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Fungal pigments inhibit the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of darkly pigmented fungi

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ABSTRACT

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) has been used to discriminate moniliaceous fungal species; however, darkly pigmented fungi yield poor fingerprint mass spectra that contain few peaks of low relative abundance. In this study, the effect of dark fungal pigments on the observed MALDI mass spectra was investigated. Peptide and protein samples containing varying concentrations of synthetic melanin or fungal pigments extracted from *Aspergillus niger* were analyzed by MALDI–TOF and MALDI–qTOF (quadrupole TOF) MS. Signal suppression was observed in samples containing greater than 250 ng/μl pigment. Microscopic examination of the MALDI sample deposit was usually heterogeneous, with regions of high pigment concentration appearing as black. Acquisition of MALDI mass spectra from these darkly pigmented regions of the sample deposit yielded poor or no $[M+H]^+$ ion signal. In contrast, nonpigmented regions within the sample deposit and hyphal negative control extracts of *A. niger* were not inhibited. This study demonstrated that dark fungal pigments inhibited the desorption/ionization process during MALDI–MS; however, these fungi may be successfully analyzed by MALDI–TOF MS when culture methods that suppress pigment expression are used. The addition of tricyclazole to the fungal growth media blocks fungal melanin synthesis and results in less melanized fungi that may be analyzed by MALDI–TOF MS.

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS)[1] is a rapid, sensitive analytical method for the mass of thermolabile, nonvolatile organic compounds, including peptides and proteins [1–3]. A number of applications of this technology have been developed, including the identification of microorganisms based on the unique mass spectra produced from a given species or strain [4]. Because different species/strains of microorganisms differentially express a host of proteins of varying masses, the MALDI–TOF mass spectrum produced from a specific species or strain tends to be unique. These MALDI–TOF mass spectral "fingerprints" can be used to discriminate microorganisms, including bacteria [5–16] and fungi [17–21]. Compared with traditional methods of microbial detection, analysis by MALDI–TOF MS is an alternative approach to the identification of bacterial and fungal species.

Although the utility of MALDI–TOF MS for the analysis of fungi has been demonstrated, several limitations have been recently reported [18,21]. Previous MALDI–TOF MS studies in our laboratory and others have demonstrated mass spectra acquired from the organic/acid extract of darkly pigmented fungi such as *Aspergillus niger* [18,21]. These spectra were characterized by a paucity of mass spectral peaks that resulted in poor statistical discrimination. Although this inhibitory effect has been noted by other laboratories, the basis of the inhibition has remained uncharacterized. A well-known characteristic of darkly pigmented fungi is the abundance of pigments in the cell wall [22–24]. Melanins are a heterogeneous group of phenolic compounds that protect fungal conidia and hyphae against cellular damage induced by solar ultraviolet radiation exposure [23] and may function as a virulence factor by neutralizing extracellular toxins, proteases, and hydrolases [25].

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[1] *Abbreviations used:* MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HPLC, high-performance liquid chromatography; MEA, malt extract agar; DDI, deionized water; FGSC, Fungal Genetics Stock Center; MEB, malt extract broth; PBS, phosphate-buffered saline; RT, room temperature; MWCO, molecular weight cutoff; UV, ultraviolet; Vis, visible; ACN, acetonitrile; TFA, trifluoroacetic acid; CHCA, α-cyano-4-hydroxycinnamic acid; ACTH, adrenocorticotropic hormone; qTOF, quadrupole TOF; FESEM, field emission scanning electron microscopy; GC, gas chromatography; DHN, dihydroxynaphthalene.

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In the current study, we hypothesized that the presence of fungal pigments (in particular the biopolymer melanin) in the MALDI sample deposit interferes with the desorption and/or ionization process. In this study, a systematic investigation of the effect of fungal melanin on acquired MALDI–TOF mass spectra is presented.

Materials and methods

Reagents

Acetonitrile (high-performance liquid chromatography [HPLC] grade), sodium citrate, and hydrochloric acid (6 N) were acquired from Fisher Scientific (Fairlawn, NJ, USA). Tricyclazole was acquired from Wako Pure Chemical Industries (Osaka, Japan). Malt extract agar (MEA) was acquired from Difco (Sparks, MD, USA). Deionized water (DDI) was produced with a Synthesis A-10 system (Millipore, Billerica, MA, USA). All other reagents were acquired from Sigma–Aldrich (St. Louis, MO, USA), and all reagents were used without further purification.

