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Influence of the Hydrophobicity of *Galleria mellonella* Antibacterial Peptides on the Parameters of Their Chromatographic Retention

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Abstract—The hydrophobicity of antibacterial peptides of *Galleria mellonella* calculated using the HyperChem and SSRCalc algorithms of the additive chromatographic separation model has been compared with experimental data on the separation of these peptides by reversed-phase high-performance liquid chromatography on SiO_2-C18. It has been found that there are some groups of peptides of different structures and functionalities, the chromatographic behavior of which can be adequately described on the basis of a preliminary assessment of their hydrophobicity. Within these groups, retention values of the peptides can be predicted for their identification.

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INTRODUCTION

Hemolymph peptides of *Galleria mellonella* (*GM*) caterpillar larvae, which exhibit antibacterial activity, are biomolecules, which have molecular masses of up to 5 kDa and contain amino acids with acidic and basic properties, different polarities, and hydrophobicities [1]. The hydrophobicity of proteins and peptides is used to assess their biological properties; it affects their interaction with each other and with cellular membranes, incorporation into nonpolar lipid media, receptor–ligand complexation, and distribution in tissues. Being one of the main factors of the stabilization of biostructures, the hydrophobic effect plays a significant role in their formation. The most common method for determining the hydrophobicity of amino acids is the analysis of their partition between two immiscible liquid phases. The octanol/water system is widely used to assess the biological activity of molecules, because it simulates the water/lipids system. The procedure for calculating partition coefficients of diverse substances has been elaborated in detail for this system [2, 3]. Additive models of chromatographic separation of peptides can more or less exactly predict their retention times and elution order under the assumption that the character of the interaction of peptides with a sorbent surface is governed by the total effect of different interacting sites distributed over amino acid chains [4]. One of the most successful methods for describing the hydrophobic effect is the LSS (Linear Solvent Strength) empirical concept, which implies that the linear correlation of chromatographic data is a consequence of the theory of linear solvent strength proposed by Snyder [5]. The discovery of the dependence of the degree of retention of small peptides and peptide-based hormones on the total degree of hydrophobicity for the majority of hydrophobic amino acid residues of a peptide [6] has made it possible to use the values of $\log P'$ (partition coefficient in the octanol/water system) for predicting retention times t_R of peptides in reversed-phase high-performance liquid chromatography (RP HPLC). However, for large macromolecules, this relation is not always fulfilled, since in this case, the separation proceeds by both the adsorption and exclusion mechanisms [4, 6].

In [7], it was proposed to use the values of relative hydrophobicity P of peptides as normalized retention times. The values of P are obtained by the SSRCalc (Sequence Specific Retention Calculator) algorithm used for calculating retention times on the basis of amino acid sequences [8, 9]. The SSRCalc algorithm is underlain by the multiparameter optimization of the free parameters of the peptide separation model for specific HPLC conditions. This approach is based the linear t_R–P dependence for a set of peptides that presumably belong to a protein being identified.

One of the promising scopes in the field of the quantitative analysis and the identification of proteins by chromatography–mass spectrometry is an approach based on the use of databases of exact masses and chromatographic retention times of peptide markers of proteins. This approach has been realized in the multipoint normalization model [10], which is underlain by the concept of the linearity of chromatographic

Table 1. Conditions for chromatographic separation of *Galleria mellonella* antibacterial peptides on Zorbax Eclipse XDB-C18

No.	Eluents	Gradient elution regime	Eluent flow rate and column T, °C	Injected sample volume, collection range
1	0.04% TFA* in water (*A*)–AcCN** (*B*)	5–80% *B* in a range of 0–30 min, 80–100% *B* in a range of 30–32 min	0.5 mL/min, 25°C	20 μL, 1–30 min (after 1 min)
2	0.04% TFA in water (*A*)–0.04% TFA in AcCN (*B*)	5–80% *B* in a range of 0–30 min, 80–100% *B* in a range of 30–32 min	0.5 mL/min, 25°C	60 μL, 3–9 min (after 3 min), 9–21.5 min (after 0.5 min), 21.5–30.5 min (after 3 min)
3	0.04% TFA in water (*A*)–0.04% TFA in AcCN (*B*)	10% *B* in a range of 0–5 min, 10–80% *B* in a range of 5–45 min	0.5 mL/min, 25°C	25 μL, 5–45 min (after 1 min)

* Trifluoroacetic acid, ** acetonitrile.

data obtained under different separation conditions typical for proteomic studies.

