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Elastic Properties of the Cell Wall of *Aspergillus nidulans* Studied with Atomic Force Microscopy

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Currently, little is known about the mechanical properties of filamentous fungal hyphae. To study this topic, atomic force microscopy (AFM) was used to measure cell wall mechanical properties of the model fungus *Aspergillus nidulans*. Wild type and a mutant strain (Δ*csmA*), lacking one of the chitin synthase genes, were grown in shake flasks. Hyphae were immobilized on polylysine-coated coverslips and AFM force−displacement curves were collected. When grown in complete medium, wild-type hyphae had a cell wall spring constant of 0.29 ± 0.02 N/m. When wild-type and mutant hyphae were grown in the same medium with added KCl (0.6 M), hyphae were significantly less rigid with spring constants of 0.17 ± 0.01 and 0.18 ± 0.02 N/m, respectively. Electron microscopy was used to measure the cell wall thickness and hyphal radius. By use of finite element analysis (FEMLAB v 3.0, Burlington, MA) to simulate AFM indentation, the elastic modulus of wild-type hyphae grown in complete medium was determined to be 110 ± 10 MPa. This decreased to 64 ± 4 MPa for hyphae grown in 0.6 M KCl, implying growth medium osmotic conditions have significant effects on cell wall elasticity. Mutant hyphae grown in KCl-supplemented medium were found to have an elastic modulus of 67 ± 6 MPa. These values are comparable with other microbial systems (e.g., yeast and bacteria). It was also found that under these growth conditions axial variation in elastic modulus along fungal hyphae was small. To determine the relationship between composition and mechanical properties, cell wall composition was measured by anion-exchange liquid chromatography and pulsed electrochemical detection. Results show similar composition between wild-type and mutant strains. Together, these data imply differences in mechanical properties may be dependent on varying molecular structure of hyphal cell walls as opposed to wall composition.

Introduction

Filamentous fungi are used to produce an exceptionally wide range of products (*6*, *18*, *26*), comprising approximately half of the world's pharmaceutical and biotechnology market (*4*). Most of these products are produced in fermentations where fungal mycelia are susceptible to shear forces from agitation (*15*) and productivity is strongly influenced by hyphal breakage or fragmentation (*17*). The mechanistic details involved in fungal fragmentation are still not completely clear (*15*), and one reason for this is the shortage of measurements of the mechanical properties (such as tensile strength and elastic modulus) of fungal hyphae.

The mechanical strength of fungal hyphae is largely attributed to the fungal cell wall, which contains primarily four structural components: α-glucan, β-glucan, chitin, and manoprotein (*13*). As the wall matures, β-glucan and chitin chains form covalent cross-links, making the wall both elastic and strong (*27*). The study of chitin synthesis in *Aspergillus nidulans* has found five chitin synthase genes (*chsA*, *chsB*, *chsC*, *chsD*, and *csmA*) encoding functionally different chitin synthases (*7*, *14*, *23*, *24*, *31*). Chitin synthesized by *csmA*-encoded enzyme appears to contribute to hyphal wall rigidity, as *csmA* deletion strains lyse when grown in low osmotic conditions, a defect that is remedied in the presence of osmotic stabilizers such as KCl (*31*).

While the chemistry of fungal cell walls is relatively well understood, few studies have reported on their mechanical properties. Recently, cell wall mechanical properties of yeast *Saccharomyces cerevisiae* were determined by compressing individual, spherical cells between two parallel surfaces and extracting elastic parameters from the force−deformation data (*29*). Cell wall elastic properties of the filamentous bacterium *Saccharopolyspora erythraea* were determined by pulling apart individual hyphae fixed between probes of a micromanipulator, allowing tensile strength and strain to be measured (*32*). In both studies sophisticated, noncommercial instrumentation was required to perform relatively difficult experiments. In contrast, atomic force microscopes are com-

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mercially available, relatively easy to operate, and have been used to measure elastic properties of a number of different types of biological materials (*1*, *2*, *36*, *37*). By monitoring the deflection of an AFM cantilever (typically 100−400 μm in length) as its tip (10−50 nm radius of curvature) deforms a sample, "force curves" (i.e., a plot of the loading/unloading force versus the sample position as it moves vertically toward and then away from the tip) are collected. Data on the relationship between the applied force and the resulting sample deformation is then fitted to mathematical models describing the mechanics of contact, to extract the elastic properties of the sample.

The elasticity of an object can be described in terms of stress and strain. Stress is defined as the force applied per unit area, while strain is the resulting amount of deformation per unit length. The ratio of stress to strain (for an elastic material following Hooke's law, i.e., $F = k \times \delta$, where k is the spring constant of the material and the deformation, δ, is proportional to the applied force, F) is defined as the elastic modulus (E) and describes the mechanical resistance of a material during elongation or compression. A large E implies a stiff or strong material, while a small E implies a softer material.

