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Reactivating Factor of *Luteococcus japonicus* subsp. *casei*: Isolation and Characterization

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Abstract—It has been shown that a producer strain of reactivating factor (RF) is identical to a typical strain of *Luteococcus japonicus* DSM 10546 from the Propionibacteriaceae family according to the physiological and biochemical properties and the sequencing of 16S rRNA fragments. A number of phenotypical differences from the model strain allowed the producer strain to be considered a subspecies of *Luteococcus japonicus*, and it was named *Luteococcus japonicus* subsp. *casei*. At cultivation of the producer, RF is secreted into the medium and plays the role of a signaling molecule. RF antioxidant activities towards various organic radicals may be a possible mechanism of its protective and reactivating effects.

Metabolites secreted by the *L. casei* producer strain into the culture medium were separated by a combination of liquid chromatographies. Four components possessing biological activities were found. The most active one was studied by MALDI-TOF mass spectrometry, which revealed that it is a polypeptide. Primary identification of some amino acid residues was performed. Sugar residues were found in the structure.

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INTRODUCTION

Microorganisms secrete a number of functionally active chemical compounds participating in various metabolic processes in the cell. Extracellular substances are often polyfunctional and species unspecific [1].

Extracellular isomers and homologs of alkylhydroxybenzenes, which are synthesized by many organisms, have a protective effect under stress conditions and play the role of signaling molecules by activating the expression of stress genes [2]. Furthermore, these extracellular autoregulators control the transition of microbial cells in the stationary phase or formation of resting forms [3]. Another example is the action of many antibiotics, the subinhibitory concentrations of which induce quorum sensing—a special type of expression regulation of bacterial genes that depends on the density of a population [4].

Some secreted compounds, for example signaling molecules, participate in chemical communication at the population level in interspecies and even interdomain interactions [5]. It plays an important role in the formation of microbial communities and has an indirect effect on the increase in cell resistance to stress.

The features and significance of chemical communication in bacteria under stress conditions were shown by the example of *Escherichia coli* strains in the remarkable works of R. Rowbury [6–8]. However, the structure of signaling molecules and the mechanism of their protective effect were not considered in these studies.

We showed earlier that chemical communication using extracellular compounds with antistress action is a characteristic of Gram-positive and Gram-negative bacteria, various yeasts, and archaea [5, 9]. Moreover, we showed for the first time a cross effect of exometabolites of prokaryotes and higher and lower eukaryotes [5, 10] and their role in both protection and reactivation of stressed cells.

The purpose of the work was to isolate, purify, and identify the reactivating factor of *Luteococcus japonicus* subsp. *casei* and to study its properties.

METHODS

Cultivation of bacteria. Reactivating factor (RF) was isolated from the culture medium (CM) of *Luteococcus casei*. Bacteria were grown under static conditions in 200 mL flasks at 32°C using a glucose-mineral medium of the following composition (%): glucose—1.0, $(NH_4)_2SO_4$—0.3, KH_2PO_4—0.1, NaH_2PO_4—0.2, $MgSO_4$—0.002, $CaCl_2$—0.002, NaCl—0.002, and yeast extract—0.1; pH was adjusted to 7.0 using a 5% NaOH solution. Inoculate was added in an amount sufficient to provide an initial optic density of 0.4–0.6 (3 mL cells; λ =540 nm; a FEK 56 PM photoelectric colorimeter, Russia).

Bacteria were grown in an agar medium in Petri dishes for 72 h at 32°C.

Stress resistance was estimated as a number of CFU formed when aliquots of a culture at the stationary growth phase (48 h) were plated into the agar medium of the composition described above, compared to the growth of a culture which had not undergone stress (control).

Stress factors. In order to study stress effects, we performed preliminary experiments to reveal the "dose-response" dependence. As a stress factor, UV irradiation was used. An instrument consisting of two paralleled BUV-15 lamps (Russia) with a power of 30 W was used as a UV irradiation source; the main emission was at 253.7 nm. Cells were irradiated with a dose of 81 J/m^2, at which the survival rate was 0.01–0.03%.