The calculation prediction methods based on models relating chromatographic retention to the hydrophobicity parameters of sorbates are efficient when detailed studies are carried out for any single limited class of compounds. Immunized hemolymph of *GM* contains representatives of different classes of organic compounds [11, 12]. Since all molecular interactions in such a chromatographic system cannot be completely taken into account, it is desirable to use a simple and universal model. The SSRCalc algorithm is currently considered to be most accurate for calculating the retention times of tryptic peptides and is widely used in proteomic studies. The application of this approach is limited by the conditions typical for proteomic investigations performed with the use of chromatography and mass spectrometry [13]. Moreover, changes in the pore sizes of a sorbent or in the gradient elution profile will require a readjustment of the entire SSRCalc program.

The combination of RP HPLC and matrix-assisted mass spectrometry (MS) with laser desorption/ionization (MALDI) when studying peptides substantially facilitates their separation and identification in complex mixtures [14, 15]. Taken together, the exact time, at which a peptide leaves a column, and the found peptide mass create the mass-time label of the peptide, with this label identifying it almost unambiguously.

Bioactive peptide products obtained using immune responses of insect underlie the development of new pharmacological agents, which are alternatives to currently used antibiotics. The mechanism of the antimicrobial activity of peptides in living organisms is, in many cases, similar to their chromatographic behavior (sorption, changes in their conformations, and interactions of hydrophobic and hydrophilic moieties). All of the aforementioned indicates that chromatographic methods are promising for studying the biological activity of peptides.

The goal of this work is to compare experimental data on the parameters of the chromatographic retention of antibacterial peptides of *GM* hemolymph on the SiO_2-C18 sorbent with the retention times theoretically calculated for these peptides on the basis of their hydrophobicity.

EXPERIMENTAL

Antibacterial peptides were induced in the hemolymph of *GM* larvae by immunizing them with *Escherichia coli* (*E. oli*) bacteria or asymmetric dimethylhydrazine [11, 12]. The methods used to obtain hemolymph of larvae and prepare samples for biochemical and physicochemical examinations have been described in detail elsewhere [16].

The antibacterial peptides were analyzed and separated by RP HPLC using a Zorbax Eclipse XDB-C18 column (Agilent Technologies, United States) with sizes of 150 × 4.6 mm. The column was filled with C18 silica gel (grain size and pore diameter were 5 μm and 80 Å, respectively) and installed into an Agilent 1200 liquid chromatograph equipped with a diode array detector and the ChemStation A.10.02 software. The gradient elution regimes are presented in Table 1. Antibacterial nisin and Galleria defensin 1 peptides were selected as references.

The mass-spectrometric study of *GM* hemolymph fractions by the MALDI method was carried out using an UltraFlex II TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a nitrogen laser (λ = 337; laser radiation energy, 110 kJ; and pulse frequency, 20 Hz), a time-of-flight mass analyzer with a reflecton, and the FlexControl 3.4 and FlexAnalysis 3.4 software packages for accumulating and processing mass spectra. The accelerating voltage was 25 kV. Mass spectra were measured in the regime of recording positive ions. The fragmentation of initial metastable protonated peptide molecules was studied in the regime of decomposition outside of the ion source using an UltraFlex II TOF-TOF mass spectrometer (Bruker Daltonics) equipped with a neodymium laser (λ = 355 nm; laser radiation energy, 105 kJ; and pulse frequency, 20 Hz) and a time-of-flight mass analyzer