In this paper we describe an AFM approach to measure cell wall elastic properties of the model fungus *Aspergillus nidulans*. Wild type and a mutant strain lacking a particular chitin synthase gene (*csmA*) were grown in shake flasks, and hyphae were immobilized on polylysine-coated cover slips. Force−displacement curves were collected from these hyphae in an aqueous environment during AFM indentation experiments and used to determine the cell wall spring constant, k_w (= dF/dδ), which is a measure of the rigidity of the cell wall. To process the data from these experiments, we used a finite element model, which allowed us to calculate the elastic modulus of the fungal cell wall. We report here the technique used as well as some unexpected results.

Materials and Methods

Strain and Culture Conditions. Wild-type *A. nidulans* (FGSC A4, Glasgow veA+) was grown on potato dextrose agar (Difco, Detroit, MI) at 31 °C, 8−10 days for sporulation and was stored as frozen stock culture (*5*). The Δ*csmA* mutant (*yA2 pabaA1* Δ*chsD*::*argB* Δ*argB*::*trpC*Δ*B trpC801 veA1*) was obtained from Dr. Peter T. Borgia (Department of Medical Microbiology & Immunology, School of Medicine, Southern Illinois University, Springfield, IL). Complete medium used for growth contained, per liter, yeast extract 5 g (Sigma, St. Louis, MO), glucose 6 g, $NaNO_3$ 6 g, KCl 0.52 g, KH_2PO_4 0.82 g, K_2HPO_4 1.05 g, and $MgSO_4 \cdot 7H_2O$ 0.52 g. To permit growth of the mutant, medium was enhanced with 0.6 M KCl (*31*). Trace element solution was added to the complete medium (∼1000 ppm) and contained, per liter, $FeSO_4 \cdot 7H_2O$ 5 g, $ZnSO_4 \cdot 7H_2O$ 22 g, H_3BO_3 11 g, $MnCl_2 \cdot 4H_2O$ 5 g, $CoCl_2 \cdot 6H_2O$ 1.6 g, $CuSO_4 \cdot 5H_2O$ 1.6 g, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 1.1 g, and EDTA 50 g. Medium osmolarity was directly measured with a vapor pressure osmometer (model 5500; Wescor, Logan, UT). Frozen spore suspension (1 mL, 6×10^6 spores/mL) was thawed at room temperature and inoculated into a 250 mL baffled Erlenmeyer flask containing 25 mL of complete medium, pH 3.3. The culture was incubated for 12 h at 32 °C on an oscillating shaker at 260 rpm and then used as inoculum for a 2.8 L baffled flask containing 1 L of complete medium, pH 6.5. Cells were further grown for 12 h at 32 °C and 260 rpm. Finally, harvested hyphae were washed in cold phosphate-buffered saline (PBS, pH 7.4) three times and centrifuged at 16000*g*, 4 °C for 5 min, and the pellet was resuspended in fresh buffer.

Dry Cell Weight and Glucose Determinations. Dry cell weights were determined by filtering 10 mL of broth through a 42 mm glass fiber filter (Fisher Scientific, Pittsburgh, PA). The cells were washed three times with 30 mL of deionized water and dried at 90 °C for 24 h before being weighed. Glucose concentration in the filtered broth was directly measured with a biochemistry analyzer (YSI 2700; YSI Inc., Yellow Springs, OH).

Electron Microscopy. The cell wall thickness and the hyphal diameter were estimated by transmission electron microscopy (TEM; Zeiss 10 CA, operating at 60 kV) following the standard glutaraldehyde−osmium tetroxide protocol (*8*).

Atomic Force Microscopy. Hyphae from submerged cultures were washed in cold PBS buffer (0.2% NaN_3 to arrest growth) three times before resuspension in fresh buffer. Biomass concentration was diluted to approximately 0.2 g·L^{-1} to prevent cell overlap. Poly-L-lysine (0.1% solution, 40 μL; Sigma, St. Louis, MO) was pipetted onto a round (12 mm diameter) glass coverslip (Fisher Scientific, Pittsburgh, PA) and removed after 30 min. The coverslip was immediately rinsed with deionized water four times and dried in ambient air. Hyphal suspension (25 μL, containing 3−5 hyphal elements) was added and dried on the coverslip, allowing hyphal elements to settle and attain a firm electrostatic attachment to the poly-L-lysine on the surface. Salt residue was then removed by gently washing with deionized water. Finally, a drop of fresh buffer was added on top of the sample to rehydrate it, and the coverslip was mounted in the AFM.

