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Antifungal Activity of Novel Hydrophobin from an Alkaliphilic Fungus *Sodiomyces alkalinus* †

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Abstact: *Sodiomyces* genus is obligate alkaliphilic ascomycetous fungi of the Plectosphaerellaceae family (Grum-Grzhimaylo et al., 2016). They prefer habitats with alkaline conditions (pH ≥ 10) such as soda salterns and the edge of the soda lakes. Such habitats are located all over the world, although, as a rule, they are small in area. In addition to pH stress, organisms, inhabiting in soda soils, have to survive under fluctuating osmotic and temperature conditions, that vary drastically with a change of drought and rain, heat and freezing. Studies of the antimicrobial activity of alkaline fungi are rare. The prime purpose of this study was to isolate and identify antimicrobial compound from an alkaliphilic fungus *Sodiomyces alkalinus.* According to previously data, high level antimicrobial culture broth was obtained for three strains of *S. alkalinus*. Analytical separation by reversed-phase HPLC for the ethyl acetate extract was carried out according to the data described earlier. It has been suggested that the molecule studied is typical to be a polypeptide; there by its initial structure analysis was conducted by automated Edman sequencing. Searching for potential homologies amongst NCBI databases (https://www.ncbi.nlm.nih.gov/) using BLASTP algorithm led to complete matching with fungal hydrophobin F11 (GenBank: ROT36721.1/NCBI Reference Sequence: XP_028464527.1). This data was previously obtained based on whole genome of *S. alkalinus* F11 typical strain. The antimicrobial activity of the novel antifungal agent hydrophobin from *S. alkalinus* was studied *in vitro*. Strong antifungal effect was against pathogenic and opportunistic fungi strains and MIC was determined. The protein showed growth inhibitory activity of filamentous and yeast fungi. The activity of hydrophobin Sa-HB1 against *Aspergillu*s spp. was comparable to reference polyene cyclic antibiotic Amphotericin B. The inhibition zones for all clinical *Candida* isolates were found to be 12-14 mm, while *C. krusei*, *C. tropicalis* were inactive to Amphotericin B. As a result, the antimicrobial compound from *S. alkalinus* is hydrophobin. It has high antifungal activity comparable to reference antibiotics. The hydrophobin Sa-HB1 responsible for the reported antifungal activity of *S. alkalinus*, and may serve as a potential source of lead compounds that can be developed as antifungal drug candidate.

Keywords: alkaliphilic fungi; antifungal peptide; *Sodiomyces alkalinus*; hydrophobin

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1. Introduction

Alkaliphiles are extremophiles typically grow well at high pH (9 to 11) [1,2]. Soda lakes, which are defined as alkaline environments, are important habitats for alkaliphilic

fungi. Fungi *Sodiomyces* genus were isolated from habitats with alkaline conditions (pH ≥ 10) such as soda salterns and the edge of the soda lakes of Russia, Mongolia, Kenya, Tanzania. All species of this genus (*S. tronii*, *S. magadii*, and *S. alkalinus*) are obligate alkaliphiles [3,4]. The demand for novel and improved medicine from biological sources to cater to the biopharmaceutical sector has increased significantly in recent years. Among the alkaliphilic microorganisms, fungi show a great potency to produce antibiotic due to their survival strategy in extreme environmental conditions. Screening of metabolites of eight obligate alkaliphilic strains of *Sodiomyces alkalinus* obtained from soda soils has revealed antifungal activity against different fungal taxons, including human pathogenic isolates [5]. In this study, we focus on the purification, identification, and antifungal activity of novel biomolecule that have been identified as hydrophobin and denominated Sa-HFB1.

