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Antimicrobial Potential of Alkalophilic Micromycetes *Emericellopsis alkalina*

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Abstract—The ability of alkalophilic micromycetes of the *Emericellopsis alkalina* to produce antimicrobial peptides has been studied. Evaluation of the spectrum and the yield of antibiotic compounds has allowed us to choose a promising producer of peptide antimycotics, *Emericellopsis alkalina* A118. The producer exhibits antifungal activity against conditionally pathogenic yeast and mold fungi, i.e., *Candida albicans*, *Aspergillus niger,* and *A. fumigatus*. The group of homologous active compounds isolated by the set of identified structural features (molecular weight, the ratio of the absorption at certain wavelengths, and the absence of initiation of Edman sequencing) may be attributed to peptaibols, which are a group of nonribosomal membrane-active antimicrobial peptides with a specificity of action primarily against fungi-micromycetes.

Keywords: antimicrobial peptides, micromycetes of *Emericellopsis* spp., peptaibols
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INTRODUCTION

Despite a significant decrease in the number of antibiotics from microbial producers that are currently used in clinical practice, the search for novel natural compounds has not lost its relevance [1, 2]. The main hopes for the discovery of unique biologically active substances are associated with producers isolated from unusual natural niches, i.e., seas, rivers, hot and salt springs, etc. Within the world screening programs, more than 20000 biologically active compounds from extremophilic organisms, including antimicrobial membrane-active peptides, were described in the last 10–12 years in different laboratories [3, 4]. Interest in these compounds is due to their rather high specificity, structural and functional diversity, and the absence of the known mechanisms of the emergence of resistance. Therefore, this group of substances is promising for the obtainment of antibiotics effective against polyresistant microorganisms [5–7]. The greatest number of antimicrobial peptides are produced by mycelial fungi from the Ascomycota, primarily the Hypocreales. Currently, about 700 peptaibiotic sequences are described for 18 ascomycete genera [8, 9]. The most promising taxons among them for searching novel antimicrobial peptides are members of the *Emericellopsis* [9, 10]. The species of this genus produce a spectrum of peptide antibiotics with antibacterial and antifungal activity. In addition to the well-studied peptaibols zervamicins, which are recommended for preclinical studies [11], emerimicins II, III, and IV (produced by *Emericellopsis microspora*) are isolated and described [12]. From the *Emericellopsis donezkii* HKI0059, Berg et al. isolated bergofungins A and B, the structure of which was determined by the X-ray analysis [13]. Their homologs, bergofungins C and D, were also found in the *E. salmosynnemata* [14, 15]. Ishiyama et al. described the isolation of heptaibin from *Emericellopsis* sp. (BAUA8289 strain) isolated from mycorrhizas of rice roots [16].

It was shown that the isolate of the *Emericellopsis minima* from the coral reef of the Similan Islands (South Thailand) is able to synthesize bicyclic sesquiterpenes and helvolic acid in addition to antimicrobial peptides [17]. The species of this genus are also known to produce β-lactam antibiotics, P, N, and C cephalosporins, which are responsible for the lysis of cyanobacteria and diatom algae phototrophic components of mats [12, 17].

The goal of the work was to evaluate the antimicrobial activity of alkalophilic isolates of the *Emericellop-*

sis alkalina and to isolate the producers of peptide antibiotics.

EXPERIMENTAL

The objects of study were 22 alkalophilic strains of the newly described fungus *Emericellopsis alkalina* Bilanenko&Georgieva (https://www.ncbi.nlm.nih.gov/nuccore?cmd=Search&dopt=DocSum&db=nucleotide&term=Emericellopsis+AND+alkalina) isolated from alkaline saline soils of different geographic regions [18, 19]. The cultures were obtained from the collection "Fungi of Extreme Conditions" of the Department of Mycology and Algology of the Biology Department (Lomonosov Moscow State University).

The antimicrobial activity of the strains was initially evaluated by the method of diffusion in agar on test cultures of the conditionally pathogenic microorganisms *Aspergillus niger* INA 00760 and *Bacillus subtilis* ATCC 6633. The cultures were considered as highly active, moderately active, or low active if they showed 25 mm or more, 10–25 mm, or less than 10 mm of the growth inhibition zone of the test organism, respectively.