Experiments were performed at 22 °C, on a multimode atomic force microscope (Nanoscope IIIa; Digital Instruments, Santa Barbara, CA) equipped with a J-type piezoscanner. AFM indentations were carried out with silicon nitride cantilevers (85 × 18 μm; Park Scientific Instruments, Sunnyvale, CA) held in a fluid cell. The spring constants of the cantilevers were determined to be 0.47 ± 0.01 N/m (n = 6) by measuring resonance frequencies of the cantilevers (*10*). PBS buffer was injected through the ports on the fluid cell during experiments to account for evaporation loss. Hyphae were scanned in contact mode to locate positions for the indentation experiments in which force curves were collected at 0.5 μm/s z scan rate, 250 nm z scan size, and approximately 40 nm cantilever deflection. The deflection of the cantilever was calibrated by taking force curves on the coverslip.

Elastic Modulus Determination. As has been used by others (*28*−*30*), we used numerical simulation via finite element analysis to determine cell wall elastic modulus as no suitable analytical models are available. By use of finite element analysis software (FEMLAB v 3.0, Burlington, MA), a mechanical model was formulated to describe the deformation of a circular cylindrical shell with finite length, under an external force normal to the shell surface (Figure 1). This external force can be idealized as a point force as the radius of the cantilever tip is approximately 2−3 orders of magnitude smaller than the hyphal diameter. Since the stress and deformation distribution were symmetrical with respect to the plane $x = 0$, only the half cylinder for $x \geq 0$ was considered with a concentrated load F/2 at the top apex. To model the symmetry, the edge of the cylinder at the plane $x = 0$ had constraints for the displacement along the x-axis and for the rotation along the y- and z-axes. In reality, the cylinder was supported by an underlying, hard substrate. Their contact was simulated with a so-

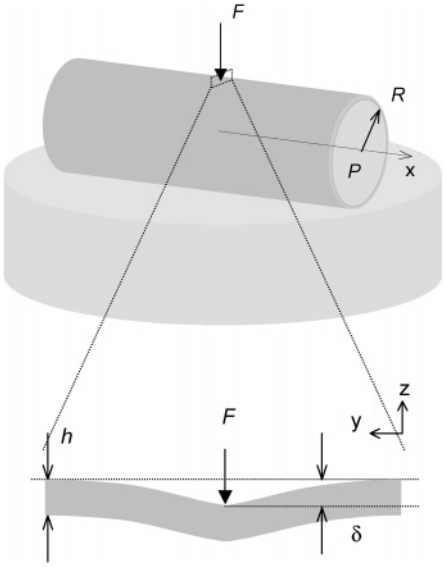

Figure 1. Schematic illustration of AFM indentation experiment. The hypha is immobilized on a polylysine-coated cover glass and submerged in aqueous buffer. The AFM cantilever tip exerts a normal force F and deforms the hyphal wall at a distance δ. The hypha is idealized as a cylindrical shell with outer radius R and wall thickness h, inflated with internal pressure P.

called penalty/barrier method, where additional stiffness acting as nonlinear springs connects the foundation and the cylinder boundary (*9*). Mesh density around the concentrated load was increased due to the singularity, where the stress and strain gradients are expected to be highest. Internal pressure was added to the cylinder boundary to simulate the cellular turgor pressure. The model was then solved by a stationary nonlinear method, and the solutions were checked for convergence by adjusting mesh density.

At the end of each simulation, δ was obtained with predefined F, E, R, and h (Figure 1). A connection between these parameters was established by fitting (least-squares) individual simulation results and was used to calculate cell wall elastic modulus of *A. nidulans*, with known F and δ determined from the force curve and R and h from TEM, respectively.

Cell Wall Composition Analysis. Cell suspension was filtrated through a milk filter (KenAG, Ashland, OH), and the cell paste was washed with cold (4 °C) deionized water three times and stored at −20 °C. As described previously (*11*), the cells were disrupted with glass beads and liberated cell walls were hydrolyzed with sulfuric acid. The separation and detection of the liberated monosaccharides was carried out on a DX-500 anion-exchange liquid chromatography system (Dionex, Sunnyvale, CA) equipped with an ED40 electrochemical detector, a CarboPac PA10 (4 mm × 250 mm) analytical column, and a CarboPac PA1 guard column. The hydrolysates were diluted with water purified by a reverse osmosis system and injected with an AS50 autosampler (Dionex) onto an injection valve (model 9126, Rheodyne, Inc., Cotati, CA) fitted with a 100-μL injection loop. Samples were eluted isocratically at 30 °C at a flow rate of 1 mL/min with 18 mM NaOH, which was prepared from a dilution of commercial 50% NaOH solution with high-purity water, followed by a wash step of 300 mM sodium acetate. All solvents were degassed and kept under pressure (N_2, ca. 10 psi). Data were collected and processed with PeakNet software (Dionex, v 5.21).

