

Research Paper

In vitro Protease Inhibition and Cytotoxicity of *Aspergillus fumigatus* Biomolecules Secreted under Long-Term Aerated Conditions

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Received: 2013.12.10; Accepted: 2014.07.01; Published: 2014.08.19

Abstract

The fatality rate of invasive aspergillosis (IA) is still very high, especially in prolonged and untreated pulmonary cases. *Aspergillus fumigatus* is the main causative agent of IA and investigation of its metabolites could provide valuable insight into virulence factor(s) associated with this organism. We evaluated the *A. fumigatus* culture filtrate (CF) products generated during short- and long-term aerated and non-aerated conditions and tested for (i) inhibition of cysteine or serine proteases and (ii) cytotoxicity. In addition, the mathematical model was determined using response surface methodology (RSM) to estimate the influence of different fermentation conditions on *A. fumigatus* CF characteristics, predict enzyme inhibition and make possible correlations with *in vivo* conditions. Biosynthesis of *A. fumigatus* low molecular weight proteinaceous products (from 6.4 to 15.4 kDa) was observed after 6 days of growth under aerated and alkaline conditions. Also, only these CFs showed significant reduction in cell lines survival (Caco-2 and WISH 35.6% and 54.6%, respectively). Obtained results provide solid starting point for further studies that would include: (i) detailed chemical characterization of *A. fumigatus* CF, (ii) activity relationships and *in vivo* correlation with pathogenicity of prolonged pulmonary IA and (iii) possible use of biomolecules as diagnostic or therapeutic markers.

Key words: *Aspergillus fumigatus*, culture filtrate, oxygen, cytotoxicity, cysteine protease inhibition

INTRODUCTION

There is a diverse set of illnesses caused by *Aspergillus*, ranging from chronic colonization, aspergilloma, invasive aspergillosis (IA) or hypersensitivity [1, 2]. *Aspergillus fumigatus* is the main causative agent of aspergillosis responsible for approximately 90% of all cases, including often chronic diseases such as IA and aspergilloma [3]. Lungs are the primary and the

most commonly affected organ making pulmonary IA a serious problem, especially in critically ill patients. The disease lasting longer than 10 days and without treatment is often fatal, especially in high risk and immunocompromised patients [2, 4]. However, aspergilloma is usually a non-invasive form, characterized by low local oxygen availability and it has better

outcome, especially after a surgical treatment [5].

A. fumigatus possesses a number of virulence factors, including metabolites secreted into the extracellular environment during mating that provide benefits for its growth [6]. More than 226 secreted compounds are detectable in *A. fumigatus* culture filtrate (CF), such as gliotoxin [7, 8] tryptacidin [9], fumigaclavin C, aurasperon C, helvolic acid, fumagilin, Asp-hemolysin and ribotoxins [10]. They might function as virulence factors, or their presence could give a competitive edge to the producing organism or enhance the survivability of spores [11].

A. fumigatus pathogenicity is quite complex [12]. It was shown that *A. fumigatus* excretes proteases or their inhibitors in later stages of infection which can lead to serious tissue damage [13]. Furthermore its pathogenic mechanisms are partially mediated through inhibition of host proteases [12]. Beside the fact that pathogen-derived protease inhibitors can significantly contribute to severity of an infection, the information about small molecule peptidase inhibitors of fungal origin is limited [14]. Previous *in vitro* studies demonstrated cytotoxicity of *A. fumigatus* CFs against macrophages [13, 15], human nasal epithelial [16] and human umbilical vein endothelial cells [17]. Anticancer activity of *A. fumigatus* metabolites [18, 19] and cytotoxicity of its spore-borne metabolite to A549 tumor lung cell line were also shown [9].