We used primary screening to study the growth rate of six strains on media of different compositions (oat agar, potato agar, alkaline agar, Czapek agar, and Saburo media) recommended for growing micromycetes of the *Emericellopsis* spp. [12, 18, 20]. With the use of linear regression, the growth rates of the colonies were evaluated by the mean values of their diameters at various moments, beginning from the stage of the linear growth. The calculated values of the slope of the regression line corresponded numerically to the colony growth rate [20].

The formation of antimicrobial compounds was studied during the cultivation of six isolated strains in three liquid media that provided a high culture growth rate. Fungi were grown in stationary mode in 500-mL flasks for 14 days. The culture fluid was removed by filtration through membrane filters on a Zeitz funnel under vacuum.

Isolation of the antibiotic substances was carried out as follows. The culture liquid (CL) of the producer was extracted with ethylacetate with a solvent–CL ratio of 5 : 1. The extracts were evaporated to dryness in a vacuum on a Rotavapor-RBüchi rotary evaporator (Switzerland) at 42°C. The residue was dissolved in aqueous 70% ethanol to obtain ethanol concentrates. The antimicrobial activity was evaluated in the initial culture liquid, ethanol concentrates, and mycelium extracts with sterile paper discs (filter paper, GOST 12026-76, Russia) wetted in extracts and dried under sterile conditions. The controls for the sensitivity of the test organism were standard discs with nystatin for fungi (80 μg/mL, Pasteur Research Institute, Russia) and with ampicillin for bacteria (10 μg/mL, Pasteur Research Institute, Russia) [21].

The subsequent fractionation of the antibiotic complex from the extract of the most active strain was performed by thin layer chromatography in a chloroform-methanol system (10 : 1), followed by separation of the fraction by direct-phase flash chromatography with the following eluents: chloroform-methanol mixtures (50 : 1, 20 : 1, 10 : 1, and 3 : 1), 96% ethanol, and ethanol-water (7 : 3).

The spectrum of antimycotic activity of the active enriched fraction was evaluated in the test cultures of mycelial and yeast microscopic fungi from the Collection of Cultures for the Search for New Antibiotics (Gause Institute of New Antibiotics, Russia). We used conditionally pathogenic fungi belonging to micromycetes of the *Aspergillus*, i.e., *A. ustus* 6K, *A. fisheri* 3K, *A. fumigatus* KBP F24, *A. flavus* 7K, and *A. niger* INA 00760; yeast conditionally pathogenic fungi *Candida albicans* ATCC 2091 and *C. tropicalis* INA 00763; phytopathogenic species *Fusarium oxysporum* VKM F-140 and *F. solani* VKPM F-890; toxigenic species of the *Penicillium*, i.e., *P. chrysogenum* VKM F-4499 and *P. brevicompactum* VKM F-4481. The antibacterial effect was evaluated with the use of the test cultures of gram-positive *Bacillus subtilis* ATCC 6633 and *B. coagulans* 429 bacterial strains and gram-negative *E.coli* ATCC 25922 bacterial strains.

The subsequent separation of the active fractions after flash chromatography was carried out by analytical reverse-phase high performance liquid chromatography (RP HPLC) with an XBridge column (5 μm 100A, 250 × 4.6 mm, Waters, United States) in a linear gradient of acetonitrile concentration (eluent A, 0.1% trifluoroacetic acid, TFA, in MQ water; eluent B, 80% acetonitrile and 0.1% aqueous solution of TFA) at a flow rate of 950 μL/min. For RP HPLC, we used ultragradient acetonitrile (Panreac, Spain) and TFA (Sigma-Aldrich, United States). The compounds were detected at three wavelengths (214, 247, and 280 nm) in a gradient of eluent B, (16–28% for 12 min; 28–55% for 27 min; 55–75% for 20 min, and 75–85% for 10 min), followed by isocratic elution for 25 min. Scaling for obtaining individual components of ethanol concentrate derived from the extract of the producer culture liquid was performed by semipreparative RP HPLC on an XBridge column (10 μm 100A, 250 × 10 mm, Waters, United States). The compounds were detected at 214 nm at a flow rate of 4 mL/min. The fractions were collected, the excess of the solvent (acetonitrile) was removed by evaporation in a SpeedVac concentrator (Savant, United States), and residual amounts of TFA were removed by lyophilization (Labcono, United States). The spectrum of antimicrobial action of isolated compounds was evaluated by the disc-diffusion method as described above.