Fungal culture

Fungal isolates were obtained from the Fungal Genetics Stock Center (FGSC, Kansas City, MO, USA). *A. niger* (FGSC A1143) was subcultured from stock sources and grown for 14 days at 25 °C on MEA in 100-mm culture plates. For submerged culture preparation, approximately 1×10^2 spores were inoculated into 50 ml of malt extract broth (MEB) and grown for 4 days in a C24KC refrigerated incubator shaker (Edison, NJ, USA) at 25 °C. For tricyclazole inhibition of melanin synthesis, cultures were grown for 14 days at 25 °C on MEA plates supplemented with 50 μg/ml tricyclazole in 1% ethanol. To ensure reproducibility, each fungal culture was performed eight times independently.

Isolation of melanin ghosts

Melanin "ghosts" were extracted from *A. niger* using a modification of the method from Wang and coworkers [26]. Briefly, cultures were grown on MEA for 10 days at 25 °C. Sporulating fungal cultures were harvested from the culture plate using sterilized DDI and then were lyophilized and weighed. The pigment sample was then suspended in 0.1 M sodium citrate buffer containing 10 mg/ml cell lysing enzymes from *Trichoderma harzianum* and 2 mg/ml cellulase from *Trichoderma reesei* and was incubated with agitation overnight at 30 °C. The sample was then pelleted by centrifugation (1000*g* for 10 min), washed with phosphate-buffered saline (PBS, pH 7.4), resuspended in 4 M guanidine isothiocyanate, and incubated at room temperature (RT) overnight with agitation. The sample was then centrifuged, and the pellet was washed with PBS, resuspended in 1 mg/ml proteinase K in DDI, and then incubated overnight at 37 °C with agitation. The sample was pelleted, washed with PBS, resuspended in 6 M HCl, and then boiled for 1 h. The sample was pelleted, washed with PBS, and then dialyzed against DDI for 10 days at 4 °C using 3500-MWCO (molecular weight cutoff) molecular porous membrane tubing (Spectra/Por, Laguna Hills, CA, USA). The sample was lyophilized and weighed, and the resulting melanin ghosts were used for further experiments.

UV spectroscopy

Spectrophotometric characterization of 1 mg/ml synthetic melanin or 1 mg/ml *A. niger* melanin ghosts was carried out using a Beckman DU 800 spectrophotometer (Fullerton, CA, USA) equipped with a deuterium (ultraviolet [UV]) and tungsten (visible [Vis]) lamp. Data acquisition and processing were carried out using the DU800 spectrometer software. The analytical wavelength range was 200 to 800 nm. Temperature control was achieved using a VWR Scientific circulating water bath (Niles, IL, USA). Fungal pigments were dissolved in 50:50 HNO_3/HF solution with sonication for 5 min. Blanking of the instrument was performed with 50:50 HNO_3/HF before absorbance readings of the melanin were taken.

Gas chromatography–mass spectrometry

Fungal melanin ghost samples were analyzed using an Agilent 6890 gas chromatograph coupled to an Agilent 5975C mass spectrometer using a 30-m HP5-MS column (Agilent Technologies, Santa Clara, CA, USA). Samples were injected (1 μl) at a 20:1 split injection ratio into a 300 °C inlet. Analytes were eluted from the column using 1.0 ml/min helium and an oven temperature profile as follows: 50 °C for 5.0 min and then ramped at 5.0 °C/min to a final temperature of 300 °C. The MS source temperature was maintained at 230 °C, and the quadrupole temperature was maintained at 150 °C.

Preparation of fungal culture samples for MALDI–TOF MS analysis

Conidia and hyphae derived from one culture plate of *A. niger* ($\sim 10^8$ spores/ml) were transferred to 100 μl of 0.1-mm zirconium beads (Biospec, Bartlesville, OK, USA) and 1 ml of 50:50 acetonitrile (ACN)/4% trifluoroacetic acid (TFA). The samples were then subjected to three 1-min bead beating cycles. Samples were cooled on ice between bead beating cycles. The resulting solution was centrifuged at 21,600*g* for 10 min, and the supernatant was retained. A 10-μl aliquot of the supernatant was combined with 10 μl of α-cyano-4-hydroxycinnamic acid (CHCA, 10 mg/ml, in 50:50 ACN/0.1% TFA), and duplicate 1.0-μl aliquots were deposited on a gold sample stage (Bio-Rad, Hercules, CA, USA) and allowed to air dry prior to MALDI–TOF MS analysis.

