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Predicting the Chemical Composition and Structure of *Aspergillus nidulans* Hyphal Wall Surface by Atomic Force Microscopy

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In fungi, cell wall plays an important role in growth and development. Major macromolecular constituents of the aspergilli cell wall are glucan, chitin, and protein. We examined the chemical composition and structure of the *Aspergillus nidulans* hyphal wall surface by an atomic force microscope (AFM). To determine the composition of the cell wall surface, the adhesion forces of commercially available β-glucan, chitin, and various proteins were compared to those of corresponding fractions prepared from the hyphal wall. In both setups, the adhesion forces of β-glucan, chitin, and protein were 25-50, 1000-3000, and 125-300 nN, respectively. Adhesion force analysis demonstrated that the cell surface of the apical tip region might contain primarily chitin and β-glucan and relatively a little protein. This analysis also showed the chemical composition of the hyphal surface of the mid-region would be different from that of the apical region. Morphological images obtained by the tapping mode of AFM revealed that the hyphal tip surface has moderate roughness.

***Keywords*:** *A. nidulans*, atomic force microscopy, hyphal wall, adhesion force

It is well established that the fungal cell wall determines the specific shape of various cells (Kapteyn *et al*., 1997). Cell wall does not only protect intracellular structures from various external damages, but plays an important role for growth and differentiation in fungi (Borgia and Dodge, 1992; Smits *et al*., 2001). Aside from the enzymatic function of cell wall protein, it also represents receptor that recognizes extracellular stimuli (Peberdy, 1994). The fungal cell wall is primarily made up of polysaccharides such as glucose, galactose, and mannose polymers (Zonneveld, 1971, 1972; Borgia and Dodge, 1992; Gow, 1994; Peberdy, 1994; Chung *et al*., 1996; Rolf *et al*., 1999; Wei *et al*., 2001; Magnelli *et al*., 2002; Rast *et al*., 2003; Pawan *et al*., 2004). The frame of the cell wall is formed by chitin, a β-1,4-N-acetyl-D-glucosamine polymer, which gives its structural strength (Borgia and Dodge, 1992). Additional components of the cell wall can be varied by different species and may include large amounts of β-1,3-glucan and chitosan (a β-1,4 linkage polymer of D-glucosamine), traces of D-galactosamine polymer, polyuronides, melanins, and lipids. All of these components have important roles in certain structural and functional aspects of the cell wall (Borgia *et al*., 1996). The hyphal wall of *Aspergillus nidulans* comprises chitin, α-1,3-glucan, β-1,3-glucan, mannose, galactose, protein, and melanin (Katz and Rosenberger, 1971; Polacheck and Rosenberger, 1975, 1977, 1978; Harsanyi *et al*., 1977; Peberdy, 1987; Kelly *et al*., 1996; Specht *et al*., 1996; Torralba *et al*., 1998). Chitin and β-glucan provide covalent bonds that enhance the mechanical stability of the cell wall (Borgia and Dodge, 1992). Because a technique that efficiently isolates the apical tips from fungal hyphae has not been available yet, it is hard to elucidate the fine composition at the apical tip area. Studies investigating cell wall biology in recent years have mainly focused on cell wall chemistry by separating it from the cell *in vitro* and on cell wall ultrastructure using an electron microscope (EM). However, there is a serious drawback in measuring biological samples with EM since the measurement requires sample treatment of coating it with metallic thin film. An atomic force microscope (AFM) has been successfully used in nanoscale surface structure analysis in order to avoid this drawback. By measuring the attractive or repulsive forces between the tip and a sample with a nanometer scale, the surface morphology, composition, and roughness of the sample are able to be acquired (Gad and Arimichiitoh, 1997; Sagvolden *et al*., 1999; Dufrene, 2001, 2003, 2004; Andrade, 2002; Green and Allen, 2002; Marszalek *et al*., 2002; Abu-Lail, 2003; Abu-Lail and Camesano, 2003; Doktycza *et al*., 2003; Touhami *et al*., 2003; Jalili and Laxminarayana, 2004; Kang and Choi, 2004; Santos and Castanho, 2004).