Table 2. Antibacterial peptides found in *Galleria mellonella* hemolymph

No.	Peptide	Amino acid sequence of a peptide*	Mass, *M*, Da	*N***	p*I****
1	Cecropin-B analog	WKVFKKIEKIGRNIRNGIVKAG-PLIAVLGEAKAL	3728	34	11.02
2	Cecropin-D-like peptide	ENFFKEIERAGQRIRDAIISAAPAVETL-AQAQKIIKGGD	4253	39	6.45
3	Proline-rich antimicrobial peptide 1	DIQIPGIKKPTHRDIIIPNWNPNVRTQP-WQRFGGNKS	4320	37	10.99
4	*Galleria* defensin 1	DTLIGSCVWGATNYTSDCNAECKRRG-YKGGHCGSFLNVNCWCE	4715	43	7.25
5	*Galleria* defensin 2	DTLIGRCVWGATNYTSDCNAE-CKRRGYKGGHCGSFLNVNCWCE	4786	43	7.25
6	Lebocin-like anionic peptide 1	EADEPLWLYKGDNIERAPTTADHPILP-SIIDDVKLDPNRRYA	4816	42	4.51
7	Proline-rich antimicrobial peptide 2	EIRLPEPFRFPSPTVPKPIDIDPILPHPWS-PRQTYPIIARRS	4929	42	9.97
8	Defensin-like peptide	DKLIGSCVWGATNYTSDCNAE-CKRRGYKGGHCGSFWNVNCWCEE	4949	44	7.46
9	Anionic antimicrobial peptide 2	ETESTPDYLKNIQQQLEEYTKNFNTQVQ-NAFDSDKIKSEVNNFIESLGKILNTEKKEAPK	6980	60	4.80

* Sequence of amino acids (single-letter code) [21], ** *N* is the number of amino acid residues in a peptide, and *** p*I* is the isoelectric point.

with reflecton. The mass spectra of ionized products were recorded by the LIFT method (Bruker Daltonics). The initial voltage and accelerating potential difference were 7 and 28 kV, respectively. The accuracy of measuring monoisotope masses $[M + H]^+$ in the reflecton regime was 0.007%, the accuracy of measuring averaged masses in the linear regime was 0.05–0.1%, and the accuracy of measuring the masses of fragments was 1–2 Da (0.02–0.1%).

Samples to be examined by MALDI were prepared on AnchorChip targets with a DHB (2,5-dihydroxybenzoic acid) matrix (Bruker Daltonics, Germany). A peptide solution (10 μL) was mixed with a 10 mg/mL matrix solution (0.3 μL) in an aqueous 20% acetonitrile (AcCN) solution containing an additive of a 0.5% of trifluoroacetic acid (TFA) solution. The resulting mixture was evaporated in air at the atmospheric pressure.

The Mascot system [17] was employed to determine the primary structure of peptides on the basis of information obtained upon their dissociation. The search was carried out using the masses of metastable protonated peptide molecules $[M + H]^+$ determined in [11] and those presented in the NCBI database [18]. The primary structure of unknown peptides was judged by the results of the de novo sequencing procedure [19].

Hydrophobicity of peptides was calculated from the partition coefficients in the octanol/water system with the help of the HyperChem 6.0 program [20].

The theoretical retention times of peptides were calculated using the SSRCalc program [9], which was based on the additive model of peptide separation and empirically determined hydrophobicity coefficients.

AcCN (for HPLC, Aldrich, United States), TFA (99%, Alfa Aesar, Germany), and triply distilled water purified with Millipore filters (Milli-P QG, Waters, United States) were used in the experiments.

RESULTS AND DISCUSSION

Antibacterial peptides identified in the immunized *GM* larvae hemolymph [11, 21] are listed in Table 2.