Many factors influence the production of *A. fumigatus* metabolites that are important for its pathogenicity, including aeration, temperature, pH level, duration of fermentation, exposure to light and medium composition [20, 21, 22]. However, *in vitro* conditions that promote production of low molecular weight molecules under long-term aerated conditions associated with biology effects have not been thoroughly elucidated. Therefore, the aim of the study was to investigate influence of O₂ tension, both under short and long-term fermentation on of *A. fumigatus* biomolecules production and their association with protease inhibition and cytotoxicity. In addition, a response surface methodology (RSM) was used to estimate the influence of different conditions on the *A. fumigatus* CFs characteristics, to predict enzyme activity and to possibly indicate correlations with *in vivo* conditions.

MATERIALS AND METHODS

Preparation of *A. fumigatus* CF samples

A. fumigatus ATCC 13073 strain was obtained from the American Type Culture Collection. It was grown for five days on Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, United Kingdom) at 30°C. After a five-day period of incubation, *A. fumigatus*

conidia were collected and resuspended in minimal essential medium (MEM) (Sigma Chemical Co., M4655, St. Louis, MO) at the concentration 1x10⁶ CFU/mL. MEM was used instead of complex and rich media, because it facilitates the detection of secreted products. Media with fetal calf serum (FCS) can mask small secreted molecules and make their detection impossible. Sixty milliliters of conidial suspension were put into 250-ml flasks, incubated under oxygen (20% O₂) and non-oxygen (0% O₂) conditions at 37°C with agitation at 140 rpm. Six different *A. fumigatus* CF samples were collected by centrifugation: (i) under aerated conditions on days 1, 3 and 6 (*A. fumigatus* CF AE-1; *A. fumigatus* CF AE-3; *A. fumigatus* CF AE-6, respectively) and (ii) under non-aerated conditions on days 1, 3 and 6 (*A. fumigatus* CF AN-1; *A. fumigatus* CF AN-3; *A. fumigatus* CF AN-6, respectively). Supernatants were filtered through a 0,22 µm Millipore filters and stored at -20°C until use.

Protein-biomolecules detection in *A. fumigatus* CF samples

A. fumigatus CFs protein concentration was determined by the Bradford method [23]. The *A. fumigatus* CF samples were concentrated 10-fold and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). An equal amount of proteins was loaded on gel in the volume of 20 µl to allow samples comparison. SDS-PAGE was performed with 75 g/L polyacrylamide gel under reducing conditions using standard protein markers (BioRad, France): aprotinin (6.5 kDa), lysozyme (14.5 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (BSA, 67 kDa), β-galactosidase (116 kDa) and myosin (200 kDa). Gels were stained with silver [24] and densitometry of stained gels was performed using Image J 1.45s software package.

A. fumigatus CF samples characterization

(i) Determination of *A. fumigatus* CFs pH was done by using the apparatus HI 9017 microprocessor pH meter (HANNA Instruments, Singapore);

(ii) Determination of *A. fumigatus* CFs effect on serine proteases: Trypsin-like proteolytic activity was determined toward low-molecular weight substrate N^α-benzoyl-arginine-p-nitroanilide (BAPNA, Merck), according to the definition that one unit hydrolyzes 1.0 µmol of BAPNA per minute at pH 6.2 at 25°C [25];

(iii) Determination of *A. fumigatus* CFs effect on cysteine proteases: Papain-like proteolytic activity was determined toward N^α-Benzoyl-L-Arginine Ethyl Ester (BAEE, Sigma Aldrich) according to the definition that one unit hydrolyzes 1.0 µmol of BAEE per minute at pH 6.2 at 25°C [25].

The effect of *A. fumigatus* CF samples on trypsin and papain like proteases, was investigated by adding 0.2 ml of CF samples in incubation mixture, and after incubation at 37°C for 15 min, the absorbance was monitored at 405 nm. Inhibition of proteases activity was expressed as the percentage of initial activity (mixture without *A. fumigatus* CFs), which was considered as 100% activity. Spectrophotometric measurements were done on Beckman DU-640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA).