Table 1. Growth rate (mm/h) of isolates on six solid media*

Nutrient medium	Strain					
	E101	A115	A117	A118	M14	M20
Wort agar	0.092	0.099	0.123	0.094	0.153	0.156
Potato dextrose agar	0.122	0.145	0.161	0.159	0.131	0.147
Oat agar	0.128	0.143	0.122	0.147	0.115	0.135
Saburo	0.178	0.185	0.115	0.153	0.156	0.154
Czapek agar	0.183	0.148	0.254	0.168	0.24	0.198
Alkaline agar	0.251	0.267	0.228	0.256	0.246	0.237

* The growth rate was evaluated by the slope of the regression line.

The molecular weights of the isolated active compounds were evaluated on an AutoSpeed MALDI TOF/TOF time-of-flight mass spectrometer (Bruker-Daltonics, Germany) equipped with a UV laser (335 nm, Nd:YAG) in the regime of positive ions with the use of reflectron. The mixture of the sample (1 μL) and 2,5-dihydroxibenzoic acid (DHB) (1 μL, 10 mg/mL in 20% acetonitrile containing 0.5% TFA) was placed on a template and air-dried.

Absorption spectra were recorded on a UV-1800 spectrophotometer (Shimadzu, Japan) in quartz cuvettes (2 mL) with an optical path of 1 cm.

The N-terminal Edman sequencing [22] was performed on a peptide and protein PPSQ-33A sequenator (Shimadzu, Japan) according to the manufacturers' protocol.

The minimum inhibitory concentration of the active peptide fraction against yeast and mold fungi was evaluated in the Saburo medium by two-fold serial dilutions in 5-mL tubes. Inoculates of the test culture with a CFU titer of 1×10^4 and active peptide fraction in ethanol at concentrations from 500 to 31.25 μg/mL were placed in each tube and incubated at 35°C without stirring. The culture growth was assessed visually and by microscopy. The minimal inhibitory concentration (MIC) was calculated for 24 h for *C. albicans* and for 48 h for *A. niger* and *A. fumigatus*. MIC is considered to be the minimum concentration of the compound that completely inhibits the growth of the test culture.

The experiments were carried out in three to five replicates. Statistical processing of the experimental results and evaluation of the significance of differences in the data were performed by Student's t-test for a level of probability of at least 95% with the use of the Microsoft Excel 2007 and Statistica 8.0 programs.

RESULTS AND DISCUSSION

Evaluation of the antimicrobial activity of 22 strains *E. alkalina* against test fungi and bacteria showed that cultures with antimicrobial action against *A. niger* INA 00760 accounted for 27% of all tested strains, whereas only 5% of all strains exhibited an antibacterial effect towards *B. subtilis* ATCC 6633. This specificity of the antimicrobial action may be caused by the competition between fungi in similar habitats, and one of the mechanisms of this competition is the production of antibiotic substances. According to the data of Carreira Cátia et al., the isolates of this genus, which numerically prevailed in the micromycete community, were isolated from salt lakes in Chile. They exhibited antagonistic properties against other genera of microscopic fungi, while they did not inhibit bacterial growth [17]. The results of the primary screening on solid media made it possible to select six active cultures of this species, which were used for the further study.

Evaluation of the culture growth rate in different synthetic and organic media showed that all strains had a high growth rate on three media, i.e., Saburo, alkaline, and synthetic Czapek media (Table 1). These liquid media were further used to assess the ability of strains to produce antibiotics. Antibiotic substances accumulated primarily in the CL of the strains but not in the mycelium. The CL extracts of all cultures were shown to have a weak antibacterial activity against *B. subtilis* ATCC 6633 and a higher antifungal activity against *A. niger* INA 00760 and *C. albicans* ATCC 2091. The A118, A117, E101, M14, and M20 strains synthesized antibiotics on the Czapek and alkaline media. The maximal antibiotic activity for these strains was found on the alkaline medium, which may be due to their alkalophilic adaptation type (Table 2).