Moreover, the reference and immunized *GM* hemolymph contained enzyme, lysozyme, and proteins, apolipophorin-3 and hemolymph protein precursor with molecular mass $M = 27$ kDa, which exhibited antibacterial axtivity [11] (Table 3).

Chromatographic separation of antibacterial peptides and *GM* hemolymph proteins in regime no. 3 has enabled us to determine factors k of their retention on SiO_2-C18 and confront their elution order with the degrees of their hydrophobicity (Table 3).

The data obtained for all peptides, except for peptide 4 (see Table 2), indicate a linear dependence of $\log k$ on $\log P$ (Fig. 1a), which may be described as

Table 3. Experimental logarithmic values of retention factor k for *Galleria mellonella* antibacterial peptides and calculated logarithmic values of their relative hydrophobicity P (SSRCalc) and partition coefficient in the octanol/water system P' (HyperChem)

Peptide no.	Peptide	M, Da	$\log k$	$\log P$	$\log P'$
3	Proline-rich antimicrobial peptide 1	4320	0.987	1.523	–15.42
6	Lebocin-like anionic peptide 1	4816	1.029	1.581	
4	*Galleria* defensin 1	4715	1.055	–	–12.24
1	Cecropin-B analog	3728	1.063	1.606	–4.89
7	Proline-rich antimicrobial peptide 2	4929	1.097	1.707	3.81
2	Cecropin-D-like peptide	4253	1.117	1.678	3.41
9	Anionic antimicrobial peptide 2	6980	1.143	1.672	
	Lysozyme	14027	1.101	1.641	
	Hemolymph protein precursor	23764	1.167	1.754	
	Apolipophorin-3	18075	1.196	1.698	

$\log k = a \log P + b$, where $a = 0.9624$ and $b = 0.5925$ (correlation coefficient is $r^2 = 0.8013$). The retention times of peptides under given chromatographic conditions can be predicted using this equation and calculated logarithmic relative hydrophobicity $\log P$.

The peculiar behavior of peptide 4 is apparently caused by its structure. Defensins (*Galleria* defensin) are characterized by the presence of three disulfide bridges that stabilize the tertiary structure. In addition, defensins have, as a rule, a short N-terminal α-helix and a tertiary structure of a β-folded sheet, which have the same lengths and relative arrangements. Due to the presence of the disulfide bonds in the primary structure of *Galleria* defensin 1 and nisin, the character of their interaction with the nonpolar surface of C18 silica gel differs from that of other peptides, which do not contain these bonds.

On the basis of their amino acid sequences and structural features, cecropins (cecropin-B analog and cecropin-D-like peptide) have been assigned to linear α-helical peptides that do not contain cysteine residues, while proline-containing peptides (proline-rich antimicrobial peptide 1 and proline-rich antimicrobial peptide 2) have been attributed to peptides with overrepresentation of proline and glycine residues [21].

The use of the HyperChem program for calculating retention factors has enabled us to obtain the linear dependence of $\log k$ on $\lg P'$ only for five antibacterial peptides (Table 3, Fig. 1b). This dependence is described by equation $\log k = 161.13 \log P' - 176.49$ (correlation coefficient is $r^2 = 0.8013$). The absence of the linear correlation for anionic peptides (lebocin-like anionic peptide, anionic antimicrobial peptide, and apolipophorin-3) results from the peculiarities of these polar compounds. Deviations from the linear dependence are observed rather often when considering groups of compounds that differ not only in hydrophobic fragments, but also in the nature of functional