Preparation of Caco-2 and WISH cell lines for cytotoxic assays

The cell lines were obtained from American Type Culture Collection. Both Caco-2 (human intestinal epithelial cell line) and WISH (contaminant of HeLa cervical cancer cells) are widely used and well validated, and they could represent general cytotoxicity with similar sensitivity as many other cell lines [26, 27]. Cells were cultured in MEM supplemented with 10% (v/v) FCS (GIBCO Laboratories, Paisley, Scotland) and grown in 80 cm² tissue culture flasks at 37°C and 5% CO₂ in a humidified atmosphere and routinely subcultured by trypsinization every 3-4 days.

Cytotoxic assay on Caco-2 and WISH cell lines with *A. fumigatus* CF samples

Caco-2 and WISH cells were seeded at initial density of 2×10^4 cells/well in flat-bottom 96-well tissue culture plates. Cells were cultivated together with series of two-fold dilutions of *A. fumigatus* CF samples (concentrations of 0.78; 1.56; 3.12; 6.25; 12.5; 25 and 50%) in quadruplicate in appropriate media (5% CO₂; 37°C; humidity). Cells without *A. fumigatus* CFs added were used as control. After 2 days, the cells were washed three times with phosphate buffered saline (PBS), and then fixed to the plates with 50 µl/well of 100% ethanol for 5 min at room temperature. Fixed cells were washed and stained with 1% crystal violet stain. Stained cells were washed, lysed and solubilized with 33.3% acetic acid solution. Optical density (OD) of developed purple color was read at 450 nm. OD was taken as index of proliferation of Caco-2 and WISH cells. OD values were expressed as inhibition rate (IR%) relative to the untreated control cells.

Mathematical model and statistical analysis

The RSM is a second-order polynomial equation, developed to correlate pH level or proteases inhibition with level of oxygen and duration of *A. fumigatus* fermentation. A design of experiments with three replications was employed.

The second-order polynomial model is presented by the following equation:

$$y = a_0 + a_1t + a_2O + a_{12}tO + a_{11}t^2 \quad \dots(1)$$

where (y) is the pH or inhibition of proteases, (O) is the oxygen level, (t) is the time, (a_i) are the parameters of equation (1) obtained using the multiple nonlinear regression method, (a_0) is the regression coefficient, and (a_i) (a_{ij}) (a_{ij}) ($i, j = 1, 2, 3, \dots$) are linear, quadratic and interactive regression coefficients, respectively. Computer software Design Expert 8.07 (Demo version) was used to perform the statistical analysis of the experimental data. The statistical significance of the models as well as the independent variables and their interactions were estimated by the analysis of variance (ANOVA). ANOVA was determined by Fisher test (F) and the performance of the developed models was statistically measured by the coefficient of determination (R). Student t test was used to analyze the data for cytotoxic assay. The statistical significance of the *A. fumigatus* CFs concentrations on the proliferation of Caco-2 and WISH cells was estimated by ANOVA. Result were considered statistically significant if $p \leq 0.05$.

RESULTS

Protein detection in *A. fumigatus* CF samples

Six *A. fumigatus* CF samples were obtained in different time points (24, 72 and 144 h) under aerated (20% O₂) (CF AE-1, CF AE-3, CF AE-6) and non-aerated (0% O₂) (CF AN-1, CF AN-3, CF AN-6) conditions and analyzed by SDS-PAGE. During the course of prolonged aerated fermentation, *A. fumigatus* generated several low weight proteinaceous biomolecules (peaks marked 1-7; Figure 1). Electropherogram and densitometric analysis showed a presence of 43.6 kDa protein in each sample (excluding *A. fumigatus* CF AN-1) (Figure 1). No significant difference was observed between proteinaceous products obtained during short fermentation of *A. fumigatus*, regardless of O₂ conditions (CF AE-1 and CF AN-1) (Figure 1a and 1e). However, several new products were detected in *A. fumigatus* CF AE-6 (Figure 1g) presented as the peaks marked with numbers 4-7, corresponding to molecular weight of 15.4; 9.3; 8.5; 6.45 kDa, respectively.