This study utilized AFM to examine the distribution of chemical constituents in the hyphal tip surface of A. nidulans in an effort to elucidate a hyperfine structure. The relative composition of hyphal tip surface was determined. Our data may suggest that the chemical composition of the specific

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surface areas in a microorganism could be estimated with AFM.

Materials and Methods

Strains and cultivation

Conidia (10^6 cells/ml) of *A. nidulans* FGSC 4A were inoculated in complete medium (CM) and incubated at 37°C for 12 h with vigorous shaking to form mycelial balls. The balls were applied to examine the AFM images. CM contained 1.5 g of yeast extract and 1.5 g of casein hydrolysate, 20 ml of minimal salt stock solution per liter (Harsanyi *et al*., 1977).

Extraction of hyphal wall components

Mycelial balls grown for 12 h in CM were harvested on nylon mesh, washed with cold distilled water, suspended in cold 0.5 M phosphate buffer (pH 6.8), and homogenized. The cell wall fraction was isolated according to Borgia and Dodge (1992). Protein was purified from the cell wall fraction as described by Masuda and Kawata (1986), and examined with the Bradford protein assay method. β-Glucan from the cell wall fraction was extracted as previously described by Borgia and Dodge (1992) and assayed using the phenol-sulfuric acid method (Dubois, 1956). Chitin was isolated according to Hackman and Goldberg method (1981).

Sample preparation for AFM

Four or five mycelial balls were prefixed in a 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 4 h on a slide glass and evenly spread. After washing five times with the same buffer, the balls were put on the solidified surface of 4% agarose, and rinsed with deionized water. Reference samples were prepared as follows: aliquots of 0.6 M hydrochloric acid were added to 50 ml of the yeast β-glucan solution (Sigma A5011, 270 μg/ml) or crab chitin solution (Sigma G9752, 100 μg/ml) and vigorously stirred until completely dissolved. A protein reference was made with 150 μg of each, bovine serum albumin (Sigma A9647), and chicken serum albumin (Sigma A5378) dissolved in 1 ml of water. Fifty-microliter of each sample was spread on silicon wafers to measure the adhesion forces between a tip and sample surface.

AFM cantilevers and measurement of adhesion forces

Adhesion forces were measured with a multimode AFM (Digital Instrument, USA) equipped with a Nanoscope controller (V. 4.31), NSC16 cantilever and NSC36 cantilever (MikroMasch, USA), as well as Noncontact FEB carbon Whisker-type cantilever (NT-MDT, Russia). All images were obtained at room temperature and collected in tapping mode under water. A force curve was generated by plotting the interaction between the AFM tip and the sample. This force curve was used to calculate the adhesion force between the AFM tip and the sample when the spring constant (k) of the cantilever was known. Adhesion force was caluclated by $F=k(\Delta z)$. The distance was then multiplied by the z piezo sensitivity to obtain Δz (Green and Allen, 2002; Dufrene, 2003).