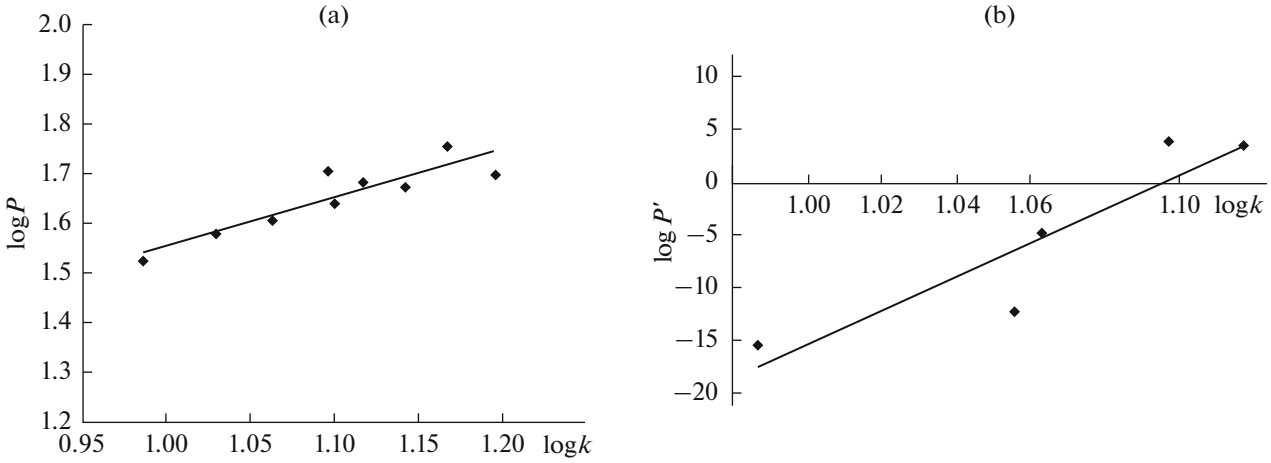

Fig. 1. Dependences of logarithmic retention factor $\log k$ on (a) logarithmic relative hydrophobicity $\log P$ and (b) logarithmic partition coefficient $\log P'$ in the octanol/water system for *Galleria mellonella* antibacterial peptides.

Table 4. Masses and amino acid compositions of protonated molecules of previously unknown peptides found in *Galleria mellonella* hemolymph immunized with 1% solution of asymmetric dimethylhydrazine

Peptide no.	$[M + H]^+$, Da	Peptide amino acid sequences
1	1105	PTDERLGYR
2	1211	CAELEKWWF
3	1233	RELESTLETR
4	1241	GLSVAQGRDALPG
5	1359	RRGNDNFRLDP
6	1523	WRSPSNPYPYKK
7	1783	KHESESEFLSESTQF
8	1590	HPTVVAYYCPMYF
9	1927	RMNYAASPFPSAHPHFV
10	1948	LYYKKKYKSNYHPNT
11	2108	DWPTTHTWLFHGKTWPP
12	2185	KAPETESGAAGGGSHSCVRSPLS
13	2340	GGASGHFNPYDYSYPNSPKWP
14	2559	CMSMWMCGKSSRIRYMMIHAH
15	2563	DHWWLSRVNEGCVRSKDLEHP
16	3256	PGAYYMMKAEMSVTYTACDEAKNYKRFS
17	3731	PVCGKCSHWAPNDFGNTTHLWVDPDWELKALHG
18	4047	RHALASLKDYTGTNKNHETHDKSKGCWWVSVVCYL

groups. It is also possible that the absence of the linear correlation is related to the features of the calculations by the HyperChem program; therefore, the search for models relating parameters $\log k$ and $\log P'$ more correctly is being continued.

In the case of immunization *GM* with highly concentrated asymmetric hydrazine or *E. coli* (Table 4), induction of unknown peptides with masses of up to 4 kDa has been detected by MALDI-MS. They seem to be formed as a result of oxidative degradation of the peptides. Using the SSRCalc algorithm, relative hydrophobicity $\log P$ was calculated for amino acid sequences of previously unknown peptides of *GM* (detected by MALDI-MS). Figure 2 shows the dependence the order of elution of these peptides on their hydrophobicity. For the separation of peptides 1–3, 6–10, 12–14, and 16 (Table 4) in the gradient elution regime no. 2, the following correlation dependence was obtained: $\log k = 1.7959 \log P - 0.2683$.