The screening of six strains *E. alkalina* for their antibiotic activity and growth rate resulted in the selection of the A118 strain (GenBank number, KC999014.1) for further study. This strain exhibited a high antibiotic activity against conditionally pathogenic mycelial and yeast fungi. The strain *E. alkalina* A118 was grown in a temperature range of 18°–35°C with an optimum at 25–28°C. The optimal temperatures for the secretion of antibiotics were 25–28°C; at

Table 2. Antimicrobial activity of strains against conditionally pathogenic fungi and bacteria in liquid media

Strain	Growth inhibition zone, mm					
	Saburo medium		Czapek medium		alkaline medium	
	A.niger	*B. subtilis*	*A.niger*	*B. subtilis*	*A.niger*	*B. subtilis*
E101	0	0	8	10	0	0
A115	0	0	8	14	0	0
A117	0	0	0	0	0	12
A118	0	0	0	13	25	13
M14	0	0	0	0	18	16
M20	0	13	0	0	12	10
Nystatin	15	–	15	–	15	–
Amoxicillin	–	22	–	24	–	24

Table 3. Antifungal activity of fractions of the A118 strain against conditionally pathogenic and toxigenic mold fungi

Test-organism	Test-organism growth inhibition zone, mm						
	flash chromatography fraction number						nystatin
	F_I	F_{II}	F_{III}	F_{IV}	F_V	F_{VI}	
Aspergillus fumigatus KBP F24	0	0	16	26	22	13	25
A. flavus 7K	0	0	10	16	13.3	12.6	18
A. ustus 6K	0	0	13.6	18	15	13	21
A. niger INA 00760	0	0	22	25	20	10	15
A. fischeri 3K	0	0	12	20	15	10	15
Candida albicans ATCC 2091	0	0	0	22	10	0	20
Penicillium brevicompactum VKM F-4481	0	0	13	13	13	9	15
P. chrysogenum VKM F-4499	0	0	23	25	15	10	17
Fusarium oxysporum VKM F-140	0	0	8	14	8	0	15
F. solani VKPM F-890	0	0	10	12	10	8	22

temperatures below 25°C, the strain produced antibiotics with lower activity. At 25°C, the strain intensively secreted metabolites with antifungal activity on 10–12 days of growth, and the activity then remained almost unchanged until the end of the cultivation.

The strain *E. alkalina* A118 exhibited a high antifungal activity against the conditionally pathogenic strains i.e., *A. niger* INA 00760. The values of the growth inhibition zones of these test-cultures reached 25 ± 2 mm and 22 ± 2 mm, respectively. The antifungal activity of the ethylacetate extracts of the A118 strain at the concentration of 80 μg/disk was higher than that of nystatin.

The successive separation of the antibiotic complex of the CL extracts on silica gel by flash chromatography made it possible to obtain five enriched fractions, which were assessed for the spectrum of antifungal activity against conditionally pathogenic fungi.

The maximal activity was observed for fractions III–V, and fraction F IV showed the maximal antifungal effect against all tested microorganisms, which inhibited the growth of all conditionally pathogenic micromycetes, i.e., *A. flavus* 7 K, *A. niger* INA 00760, and *A. fumigatus* KBP F24 *A. ustus* 6K. This fraction was also active against phytopathogenic strains of the *Fusarium* genus and toxigenic strains of the *Penicillium* genus (Table 3), with the growth inhibition zones of the test fungi being larger than for nystatin.

We further developed the scheme of the separation of active F IV fraction by RP HPLC after direct-phase flash chromatography of the antibiotic complex. This separation resulted in the component profile of the

