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Characterization of nigerlysin ©, hemolysin produced by *Aspergillus niger*, and effect on mouse neuronal cells in vitro

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Abstract

Aspergillus niger produced a proteinaceous hemolysin, nigerlysin ©when incubated on sheep's blood agar (SBA) at both 23 and 37 °C. Nigerlysin was purified from tryptic soy broth (TSB) culture filtrate and found to have a molecular weight of approximately 72 kDa, with an isoelectric point of 3.45. Nigerlysin is heat stable up to 65 °C but unstable at 75 °C when incubated for 10 min. Circular dichroic analysis revealed that nigerlysin has an alpha helical structure. Exposure of mouse primary cortical neuronal cells to 0.1 $\mu g\,ml^{-1}$ of nigerlysin resulted in the rapid loss of their viability, approximately 50% in 24 h. The IC_{50} is estimated to be 0.037 $\mu g\,ml^{-1}$, or between 0.034 and 0.041 $\mu g\,ml^{-1}$ at the 95% confidence level.

Keywords: *Aspergillus niger*; Nigerlysin; Hemolysin; Neuronal cells

1. Introduction

Aspergillus niger is an opportunistic fungal pathogen (Richardson and Warnock, 2003) and a common indoor contaminant (Summerbell et al., 1992) even in hospitals (Curtis et al., 2005). *A. niger* causes infections of the brain and other organs (Denning, 1998). Infections of the central nervous system occur in 10–20% of all cases of invasive aspergillosis resulting in nearly 100% mortality (Denning, 1998). Culture filtrates from *A. niger* were toxic to human neuroblastoma and microglial cell lines (Speth et al., 2000). However, the toxic protein in this filtrate was not identified. In this study, we suggest that this neurotoxic protein is the hemolysin, nigerlysin.

2. Materials and methods

2.1. Characterization and hemolytic activity of strains of *A. niger*

Strains of *A. niger* used in the study and their sources are shown in Table 1. These strains were grown on potato dextrose agar (PDA) (Becton Dickinson, Sparks, MD) and conidia were

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Table 1
Aspergillus niger strains and sources, as well as growth and hemolysin production on sheep's blood agar.

Culture collection and number	Environmental source	Hemolysis	
		23 °C	37 °C
ATCC[a] 16888	Fermentation	+	+
EPACC 1[b]	Clinical	+	+
EPACC 10	Air	+	+
EPACC 655	Lake Michigan	+	+
EPACC 685	Dust	+	+
EPACC 733	Ceiling tile	+	+

[a] ATCC, American Type Culture Collection, Rockville, MD.
[b] EPACC, Environmental Protection Agency Culture Collection, Cincinnati, OH.

collected using a sterile cotton tipped swab and placed on two sheep's blood agar plates (SBA) (Becton Dickinson) which were then incubated at either 23 or 37 °C. The plates were observed daily for evidence of growth and hemolysin production.

2.2. Purification of nigerlysin

Nigerlysin was purified using the following procedure. Strain ATCC 16888 was grown on PDA and the conidia recovered. Approximately 1×10^5 spores were added to 500 ml of tryptic soy broth (TSB) (Becton Dickinson). The cultures were incubated at 23 °C for 48 h on an incubator shaker at 100 rpm. Subsequently, the temperature was raised to 35 °C and the incubation continued for an additional 72 h. The fungal mass was then removed by filtering through Whatman 541 filter paper in a Büchner funnel. The recovered filtrate was centrifuged in a Millipore Centricon plus 70 filter apparatus with a MW cut-off of 30 kDa (Millipore, Bedford, MA), following the manufacturer's instructions. The concentrate from the filtration was then subjected to ion exchange chromatography.