2. Materials and Methods

The objects of study were two alkaliphilic strains of *S. alkalinus* Grum-Grzhimaylo, Debets & Bilanenko (Plectosphaerellaceae, Glomerellales, Hypocreomycetidae, Sordariomycetes, Pezizomycotina, Ascomycota): ex-type strain F11 (=CBS 110278) [4–7] and strain 8KS17-10 (=SLF 0117.0810). The strain 8KS17-10 was identified as *S. alkalinus*, based on morphological features and on ITS region sequence comparison with the GenBank database (NCBI ID ON146325). Fungi were cultivated according to the previous protocol on a special alkaline medium with the following composition (g/L): Na_2CO_3, 24; $NaHCO_3$, 12; NaCl, 6; KNO_3, 1; K_2HPO_4, 1; malt extract (15^0Balling), 200 mL; yeast extract, 1; and distilled water, 800 mL. The culture liquid (CL) was separated by filtration through membrane filters on a Seitz funnel under a vacuum. To isolate the antibiotic substances, the CL of the producers was extracted three times with ethyl acetate in an organic solvent/CL ratio of 1:5. The obtained extracts were evaporated in a vacuum on a Rotavapor rotary evaporator (Buchi, Flawil, Switzerland) to dryness at 42 °C, the residue was dissolved in aqueous 70% ethanol, and the alcohol concentrates were obtained.

Further separation was carried out via analytical reversed-phase high-performance liquid chromatography (RP-HPLC) with an XBridge 5 μm 130 A column with a size of 250 × 4.6 mm (Waters, Ireland) in a growing linear gradient of the acetonitrile concentration as a mobile phase (eluent A, 0.1% trifluoroacetic acid (TFA) (HPLC, Sigma-Aldrich, Darmstadt, Germany) (in water MQ; eluent B, 80% acetonitrile in 0.1% aqueous TFA) at a flow rate of 950 L/min. Ultragradient acetonitrile (Panreac, Barcelona, Spain) and TFA (HPLC, Sigma-Aldrich, Darmstadt, Germany) were used for RP-HPLC. The substances to be separated were determined at the wave-length of 214 nm in the concentration gradient of eluent B: 16–28% in 12 min; 28–55% in 27 min; 55–75% in 20 min; and 75–85% in 10 min. This was followed by isocratic elution for 25 min. Re-chromatography of an active compound was made using a Jupiter C5 4.6 x 250 mm analytical HPLC column (Phenomenex, Torrance, CA, USA) at the same gradient conditions and a flow rate of 1 mL/min. The absorbance (D) was determined at a wavelength of 214 nm and a mobile phase flow rate of 4 mL/min. The fractions obtained during RP-HPLC, which correspond to individual peaks, were collected manually, and the excess of organic solvent (acetonitrile) was then removed via evaporation in a SpeedVac vacuum centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) and lyophilized (Labconco, Kansas, MO, USA) to remove residual amounts of TFA. The spectrum of antimicrobial action of the substances contained in the fractions was determined via disk diffusion test. An aliquot of peptides solution (1 μL) obtained with a trypsinolisys employment was applied on the steal target where it was mixed up with 0.3 μL of 2,5-dexidroxobenzoic acid (Sigma-Aldrich, Darmstadt, Germany) in solution of 20 mg/mL 20% acetonitrile (Sigma-Aldrich, Darmstadt, Germany) in 0,5% TFA (Sigma-Aldrich, Darmstadt, Germany).

Analysis was performed on a MALDI-time of flight (ToF)-ToF mass spectrometer (Ultraflex II Bruker, Bremen, Germany), equipped with a neodymium-doped (Nd) laser.

The $[M+H]^+$ molecular ions were measured in reflector mode; the accuracy of mass peak measurement was within 0.005%. Identification of proteins was carried out by a peptide fingerprint search using Mascot software through the NCBL mammalian protein database with the indicated accuracy. The search allowed for possible oxidation of methionine by environmental oxygen and modification of cysteine with acrylamide, and where a score was >71, protein matches were considered significant (pb0.05).