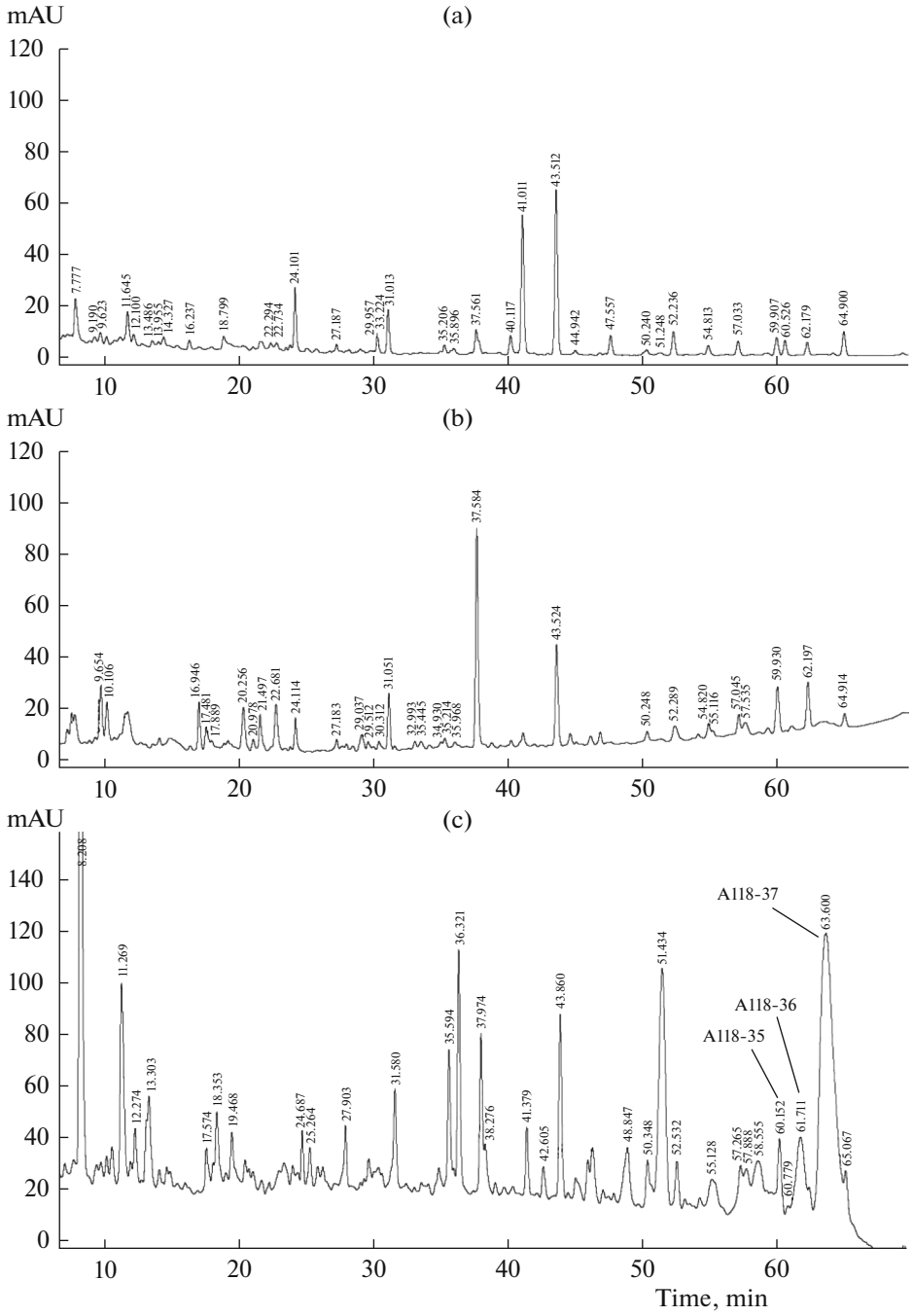

Fig. 1. Comparative quantitative analysis of the composition of the active fraction after direct-phase flash chromatography by analytical RP HPLC. Absorption at 280 (a), 247 (b), and 214 nm (c). Retardation times are indicated for the most intensive peaks (min). The target fractions are designated as A118-35, A118-36, and A118-37.

active concentrate consisting of about 30 main fractions (Fig. 1b) with different degrees of hydrophobicity. The primary structural characteristics were performed by comparative quantitative analysis of the sample by RP HPLC at three wavelengths (214, 247, and 280 nm). It is known that the main antimicrobial components that are secreted by fungi micromycetes, in particular, species of the *Emericellopsis*, are peptaibols. These short lipophilic polypeptides have the characteristic modification of the C-terminal amino acid residue in the form of the reduced carboxyl group of phenylalanine to the alcohol group, which is determined by the presence of the characteristic absorption maximum at 247 nm [23].

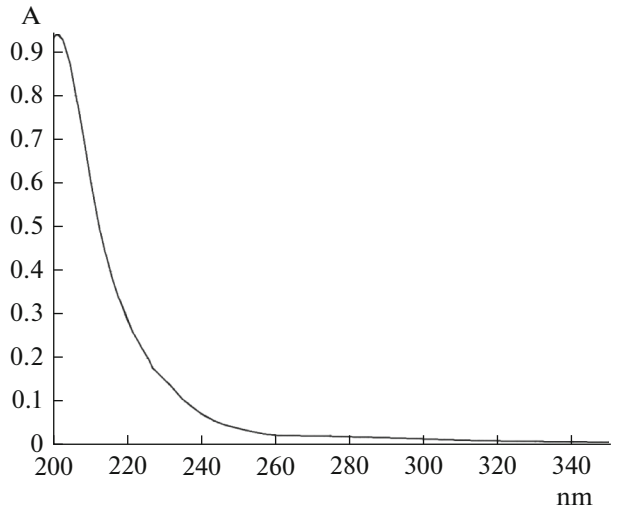

Fig. 2. UV absorption of fraction A118-37 in the range of 200–340 nm.