Submerged cultures were transferred to 50-ml conical tubes and centrifuged at 41*g* for 5 min. The mycelia were washed three times with PBS to remove residual MEB and then forced through a 70-μm mesh filter (BD Biosciences, Bedford, MA, USA) using a syringe plunger in 20 ml of PBS. This solution was centrifuged at 41 g for 5 min, the supernatant was collected and lyophilized, and the dry weight was recorded for quantification (the average mass of pigment per fungal culture plate was ~3 mg). The resulting dry mass was suspended in 1 ml of 50:50 ACN/4% TFA. A 10-μl aliquot of supernatant fluid was combined with 10 μl of CHCA (10 mg/ml in 50:50 ACN/0.1% TFA), and duplicate 1.0-μl aliquots were deposited on a gold sample stage (Bio-Rad) and allowed to air dry prior to MALDI–TOF MS analysis.

Preparation of melanin samples for MALDI–MS analysis

Varying concentrations of both commercially available synthetic melanin and purified *A. niger* melanin ghosts were prepared over the range of 10 ng/μl to 20 μg/μl in 50/50 ACN/DDI. A 10-μl aliquot of each melanin solution was combined with 10 μl of 100 pmol/μl adrenocorticotropic hormone (ACTH) fragment 18–39 (RPVKVYPNGAEDESAEAFPLEF, m/z 2465.1983). The resultant mixture was combined with 20 μl of CHCA (10 mg/ml in 50:50 ACN/0.1% TFA), and triplicate 1.0-μl aliquots were deposited on a stainless steel sample stage (Waters, Milford, MA, USA) and allowed to air dry prior to MALDI–TOF MS analysis.

MALDI–MS

MALDI–TOF mass spectral fungal fingerprints were acquired using a Ciphergen PBS-IIc linear TOF mass spectrometer (Bio-

Rad) with a flight path of 0.8 m, capable of mass resolution ($m/\Delta m$) of 1000 and mass accuracy of ±1000 ppm. Spectra were acquired over the m/z range of 0 to 100,000 u, with delayed extraction parameters set to optimally focus the range of 10,000 to 20,000 u. Composite mass spectra were the average of 100 laser shots taken from 20 distinct positions across the sample deposit. These positions were held constant for all samples used in this study, and 337-nm N_2 laser pulse energy was maintained just above the threshold for ion production. Mass spectra were externally calibrated using a set of peptide and protein calibrants that covered a range of 1 to 66 kDa.

Peptide MALDI–qTOF (quadrupole TOF) mass spectra were acquired using a MALDI–SYNAPT MS (Waters) qTOF mass spectrometer capable of mass resolution ($m/\Delta m$) of 14,000 and mass accuracy of ±5 ppm. Spectra were acquired over the m/z range of 500 to 4000 u. Composite mass spectra were the result of a 120-s acquisition with the frequency-tripled Nd:YAG laser (355 nm) operating at 200 Hz, with the laser pulse energy maintained just above the threshold for ion production. Mass spectra were acquired using a predetermined "spiral" pattern that was held constant for all sample deposits, ensuring that a reproducible surface area was irradiated for each sample.

Statistical analysis

MALDI–TOF MS data were statistically analyzed to determine whether there were differences in mean peak heights between concentrations and pigment levels. Univariate analyses were performed to ensure that all data met analytical assumptions. Analysis of variance tests were performed using mixed linear models followed by a Bonferroni posttest to determine whether there were differences in the mean ion counts between concentrations within each pigment when compared with the control value. Results were considered as significantly different at an alpha level of 0.05. Data were analyzed using SAS 9.1 (SAS Institute, Cary, NC, USA) or GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