DEAE-cellulose (Sigma, St. Louis, MO) was hydrated in 20 mM Tris–HCl, pH 6.5, for 1 h and then poured into a column giving a final bed of 3 cm × 0.5 cm (height × diameter). Then 0.5 ml of the concentrate was introduced on the top of the column. The bed was eluted with 10 ml of the 20 mM Tris–HCl buffer, followed by 10 ml of 0.1 M NaCl in 20 mM Tris–HCl buffer. Fractions of the elution with 0.2 M NaCl in 20 mM Tris–HCl buffer were collected (five drops each) throughout the elution. Ten microliters of each fraction was plated on SBA and hemolysis noted at 24 h.

The five hemolytically active fractions from the ion exchange chromatography were subjected to gel filtration using Sephadex G 100-50 (Sigma, St. Louis, MO) hydrated for 72 h in the running buffer containing 0.2 M NaCl and poured into a chromatography column to give a final bed 24 cm × 0.25 cm (height × diameter). Fractions of five drops each were collected at 1.5 ml h^{-1} using a fraction collector (ISCO, Lincoln, NE). Ten microliters of each fraction was plated on SBA and incubated at 37 °C, and hemolysis noted at 24 h. The process was repeated through a Sephadex G 200 column.

The five most hemolytically active fractions from the gel filtration were combined and dialyzed against sterile type 1 water. The desalted solution was frozen at −80 °C and lyophilized using a Spin Vac (Savant Instruments, Farmingdale, NY) resulting in a lyophilized pellet containing the nigerlysin.

2.3. Test of nigerlysin heat stability

Purified nigerlysin in 20 mM Tris–HCl buffer was incubated at 55, 65, 75 or 85 °C for 10 min then 10 μl aliquots of each was placed on SBA. Lysis of the RBCs was photographed at 24 and 48 h.

2.4. Gel electrophoresis

Methods described by Donohue et al. (2005) were used for native protein electrophoresis, SDS gel electrophoresis and isoelectric focusing (IEF) analysis.

2.5. Mass spectrometry

Samples were analyzed using a PBSIIc matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) operated in positive linear mode (Ciphergen Biosystems Inc., Fremont, CA). Samples were prepared as conventional dried droplets, on a gold sample support, using a-cyano-4-hydroxycinnamic acid (CHCA, 10 mg/ml in acetonitrile/0.1% TFA) as a matrix. All mass spectra were acquired between 0 and 150,000 m/z at a laser setting slightly above the threshold for ion production (laser step 140). The spectra were externally calibrated using bovine serum albumin $[M + H]^{2+}$ at m/z 33,217 and $[M + H]^+$ at m/z 66,433. The acceleration voltage was maintained at 20 kV, and a low mass gate was used to reject ions below m/z 500.

2.6. Circular dichroic analysis of nigerlysin

Circular dichroic analysis of an aqueous solution of nigerlysin (1 μg μl^{-1}) was carried out at room temperature on a Jasco J-810 CD spectrophotometer (Jasco Inc., Easton, MD). Quartz cells of 5 mm path length were used for measurements in the far (190–280 nm) UV spectra.

2.7. Neuronal tissue culture and exposure to nigerlysin

All animal experiments were performed under a protocol approved by an institutional animal care and use committee (IACUC) of the University of Rhode Island. Mice fetuses (C57BL/6) (Charles River Laboratories, Wilmington, MA, USA) were used to generate primary cortical neuronal cultures. Pregnant mice were sacrificed on Day 15 of gestation. Fetuses were removed and the cortexes were separated from the rest of the brain under the microscope. After removing the meninges, the cortices were placed in 15 ml tube containing 5 ml Hanks'

Balanced Salt Solution (HBSS). The cortices were then dissociated by papain (2 mg ml^{-1}) while incubating in a 37 °C water bath for 15 min. After incubation, the cells were centrifuged at 1000 rpm and the supernatant was aspirated leaving the pellet. Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum was added to triturate with a fire polished Pasteur pipette; centrifuged again and then plating medium was added to the cell pellet. Cells were then counted with trypan blue and plated at a constant density of 6.5×10^4 cells per well, in 96-well pre-coated plates. The plating medium contained 2% B27, 0.5 mM L-glutamine and 25 μM glutamic acid in NEUROBASAL medium (Invitrogen). On Day 4, half of the medium was replaced with fresh medium that did not contain glutamic acid. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Modified NEUROBASAL medium with 2% B27 and 0.5 μM L-glutamine is an optimized serum-free substitute formulated to meet the special requirement of neuronal cells and gives optimal long-term maintenance of the normal phenotype of neural cells without the need of an astrocyte feeder layer. In addition, the low concentration of L-glutamine does not support the survival and growth of glia cells. These conditions are designed to limit glial growth to less than 0.5%, preserving a nearly pure neuronal culture (Brewer et al., 1993).