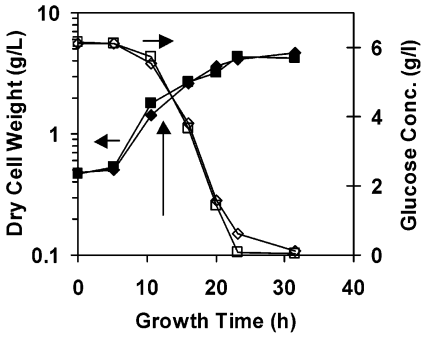

Figure 2. Dry cell weights and glucose concentrations of *A. nidulans* wild type (■ and □) and Δ*csmA* mutant (◆ and ◇) grown in CM + KCl medium in shake flasks. Cells were harvested at 12 h for sample preparation as indicated by the vertical arrow.

Monosaccharides were quantified by use of the response factors determined from peak areas of standard solutions.

Statistics Information. Cell wall physical properties and composition were determined in multiple tests. Mean and 95% confidence interval (CI) were calculated. Analysis of variance (ANOVA, single factor) was performed between means from different strains and/or growth conditions and the result was expressed with a P value. These means were considered to be significantly different if P was smaller than 0.01. Otherwise they were considered to be not significantly different from one another.

Results

Cellular Growth. Figure 2 shows biomass, measured as dry cell weight, and glucose concentration versus time for the two strains used in this study. Both the wild type and mutant exhibit similar growth profiles. Note that the wild-type (WT) hyphae were grown both with and without KCl but that Δ*csmA* hyphae could only be grown with KCl (*31*). Hyphae for subsequent AFM and TEM measurements were harvested at 12 h.

Hyphal Wall Thickness and Diameter. Cell wall thickness (h) and hyphal diameter ($2R$) were determined from electron micrographs, and results are shown in Figure 3. We observed no significant differences in wall thickness between WT and WT/KCl (ANOVA, $P = 0.09$) and between WT/KCl and Δ*csmA*/KCl ($P = 0.21$). Overall mean thickness was 100 ± 10 nm (95% CI). Similarly, we observed no significant differences in hyphal diameter between the different conditions/strains ($P = 0.36$), and overall mean diameter was 2.6 ± 0.2 μm (95% CI). Alternatively, hyphal diameter was also estimated from the topographical measurements with AFM. The measurements were in the range of 2.5−3.0 μm, showing no significant difference between strains, and were nearly identical to those determined by TEM. This implies there was little to no shrinkage of hyphal shape due to dehydration in TEM sample preparation, and thus TEM measurements for wall thickness and hyphal diameter are reasonable substitutions for those at physiological conditions.

Cell Wall Spring Constant. An essential requirement for the AFM experiments described here is that hyphal elements must be immobilized on a substrate for imaging and indentation experiments. Typically, hyphae consisted of many branches with different orientations that moved around above the substrate, making AFM tests impossible. This morphology also disallowed hyphae from being held in filter pores as has been done for bacteria (*38*) and fungal spores (*12*). We solved this difficulty by allowing hyphae to first dry in air on the

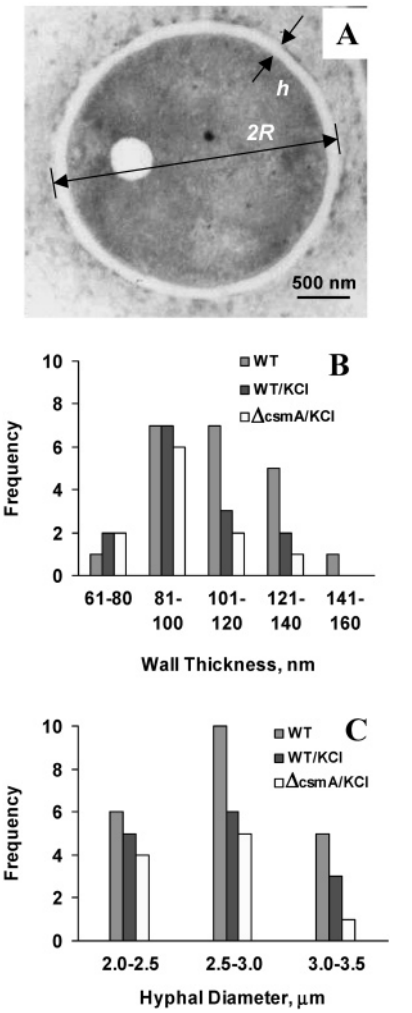

Figure 3. Physical parameters measured via electron microscopy: (A) typical cross section of wild-type *A. nidulans*; (B) histogram of measured cell wall thickness; (C) histogram of measured hyphal diameter. See Table 1 for legend definitions.

polylysine-coated coverslip so that all hyphal branches were firmly attached to the substrate. We then immediately rehydrated the hyphae by adding fresh buffer. The imaging and indentation experiments in the aqueous milieu were performed without difficulty.