Results and discussion

Darkly pigmented fungi, such as *A. niger*, yield poor fingerprint mass spectra that contain only a few peaks of low relative abundance (Fig. 1). It is hypothesized that the presence of photo- and chemoprotective pigments, such as melanin, in the cell wall may suppress ionization and ultimately the MALDI-based detection of expressed proteins in the sample. To test this hypothesis, we examined the effect of spiking synthetic melanin into standard protein and peptide solutions. We observed suppression of MALDI signal (by both MALDI–TOF MS at 337 nm and MALDI–qTOF MS at 355 nm) for all proteins and peptides tested over the m/z range of 700 to 66,000 u. A range of melanin concentrations, from 2.5 to 5000 ng/spot, was examined. The molar concentration of melanin in these solutions cannot be determined from the mass of suspended melanin because melanin is a highly disperse polymer that is only slightly soluble in ACN/4% TFA. However, the stock melanin solutions prepared for this study were empirically chosen to produce CHCA dried droplet MALDI sample deposits that match the color of sample deposits obtained from darkly pigmented fungi such as *A. niger*, which range in color from dark yellow to black. Furthermore, calculations based on pre- and postmelanin ghost extraction suggest that fungal fingerprint samples contain approximately 3 μg of fungal pigment per sample deposit. Fig. 2 demonstrates the effect of increasing melanin concentration on the observed MALDI–qTOF mass spectrum of 25 pmol ACTH. The control mass spectrum (Fig. 2A) consisted of a strong $[M+H]^+$ ion (mean ion counts ± standard error of measure = $1.12 \times 10^5 \pm 7572$ counts/120-s acquisition) at a monoisotopic mass of 2465.1983. The addition of 25 ng synthetic melanin to the sample deposit (Fig. 2B) resulted in a decrease in peak height of approximately 20% to $9.26 \times 10^4 \pm 3110$ counts, whereas the addition of 1250 ng melanin resulted in suppression of greater than 99.5% (589 ± 123 counts) (Fig. 2C).

Since the synthetic melanin examined in the preceding experiments was not chemically identical to that obtained from fungal sources, we isolated and characterized fungal pigments from *A. niger* (a darkly pigmented fungal species) to determine whether these naturally obtained fungal pigments would also suppress analyte $[M+H]^+$ ion formation in MALDI–TOF mass spectra. These pigments exhibited strong nonzero absorption in the range of 200 to 800 nm when analyzed by UV–Vis spectroscopy; absorption of a 1-mg/ml solution approaches 1.0 at 337 and 355 nm, the emission wavelengths of the nitrogen and Nd:YAG lasers used for these experiments. This observation is consistent with the fact that melanin has photoprotective properties, absorbing UV radiation and dissipating energy as heat via rapid internal conversion [25]. Field emission scanning electron microscopy (FESEM) analysis (data not shown) suggested that fungal pigments isolated from *A. niger* maintained the ultrastructural architecture of the fungal spores following melanin extraction as observed previously in *Cryptococcus neoformans* [23,26,27]. The gas chromatography (GC)–MS analysis indicated the presence of cell wall components, including long chain fatty acids and aliphatic compounds in the *A. niger* pigment preparation. These results are consistent with previous reports of dihydroxynaphthalene (DHN)–melanins that are known to be extensively bound to the cell wall of *Aspergillus fumigatus* conidia [28,29].

Spiking purified *A. niger* pigment into peptide and protein standards resulted in a suppressive effect similar to that observed for synthetic melanin. The results of both experiments for the standard peptide ACTH are summarized in Fig. 3. Although the addition

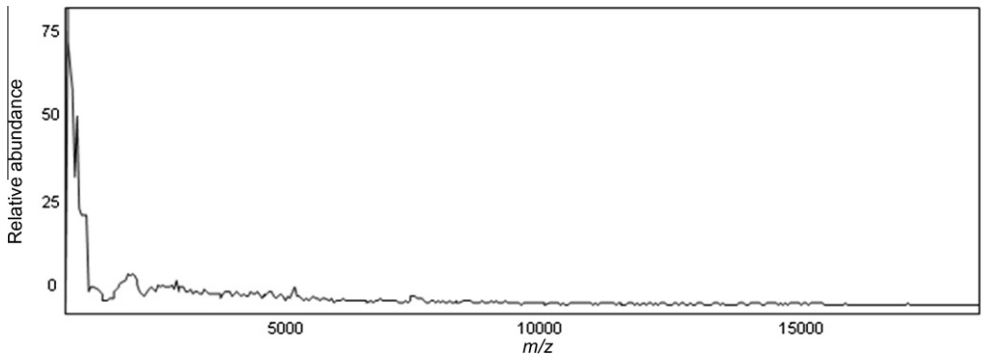

Fig.1. MALDI–TOF MS fingerprint mass spectrum of *A. niger* cultured on MEA and acquired using previously published cellular extraction methodology [18].