Determination of protective and reactivating activities. In order to determine activities, cell suspensions were incubated with RF before (protective effect) or after (reactivation) stress for 10 and 15 min, respectively.

Reactivating and/or protective effects were estimated by the comparison of CFU numbers of cells grown in an experimental variant and a control (irradiated but not protected). The survival rate of bacteria was expressed in percentage with respect to the unirradiated control. The division index was determined as the number of cells forming colonies after incubation with RF as related to a number of colonies grown from cells without pre- or postincubation.

Detection of quantities of volatile fatty acids. Chromatographic separation and detection of quantities of fatty acids formed in bacteria during glucose fermentation was performed in a Kristall 2000 M gas-liquid chromatograph (Khromotek, Russia) using a 2B-FFAP column (15000 mm × 0.32 mm × 0.5 μm) with nitroterephthalic acid modified with polyethylene glycol in a temperature range of 70–150°C as a carrier.

Determination of genetic affinity of a strain. DNA was isolated from bacteria according to the method described in [11]. The obtained preparations contained 30–50 μg/mL of DNA (the RNA content in preparations was less than 1%). Two independent DNA preparations were obtained for each studied strain.

A universal set of primers was used for PCR and the following sequencing of the 16S rRNA gene [12]. The reaction mixture included: primers—25 pmol each; a 10-fold buffer for Taq polymerase (Taq DNA polymerase Dream™)—2.5 μL; 2 mM dNTP—2.5 μL; BioTag polymerase (Dialat, Moscow, 5 U/μL)—0.2 μL; DNA template—50 ng; H_2O—25 μL. The reaction was performed as follows: 30 cycles; 94°C 0.5 min, 45°C 1 min, 72°C 1 min; final polymerization of 7 min.

PCR products were analyzed by electrophoresis in a 2% agarose gel at 6 V/cm. Results were documented using a BioDocII system (Biometra, Germany). PCR products corresponding to various regions of the gene were isolated and purified from low melting point agarose using a Wizard PCR Preps kit (Promega, United States) according to the manufacturer's manual.

PCR products were sequenced using a Silver Sequencing kit (Promega, United States) according to the manufacturer's manual with insignificant modifications. Sequencing was performed in both directions; both external and internal primers were used.

Nucleotide sequences of 16S rRNA genes of the studied strains were primarily analyzed using a BLASTA algorithm. Multiple alignments with corresponding sequences of closest bacterial species were performed using a CLUSTAL W program [12]. The building of rootless phylogenetic trees of the studied bacteria was carried out using TREECON software [13].

Isolation and identification of RF. After 48 h of cultivation in the medium of the aforementioned composition, the bacterial cells were centrifuged for 20 min at 10000 g, washed twice with a 0.05 Na-phosphate buffer, pH 7.4, and suspended in the same buffer up to an optical density of 0.4–0.6 at 540 nm.

Cell suspension was the object of the stress studies, and a supernatant (CM) served as a RF source. CM was passed through a nitrocellulose membrane filter with pores of 0.22 μm (Millipore, United States). Components adsorbed on the filter were eluted with a 3% NaCl solution and then passed through a membrane filter with low protein affinity (Pall, United States) to remove cells completely. After that the solution was desalted by reversed phase low-pressure chromatography in a Synchroprep RP-P C_8 column (SynChrom Inc., United States) with a volume of 10 mL. The column was preliminarily equilibrated with a 0.1% solution of trifluoroacetic acid (TFA). The speed of a mobile phase was about 1.5 mL/min. After elution of organic salts—the components of the culture medium—and other unbound substances, the protein-peptide fraction was desorbed by a 70% water solution of acetonitrile in 0.1% TFA. Optical densities of fractions were detected at 280 nm. The obtained fractions were stripped in a vacuum and freeze-dried.