Change of pH value in *A. fumigatus* CF samples

Prior to inoculation of *A. fumigatus*, the pH of MEM broth was adjusted to pH 7.4. Under aerobic fermentation conditions the value increased to 8.57, 8.34 and 8.32 after 24 h, 72 h and 144 h, respectively. Comparing to this, under anaerobic conditions pH value changed to 8.31, 8.15 and 8.07 after 24 h, 72 h

and 144 h, respectively.

Inhibitory effect of *A. fumigatus* CF samples on trypsin and papain like proteinases

Inhibitory effect of six *A. fumigatus* CFs on trypsin and papain like proteinases was examined. Inhibitory effect of *A. fumigatus* CFs on papain like proteinases was significant ($p < 0.05$) and most pronounced when *A. fumigatus* CF-AE3 and *A. fumigatus* CF-AE6 were used (41.95 % and 43.21 %, respectively). No significant inhibitory effect was observed for trypsin like proteinases.

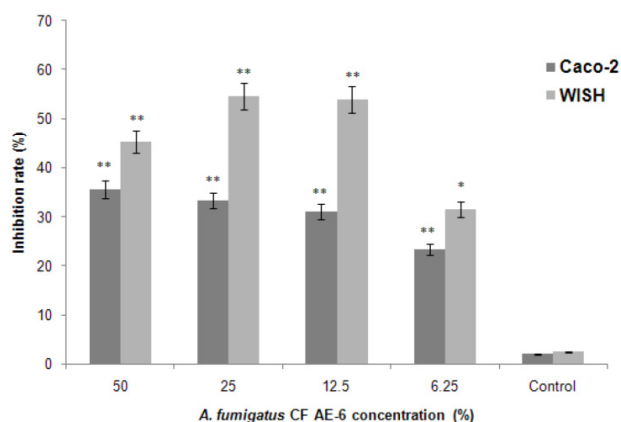


Figure 2. Long-term aerated conditions influence biomolecule generation in *A. fumigatus* CF AE-6 sample which exposed a statistically significant inhibition rate (IR, %) on Caco-2 and WISH cell line proliferation, comparing to cell IR in the media only (t-test; * $p < 0.05$; ** $p < 0.01$). Assays were performed in triplicate and the data represent mean value of IR \pm SD.