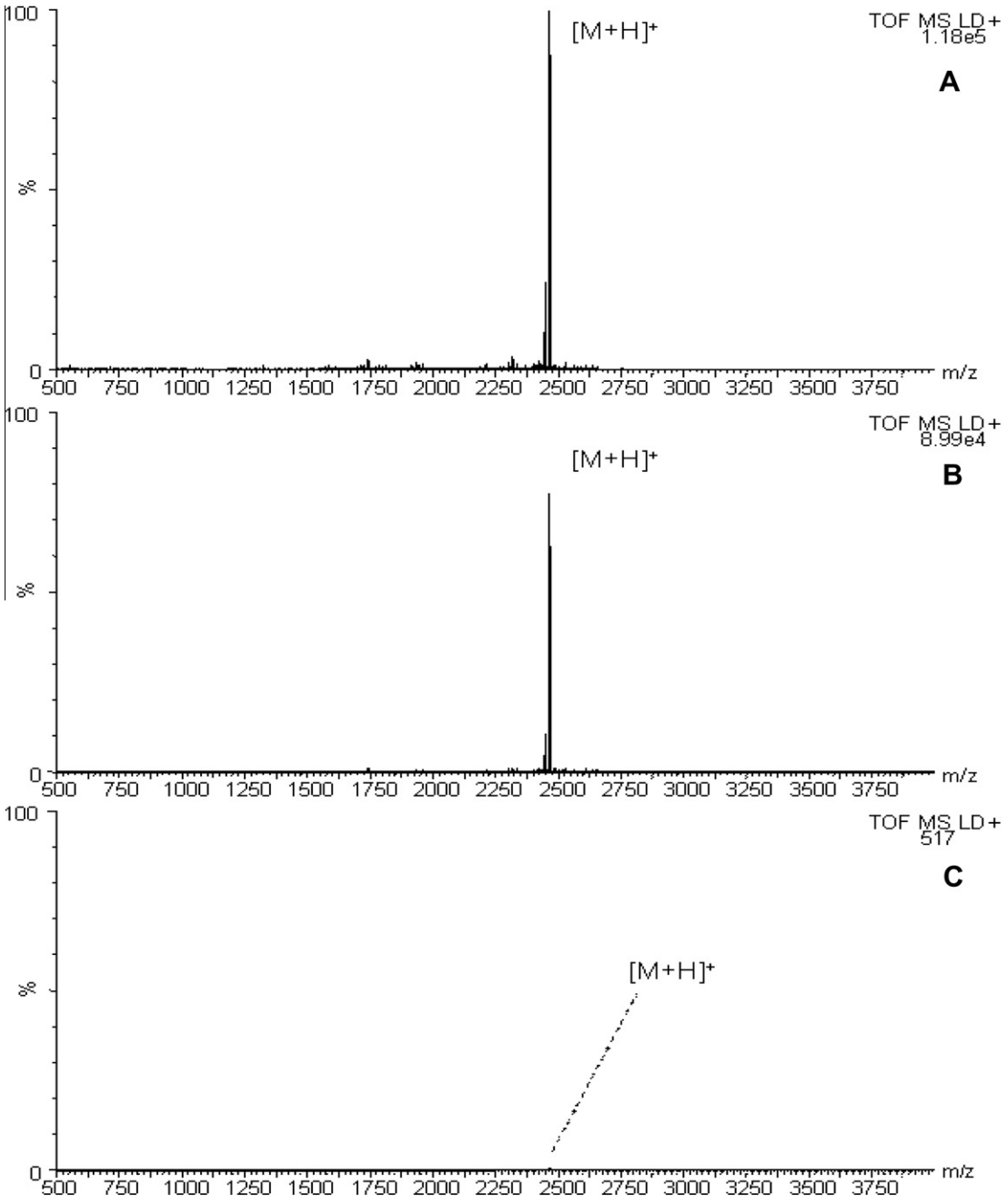

Fig.2. MALDI–qTOF mass spectrum of 25 pmol ACTH spiked with 0 ng of synthetic melanin (A), 25 ng of synthetic melanin (B), and 1250 ng of synthetic melanin (C). Reported spectra are representative of three independent acquisitions.

of synthetic melanin to the sample deposit had a significant suppressive effect at concentrations as low as 25 ng/spot, both synthetic melanin and purified *A. niger* pigment exhibited a sharp threshold effect at a concentration of 1000 ng/spot, with complete suppression of MALDI ion signal at concentrations above 1250 ng/spot for synthetic melanin and 5 μg/spot for purified *A. niger* pigment. This observation is in agreement with the near-complete MALDI ion suppression in *A. niger* fungal fingerprinting experiments, where an estimated 3000 ng/spot fungal melanin is present. Differences in the suppressive effects of synthetic melanin and *A. niger* fungal pigment may be due to physical characteristics, including the retention of spore morphology in the *A. niger* pigment extract that may interfere with crystallization within the matrix and subsequent spot homogeneity.