Primary fractionation of desalted CM was performed by affinity low-pressure chromatography in a Heparin HiTrap-Sepharose column (GE Healthcare, Sweden) with a volume of 5 mL. Freeze-dried column fractions were diluted in 10 mL of the start 10 mM Tris-HCl buffer, pH 7.2, and applied to a column preliminarily equilibrated with 5 volumes of the same buffer. After the elution of unsorbed compounds (fraction SF 1), proteins and peptides were desorbed by 1.0 M NaCl in the start buffer (fraction SF 2). Elution was performed with a speed of 1 mL/min. Optical density was measured at 280 nm.

Separation of the two fractions after affinity chromatography was performed by reversed phase liquid chromatography (RP-HPLC) in a Luna C_{18} 4.6 × 250 mm column (Phenomenex, United States). Chro-

matography was performed using two linear gradients of solvent B (an 80% solution of CH_3CN, 0.1% of TFA) in relation to solvent A (0.1% TFA solution) from 0 up to 50% for 40 min (main) and 50–70% for 20 min (additional) at a flow speed of 0.75 mL/min. Peptides were detected at 210 nm.

Molecular weights of peptides were measured using an Ultraflex MALDI time-of-flight mass spectrometer (Bruker Daltonics, Germany) equipped with a UV laser (337 nm) in the regime of positive ions. As a matrix, 2,5-dihydroxybenzoic acid was used. Equal volumes (0.7 μL each) of samples and the matrix (15 mg of the matrix/mL in 80% of acetonitrile and 0.1% of TFA in MQ water) were mixed on a target. The obtained mixture was air-dried. Mass spectra were analyzed using a Bruker DataAnalysis for TOF program. The measurement error was 0.015%.

Determination of antioxidant capacity of RF. Antioxidant capacity (AOC) was determined by quenching of a radical cation of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ABTS (Trolox Equivalent Antioxidant Capacity, TEAC), and a peroxyl radical (Oxygen Radical Absorbance Capacity, ORAC).

The ABTS radical cation was obtained by the method of Re et al. [14]: a 7-mM ABTS solution was incubated with 2.45 mM potassium peroxodisulfate for 12 h at room temperature in the dark. The obtained concentrated solution of the ABTS radical cation was diluted with 50 mM phosphate-buffered saline (100 mM NaCl), pH 7.4 (PBS), until $OD_{734} = 0.70 \pm 0.02$, which corresponded to a final ABTS radical cation concentration of ~47 μM ($\varepsilon_{734} = 1.5 \times 10^4$ $mol^{-1}L\ cm^{-1}$). In order to determine AOC, 20 μL of the studied RF or the standard solution, trolox, (10–100 μM) and 180 μL of the ABTS radical cation solution were added into a 96-well unsorbing polystyrol plate with a flat bottom (Greiner BioOne, Germany). Control wells contained 20 μL of PBS and 180 μL of the ABTS radical cation solution. The reaction was registered by a diminution of OD_{734} for 40.5 min with a measurement interval of 60 s at 25°C in a Synergy 2 multi-mode microplate reader (BioTek, United States). Measurements of each concentration of the standard and the studied sample were repeated 4 times. The equivalent concentration of antioxidants in a sample was determined by a decrease in optical density of the reaction medium in the presence of RF and expressed in equivalents of a water-soluble analogue of vitamin E—trolox equivalents (TE).

The AOC of RF towards the peroxyl radical was performed according to the method of Ou et al. as modified by Moore et al. [15, 16]. The peroxyl radical was generated directly in the reaction medium in the course of thermal degradation of 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH). The reaction mixture contained 15 μL of either RF or deionized water (control C1) or a trolox water solution (5–75 μM) and 115 μL of a 8.16×10^{-8} M freshly prepared solution of fluorescein sodium salt in a 75 mM Na-phosphate buffer (Na-PB), pH 7.4. The reaction mixture in control wells (without fluorescence, C2) contained an analogous amount of Na-PB instead of fluorescein sodium salt. The analogous amount of Na-PB was added into the control wells with 100% fluorescence intensity (C3) instead of AAPH solution. The reaction was initiated by the addition of 15 μL of a freshly made 0.6 M AAPH solution in Na-PB into all wells except the control C3. A decrease in fluorescence was registered for 1 h with a measurement interval of 60 s at 37°C in a Synergy 2 multimode microplate reader (BioTek, United States) in the regime of fluorescence registration (excitation wavelength of 485 nm, emission wavelength of 528 nm). AOCs were calculated from the difference of squares under kinetic curves of diminution of relative fluorescence intensity of fluorescein in the presence of RF and expressed in μM of TE.