Force curves collected in the AFM indentation tests record the relationship between the deflection of the cantilever and sample position. Figure 4 shows a representative force curve. A linear response of the cell wall is observed, and the slope m is less than 1, indicating the softness of the cell wall as the AFM tip indents it. The AFM can measure two quantities from force curves: the applied force, F, and the indentation, δ. The spring constant of the cell wall, k_w (= dF/dδ), which characterizes rigidity, can be calculated according to (*2*)

$$k_w = \frac{k_c m}{1 - m} \tag{1}$$

where m is the slope of the force curve and k_c is the spring constant of the cantilever. The results are shown in Table 1.

Validation of Finite Element Modeling. To validate our finite element model, we compare our simulation results with theoretical results from two cases where analytical models are available: shell inflation (*35*) and indention (*22*). If the edges of the shell are free from

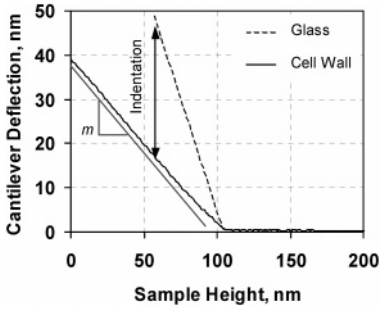

Figure 4. Typical AFM force curve taken on an *A. nidulans* hypha. The cantilever deflection is zero in the noncontact region. In the sloped contact region the cantilever tip indents the hyphal wall. The curve is linear with slope m in the contact region. The spring constant of the cantilever is 0.47 N/m.

Table 1. Summary of Growth Medium Osmolarity, Cell Wall Spring Constant (k_w), and Elastic Modulus (E) of *A. nidulans*[a]

strain	medium osmolarity (Osm/L)	k_w (N/m)	E (MPa)
WT[b]	0.25	0.29 ± 0.02 (71)	110 ± 10 (71)
WT/KCl[c]	1.4	0.17 ± 0.01 (58)	64 ± 4 (58)
Δ*csmA*/KCl[d]	1.4	0.18 ± 0.02 (63)	67 ± 6 (63)

[a] Values are expressed as mean ± 95% confidence interval. The number of measurements is given in parentheses. [b] Wild type grown in complete medium (CM). [c] Wild type grown in CM containing 0.6 M KCl. [d] Mutant grown in CM containing 0.6 M KCl.

constraints, internal pressure, p, will produce only a hoop stress and the radius of the cylinder will increase by the amount (*35*)

$$\delta = \frac{pR^2}{Eh} \tag{2}$$

where δ is the radial displacement and E is elastic modulus. Figure 5B shows results generated with this analytical solution match those generated with our finite element model well.

In a second example the cylinder is subjected to equal and opposite radial loads, as shown in Figure 5C, and the equation for the radial indentation has been expressed as follows (*22*):

$$\delta(x, \theta) = \frac{F}{Eh} f\left(\frac{R}{h}, x, \theta\right) \tag{3}$$

where f is a complicated function of R/h and coordinate parameters x and θ. The maximum indentation appears at the point of the applied force and its analytical solution is determined to be (*16*)

$$\delta(0,0) = 0.74 \frac{F}{Eh}\left(\frac{R}{h}\right)^{1.5} \tag{4}$$

The radial indentation in the plane $\theta = 0$ is presented in Figure 5D, showing a close agreement between the numerical solution and the analytical solution. The close agreement for both the inflation and the indentation models provides a validation for the finite element approach and shows that it accurately represents the physical situations.