2.8. Cytotoxicity assays

On Day 7 of culture incubation, the cell suspension in each well was changed to 40 μl of old medium and 40 μl of new medium. Nigerlysin was dissolved in water to give a final concentration of 0.10 μg ml^{-1}, measured with the Micro BCA kit (Pierce, Rockford, IL). The nigerlysin was diluted 200× with the cell medium NEUROBASAL containing 2% B27, 0.5 mM L-glutamine and 1% antibiotic, as working solution. Then 20 μl of the working solution was added to each well.

In the dose response studies, cells in each well were exposed to 0, 0.025, 0.033, 0.05 and 0.1 μg ml^{-1} of nigerlysin for 72 h. For the time course studies, cells in each well were exposed to 0.1 μg ml^{-1} of nigerlysin for 0, 4, 24, 48 and 72 h. Sterile water was added to the NEUROBASAL medium as controls. During exposure, cells were assessed for viability using the MTT (3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay, CellTiter 96 Non-Radioactive cell Proliferation Assay (Promega, Madison, WI). Either three or five replicate treatments were assayed at each time point.

3. Results

A. niger is readily isolated from many environments, including lake water, ceiling tiles, indoor air and dust. All strains of *A. niger* in this study grew at 23 °C and at 37 °C on SBA and all produced hemolysis within 4 days (Table 1).

After purification, nigerlysin in a native gel revealed a single band which when place on SBA lysed the blood cells underneath (Fig. 1(A and B)). Under reducing conditions, the purified nigerlysin showed a single silver staining band with a MW of about 72 kDa in an SDS-PAGE gel (Fig. 1(C and D)), in good agreement with MALDI-TOF MS (Fig. 2). The isoelectric point of nigerlysin is 3.45 with four isomers (Fig. 1(E and F)). Nigerlysin is stable at 65 °C for 10 min but unstable at 75 °C for 10 min (Fig. 3). The circular dichroic analysis showed that nigerlysin forms an alpha helix (Fig. 4).

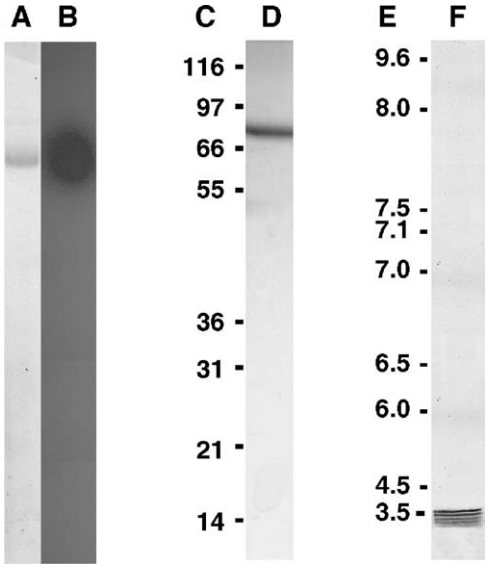

Fig. 1. Characteristics of nigerlysin. Native gel electrophoresis of purified nigerlysin (A); and SBA plate after native nigerlysin gel exposure for 24 h (B); molecular weight standards in kDa (C); for SDS-PAGE of nigerlysin, after treatment with dithiothrietol (D); isoelectric focusing gel standards (E); and nigerlysin (F).