RESULTS AND DISCUSSION

***L. casei* is a producer of exometabolites with reactivating and protective properties.** The culture medium (CM) of bacteria described earlier, isolated from Sovetskii cheese from submontane and steppe areas of the Altai Territory [17, 18], was the source of the active factor.

The phylogenetic position of the isolated strain (B2 at the phylogenetic tree) is shown in Fig. 1. It implies that the bacteria form a cluster with the *Luteococcus* genus. According to the preliminary screening of a sequenced fragment of the 16S RNA gene with a length of 1377 bp corresponding to positions from 26 to 1427 (*E. coli*) and the use of the GenBank database, the B2 strain belongs to the *Luteococcus* genus of the Propionibacteriaceae family. A more detailed comparative phylogenetic analysis revealed that the B2 strain is identical to a typical strain of *L. japonicus* DSM 10546 (100% of nucleotide similarity). The level of nucleotide similarities of the B2 strain with other species of the *Luteococcus* genus was 96.7–97.6% and did not surpass 90% with other *Propionibacteriaceae* representatives. *P. innocua* (the modern name of *Propioniferax innocuum*) is the nearest relative strain among the *Propionibacteriaceae* genera. According to present-day ideas [19], the revealed high similarity of the 16S rRNA sequences of the studied B2 strain and the typical strain of *L. japonicus* allows us to classify it as the same species.

The MALDI–TOF analysis of the studied strain did not reveal peaks characteristic of the *Propionibacterium* genus [20] but detected the presence of well-marked peaks with the m/z of 3377, 5783, and 6972, with a dominating peak of 6764. Luteococci mainly synthesize monounsaturated long chain fatty acids, in contrast to all other genera of the *Propionibacteriaceae* family, which form the largest quantities of methyl-branched fatty acids [21].

The study of phenotypic characteristics revealed differences of the isolated strain from the typical *L. japonicus*: it was not able to ferment ribose, adoni-