Elastic Modulus of the Cell Wall. Using our validated finite element model, we performed simulations on the mechanical model depicted in Figure 1. As has been routinely assumed by others (*1*, *28*, *36*), we assumed the

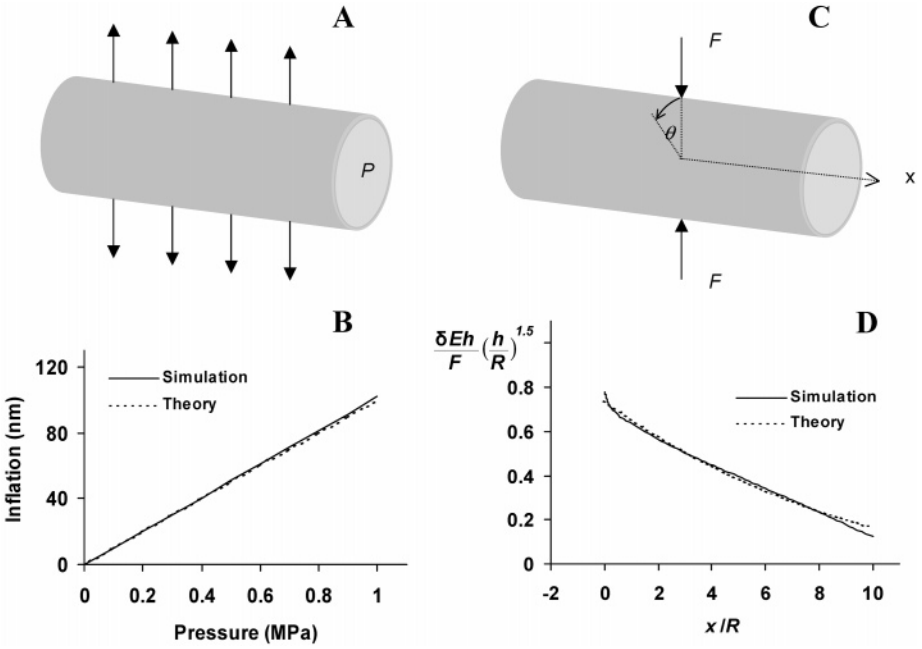

Figure 5. Validation of the numerical simulation of the finite element analysis for a cylindrical shell (A, B) inflated with an internal pressure and (C, D) indented by two equal and opposite radial point loads.

cell wall to be an incompressible material (Poisson ratio = 0.5) as it was tested in a hydrated condition during the indentation. At the end of each simulation the maximum indentation $\delta(0,0)$ (simplified as δ below) was obtained with predefined F, E, R, and h. We found F and δ showed a linear relationship with each other while k_w ($= F/\delta$) was dependent on the material properties and dimensions of the cell wall (e.g., E, R, and h). However, k_w was found to be independent of cellular turgor (internal) pressure. This finding is consistent with our experimental observations that force curve slopes do not change (i.e., constant k_w, according to eq 1) with varying concentrations of KCl (0.01−1 M) during the indentation tests (data not shown). This indicates that indentation tests do not probe the effect of turgor pressure but instead the mechanical properties of the cell wall.

The dependency of k_w on the other parameters is expressed with a correlation obtained by fitting individual simulation results, as shown in Figure 6. The dimensionless group Eh/k_w (representing the reciprocal of a dimensionless spring constant, $1/\hat{k} = \hat{\delta}/\hat{F}$, where $\hat{\delta} = \delta/h$ and $\hat{F} = F/Eh^2$) was calculated after each simulation and plotted as a function of R/h. The value of R/h is 10−60, a sufficient range to cover most biological systems. The fitting exhibits a power correlation with an index of 1.5, identical with that in eq 4. This finding can be regarded as another validation of our model. By transforming the correlation, cell wall elastic modulus, E, can be calculated according to

$$E = 0.80\frac{k_w}{h}\left(\frac{R}{h}\right)^{1.5} \quad (5)$$

With the known k_w and R/h, determined from AFM and TEM, respectively, eq 5 was used to calculate E. The results are summarized in Table 1. It is noted that wild-type hyphae have a significantly lower elastic modulus (from 110 to 64 MPa) when grown in the presence of 0.6 M KCl, implying high osmotic condition has a strong effect on cell wall elasticity. At the same time it is noted that wild-type and mutant strains have similar elastic moduli in high osmotic condition.

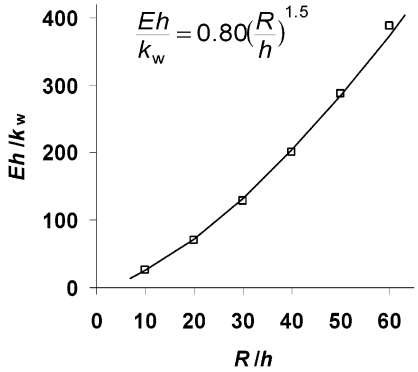

Figure 6. Plot of the dimensionless group Eh/k_w as a function of R/h, the dimension of the cylindrical shell. Data points represent the results from individual numerical simulations.

