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MALDI-TOF Mass Spectrometric Identification of Novel Intercellular Space Peptides

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We performed the matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry (**MALDI-TOF**) analysis of the peptides entering into the composition of not yet explored bioregulators derived from the extracellular matrix of the tissues of the various organs of the mammals, and also plants and fungi. The study included 15 different mammalian tissues, 13 species of plants, and 2 species of fungi. Exploring the bioregulators derived from eye tissues, we demonstrated that their composition includes peptide components with the same values of the molecular weight. The composition of the bioregulators derived from the tissues of various organs of mammals or different species of plants and fungi includes the peptides with different values of molecular weight. Obtained data indicate the growing evidence of the assumptions about the major function of the bioregulators of this group—their involvement in the regulation of tissue-organ homeostasis in the biological systems.

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In the different tissues of mammals and plants, we found the new, not explored group of bioregulators that in minute doses (10^{-8}–10^{-15} mg protein/ml) influence migration and adhesion of the cells and cell proliferation and differentiation. We demonstrated that the feature of bioregulators of this group to stimulate restoration and reparation in the pathologically modified tissues is connected to their ability to additionally activate the cell sources of regeneration [1–10]. Based on the singularity of their properties and activities, these bioregulators are allocated into a separate group of membrane–tropic homeostatic tissue-specific bioregulators (**MHTBs**).

It is determined that MHTBs in minute doses stimulate the restoration processes in the pathologically modified tissues, and their biological activity is characterized with the absence of species- but the presence of the tissue-specificity. On the basis of the MHTBs, pharmacological preparations have been developed and applied in medicine, for example, Adgelon for the treatment of the keratopathies of different aetiology, joint diseases, and fractures. The big perspectives of application of the MHTBs is causing great interest in the search and isolation of them from different biological objects (mammals, plants, microorganisms, etc.).

Now it is known that MHTBs have a complex composition: NMR showed the presence in them of not only protein but also carbohydrate and lipid components; however, their functional role in the composition of MHTBs remains unknown [6, 11–13]. The most studied components of the MHTBs are peptides (mol. weight 1–8 kDa) responsible for the whole composition activity, and also modulator proteins, influencing the biological action of peptides and interacting with them by the Ca^{2+} dependent mechanism [13, 14]. Moreover, we determined the extracellular localization of the MHTBs, for instance, MHTBs obtained from rat liver are localized in the sinusoidal area, MHTBs obtained from bovine serum are localized in the interhepatocyte space, MHTBs obtained from bull retina are localized on the surface of the photoreceptor processes, and MHTBs obtained from the common plantain are localized in the intercellular spaces of the leaf tissues [8, 15–17]. For isolation and purification of the bioregulators of this group, we developed an original method, including the biochemical methods, the method of biotesting, and methods of physical chemical protein research [1, 18]. Application of this methodology allows us to obtain small quantities of the refined peptides entering into the composition of MHTBs, which defined an application of the mass-spectrometric methods for studying them. High sensitivity of MALDI-TOF mass-spectrometry (10^{-4}–10^{-8} M) allows determination of the minor components of the

complex protein mixtures and determination of the molecular weights of these proteins on every stage of research.

We should note that the main stage of MHTB purification is the separation of them from the doped proteins salting out the tissue extract in the saturated solution of the ammonium sulfate. In this condition, MHTBs stay in the solved state and other proteins precipitate. Thus, the fractions of the supernatant fluid (supernatants) derived after the salting out of the tissue extracts contain, in general, MHTBs that are presented as a complex of the biologically active peptides and the modulator, consisting of proteins with molecular weight of 15–66 kDa [14]. Therefore, research of the peptide composition now appears to be urgent, because this allows us to determine more thoroughly the peptide components of the MHTBs.

In the current research, we identified the peptides entering into the composition of MHTBs obtained from the diverse sources: tissues of mammals, plants, and also fungi. Using MALDI-TOF mass spectrometry, we analyzed supernatants of 15 tissue extracts of animals, 18 of plants, and of 2 fungi species.

The goal of the work was to establish the molecular weights of the peptide components entering into the composition of the supernatant fractions of MHTBs.

METHODS

Biological material. Eye tissues (the sclera, cornea, lens, iris, vitreous humour, retina, pigment epithelium, ciliary body) were obtained from freshly isolated eyes of *Bos taurus Taurus* L. young bulls of less than 1 year (total amount of eyes was 60) that were, as well as liver (1.5 kg), presented by the Tagansk Meat Processing Complex, Moscow. The liver, brain, heart, and bones were isolated from Wistar rats of both sexes weighing 180–250 g from the vivarium of the Koltzov Institute of Developmental Biology (Russian Academy of Sciences). We also used the preparation "Sterile inactivated bovine serum, the nutritious addition for cell and tissue culturing" produced in the Chumakov Institute of Poliomyelitis and Viral Encephalitis (Russian Academy of Medical Sciences) and fetal bovine serum from Serva (Germany).