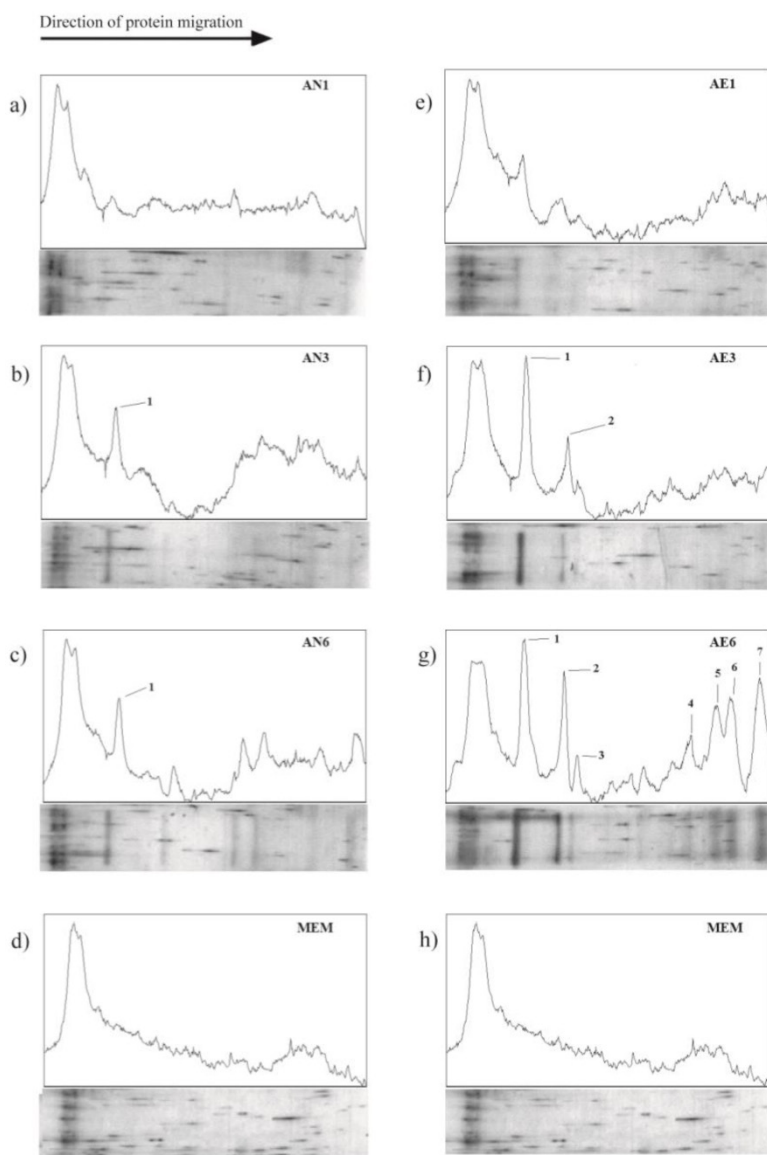


Figure 1. Electropherogram and densitogram of the products detected in *A. fumigatus* CFs obtained in non-aerated conditions (0% O₂) after 24 h (AN-1; a), 72 h (AN-3; b) and 144 h (AN-6; c) and in aerated conditions (20% O₂) after 24 h (AE-1; e), 72 h (AE-3; f) and 144 h (AE-6; g). Protein traces are presented as the peaks marked with numbers 1-7, corresponding to molecular weight of 43.6, 33.8, 31.2, 15.4, 9.3, 8.5 6.45 kDa, respectively.

Cytotoxicity of *A. fumigatus* CF samples on Caco-2 and WISH cell lines

Among all the six *A. fumigatus* CFs tested only *A. fumigatus* CF AE-6 exerted significant cytotoxic effect on Caco-2 ($p < 0.05$) and WISH ($p < 0.01$) cell lines (data not shown). Results were expressed as percentage of inhibition rate (IR%) on cell lines proliferation in comparison with cell proliferation in media alone (Figure 2). However, IR higher than 50% was demonstrated only for the WISH cell line and for CF AE-6 concentrations 25% and 12.5% (Figure 2), while the lower IR was registered for Caco-2 cell line ($p < 0.05$) for the same extract.

RSM correlation between *A. fumigatus* CFs pH value and proteases inhibition with oxygen saturation and fermentation time

The obtained values of pH and inhibition of proteases were analyzed by RSM applying the multiple regression method to fit the second-order polynomial equation (equation 1). The statistical significance of the model equation, individual factors and their interactions were analyzed by the ANOVA. *F*- and *p*-values for the fitted models indicated that they were statistically significant at the 95% confidence level, suggesting that this function is appropriate for fitting the data for both pH and inhibition of tested enzymes (Table 1).