One of the benefits of AFM over alternative methods for measuring E is the ability of the AFM to measure spatial variations in elastic modulus. This is achieved by generating force curves at different positions on a given sample. To illustrate this, we recorded force curves at equal intervals along individual hyphae beginning at the hyphal tip. Calculated elastic moduli are plotted as a function of position in Figure 7. The calculations assume cell wall thickness and cell radius are constant along hyphae. We found the variation of E along the hyphae is small, indicating that for hyphae grown under these conditions cell wall strength is homogeneous.

Cell Wall Composition. To determine how composition is influenced under high osmotic conditions, cell wall materials were hydrolyzed by sulfuric acid and the composition was measured by anion-exchange liquid chromatography followed by pulsed electrochemical detection. Four analytes from the hydrolysates were determined: glucosamine, galactose, glucose, and mannose. Although showing differences in mechanical properties, wild-type and mutant strains were consistent in cell wall composition (Table 2; ANOVA, $P > 0.58$), and their cell wall chitin contents (i.e., glucosamine) showed no significant difference ($P > 0.87$).

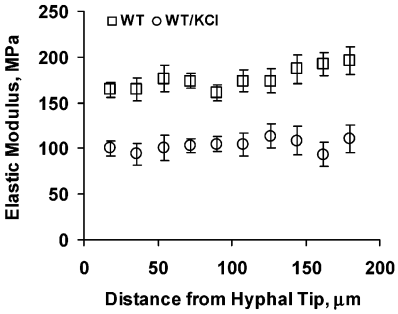

Figure 7. Spatial variation in elastic modulus along the length of a hyphal branch. Measurements for each position were performed on 3−10 hyphae grown in two independent experiments. Error bars represent standard error of the mean.

Table 2. Cell Wall Composition of *A. nidulans* Wild-Type and Δ*csmA* Mutant Hyphae[a]

strain	glucosamine (mg/g DCW)	galactose (mg/g DCW)	glucose (mg/g DCW)	mannose (mg/g DCW)
WT	46 ± 28	18 ± 8	260 ± 80	13 ± 2
WT/KCl	45 ± 6	17 ± 5	300 ± 100	17 ± 7
Δ*csmA*/KCl	48 ± 34	18 ± 11	300 ± 160	15 ± 6

[a] Values are expressed as mean ± 95% confidence interval and are presented in milligrams per gram of dry cell wall. Data were generated from three independent experiments.

Discussion

In filamentous fungal fermentations, hyphae are known to be susceptible to the shear forces from agitation, with hyphal fragmentation depending on both the hydrodynamic stresses and the mechanical properties of hyphal cell walls (*15*). To date, fungal cell wall mechanical properties and how environmental conditions affect these properties remain poorly studied. In this study, the elastic properties of filamentous fungal hyphae have been determined with the atomic force microscope under physiological conditions. We find that the osmotic conditions in growth medium have a strong effect on cell wall elasticity.

The elastic modulus of *A. nidulans* was calculated from eq 5, determined by use of finite element modeling. While direct validation of this model was limited by the fact that no analytical solutions are available, indirect validation was achieved under similar physical situations where analytical models are available. Our validation is corroborated by the fact that eqs 4 and 5 are nearly identical to each other. While it would be a valuable exercise to compare results from eq 5 with experimental results from other fungi or biological systems, this is problematic as none of the available literature studies include the complete experimental data needed for eq 5. The only correlated study, to our knowledge, is the elasticity measurement of the Gram-negative bacterium *Magnetospirillum gryphiswaldense* (*2*, *3*). The elastic modulus of this bacterium was estimated to be approximately 30 MPa (2), the same order of magnitude as that of the cell wall of the bacterium *Bacillus subtilis* determined from tensile tests (*34*). When we use eq 5 to estimate E for this bacterium, we arrive at a value of 70 MPa, which is in good agreement with the estimated value. This reasonable agreement convinces us that eq 5 correlated from the numerical simulation of finite element analysis is applicable for calculating the cell wall elastic modulus of filamentous fungi or other rod-shaped cells such as bacteria.

In contrast to the numerical simulations in this paper, theoretical considerations of an AFM indentation on microbial cell walls have been made for cylindrical bacteria such as *Magnetospirillum gryphiswaldense* (*2*). Arnoldi et al. (*2*) suggested that the free energy describing the state of the bacterial wall when deformed by the AFM tip included the contributions from wall tension and additional stretching by the tip, while wall bending played a negligible role. By minimizing the free energy, a theoretical expression was derived for the applied force as a function of the indentation distance of the bacterial wall. Although the indentation framework is similar between cylindrical bacteria and fungi, there are two uncertainties preventing the application of the bacterial study to fungal indentation. First, using the method described by Arnoldi et al. (*2*), we find that the energy of fungal wall bending is of the same order as the energy of wall tension and thus may not be ignored. Second, the theoretical expression of the spring constant of the bacterial wall contains an unknown parameter for fungal indentations: a cutoff distance that is a measure of the range of the elastic deformation of the wall. Due to these reasons, the finite element analysis was chosen in this study to simulate the fungal indentation that allows the cell wall elastic modulus to be determined.