The revealed correlation dependences between logarithmic values of k and P, P' may underlie a priori calculations of both chromatographic retention and hydrophobicity. Moreover, they enable one to describe the retention of compounds of different classes using a single model and may become the basis for the development of a method for identifying new antibacterial peptides.

Results of experimental determining retention times t_R of peptides in different regimes of separation of their mixtures were compared with the t_R values calculated by the SSRCalc method to describe the regularities of the chromatographic separation of peptides by gradient RP HPLC (Tables 5, 6).

For chromatographic regime no. 1 (Table 1), satisfactory correlations have been found between the experimental and calculated retention times of antibacterial peptides of *GM* (Table 5). The SSRCalc program adequately describes the sorption behavior of

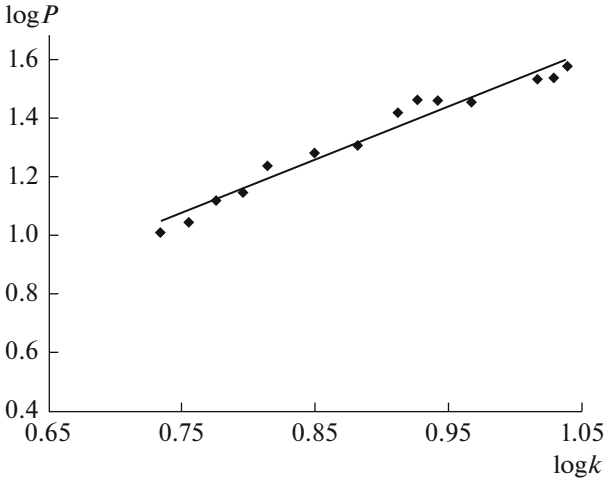

Fig. 2. Dependence of logarithmic retention factor on the calculated logarithmic relative hydrophobicity for previously unknown peptides 1–3, 6–10, 12–14, and 16 (Table 4) identified by MALDI-MS.

Table 5. Physicochemical characteristics for the separation of a mixture of *Galleria mellonella* antibacterial peptides in chromatographic regime no. 1

Peptide no.	Peptide	*M*, Da	*k*	t_R, min	
				experiment	calculation (SSRCalc)
3	Proline-rich antimicrobial peptide 1	4320	6.6	13.8	15.2
2f	Cecropin-D-like peptide	2078	6.9	14.3	11.7
5	*Galleria* defensin 2	4786	7.2	14.8	14.4
6	Lebocin-like anionic peptide 1	4816	7.4	15.2	16.6
4	*Galleria* defensin 1	4715	8.0	16.2	14.6
9f	Anionic antimicrobial peptide 2	3420	8.5	17.2	15
2f	Cecropin-D-like peptide	3031	8.9	17.9	15.8
9	Anionic antimicrobial peptide 2	6980	9.4	18.8	17.7
2	Cecropin-D-like peptide	4253	9.6	19.2	18.1

Table 6. Physicochemical characteristics for the separation of a mixture of *Galleria mellonella* antibacterial peptides in chromatographic regime no. 3

Peptide no.	Peptide	*k*	t_R, min	
			experiment	calculation (SSRCalc)
3	Proline-rich antimicrobial peptide 1	9.6	26.8	26.5
6	Lebocin-like anionic peptide 1	10.7	29.2	28.5
4	*Galleria* defensin 1	11.4	30.9	25.5
	Nisin	11.5	31.2	22.5
1	Cecropin-B analog	11.6	31.4	30.5
7	Proline-rich antimicrobial peptide 2	12.2	33.7	31.1
2	Cecropin-D-like peptide	13.1	35.2	30.6
9	Anionic antimicrobial peptide 2	13.9	37.2	30.1
	Apolipophorin-3	15.7	41.7	33.4

peptides 2, 3, 5, 6, and 9, and somewhat worse—the behavior of fragments of these peptides (2f, 9f) (Table 5).