Table 1. The results of ANOVA for RSM: pH, inhibition of cysteine and serine proteases

	Source of variance	Suma of Squares	De-grees of freedom	Mean Square	F-Value	p-value
pH	Model	1.038884	4	0.259721	11.72892	0.0002*
	<i>t</i>	0.007789	1	0.007789	0.351748	0.5626
	<i>O</i>	0.815504	1	0.815504	36.82794	< 0.0001*
	<i>tO</i>	0.149564	1	0.149564	6.754272	0.0210*
	<i>t²</i>	0.032836	1	0.032836	1.482849	0.2435
	Residual	0.310011	14	0.022144		
	Cor Total	1.348895	18			
Inhibition of cysteine proteases	Model	3751.416	4	937.8541	58.90252	< 0.0001*
	<i>t</i>	1739.072	1	1739.072	109.2235	< 0.0001*
	<i>O</i>	1365.446	1	1365.446	85.75772	< 0.0001*
	<i>tO</i>	247.9979	1	247.9979	15.57567	0.0015*
	<i>t²</i>	346.0738	1	346.0738	21.73538	0.0004*
	Residual	222.91	14	15.92214		
	Cor Total	3974.326	18			
Inhibition of serine proteases	Model	221.7724	4	55.44311	19.11203	< 0.0001*
	<i>t</i>	0.480761	1	0.480761	0.165725	0.6901
	<i>O</i>	6.261603	1	6.261603	2.158464	0.1639
	<i>tO</i>	0.057737	1	0.057737	0.019903	0.8898
	<i>t²</i>	170.4013	1	170.4013	58.73975	< 0.0001*
	Residual	40.61334	14	2.900953		
	Cor Total	262.3858	18			

*Statistically significant at the confidence level of 95%.

Table 2. RSM - the values of parameters of the second-order polynomial equation

Term of equation	pH	Inhibition of cysteine proteases	Inhibition of serine proteases
Intercept	7.78	-3.33	4.20
<i>t</i>	5.49x10 ⁻³	0.48	0.24
<i>O</i>	3.39x10 ⁻²	0.34	-6.63x10 ⁻²
<i>tO</i>	-1.80x10 ⁻⁴	7.23x10 ⁻³	1.10x10 ⁻⁴
<i>t²</i>	-2.50x10 ⁻⁵	-2.32x10 ⁻²	-1.63x10 ⁻³
R	0.877	0.972	0.919
R-adj	0.850	0.963	0.894

The level of oxygen and two-way interaction was statistically significant for pH values (Table 1). Furthermore, *p*-values of model terms showed that independent variables (time and the level of oxygen), their interaction and the time squared significantly affected the inhibition of cysteine protease, while only terms the time squared was statistically significant for the inhibition of serine proteases (Table 1). Values of the parameters of Equation (1) are given in Table 2. The value of R and the adjusted R showed a good correlation between the observed values and the predicted ones (Table 2).

The RSM curves were plotted to determine the optimum pH value and optimum inhibition of cysteine and serine proteases. At lower oxygen level, the pH value reached a plateau during long-term fermentation, while at higher oxygen level pH value reached its maximum and then slightly decreased in the later period of time (Figure 3). Inhibition of cys-

teine proteases was found to increase rapidly at the beginning but at slower rate toward the end. Also as the time increased, the inhibition of these proteases increased in parallel, especially at the highest oxygen level (Figure 4a). With time the inhibition of serine proteases increased, especially at the lowest oxygen level, and decreased in the later period of the time (Figure 4b). Within the ranges of the extraction variables applied for development of the RSM, the maximum pH value (8.5) was predicted after 42 h (20% O₂) (Figure 3) and the maximum inhibition of cysteine (45.6%) and serine proteases (12.8%) was predicted after 135 h (20% O₂) and 42 h (0% O₂), respectively (Figure 4).

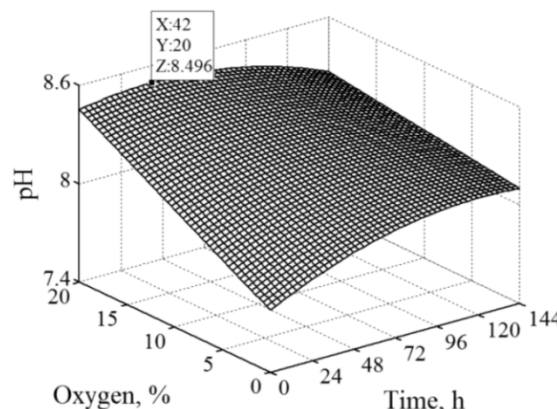


Figure 3. The response surface for pH as a function of time and the level of oxygen by RSM. Maximum values: time (X), level of oxygen (Y) and pH (Z).