Results and Discussion

Observation of the hyphal tip surface using an AFM

To establish the surface structure of the apical tip of hyphae, conidia were cultivated in CM broth for 12 h. A mycelial ball from the culture was placed on a slide glass which had been covered with 4% agarose, and the edge of the ball was examined through an optical microscope and an atomic force microscope (Fig. 1). The surface structure observed by atomic force microscopy rendered more detail in roughness than previous studies conducted with scanning electron microscopy (SEM), indicating that imaging by AFM is more powerful to observe native surface than conventional approaches using SEM (Kowal *et al*., 2000). The horizontal resolution of an SEM approaches atomic levels (0.1-0.5 nm), but the vertical resolution is considerably lower. With AFM, the measurement of surface roughness could be achieved by a vertical resolution of 0.01 nm and a horizontal resolution of 0.1-0.5 nm (Santos and Castanho, 2004). Figure 1A depicts an optical micrograph of the margin of mycelial ball. Figures 1B through 1D show the images of the center of hyphal tips acquired with AFM. The 20×20 μm area representing the hyphal tip is shown in Fig. 1B (arrow) obtained by the tapping mode of AFM. The surface features at hyphal in height image were similar in size and shape to those at hyphal in optical image. Detailed surface structures of the apical tip were also acquired as a height image (Fig. 1C) and a phase image (Fig. 1D) at higher magnification (500×500 nm). Phase images are based on changes in resonance frequency and shown by the differential imaging of different chemical constitution according to the elasticity and viscosity of the sample substance (Nguyen *et al*., 2001). The various intensities in the phase image allow us to discriminate the chemical properties of components which are composed of hyphal surface. As illustrated in Fig. 1D, four or more areas indicated by arrows showed differences in phase of the initial resonance frequency, which may reflect the

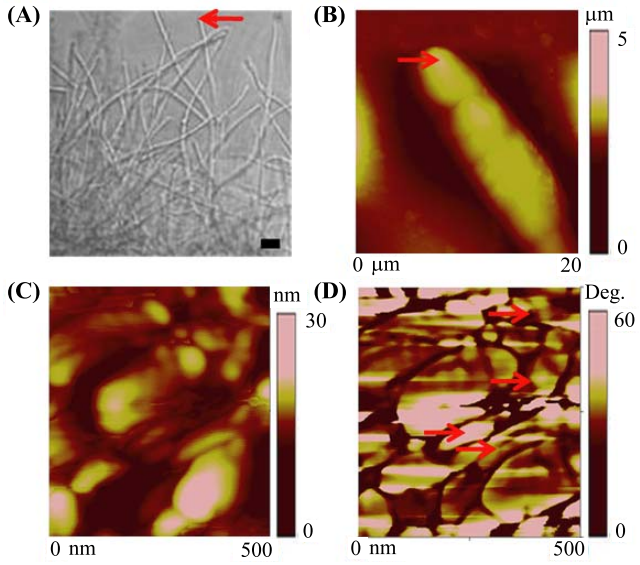

Fig. 1. AFM images of hyphal tip. (A) An optical microscope image of the margin area of a single *A. nidulans* mycelial ball. The arrow indicates an apical tip. (B and C) The height images of apical tip were taken by the tapping mode of AFM. The scanned areas are 20 μm×20 μm in panel (B) and 500 nm×500 nm in panel (C) of hyphal tip which is indicated by an arrow in panel (B). (D) The phase image of the same area of hyphal tip as in panel (C) were obtained by tapping mode under water at room temperature. Arrows in panel (D) indicate different resonance frequency. Bar in panel (A) is 20 μm.

presence of different components on the cell wall. AFM images of tip and matured hyphae surfaces that showed distinguishable structures suggested each surface could have different mechanical properties (Ma *et al*., 2005). Differences in rigidity and adhesion with regard to hyphal position also could imply that they were due to the differences of their physical and chemical properties (Ma *et al*., 2005).

Analysis of the *A. nidulans* hyphal wall surface

As previously reported, the hyphal wall of *A. nidulans* consists of various substances such as chitin, α-1,3-glucan, β-1,3-glucan, mannose polysaccharide, galactose polysaccharide, protein, and melanin (Polacheck and Rosenberger, 1977; Peberdy, 1987; Torralba *et al*., 1998). Among these substances protein, β-glucan, and chitin were determined in this study because they are the major components of the hyphal wall and likely exist on the surface. These constituents were extracted from the cell wall and spread onto silicon wafers for measuring the adhesion forces with AFM. For a reference, the measurement of adhesion force was accomplished on the substances: chicken or bovine serum albumin, β-glucan, and chitin obtained commercially. The resulting reference values of adhesion force were used to differentiate the chemical composition of the hyphal tip surface. Although the structure of extracted substances might not have the intact state, the ranges of adhesion forces of each substance were not different from those of commercial substance (Figs. 2, 3, and 4 and see the followings). Because adhesion forces vary with friction and elasticity between the AFM tip and the sample, certain chemical, physical (e.g., hardness), and surface properties (e.g., roughness) can be determined (Green and Allen, 2002). This technique has been successfully used to establish the surface characteristics of yeast cells, rendering valuable information about ion content, hardness, and surface charge (Jalili and Laxminarayana, 2004). Additional information about the presence of certain functional groups such as hydroxyl, carboxyl, sulfonyl, and amino groups may also be collected at the same time. To minimize the bias of the adhesion force values depend on the kind of AFM tips, NSC16 cantilever, NSC36 cantilever, and Whisker-type cantilever, they have their own spring constant, were used in the experiment. The respective adhesion forces of protein, β-glucan, and chitin obtained with three cantilevers showed no significant differences (data not shown).

