fig. 6). When these DNA fragments were sequenced, analyzed, and compared with other sequences, they were consistent with similar fungi from GenBank. The fragments were deposited in GenBank with accession numbers DQ363379–DQ363382.

DISCUSSION

All of the enzyme treatments produced some sphaeroplasts from sporangiospores that had been disrupted. With each treatment some empty cell walls were observed, other spores remained intact. Nearly all of the sporangiospores were broken in some samples, whereas others contained fewer disrupted spores. Treatments utilizing multiple enzyme treatments, with different conditions of temperatures and pH, were more labor intensive and less productive than a combined treatment. Although we did not count the number of intact and disrupted spores, it was clear that a combination of chitinase and chitosanase worked very well to release sphaeroplasts from *Pilobolus* sporangiospores. Relatively small amounts of DNA were released from these spores; however, there was sufficient DNA after PCR amplification to sequence the fragments for comparative study.

It appeared that the disruption of particular cells by enzyme activity was related to the distribution of the sporangiospores within the enzyme suspension. *Pilobolus* sporangiospores adhere to each other in a common matrix. Within the sporangium, an adhesive matrix causes the several thousand sporangiospores to form a multicellular mass. This matrix causes the sporangium to stick to herbaceous material when discharged, but makes it difficult to treat the individual spores with enzymes. Tween 20® was used to reduce adhesion within the matrix; yet many spores adhered as a mass. The regimen of enzymes appeared to disrupt spore cell walls upon contact with the surface of the spore wall. Thus, regardless of the effectiveness of the method for degrading spore cell walls, many of the spores remained intact. This inter-spore adhesion is an impediment to sphaeroplast production from *Pilobolus* sporangiospores.

Since this technique was used with multiple isolates of *Pilobolus* of two species, it should work with all species of this particular genus. It is a relatively quick, easy, straightforward technique to disrupt sporangial cell walls with the release of DNA. A major asset of this technique is that it works with relatively small numbers of cells. There are approximately 50,000 spores within a single sporangium producing sufficient DNA for sequencing. By using sporangiospores from a single sporangium to provide DNA, isolates of these fungi, which are often difficult or impossible to maintain in the laboratory, can be studied.

Of the various enzyme regimens used, we found a combination of chitinase and chitosanase worked best to remove the cell walls from *Pilobolus* sporangiospores. Because of the high proportion of lysed spores, we conclude that the mode of action is appropriate for the composition of the sporangiospore cell wall and speculate that the sporangiospore wall is comprised of, at least in large part, chitin and chitosan.

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