DISCUSSION

Up to now, it was showed that *A. fumigatus* soluble metabolite profiles depended on different factors such as pH levels, temperature, light, substrates and media used (20, 21, 22). Our results clearly demonstrated the importance of time and aeration for *A. fumigatus* metabolite synthesis and their cytotoxicity. The quantity and diversity of *A. fumigatus* extracellular low molecular weight biomolecules increase with time and aeration (Figure 1g). We confirmed the significance of our finding by using a mathematical model which corroborated the importance of aeration on pH value. The model suggested that pH remained alkaline, regardless of the presence of low molecular weight proteins which were secreted in aerated conditions. Many studies reported that *A. fumigatus* produces different active alkaline proteases during submerged fermentation (28, 29, 30), which is consistent with our data. Our data demonstrated the change of *A. fumigatus* CFs protease inhibition profile based on different conditions. Model predicted that the maximum inhibition cysteine protease was 3.6 times higher than inhibition of serine protease (Figure

4), suggesting that long-term aerated conditions have an influence on generation of biomolecules with inhibitory activity on cysteine proteases. These metabolites are potential compounds capable of reacting with free thiol groups and consequently inhibiting different classes of enzymes with functional thiol group at their active sites. In this study we demonstrated some *in vitro* activity which may be associated with *A. fumigatus* protease or protease inhibitors that can be significant during infection and may interplay with the host cells. Importance of proteases and/or proteases inhibitors resulted in formation of database that contains information for about 2000 proteases and 400 inhibitors [31].

Having in mind the importance of microbial protease and/or protease inhibitors for host cells cytotoxicity, we screened *A. fumigatus* CFs samples for cytotoxic effect against Caco-2 and WISH cell lines. Our data demonstrated significant inhibition of cell proliferation when extracts after prolonged aerated conditions were used (*A. fumigatus* CF AE6) (Figure 2). It is shown that *A. fumigatus* CF expressed a high cytotoxicity on murine macrophages under highly aerated conditions [32]. However, in our study cytotoxic effect was expressed by CFs obtained on day 6 and condition with 20% O₂, while other study showed cytotoxicity with *A. fumigatus* CF obtained after 15 h [32]. The samples obtained during short-term fermentation (day 1 and 3) did not express a significant cytotoxicity in this study. It may be concluded that biomolecules produced under long-term aerated conditions corresponding to molecular weight of 15.4; 9.3; 8.5 and 6.45 kDa are at least partially responsible for expressed cytotoxicity. This is in correlation with the fact that biosynthesis of low weight biomolecules is complex and they are often produced late in fungal development [11]. This is known for oxylipins which regulate *Aspergillus* development and mycotoxin

production, therefore all this indicates that observed small biomolecules can be further investigated as *Aspergillus* quorum sensing molecules [33].

The oxygen availability during fungal pathogenesis may play a critical role in the outcome of infection from the perspective of both the host and the fungus [34]. It can be suggested that the ability of *A. fumigatus* to adapt to long-term aerated conditions can be significant mechanism for virulence factors generation during infection. These finding may prove to be clinically beneficial, indicating that manipulation of oxygen (and/or CO₂) levels at sites of human fungal infection may be a promising therapeutic approach [35]. It is also known that ventilator-associated pneumonia is a common hospital infection, especially in intensive care units (ICU) patients. Study showed that patients with pulmonary IA and high mortality rate had a longer ICU stay and a more extended period of mechanical ventilator dependency [36]. Among numerous airborne molds, *A. fumigatus* is the most common agent responsible for causing diseases in critically ill mechanically ventilated patients [37] highlighting the most important finding of this study related to biological activity of *A. fumigatus* samples obtained under long-term aerated experimental conditions, similar to the lungs of the mechanically ventilated patient without applied therapy. *A. fumigatus* metabolite production is often strain-specific and may tolerate different oxygen levels so further study can be improved by testing the influence of different oxygen concentrations and more fungal strains (including clinical isolates) in the same way. Also, the onset and the outcomes of *A. fumigatus* infection depend on exogenous and endogenous conditions and their interaction [2], so the animal models will prove significant contribution to further investigations of described biomolecules [38, 39].