Adhesion forces of protein

Hyphal wall protein was extracted and purified to 150 μg/ml (Bradford assay) using Masuda's method (1986). Reference values of adhesion forces were determined with 50 μl of commercial bovine and chicken serum albumin (150 μg/ml) spread on a silicon wafer. The AFM images of the purchased and the extracted proteins showed a highly similar shape in terms of surface roughness (Figs. 2A and B), although there are a few white spots on the image of extracted protein which are likely to be some contaminated materials during the extraction process. Adhesion force values obtained from the commercial protein ranged in 125-300 nN (Fig. 2C), which were corresponding more than 92% to the values of hyphal wall-extracted protein (Fig. 2D). However, some of the extracted protein had adhesion forces lower than 125 nN (Fig. 2D), likely representing that it contained some additional substances introduced during the protein extraction process.

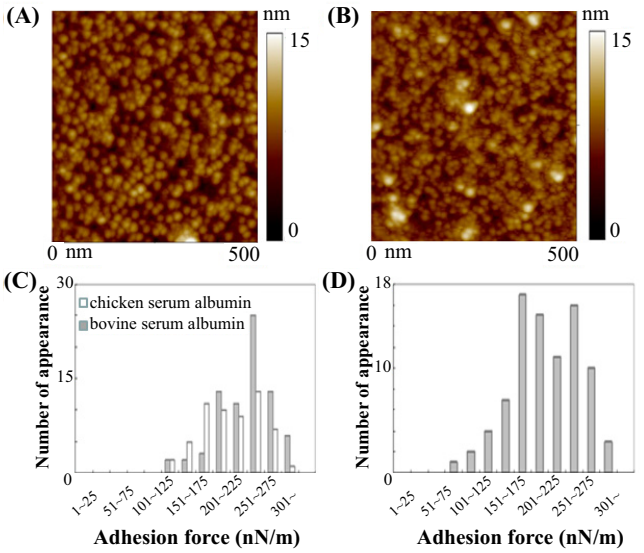

Fig. 2. AFM images and adhesion forces of proteins. Images were obtained by tapping mode under water at room temperature from purchased bovine serum albumin (A) and protein fraction prepared from hyphal wall (B). The scanned areas are 500 nm×500 nm. Distribution of adhesion force values of bovine serum albumin and chicken serum albumin (C) and protein fraction prepared from hyphal wall (D). Adhesion forces were calculated from the data of AFM force curve that were measured in tapping mode at room temperature.