The analysis of equal amounts (about 250 μg) of most components of fraction F IV showed the predominant absorption at 214 nm, whereas the compounds of the so-called hydrophobic part of the profile (retardation time, 40–65 min) containing primarily peptaibols showed a low absorption at 247 and 280 nm [24].

Scaling of the obtained components of the active concentrate of fraction F IV was performed by semipreparative RP HPLC with the detection of the absorption at 214 nm. We manually 37 collected fractions and tested the resulting compounds for antimicrobial properties. We revealed pronounced activity only in three most lipophilic fractions (retardation time, 60.152, 61.711, and 63.6 min) named A118-35, A118-36, and A118-37, respectively, and component 37 was the most active (Fig. 1c). To find the similarity or difference in the chemical nature of the active components, we recorded their absorption spectra in the short-wavelength UV region (210–340 nm). The spectral composition of three compounds was identical with the minimal absorption at 240–280 nm, which indicated the absence of the carbo and heterocyclic elements in their structure and allowed one to draw a conclusion about their structural identity (Fig. 2).

Regardless of the location of the component, the absorption at 214 nm may likely indicate the polypeptide nature of the individual compounds, including those with the maximum absorption at 280 nm, which contain aromatic amino acid residues. The hydrophobic compounds with absorption at all three wavelengths with maximums at 214 and 247 nm may probably be attributed to peptaibols. The structure of three active compounds was analyzed by mass spectrometry, which showed that the m/z values were in the range of 1015–1230 Da (Fig. 3). These values are lower than those for the overwhelming majority of known peptaibols from fungi micromycetes [25, 26].

The N-terminal sequencing of the predominant A118-37 fraction did not detect the presence of the phenyl thiohydantoin derivatives of amino acids. This probably indicated the protection of the amino group at the N-terminal amino acid residue, which prevented the reaction with phenylisothiocyanate (data not shown) [26]. It should be noted that the posttranslation blocking of the amino group (as a rule, acetylation) is also a feature of peptaibols [27]. Thus, the isolated active component apparently belongs to a new structural subtype of peptaibols that probably has a polypeptide sequence consisting of fewer amino acid residues.

We evaluated the minimal inhibiting concentration for fraction A118-37 against the conditionally pathogenic mold and yeast fungi and bacteria. It was shown that the fraction completely inhibited *C. albicans* and *A. niger* at the concentration of 31.25 μg/mL, inhibited the growth of *A. fumigatus* at a lower concentration (62.5 μg/mL) as compared to the known antimycotic nystatin, and had no pronounced bactericide effect on *B. subtilis* at concentrations up to 500 μg/mL.

It was shown earlier that the species of the *Emericellopsis* and the produced antibiotic compounds primarily exhibited the antibacterial activity towards gram-positive bacteria [27, 28]. Two endophyte isolates of *E. donezkii* (UFMGCB 1966 and UFMGCB 2001) isolated from the leaves of *Alchornea castaneifolia* exhibited a high antibacterial activity against *Bacillus cereus* ATCC 11778, *Salmonella typhimurium* ATCC 14028, and *Staphylococcus aureus* ATCC 12600 [29]. The methanol extracts of *E. donezkii* UFMGCB 2001 had a weak antifungal effect towards the yeast *Candida albicans* ATCC 18804 and *C. krusei* ATCC 2159 fungi (MIC 62.5 μg/mL) but were inactive against mold fungi [30].