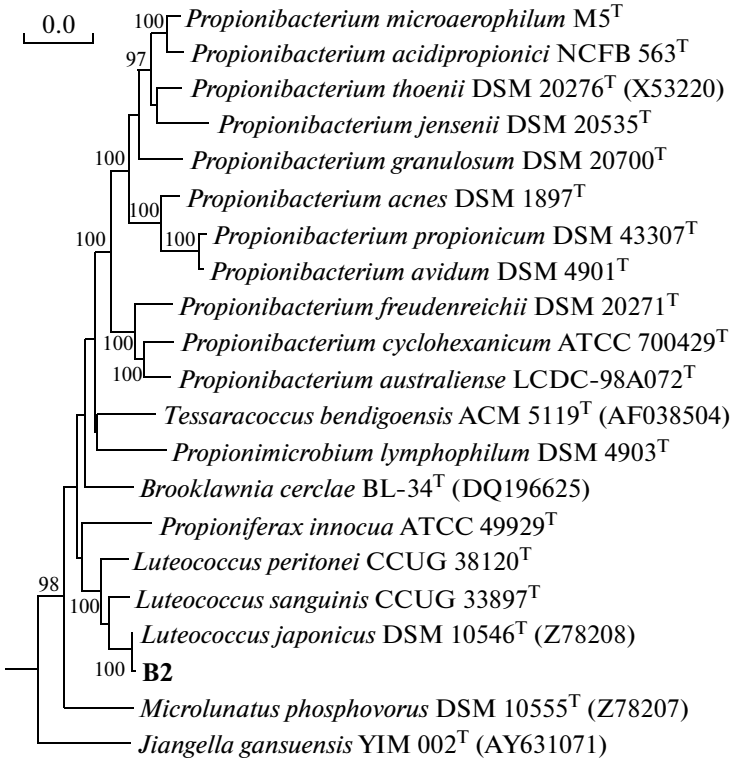

Fig. 1. Phylogenetic position of the RF producer—*Luteococcus casei* B2 strain. The scale shows evolutionary distance corresponding to 5 nucleotide substitutions per every 100 nucleotides. Numbers designates reliability of branching revealed using bootstrap analysis of 100 alternative trees (values more than 95 are considered as significant).

tol, L-sorbose, inositol, α-methyl-D-glucoside, *N*-acetylglucosamine, salicin, or cellobiose [2]. The B2 strain also differed in the ability to utilize ornithine and possessed urease activity. Taking into account phenotypic differences, we considered the isolated strain as a subspecies of *L. japonicus* and named it *L. japonicus* subsp. *casei* (*L. casei*). The strain is stored in the Russian Collection of Microorganisms (RKM) under the number of AS-1910.

The ability of luteococci to grow at low temperatures (8–10°C), their salt resistance (ability to grow at NaCl contents up to 6.5%), their growth in both aerobic and anaerobic conditions [18], and their antistress properties provide a wide range of opportunities for adaptation in the environment [22]. Luteococci were isolated from water and soil of Tokara island (Japan), from water used in brewing [21], and from nonmotile Gram-positive cocci, which were described as new [23]. Furthermore, they were isolated from milk and cheese [18], clinical samples (*L. peritonei*, rod-like cells) [24], and human blood (*L. sanguinis*) [25]. The pathogenic properties of strains were not found [24].

The studied *L. casei* strain was isolated from hard cheese (Sovetskii), which defines its ability to exist under conditions of low concentrations of fermented carbohydrates and high salt contents in the environment. *L. casei* forms orange glossy colonies and retains a spherical shape of cells, with the diameter varying from 0.5 to 0.9 μm at all developmental stages. When cells are cultured in liquid media, they precipitate on the bottom of a fermenter several hours after the beginning of growth and form a drawable yellow-orange precipitate. At the early stationary phase, the main fermentation products include propionic and acetic acids. Caprylic, isocaproic, butyric, and caproic acids are formed in trace amounts (Table 1). At the end of the stationary phase (72 h), the amounts of propionic and acetic acids slightly increased, and isovaleric acid appeared in the medium; other fatty acids with average chain lengths were not found. Carbon dioxide

Table 1. Metabolites of *Luteococcus casei* formed during glucose fermentation

Organic acid	Concentration (mM)	
	48 h	72 h
Acetic	6.82	8.35
Propionic	11.45	14.60
Butyric	0.003	–
Isocaproic	0.012	–
Caproic	0.020	–
Caprylic	0.024	–
Isovaleric	–	0.004

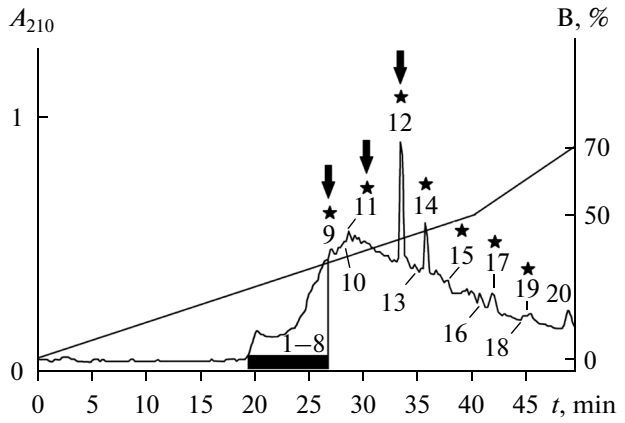

Fig. 2. RP-HPLC of the total fraction SF2 after cation-exchange chromatography. Joined fractions 1–8 (black rectangular). In Figs. 2 and 3, the fractions in which biological activity was tested are asterisked; fractions that showed reactivating activity are marked with arrows. B is a solvent concentration (%).

accumulated in the amount of 50 mg/L (240 mL/L) after 10-day cultivation.