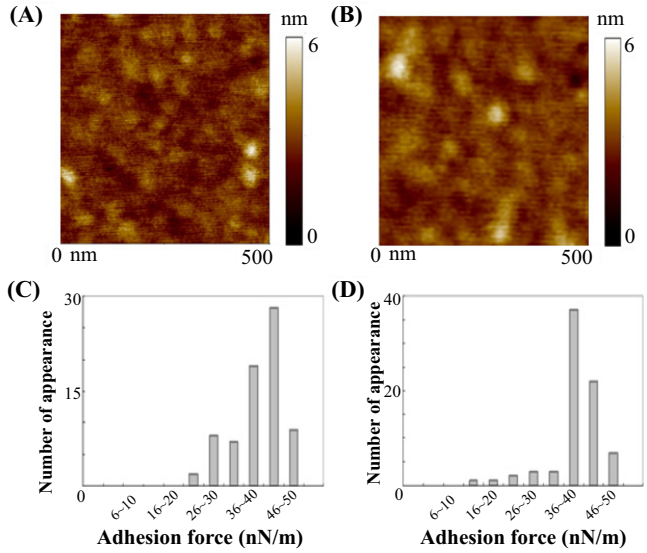

Fig. 3. AFM images and adhesion forces of β-glucan. Images were obtained by tapping mode under water at room temperature from purchased yeast β-glucan (A) and β-glucan fraction prepared from hyphal wall (B). The scanned areas are 500 nm×500 nm. Distribution of adhesion force values of yeast β-glucan (C) and β-glucan fraction prepared from hyphal wall (D). Adhesion forces were calculated from the data of AFM force curve that were measured in tapping mode at room temperature.

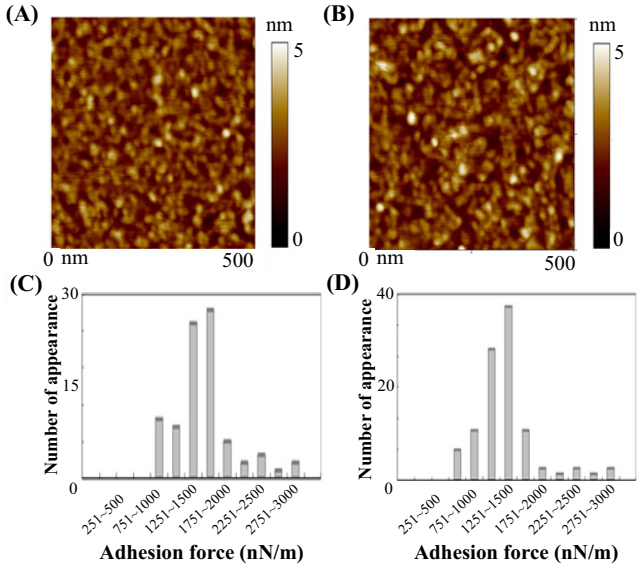

Fig. 4. AFM images and adhesion forces of chitin. Images were obtained by tapping mode under water at room temperature from purchased crab chitin (A) and chitin fraction prepared from hyphal wall (B). The scanned areas are 500 nm×500 nm. Distribution of adhesion force values of crab chitin (C) and chitin fraction prepared from hyphal wall (D). Adhesion forces were calculated from the data of AFM force curve that were measured in tapping mode at room temperature.

Our data suggest that it is possible to identify the protein composition of the *A. nidulans* surface by comparing the adhesion forces of reference protein to those of hyphal surface.

Adhesion forces of β-glucan

Cross connection between β-glucan and chitin at the apical tip provides mechanical stability for the cell wall (Borgia and Dodge, 1992). We isolated β-glucan from the hyphal wall using Borgia's method (1992), determined the concentration (270 μg/ml) with phenol-sulfuric acid (Dubois, 1956), and measured the adhesion forces via AFM. Figure 3 shows the results of the β-glucan analysis which were referenced to a 50-μl aliquot of commercial β-glucan (270 μg/ml). The AFM images of β-glucan (Figs. 3A and B) are technically challenging in determining the surface structure of hyphal wall. Since the image would be limited to discriminate the substances on the hyphal wall surface, the adhesion forces of commercial β-glucan and extracted β-glucan from the hyphal wall were examined whether their adhesion force values were in the same range. Adhesion forces in the commercial sample ranged from 25 to 50 nN (Fig. 3C). Comparison to β-glucan extracted from the hyphal wall (Fig. 3D) showed that more than 95% of the extract sample presented in the same range of commercial β-glucan's adhesion force.