Thus, the third of the tested extremophilic *E. alkalina* isolates demonstrated the ability to produce antimycotic compounds with high and moderate activity, which may indicate a promising strategy for the search for antimycotic producers among this species. We selected the strain *E. alkalina* A118, a producer of antimicrobial peptides with antifungal activity against mold and yeast fungi. The group of the homologous active compounds isolated by the set of identified structural features (molecular weight, the ratio of the absorption at certain wavelengths, and the absence of initiation of Edman sequencing) may be attributed to peptaibols, which is a group of nonribosomal membrane-active antimicrobial peptides with a specificity of the action against primarily fungi micromycetes. Their further structural identification will be performed by the combination of physical and chemical

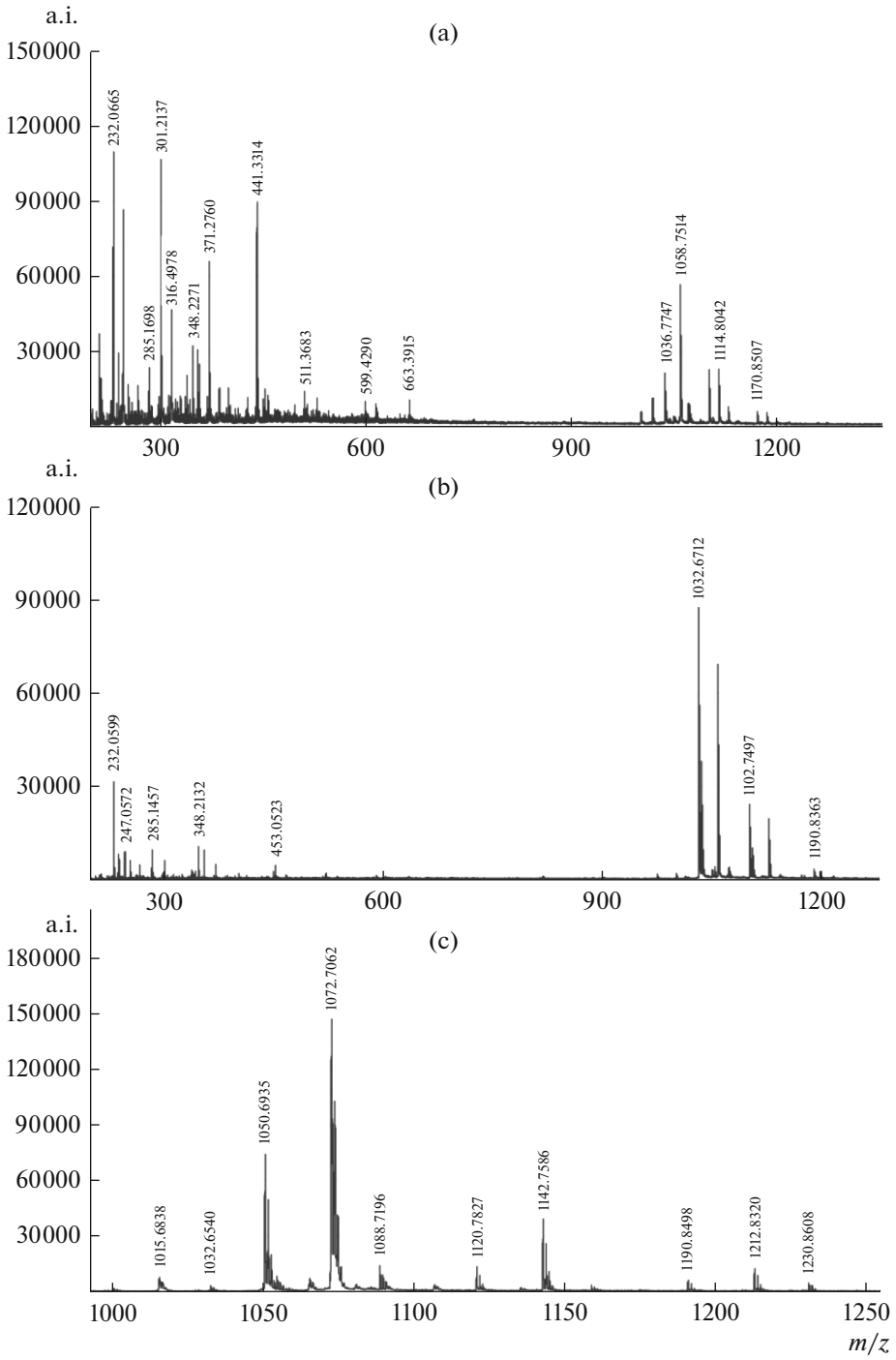

Fig. 3. MALDI mass-spectrometry analysis of individual fractions A118-35 (a), A118-36 (b), and A118-37 (c).

methods, i.e., high-resolution mass spectrometry and nuclear magnetic resonance spectroscopy (NMR).

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