For previously unknown induced peptides of *GM* (Table 4), satisfactory correlations have also been obtained between the experimental and calculated retention times in separation regime no. 1 (Fig. 3).

Under the conditions of chromatographic regime no. 1, antibacterial peptides are eluted in an AcCN concentration range of 35–55%, while, in regime no. 3, they are eluted in a range of 45–75%. The difference as large this is due to the presence of TFA in AcCN (Table 1). TFA is an ion-paired agent, which maintains a constant pH value throughout the separation process. When separating antibacterial peptides in regime no. 3 (Table 1), the chromatographic behavior of peptides 2 and 9 is not described quite adequately by the SSRCalc model (Table 6) in contrast to the separation in regime no. 1. However, for other antibacterial peptides, good correlations between the experimental and calculated retention times have been observed. Linear dependences (Fig. 4) were obtained for peptides 1, 3, 6, and 7 and Lysozyme (Table 2), as well as for previously unknown peptides 14 and 16 (Table 4).

Thus, the additive scheme of calculations using the SSRCalc algorithm makes it possible to achieve practically acceptable reliability of predicting the retention of peptides, while the obtained correlation dependences may become the basis for developing a method for identifying unknown antibacterial peptides.

Additive models for calculating chromatographic separation parameters on the basis of the summation of increments are especially urgent for peptides containing a large number of different amino acids, with each of them having different reactive groups distributed throughout the molecule. Antimicrobial cecropins (cecropin-B analog and cecropin-D-like peptide) are cationic α-helical peptides that do not

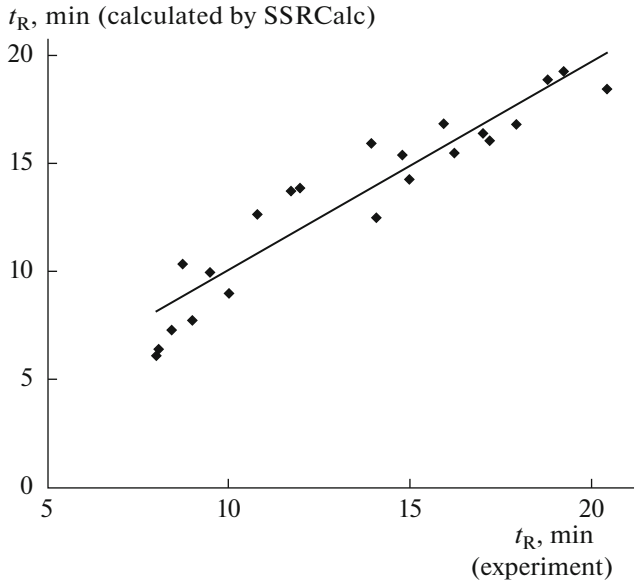

Fig. 3. Comparison between experimental (separation regime no. 1) and calculated (SSRCalc algorithm) retention times of previously unknown induced antibacterial peptides of *Galleria mellonella* (see Table 4).