Visual observation of the *A. niger* pigment-containing MALDI sample deposits via light microscopy demonstrated that these pigments were heterogeneously dispersed across the sample deposit. To examine the effect of this heterogeneity on the acquired MALDI mass spectrum, a sample of 25 pmol ACTH was spiked with 1250 ng of pigment extract and analyzed by MALDI–qTOF MS (Fig. 4). Using this instrument, a camera imaged the sample deposit and the exact spatial location of the MALDI laser output could be guided in real time. Spectra were acquired from regions of high and low *A. niger* pigment concentration (empirically determined by black pigment coloration). The ACTH $[M+H]^+$ ion signal from a region of low pigment concentration (Fig. 4B) was approximately 60 times higher (571 counts) than that from a darkly colored region of high pigment concentration (9 counts) (Fig. 4A). Attempts

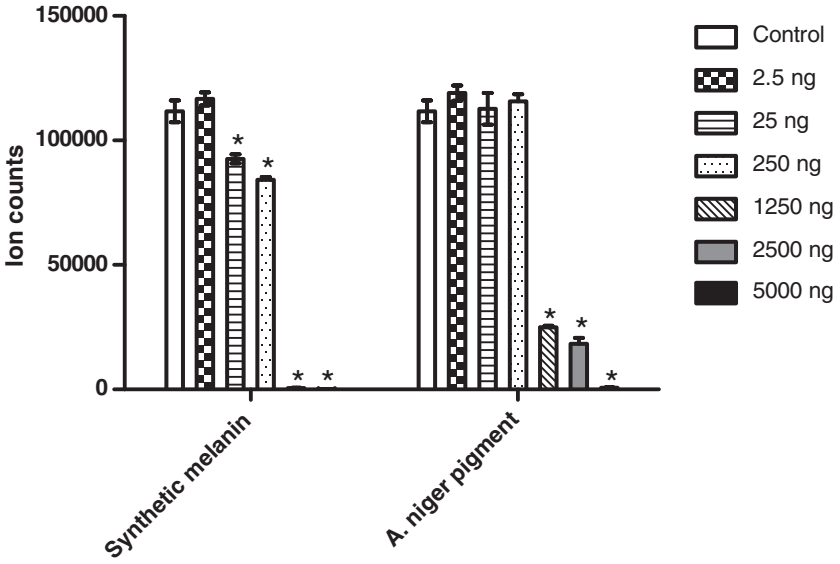

Fig.3. Effect of synthetic melanin and fungal pigment concentration in the sample deposit on total ACTH ion counts. Each bar represents the average of three independent sample spots. Error bars represent the standard error of measure of the three sample spots. $^{*}P < 0.001$ when compared with control.