Nigerlyisn is toxic to about 60% of the cultured neuronal cells but about 40% are more resistant (Fig. 5). The loss of viability time course shows that cell death begins

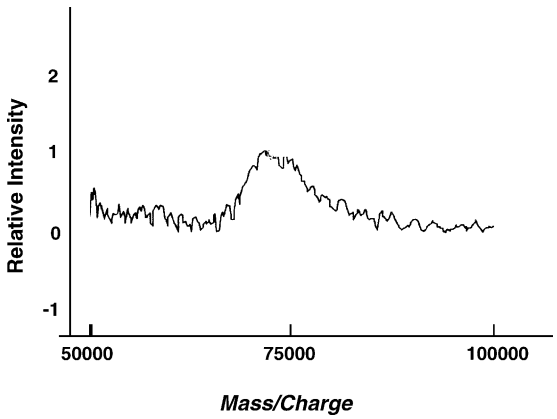

Fig. 2. Matrix-assisted laser desorption/ionization time-of-flight mass spectrum of nigerlysin.

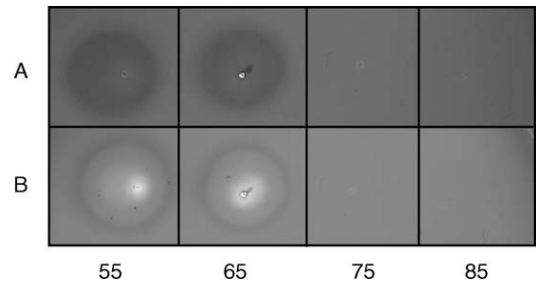

Fig. 3. Test of heat stability of nigerlysin. Purified nigerlysin in 20 mM Tris–HCl buffer was incubated at 55, 65, 75 or 85 °C for 10 min then 10 μl placed on sheep's blood agar and incubated at room temperature for 24 h (A) and 48 h (B).

almost immediately and proceeds rapidly (Fig. 6). Since these are primary neuronal cultures, there is some loss of cell viability (less than 5%) even in the controls. The percent cell viability should be considered relative and not absolute. Robust and differentiated neurons with synaptic connections are seen prior to exposure (Fig. 7(A)) and mostly dead and floating cells are seen post-exposure (Fig. 7(B)).

One-way analysis of variance on the difference in absorbance at 690 nm from 570 nm among the five concentrations, including zero, was performed and one-sided Bonferroni-adjusted *p*-values were used to determine whether survival (which is linearly related to the difference in absorbance) declined significantly from one concentration to the next (four comparisons, including the control). Parametric determination of an IC_{50} using logistic regression in either the raw or log-transformed dose failed to result in an adequate fit to these data, therefore a natural cubic spline interpolation was used. A 95% confidence interval for the IC_{50} was estimated using a boot-strap and Monte Carlo sampling of the original data.

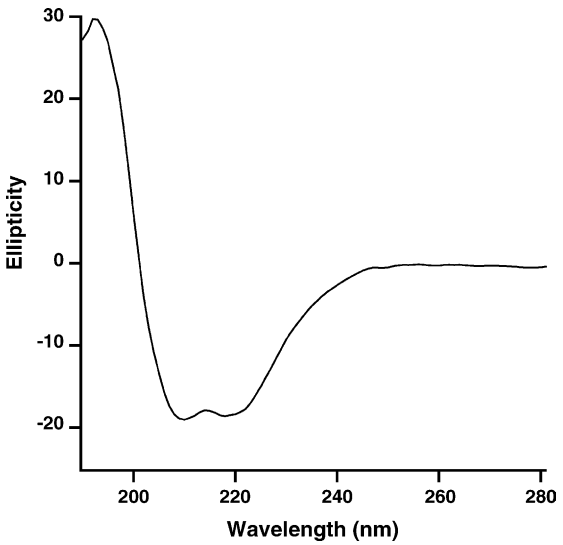

Fig. 4. Circular dichroic analysis of nigerlysin. Circular dichroic analysis of an aqueous solution of nigerlysin (1 μg μl^{-1}) was carried out at room temperature on a Jasco J-810 CD spectrophotometer. Quartz cells of 5 mm path length were used for measurements in the far (190–280 nm) UV spectrum.