Bacteria showed antioxidant properties: they possessed superoxide dismutase (SOD), catalase [26], and peroxidase [27] activities. The bacterial genome was not decoded, and their molecular biological properties remain understudied, though the bacteria possess a number of unique peculiarities. They include, for example, bifidogenic and prebiotic activities [28] and the formation of molecules with protective and reactivating properties active towards cells of various organization levels which underwent various stresses. Active compounds are formed by cells at the early stationary phase when cultured under conditions described in "Methods" and show an antistress effect for 10–15 min, presenting in cell suspension in trace amounts. Protein exometabolites are secreted into the medium and localize on the surface of producer cells, because protective activity was found in cell lavage (data not shown).

Isolation and identification of RF. RF was isolated from 130 L of CM. At the first stage of fractionation, a RF salt solution was desalted by reversed phase low - ressure liquid chromatography with the stepwise gradient of acetonitrile with the addition of TFA at a pH of 2.0 using a column with the C_8 phase. This stage allowed the performance of the first stage of fractionation, separation of proteins and peptides from hydrophilic low-molecular components, and the concentration of hydrophobic components in the solution of smaller volume (about 30 mL). The obtained fraction containing mainly proteins and peptides (named RF-S) and showing biological activity (1.8-fold) was stripped in a rotary evaporator to remove excess solvent and then freeze-dried.

The following steps of isolation of homogenous components from desalted total extract of the culture medium of *L. japonicus* subsp. *casei* included multistage separation using ion-exchange chromatography and analytical RP-HPLC. At the first stage the lyophilized total extract was separated by cation-exchange chromatography in a Heparin-Sepharose column. The separation resulted in the obtainment of two subfractions: fraction SF1, which did not bind the column, and SF2 eluted from the sorbent using 1.0 M NaCl (data not shown). Quantitative estimation of the obtained total fractions by the optical density at 280 nm showed the predominant content of subfractions not binding the column sorbent. Both subfractions were desalted by reversed phase low-pressure chromatography as described above, evaporated, and freeze-dried.

Reactivating activity was revealed in both desalted subfractions; however, it was more pronounced in the sorbent-binding subfraction SF2.

At the second stage of the analysis, subfractions were separated by analitycal RP-HPLC in a Luna C_8 column with a linear gradient of acetonitrile in TFA. Fractionation of SF2, which had been previously eluted with 1.0 M NaCl, resulted in the obtainment of 20 main fractions, with the maximum absorbance at 210 nm (Fig. 2). All fractions were freeze-dried to remove acetonitrile and residual TFA and studied for the presence of reactivating activity. Activity was tested in the fractions that had the most optical densities (peaks 9, 11, 12, 14, 15, 17, and 19); it was found only in three peaks (9, 11, and 12, Fig. 2) out of seven.

The analogous separation of the fraction not bound with the column sorbent at the first separation stage (SF1) resulted in the obtainment of 25 main subfractions absorbing at 210 nm (Fig. 3). All obtained subfractions were also freeze-dried to remove acetonitrile and residual TFA and checked for the presence of biological activity. Activity was tested in the fractions that had the most ODs (peaks 2–5, 14–20, and 23–25); the reactivating effect (2.5-fold) was found only in fraction 25, which was the most hydrophobic out of all of those presented in the profile.

Subfraction 12 from SF2 had the most pronounced reactivating effect among all of the studied subfractions. That is why further studies of the nature and physicochemical properties of components comprising it were performed.

Mass spectra of the fraction in a range of 1–10 kDa were registered by MALDI-TOF mass spectrometry (MS) in the regime of positive ions. The results showed that this component included substances with molecular weights of 0.4–2.5 kDa (Fig. 4). Further MS/MS fragmentation of the sample revealed a set of monoisotopic molecular weights that differed in the masses of certain amino acid residues. This fact allowed us to suppose that this compound has a peptide nature. In a similar way, we performed preliminary identification of sugar residues—fucose and hexose, which are probably attached in different positions to amino acid residues by glycosidic bonds.