The antifungal activity was measured by the disc-diffusion method. Discs of 6 mm diameter containing 40 μL of sample were deposited on PDA agar plates (Sigma-Aldrich, St. Louis, MO, USA). The spectrum of antifungal activity of the CL, extracts, and individual compounds was determined on test cultures of mycelial and yeast fungi from the collection of cultures of the Gause Institute of New Antibiotics (Moscow, Russia). Opportunistic mold and yeast test cultures of the fungal species *Aspergillus fumigatus* KPB F-37, *A. niger* INA 00760, and *Candida albicans* ATCC 2091 were used as tests. The diameter of the inhibition zones was measured after 24 h at 28 °C. The sensitivity of the test organism was controlled with standard discs containing amphotericin B (AmpB), itraconazole (IZ), fluconazole (FZ) and voriconazole (VOR) (40 μg/disk) were used as a positive control.The spectrum of antifungal action was also evaluated on clinical isolates of molds and yeasts—pathogens of opportunistic pneumomycosis of the bronchi and lungs—in tuberculosis patients with multiresistance to the antibiotics-azoles used in clinical practice. The MIC was determined after cultivation at 37 °C at 24 h for the yeast fungi *Candida albicans* ATCC 2091, *C. albicans* 1582m, *Cryptococcus neoformans* 297m at 48 h for the molds *Aspergillus niger* INA 00760, *A. fumigatus* KPB F-37, *A. fumigatus* 390m. The MIC was defined as the minimum concentration of a substance that completely suppresses the growth of the test culture [8,9].

3. Results

The production **Sa-HFB1** from the culture liquid and mycelium extracts of *S. alkalinus* **8KS17-10** strain was achieved in a period of fermentation in 14 days about 25.54 ± 1.4 mg/L. To purify compound for further analysis we made three single-type chromatographic separations, and the target peak was collected manually (Figure 1). Known compounds were removed from further analysis at this step, leaving only compound with a high probability of novelty for further structural characterization.

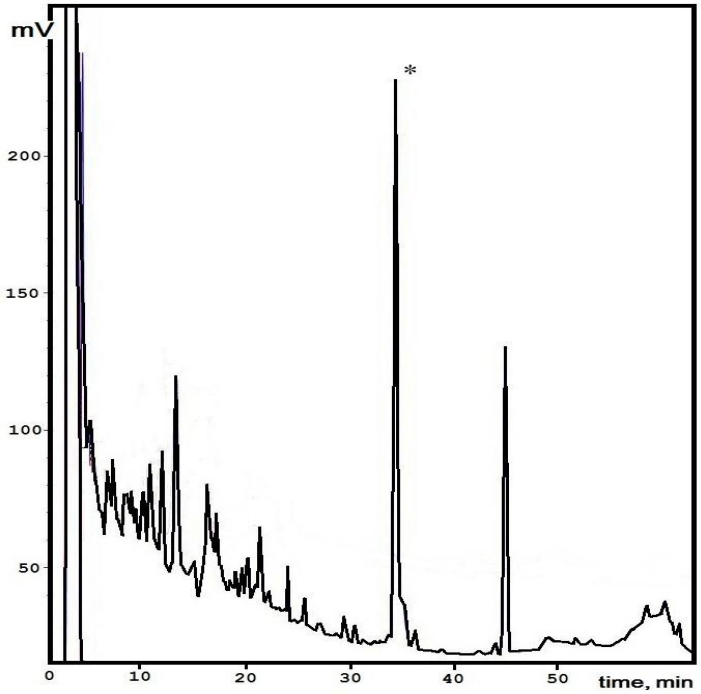

Figure 1. Isolation of Sa-HFB1 from *S. alkalinus* ethyl acetate concentrate. The target peak is signed.

Individual fraction was analyzed with MALDI-TOF Ms method to obtain information about masses, consisted in matrix. The result of mass-spectrometry is depicted in Figure 2a. Several peaks on the specter were received, but only the major one with a mass of 7588 Da ($[M+H]^+$) was of interest tous. One of the rest peaks with a mass of 3794 Da is a binary ionized form of the major peak ($[M+H]^{++}$] and the others were considered to be any homologues of hydrophobin.