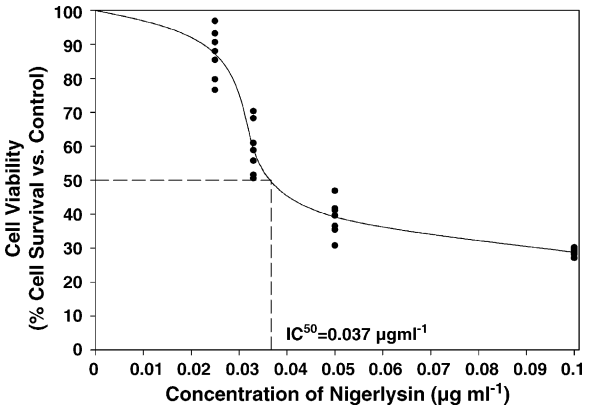

Fig. 5. Dose response (mean percent viability ± standard deviation) of cortical neuronal primary cell cultures following exposure to nigerlysin at different concentrations. Cells were exposed to 0, 0.025, 0.033, 0.05 and 0.1 μg ml^{-1} of nigerlysin for 72 h. Each data point represents the mean and standard deviation of relative cell viability of exposed cells vs. relative cell viability of control cells. (Viability of control cells is greater than 95% during the experiment.)

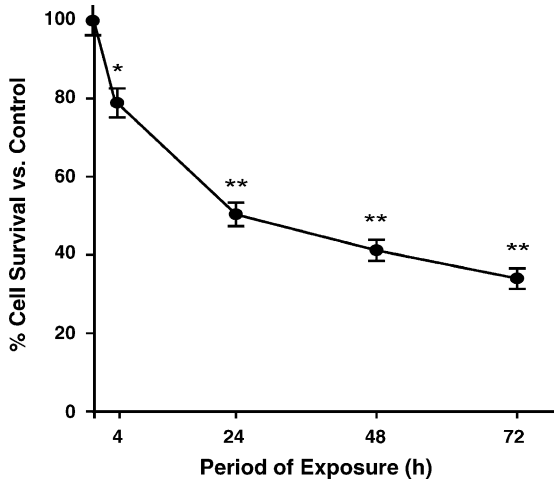

Fig. 6. Time course of toxicity (mean percent viability ± standard deviation) of cortical neuronal primary cell cultures following exposure to 0.1 μg ml^{-1} nigerlysin for 0, 4, 24, 48 and 72 h. Each data point represents relative cell viability of exposed cells vs. relative cell viability of control cells. (Viability of control cells is greater than 95% during the experiment.) Values marked with an asterisk or asterisks are significantly different from their corresponding controls ($^{*}p < 0.05$, $^{**}p < 0.001$).

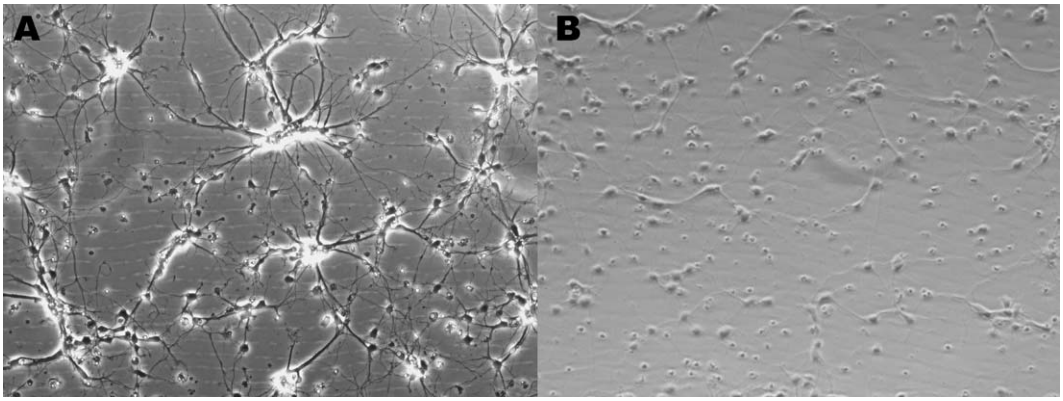

Fig. 7. Appearance of control cortical neuronal cells (A) and cells after exposure to 0.1 μg ml^{-1} nigerlysin for 72 h (B), approximate magnification 400×.