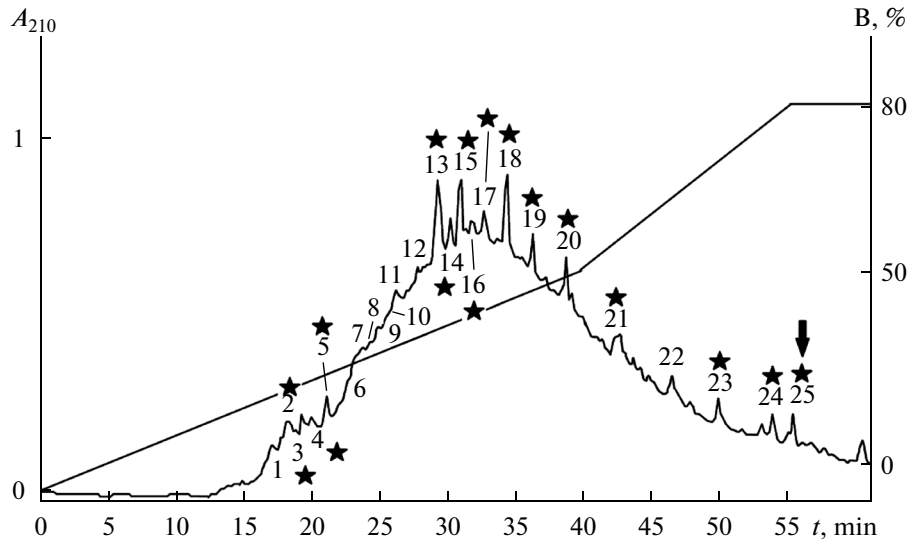

Fig. 3. RP-HPLC of the total fraction SF1, which did not bind the sorbent during affinity chromatography.