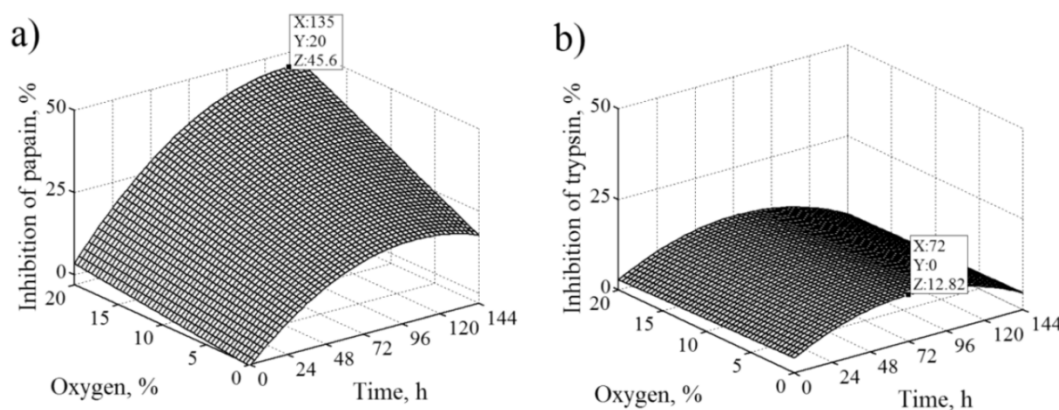


Figure 4. The response surface for inhibition of cysteine proteases as a function of the time and the level of oxygen (a) by RSM; Response surface for inhibition of serine proteases as a function of the time and the level of oxygen (b) by RSM. Maximum values: time (X), level of oxygen (Y) and inhibition of proteases (Z).

In conclusion, the genus *Aspergillus* represented with *A. fumigatus* is a threat to public health because is associated with high mortality rate in high risk patients, especially in those with cystic fibrosis, asthma, chronic obstructive lung disease and/or immunosuppression [3]. Here we demonstrated that *A. fumigatus* excretes low molecular weight biomolecules in positive correlation with time and oxygenation associated with inhibition of cysteine proteases and cytotoxicity against WISH and Caco-2 cell lines. These *in vitro* findings may support a presumption that during *in vivo* conditions similar products are expressed and may act as virulence factors important for *A. fumigatus* pathogenicity and severity of diseases, which can contribute to better understanding of these diseases. Further investigations are needed to select, research and analyze low molecule weight biomolecules produced by *A. fumigatus* under long-term aerated conditions, including their examination as virulence factors, diagnostic biomarkers or therapeutic targets.

ACKNOWLEDGMENT

This study was supported by Ministry of Education, Science and Technological development Republic of Serbia (grants no 175034 and 175033) and by the Ministry of Education Science and Technology Republic of Slovenia – Scientific and Educational Foundation of Republic of Slovenia (*Ad Futura*). We thank Jasmina Nikodinovic-Runic, PhD (Institute of Molecular Genetics and Genetic Engineering, Belgrade, Serbia) for manuscript review and English correction.

CONFLICT OF INTEREST

V. A. A. has served as a consultant for Pfizer and MSD and has served on the speakers' bureaus of Pfizer, MSD and Providens and received grant support from Pfizer. All other authors: no potential conflicts of interest.

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