Survival at the lowest dose level, 0.025 μg ml^{-1}, was significantly lower than that of the control ($p = 0.005$). Survival further declined at 0.033 and 0.05 μg ml^{-1} compared to the next lower dose, $p < 0.0001$ and $p = 0.017$, respectively. The IC_{50} is estimated to be 0.037 μg ml^{-1}, or between 0.034 and 0.041 μg ml^{-1} at the 95% confidence level.

4. Discussion

Hemolysins lyse RBCs by creating pores or holes in red blood cell membranes resulting in the release of iron that promotes microbial growth (Bullen, 1981). Many bacterial hemolysins are critical virulence factors (Bhakdi et al., 1996; Cavalieri et al., 1984; Doran et al., 2002; Johnson et al., 1985; Ou Said et al., 1988) The hemolysin produced by *A. fumigatus* (asp-hemolysin) promotes aspergillosis (Ebina et al., 1982). Nigerlysin may also promote opportunistic infections, such as cerebral aspergillosis.

Invasion of the central nervous system by fungi can occur through an anatomically adjacent site like the nasal sinus (Young et al., 1970). Human nasal sinus is colonized by many indoor molds, including *A. niger* (Ponikau et al., 1999). Sinus colonization by *A. niger* may be accompanied by release of nigerlysin and could promote infections of many organs including the brain. Patients with cerebral aspergillosis present with symptoms like fever, altered mental status, headache, hemiplegia and seizures (Hagensee et al., 1994). If nigerlysin can cross the blood brain barrier, it may cause some neurological effects independent of the presence of the fungus in the brain.

In this study we have demonstrated the rapid loss in the viability of primary mouse cortical neuronal cells, after the in vitro exposure to nigerlysin (Fig. 6). The destruction begins quickly and after 24 h, approximately 50% of the cells were no longer viable compared to the controls. If such destruction occurs in humans, then some cognitive effects might be expected.

Human exposure to indoor environmental fungi purportedly causes cognitive dysfunction (Gordon et al., 2004). Since *A. niger* can colonize the human nasal sinuses and potentially produce nigerlysin there, nigerlysin has the potential to enter the central nervous system and damage neurons. Further work is required to determine the effects of nigerlysin on human neuronal cells but earlier work may already have provided some answers.

Speth et al. (2000) demonstrated that a "toxic factor" from *A. niger* filtrates killed cultured human neurons and glial cells. They described this toxic factor as a protein with a MW between 50 and 100 kDa, heat stable to 65 °C but labile at 85 °C, and toxic to neuronal cells. These characteristics match those of nigerlysin and we suggest that their "toxic factor" was probably nigerlysin. If so, then nigerlysin is a toxin similar to other known microbial toxins.

Since nigerlysin has an alpha helical structure, it may behave like the alpha helical bundle toxins, e.g., diphtheria toxin (MW 58 kDa), colicins (MW 60 kDa), δ-endotoxins (MW 70–135 kDa) and *Pseudomonas aeruginosa* exotoxin A (MW 67 kDa) (Parker and Pattus, 1993). The alpha helical structure of nigerlysin differs from the beta-sheet structure of many bacterial hemolysins, such as staphylococcal alpha toxin (Tobkes et al., 1985) and perfringolysin-O (Shimada et al., 1999). Alpha helical toxins damage susceptible cells by creating pores in membranes. It seems likely that nigerlysin has a similar mode of action. Therefore, the common occurrence of *A. niger* in the environment, along with

the toxic characteristics and the stability of nigerlysin, highlight a potential threat to human health.

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