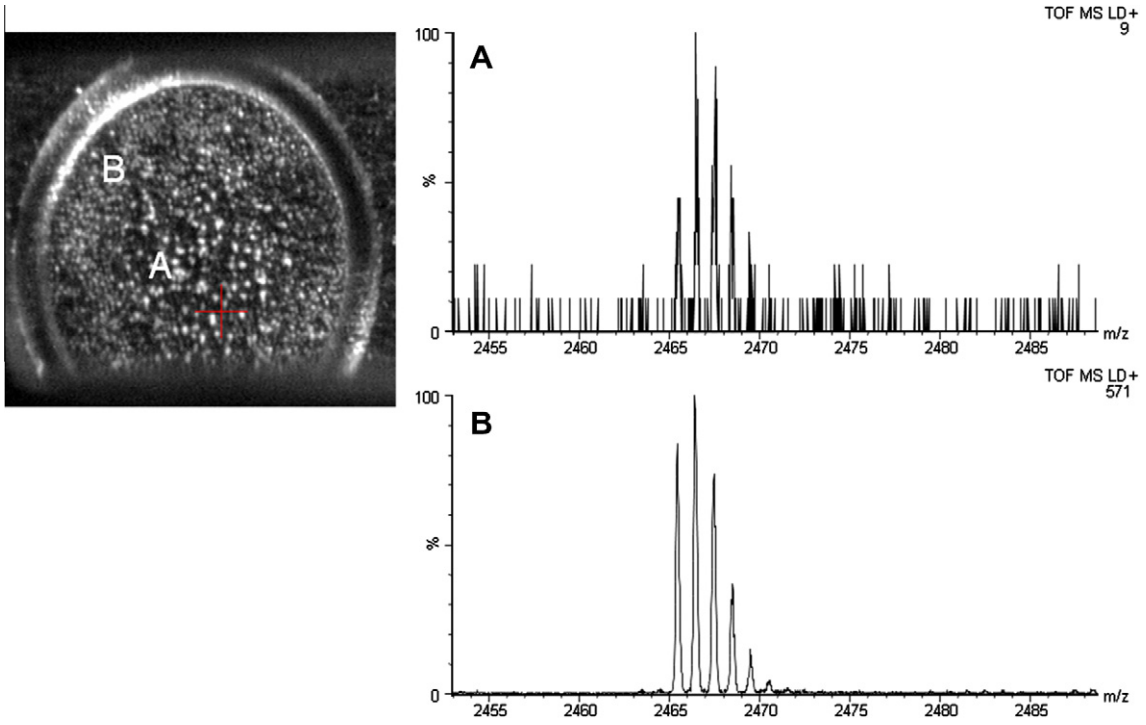

Fig.4. MALDI–qTOF mass spectrum of ACTH $[M+H]^{+}$ isotopic distribution from sample deposit containing 1250 ng of *A. niger* pigment. Spectra are the result of 200 laser shots at point A (high relative pigment concentration) and point B (low relative pigment concentration) within a heterogeneous sample deposit. Base peak intensity in spectrum B is approximately 60 times higher than that in spectrum A.

to increase the ion intensity by increasing the desorption laser fluence resulted in increased fragmentation of the analyte, rather than increases in $[M+H]^{+}$ signal, as reported previously [30,31].

Although the precise mechanism(s) by which the MALDI event proceeds remains the subject of much discussion and active research [32,33], the unique properties of melanin and fungal pigments may interfere with MALDI in a number of fundamental ways. One plausible explanation for the observed inhibition of MALDI–TOF is that pigments in fungal samples (particularly at high concentrations) directly compete with the organic acid matrix for irradiating photons. Following the MALDI laser pulse, ionization in the dense plume above the sample deposit can occur only where sufficient populations of electronically excited matrix molecules exist. If a photoexcitation/pooling mechanism for MALDI is assumed [33], mobile excitons in the bulk sample deposit could be quenched by melanin and fungal pigments.

The preceding experiments demonstrate that melanin pigments produced by darkly pigmented fungi are at least partly responsible for the observed suppression of MALDI ionization. We further hypothesize that if melanin production could be inhibited in darkly pigmented fungi, darkly pigmented species, such as *A. niger*, would yield fingerprint mass spectra similar to moniliaceous species. To