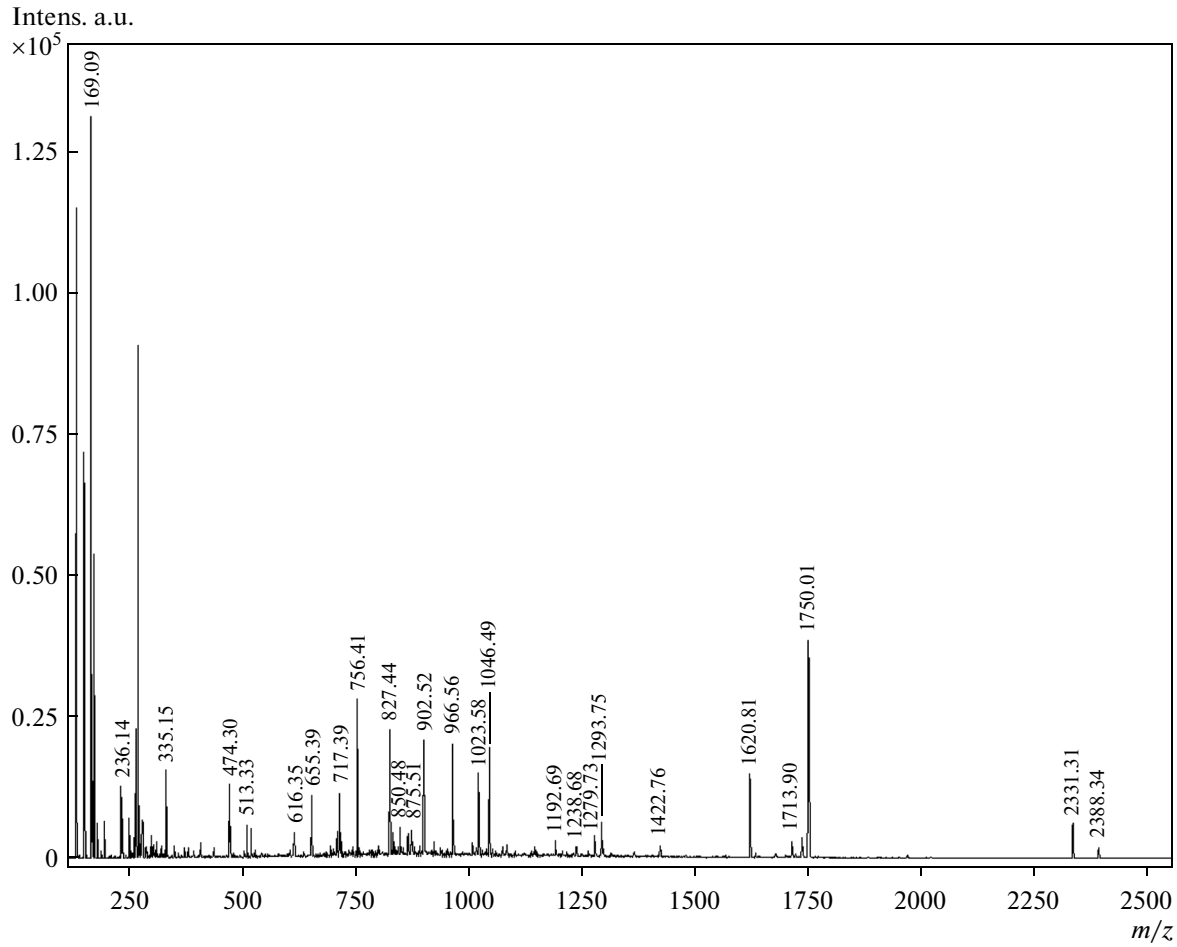

Fig. 4. Mass spectrometric analysis of the most active fraction, #12, resulting from RP-HPLC of the fraction SF2.

Table 2. Antioxidant capacity (AOC) of RF fractions

Subfraction	Protein concentration (mg/mL)	AOC for the peroxyl radical		AOC for the ABTS radical cation	
		μmol of TE/L	μmol of TE/g of protein	μmol of TE/L	μmol of TE/g of protein
RF-S	0.043	93.16 ± 8.12	2166.5 ± 188.8	39.01 ± 1.94	907.3 ± 45.2
∂ 9	<0.02	5.94 ± 0.17	–	<10	–
∂ 11	<0.02	6.67 ± 0.24	–	<10	–

Antioxidant activity of RF. The study of antioxidant properties showed that RF is able to quench organic radicals that significantly differed in reactivity (Table 2). Thus, for the ABTS radical cation oxidation-reduction, the potential was 680–700 mV, while for the peroxyl radical it was about 1000 mV [29, 30]. RF AOC values for the ABTS radical cation and the peroxyl radical were 91.2 and 70.0 μM of TE, respectively. The analogous AOC values are characteristic of a L-tryptophane solution with a concentration of 25–27 μM [31, 32]. It is known that only 5 (Trp, Tyr, Cys, Met, and His) out of 20 protein amino acids show antioxidant properties toward the peroxyl radical and 3 (Trp, Tyr, and Cys) are active in relation to ABTS [33] at concentrations up to 1.0 μM. It allowed us to propose the presence of residues of the redox-active amino acids mentioned above in RF oligopeptides. Since oxidative stress is a common response for various stress factors [34], including UV irradiation, RF antioxidant properties indicate that the mechanism of its biological effects can involve its participation in the regulation of antioxidant status and redox signaling. The literature contains descriptions of more than 100 antioxidant peptides isolated from various sources and/or obtained by protein conversion using microorganisms and enzymes [35, 36]. Antioxidant activity is believed to be the reason [37] for multifunctionality (prevention of dysbacteriosis, protective and antiulcer actions) of low-molecular peptides of lactoferrin and α-lactalbumin, because they contain residues of redox-active amino acids. Investigation of the mechanism of RF action is impossible without the discovery of the structures of its active components, which will be the purpose of our further studies.

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