As few studies are available in the literature on the mechanical properties of fungal cell walls, comparisons are drawn with other biological systems such as yeast and bacterial cell walls. Determined by different experimental techniques, the elastic moduli of the cell walls of *Saccharomyces cerevisiae*, *Saccharopolyspora erythraea*, and *Bacillus subtilis* have been estimated to be 135−165 MPa, 109−167 MPa, and ∼30 MPa, respectively (*29*, *32*, *34*), while our AFM determinations in E for wild-type *A. nidulans* are 100−120 MPa (Table 1), being in the same range. Perhaps the important comparison is between *S. cerevisiae* and *A. nidulans* as the cell walls of both organisms probably possess similar chemistry and structure (*27*). It is seen that their elastic moduli are quite close, indicating the estimate for the elastic modulus of *A. nidulans* is reasonable.

The effect of osmotic stress on cell wall strength during growth has been reported previously for the oomycetes *Achlya bisexualis* and *Saprolegnia ferax* (*19*, *20*). For both species, an increase in growth medium osmotic pressure corresponded to a reduction in the tensile strength of the apical cell wall. Similarly, we found osmotic stress had a strong effect on cell wall elasticity. Table 1 and Figure 7 show that *A. nidulans* wild-type hyphae have lower cell wall elastic properties in response to increasing osmolarity in growth media. The mechanism for controlling wall compliance in osmotic regulation is still not clear. However, as discussed by Money and Hill (*21*), potential mechanisms center on either the hydrolysis of the cross-links between wall polymers or the decreasing order in the orientation of the polymers. In both cases weaker cell walls are generated. Therefore, we suggest that the increasing growth medium osmolarity induces wild-type hyphae of *A. nidulans* to assemble the cell wall with a weaker molecular structure (i.e., smaller tensile strength and elastic modulus). This implies that cells may fragment more easily in response to shear stresses in fermentors. With the support from additional analytical tools, the above suggestion may be helpful in understanding mechanisms of hyphal fragmentation and how they are affected by environmental conditions.

The growth of wild-type and Δ*csmA* strains were successful, except that the mutant would lyse if grown in low osmolarity medium. This has been shown and concluded to be due to the lack of the chitin synthesized by *csmA*-encoded chitin synthase, in the mutant cell wall (*31*), indicating *csmA* product (CsmA) is particularly

important in the maintenance of cell wall integrity under low osmotic conditions. However, the defect in growth of the *csmA* mutant has been remedied with osmotic stabilizers (*31*), and additional results have shown that the yield of CsmA is significantly reduced under high osmotic conditions (*33*), indicating CsmA does not play an important role under high osmotic conditions. As a result, wild-type and mutant hyphae of *A. nidulans* may generate their cell walls with high similarity. Evidence for this speculation is that both strains have approximately same cell wall spring constants and elastic moduli, as shown in Table 1.

Despite the deletion of the *csmA* chitin synthase gene, chitin content in the mutant strain remains at the same level as that in the wild type (Table 2). This finding is consistent with wall composition testing for *A. oryzae* in which the same methodology (i.e., sulfuric acid hydrolysis) was used (*25*). This implies one of two possibilities. Either the chitin synthesized by the *csmA*-encoded chitin synthase accounts for only a small percentage of total amount of chitin, or as mentioned in ref *25*, the lack of *csmA* chitin synthase is compensated for by other chitin synthases. We note that in one report a lower chitin level was reported in the Δ*csmA* strain (~35% reduction); however, chitin contents were measured on the basis of enzymatic degradation of the cell wall (*31*). This apparent inconsistency may result from the possibility that enzymatic wall hydrolysis is not as complete as chemical hydrolysis, resulting in underestimation of chitin content.

Conclusion

In this paper we describe an AFM approach to measure cell wall elastic properties of the model fungus *Aspergillus nidulans*. The results presented in this paper show that elastic modulus of the cell wall responds to osmotic conditions in growth media. We suggest that the increasing growth medium osmotic pressure induces *A. nidulans* hyphae to assemble the cell wall with a weaker molecular structure as cell wall composition does not change with osmotic conditions. This could be an important finding in understanding cell−liquid interactions in fungal fermentations. For a better understanding, further studies need to be carried out on how mechanical properties of the cell wall respond to various environmental factors.

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