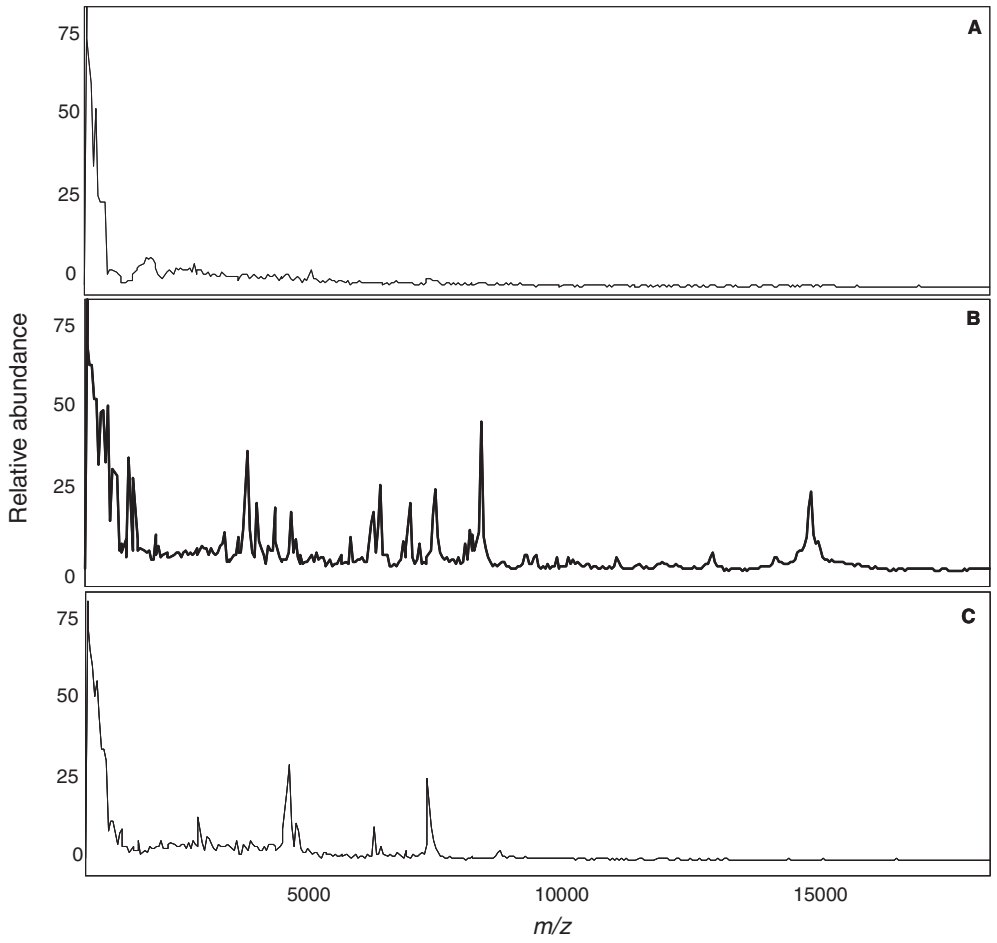

Fig.5. MALDI–TOF fingerprint mass spectra of *A. niger* conventional MEA extraction (A), fingerprinting following MEB culture (B), and fingerprinting following culture on MEA supplemented with 1% EtOH + 50 μg/ml tricyclazole (C).

test this hypothesis, melanin-free *A. niger* hyphae were produced by submerged culture in MEB, and spores were produced in MEA supplemented with tricyclazole. The results of these experiments are summarized in Fig. 5. *A. niger* conidia inoculated in MEB produced unpigmented hyphae that yielded a diverse fingerprint mass spectrum (Fig. 5B). However, due to the differences in growth characteristics of fungi grown on solid and liquid media, *A. niger* was grown on solid nutrient media supplemented with tricyclazole to inhibit pigment production and to determine whether MALDI–TOF MS fingerprint mass spectra could be acquired from this darkly pigmented species. *A. niger* was grown under sporulating conditions identical to the previous fungal mass fingerprinting methodology [18]; however, the MEA was supplemented with tricyclazole, an antifungal agent that inhibits DHN–melanin formation by down-regulating two reductases critical to the pentaketide DHN–melanin synthesis pathway. Compared with *A. niger* grown on MEA (Fig. 5A), *A. niger* grown on MEA supplemented with tricyclazole resulted in a fingerprint mass spectrum with several prominent mass signals (Fig. 5C).

Conclusions

The presence of fungal melanin in MALDI sample deposits suppressed peptide and protein ion signals during the acquisition of MALDI–TOF mass spectra. Suppression of standard peptide and protein signals was observed on the addition of both commercially available synthetic melanin and melanins purified from *A. niger* to the MALDI sample. Suppression of peptide and protein $[M+H]^+$ ion signals was observed at melanin concentrations greater than 1250 ng/spot, a concentration significantly less than the level of melanin obtained from a fungal culture. Ion suppression occurs in a concentration-dependent manner, approaching 100% at high melanin concentrations (>5000 ng/spot). Culture conditions may be manipulated to block fungal melanin synthesis and obtain acceptable MALDI–TOF fingerprint mass spectra from darkly pigmented species such as *A. niger*. MALDI–TOF fingerprint mass spectra were acquired from *A. niger* grown under liquid culture conditions as well as from cultures grown on MEA supplemented with 50 μl/ml tricyclazole, an antifungal agent used to block DHN–melanin synthesis. To our knowledge, this is the first report demonstrating the inhibitory effects of fungal pigments on the acquired MALDI–TOF mass spectrum as well as the first report of successful fingerprinting of darkly pigmented fungi such as *A. niger*. These data represent an important advance for the use of MALDI–TOF MS for the identification of darkly pigmented fungi.

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