Adhesion forces of chitin

Chitin, the frame-defining compound of the cell wall, was extracted from the hyphal wall using Hackman and Goldberg method (1981). As same as in the experiments of protein and

Table 1. Measurement of adhesion force at hyphal wall

(A) Values are the number of appearance in each range of adhesion force at apical tip region

Range of Adhesion force \ Exp.	I	II	III	IV	V	VI	VII	VIII	Mean±SD	Proposed Chemical[a] (%)
1-24							2		0.25±0.71	Unknown (0.4)
25-50	22	20	19	27	17	21	23	19	2.1±3.07	Glucan (35.2)
51-124			2	2		3			0.88±1.25	Unknown (1.4)
125-300	4	2	4	2		3	4	6	3.5±1.81	Protein (5.8)
301-999		3			3		2	3	1±1.51	Unknown (1.6)
1000-3000	31	39	32	28	37	38	19	39	32.88±6.96	Chitin (55.6)
Total	57	64	57	59	57	65	50	67		

(B) Values are the number of appearance in each range of adhesion force at mid-hyphal region

Range of Adhesion force \ Exp.	I	II	III	IV	V	VI	VII	Mean±SD	Proposed Chemical[a] (%)
1-24		4			2			0.86±1.58	Unknown (2)
25-50	17	7	18	15	8	14	13	13.15±4.22	Glucan (31)
51-124		7		6	8	3	4	4±3.21	Unknown (9.4)
125-300	4		6		9	3	13	5±4.76	Protein (11.8)
301-999		6		3		8		2.43±3.36	Unknown (5.8)
1000-3000	19	19	14	12	30	10	14	16.86±6.69	Chitin (40)
Total	40	43	38	36	57	38	44		

[a] Determination of chemical was proposed by the ranges of adhesion force value obtained from yeast β-glucan (Sigma G5011), proteins such as BSA (Sigma A9647) and CSA (Sigma A5378) and crab chitin (Sigma G9752).

glucan, there were no clear differences in AFM images between commercial and extracted chitins (Figs. 4A and B). The reference adhesion force values determined with a 50-μl commercial sample (100 g/ml) were in the range of 1,000 and 3,000 nN (Fig. 4C). The adhesion force values of extracted chitin were comparable as 70 out of 74 trials were in the same range of commercial's (Fig. 4D). Although we do not know the reason why adhesion value for chitin showed such a large range, a possible explanation could be that the physical properties of chitin spot on which the AFM probe interacted might affect the adhesion force.

Compositional analysis of hyphal surface by adhesion force

The adhesion forces measured for the substances extracted from the hyphal wall fell into the same range as the values determined for the corresponding substances obtained commercially. It was therefore deemed to establish their distribution in the apical region of *A. nidulans* hyphae. The moisture content of the air was maintained at a low level of 25 ±2% to minimize experimental deviation. All measurements were accomplished with the same AFM cantilever. The adhesion forces at the randomly selected spots on the apical tip (476 spots) and the mid-hyphal region (296 spots) were measured (Table 1). Compositional analysis of the measurements was based on the adhesion force values which were separately obtained from the commercial compounds of β-glucan (25-50 nN), albumin (125-300 nN), and chitin (1,000-3,000 nN). Approximately 35.2% of the adhesion forces measured at the apical tip of hyphae fell into the β-glucan range. The remainder was consistent with protein (5.8%), chitin (55.6%), and other unidentifiable substances (3.4%). Because the cell wall is synthesized at the apical tip of hyphae, cell wall composition in this region may differ from other areas. The main components of the apical tip cell wall were chitin and β-glucan, with only a small amount of protein at an approximate ratio of 18:12:2:1 (one part of unidentifiable compounds). In comparison, the mid-hyphal area presented with a ratio of 16:12:4:6, showing a 17.2% increase of unidentifiable constituents over the apical region possibly reflecting different areas of cell wall construction. These results showed that the chemical composition and structure of the hyphal wall surface is changed during growth and maturation by rearrangement or formation of chitin, β-glucan, protein, and other materials.

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