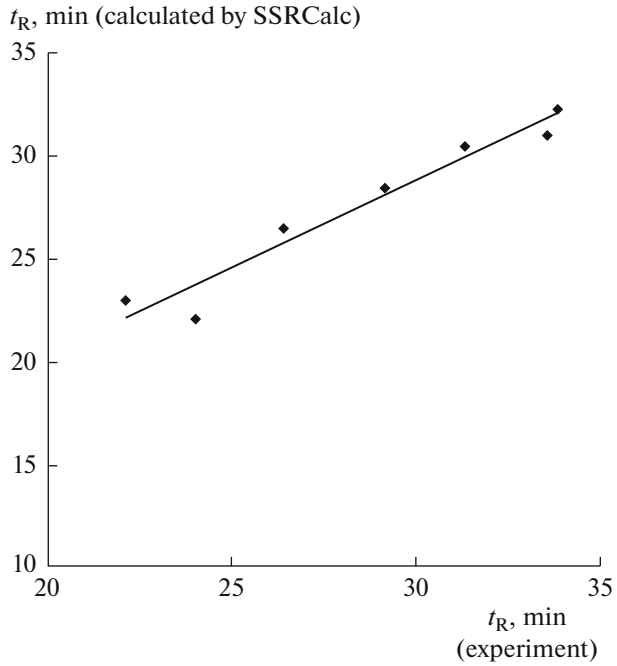

Fig. 4. Comparison between experimental (separation regime no. 3) and calculated (SSRCalc algorithm) retention times of *Galleria mellonella* antibacterial peptides.

contain cysteine residues; anionic peptides include lebocin-like anionic peptide, anionic antimicrobial peptide, defensin-like peptide, and apolipophorin-3. The structure of peptides *Galleria* defensin and nisin is stabilized by disulfide bridges, proline-rich antimicrobial peptides 1 and 2 are peptides with overrepresentation of proline and glycine residues [21]. The comparison between experimental and calculated data has shown that the retention times determined experimentally and calculated theoretically for peptides do not always correlate with each other apparently because of the interaction of the peptides with C18 sorbent not only due to dispersion forces. As a result, none of the considered algorithms of the additive model (SSRCalc and HyperChem) does not completely describe the separation of all antimicrobial peptides of *GM* and the products of their oxidative degradation; however, the predictions obtained using the SSRCalc algorithm are experimentally confirmed more often. The largest approximation errors are observed for peptides with unprotected end groups, because the contributions of these polar functional groups to the balance of hydrophilic and hydrophobic properties in a system comprising an aqueous AcCN solution and C18 alkyl groups differ from these contributions in the octanol/water system. However, the chromatographic behavior of some peptides in different separation regimes has been described with a high accuracy. At the same time, when the separation conditions are changed (e.g., upon the passage from regime no. 1 to regime no. 3), the accuracy of the model description of the chromatographic behavior of different peptides changes as well. The data obtained on the most accurate and adequate description of some peptides in separation regime no. 1 and other peptides in regime no. 3 supplement each other and can be considered as one of the physicochemical characteristics for the separation of these peptides. The accumulation of experimental data on the retention of peptides under the RP HPLC conditions will make it possible to further correct the existing models of the physicochemical behavior of these compounds; create new models; substantiate the optimal conditions for the separation of mixtures; and, when combining the RP HPLC and MALDI-MS methods, identify the components of complex mixtures of peptides.

CONCLUSIONS

The chromatographic behavior under the conditions of RP HPLC on C18 silica gel has been experimentally studied for *Galleria mellonella* antibacterial peptides, their fragments, and modifications induced by the action of asymmetric dimethylhydrazine or *E. coli* bacteria on hemolymph of *GM* larvae. It has been found that there are groups of peptides with different structures and functionalities, the chromatographic behavior of which can be adequately described on the basis of the preliminary assessment of their hydrophobicity using the HyperChem and SSRCalc algorithms of the additive model of chromatographic separation. Within these groups, linear dependences of the retention factors of peptides on their hydropho-

bicity have been obtained, which has made it possible to predict the order of their elution.

The calculation methods of prediction that relate chromatographic retention with peptide hydrophobicity parameters are efficient in the case of narrow classes of substances, because the systems of increments in the additive model used for the calculations have been developed for compounds of the same type. The immunized *GM* hemolymph contains organic compounds of different classes; therefore, none of the considered additive models completely describes the separation of all antimicrobial products of *GM*; however, the predictions obtained using the SSRCalc algorithm are experimentally confirmed most often. The observed deviations from the linear dependences are probably associated with the features of peptides, which differ from each other not only in hydrophobic fragments, but also in the nature of functional groups. At the same time, the entire set of the obtained regularities provides the basis for describing the chromatographic behavior of antibacterial peptides from different points of view, thereby revealing the factors that primarily govern their retention.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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