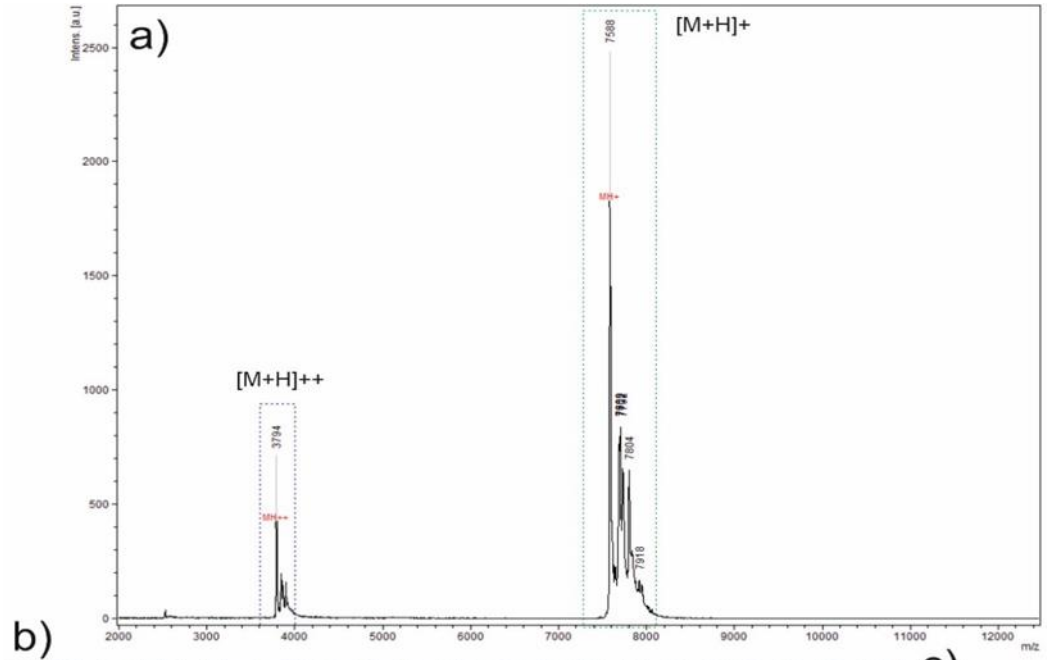

Figure 2. Analysis of probe with MALDI-TOF Ms method. (**a**) The specter with two fields of peaks is presented. The main peak with a mass of 7588 Da is highlighted with others in one set in dotted rectangle colored in green and another set of peaks with the main peak mass of 3794 Da is highlighted with blue dotted rectangle. Symbols $[M+H]^+$ and $[M+H]^{++}$ mean the masses of molecular ions plus proton, the single charged and double charge ions respectively. (**b**) The table, including information about molecular mass differences between each of nature amino acid; results are presented in Da units and the goal 14 Da meaning between corresponding amino acids are colored in red. (**c**) The set of possible amino acid single changes in hydrophobin, calculated over the molecular masses, is presented.

The complete matchup for the N-terminal sequence of the isolated peptide with *S. alkalinus* fungal hydrophobin F11 allowed us to specify that the fifth amino acid residue is cysteine which can be visualized as a PTH-s-beta-4-pyridylethylated derivative. The isolated polypeptide is found and denominated Sa-HFB1. Hence, the complete amino acid sequence for precursor of *S. alkalinus* the class II hydrophobin F11 (NCBI ID XP_028464527.1) [19] is [1]MKFIAVVAALTASLAMAAPTESSTDTTYIA**CPISLYGNAQCCATDILGLANLD-CESPTDVPRDAGHFQRTCADVGKRARCCAIPVLGQALLCIQP**AGAN[99]. Based on NCBI annotation data for ID XP_028464527.1 the current protein-precursor has a

calculated molecular mass of 10181.6 Da, residues 1-30 are represented a signal peptide, whereas residues 31-95 form a mature hydrophobin sequence (marked in bold), at that primary peptide bond is cleaved located between Tre26-Tre27. This allowed the identification of 1 new hydrophobin of Class II denominated Sa-HFB1. Thus, Sa-HFB1 is typical with up to five additional amino acid residues at the N-terminus. For identification of any possible homologous for the complete hydrophobin sequence in genome NCBI ID PRJNA196044 [7], we used a local blast. As a result of the search, only one gene was found corresponding to hydrophobin (located in the genomic scaffold SODALscaffold8). Further search in the transcriptional data confirmed this fact (according to scaffold SODALDRAFT_334916, NCBI ID XM_028612330.1).

As was shown the mass difference between annotated hydrophobin with a mass of 7609 Da (with disulfide bonds formation—7601 Da) and 7588 Da, presented at the specter. For analysis, the table with molecular mass differences between different nature amino acids was created and the goal mass of 14 Da was discovered and colored in red in Figure 2b. It gave us an opportunity to propose a set of appropriate amino acid changes, presented in Figure 2c.

The antifungal activity of Sa-HFB1 against multi drug resistant clinical isolates is shown in Table 1. The clinical pathogenic isolates *Cryptococcus neoformans*, *Candida krusei*, and *C. tropicalis* were selected based on their resistance phenotype, including resistance to caspofungin, micafungin, fluconazole and flucytosine. The antifungal AmpB, FZ, and VOR were used as reference drugs. Sa–HFB1 inhibits opportunistic and clinical isolates with the broth twofold microdilution method. The activity of Sa-HFB1 against *Aspergilllus* spp. showed potent growth inhibition which are comparable to reference standards. The inhibition zones for all clinical *Candida* isolates were found to be 12–14 mm for Sa-HFB1, while *C. krusei* 1447m, *C. tropicalis* 156m were inactive to AmpB, FZ, and VOR respectively.

Table 1. The activity of purified Sa–HFB1 on growth of clinical pathogenic fungi isolates (mm) measured by disc-diffusion assays.

Zone, mm				
Compound, 40 mg/mL				
Strain	**Sa-HFB1**	**AmpB**	**FZ**	**VOR**
Candida albicans 1582m	18 ± 0.1	10 ± 0.6	0	10 ± 0.6
C. glabrata 1402m	16 ± 0.3	15 ± 0.1	0	10 ± 0.6
C. krusei 1447m	12.5 ± 0.2	0	0	0
C. tropicalis 156m	14 ± 0.1	0	0	0
C. parapsilosis 571m	14 ± 0.2	18 ±0.3	0	0
Cryptococcus neoformans 297m	30 ± 0.1	18 ± 0.6	0	0
Aspergillus fumigatus 390m	12 ± 0.5	9 ± 0.6	0	0
A. niger 219	14 ± 0.1	15 ± 0.8	0	11 ± 0.6

The MICs of Sa-HFB1 against opportunistic and clinical fungi ranged from 0.25 to 2 μM/mL and confirmed the higher activity of against both opportunistic and clinical isolates that has been previously reported (Table 2). The highest level of antifungal activity (MIC 0.25) was demonstrated for *Cryptococcus neoformans* 297m.

Table 2. Minimal Inhibitory Concentration of Sa-HFB1 compared with target antifungal drugs.

Minimal Inhibitory Concentration (MIC, μM)				
Compound				
Strain	**Sa-HFB1**	**AmpB**	**FZ**	**IZ**
Aspergillus niger INA 00760	1	-*	-	-
A. fumigatus KPB F-37	1	-	-	-

A. fumigatus 390m	1	1	106.7	1.4
Candida albicans ATCC 2091	2	-	-	-
C. albicans 1582m	2	2	213.3	1.4
Cryptococcus neoformans 297m	0.25			

* non tested.

4. Conclusions

Hydrophobins (HFBs), small proteins found only in filamentous fungi (Dikarya) and reported to be some of the most surface-active proteins in nature [10–14]. HFBs are characterized by their amphipathic nature at hydrophilic/hydrophobic interfaces are currently attracting great interest from the biotechnology industry. Among biocontrol fungi, some studies revealed that the participation of HFBs in biocontrol processes could contribute to plant growth and elicit plant defense reactions. *T. asperellum* mutants that lack the hydrophobin gene TasHyd1 are severely impaired in terms of root attachment and colonization, and these phenotypes are recovered by complementation of TasHyd1, indicating that this protein contributes to the interactions between *Trichoderma* and its host plant [12–15]. In *Clonostachys rosea*, the class II hydrophobin Hyd3 can influence the root colonization ability [13]. Similarly, the class II hydrophobin HYTLO1 in *T. longibrachiatum* can directly inhibit both spore germination and hyphal elongation of *Botrytis cinerea* and *Alternaria alternata in vitro*, and enhance tomato plantlet development HYTLO1, which has multiple roles and effects on treated plants, was able to trigger a nicotinic acid adenine dinucleotide phosphate-mediated Ca2+ signaling pathway in *Lotus japonicus*, highlighting a possible mechanism underlying its action. Zhang et al. demonstrated biocontrol functions of the class II hydrophobin HFB2-6 of *T. asperellum* ACCC30536 [14–18]. The hydrophobin gene HFB2-6 was expressed in *Escherichia coli* and the recombinant protein rHFB2-6 was found to affect the transcription of poplar defense-related genes. HFBII-4 from *T. asperellum* can enhance the resistance of *Populus davidiana* × *P. alba* var. *pyramidalis* to phytopathogenic fungi *A. alternata*. In summary, the class II hydrophobin gene HFBII-4 upregulated the expression of growth-related, disease resistance and defense-response genes in PdPap poplar [19].

Sodiomyces species are fundamentally different from alkalitolerant ones in terms of mechanisms of adaptation. Life cycle of *S. alkalinus* and some possible mechanisms of its adaptation to high pH combined with salinization have been investigated recently at cytomorphological level [3,4,6], while the biochemical mechanisms have not been yet studied sufficiently. Our experiments to evaluate antifungal activity and identification of active hydrophobin might offer a clue as to the possible ecological role and biological activity of alkaliphilic *S. alkalinus* in alkaline soils. The structure, MALDI Ms|Ms and NCBI annotation data analysis helped to identify Sa-HFB1 as hydrophobin II class. Hydrophobin gene may have SNPs, resulting in different amino acid sequences in synthesized HFBs of different strains.

The Sa-HFB1 production yield was achieved in a period of fermentation in 14 days about 25.54 ± 1.4 mg/L. This value has been found to be typical of Class II HFBs secreted by wild type fungi into the extracellular medium [14–20]. Previous research has reported the amount of HFBII of wild type *T. reesei* around 30 mg/L and around 200 mg/L with genetically engineered *T. reesei* [16,20]. It is the first time that *S. alkalinus* was explored for isolation of HFBs. Afterward, extraction methods have been applied to isolate HFBs of Class II from culture liquid. This allowed the identification of 1 new hydrophobin of Class II denominated Sa-HFB1 with promising properties for biotechnological applications. Due to the strong antifungal property exhibited by compound, it has been evaluated to further the antifungal activity against pathogenic fungi. This is the first report of the in

vitro antifungal activity of HFBs of Class II from any filamentous fungi, especially from alkaliphilic fungi.

The hydrophobin Sa-HFB1, produced by *S. alkalinus* 8KS17-10 strain, was isolated, purified chromatographically, and sequenced using a trypsinolisys assay with the further MALDI-TOF Ms and Ms|Ms methods. As a result, we report about convergence of amino acid between experimental and annotated sequence, obtained by full genome sequencing of another strain of *S. alkalinus* F11, to within one amino acid in 29 position. Thus, we declare more concretely about glutamic acid change to aspartic acid in 29 position of protein (E29D) and that this region has a low level of residues conservation, so amino acids can differ there from strain to strain.

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Conflicts of Interest: The author declares no conflict of interest

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