The plants (onion *Allium cepa* L., garlic *Allium sativum* L., aloe *Aloe arborescens* L., celandine *Chelidonium* L., beet *Beta vulgaris* L., watermelon *Citrullus lanatus* Thunb., melon *Cucumis melo* L., pumpkin *Cucurbita pepo* L., horseradish *Armoracia rusticana* Gilib., lemon *Citrus limon* L., plantain *Plantago major* L., wormwood *Artemisia absinthium* L., and dandelion *Taraxacum officinale* Wigg.) were received from Tsitsin's Botanical Garden (Russian Academy of Sciences); fungi (chanterelle *Cantharellus cibarius* Fr. and tinder *Fomes fomentarius* L.) were picked up in the ecologic region of the Vladimir oblast.

Bioregulator isolation. Mammalian tissues including bulls' eyes were separated microsurgically and, the same way as plants' and fungal tissues, were cut into fragments of 1–1.5 cm. Fragments then were extracted for 2 hours at 4°C in aqueous salt solution (0.15 M NaCl, 1mM $CaCl_2$, 1 mM Hepes). After the centrifugation of the tissue extract (3000 g, 30 min), the supernatant was collected, salted out with dry ammonium sulfate until saturation (salt concentration of 780 g/l) in the presence of 10^{-2} M EDTA and leaved for 80–90 h at 4°C. The obtained salt solution of the protein mixture was centrifuged (10500 g, 45 min). After that, the supernatant was separated from the pellet and dialysed against water until the full removal of the salt. Obtained salt-free supernatants of the extracts were concentrated using the vacuum rotary evaporator at 40°C; protein content was determined quantitatively by the Warburg and Christian method [19]; membrane tropism was determined by measuring the adhesion [2] and it wa researched by the MALDI-TOF mass spectrometry method.

Mass spectrometry analysis. Mass spectrometry analysis was carried out with the time-of-flight MALDI-TOF mass spectrometer UltraFlex 2 (Bruker Daltonics, Germany), equipped with a nitrogen laser 337 nm at the impulse frequency up to 20 Hz. All the measurements were implemented in the linear mode, detecting the positively charged ions. For the accumulation of the mass spectrums, the powerfulness of the laser emission was established on the level of the minimal threshold value sufficient for the adsorbtion–ionization of the sample. The parameters of the mass spectrometer were optimized for the m/z range from 1000 to 20000. The outer calibration was conducted with use of the exact values of masses of the known proteins. The sample was added to three wells of the plate, and the spectrum derived from the summation of the 10 series of spectrums with 50 laser impulses for each was read. For the writing, processing and analysis of mass spectrums the following Bruker Daltonics software was used (Germany): flexControl 2.4 (Build 38) and flexAnalysis 2.4 (Build 11). The accuracy of mass measurements was ±2 Da. As a matrix, we used α-cyano-4-hydroxycinnamic acid Sigma-Aldrich (Germany) as a saturated solution in the mixture of 50% acetonitrile and 2.5% trifluoroacetic acid. All the reagents used, including the water, were of analytical grade or specialized for mass spectrometry.

Interpretation of the mass spectra. Interpreting the mass spectra we proceed from the assumption that most of the registered signals correspond to the protein molecules, while the determined masses correspond to the masses of the whole (not a fragmented) proteins. Identification of the proteins was implemented using the search of the matches of the experimental masses values with the masses of the proteins annotated in the SwissProt/TrEMBL databases with use of the resources of ExPASy server (http://www.uniprot.org.). Introducing the parameter of the "molecular mass," we used the experimental value of the mass measured with an accuracy of ±2 Da. In the case of failure, we ran the

Table 1. Signals of the mass spectra of peptides identified in the rat and bull tissue supernatants

Source	M, *m/z*	Protein concentration, mg/ml
Bos taurus Tauris L. sclera	4171, 4302, 4531, 4819	0.039
Bos taurus Tauris L. cornea	1442, 3376, 3973, 4302, 4418, 4531, 4817, 8604	0.0105
Bos taurus Tauris L. lens	4302, 4529, 4817, 8604	0.0041
Bos taurus Tauris L. iris	3944, 4301	0.083
Bos taurus Tauris L. ciliary body	4301	0.068
Bos taurus Tauris L. vitreous humour	4300, 4370, 4420	0.081
Bos taurus Tauris L. retina	4302, 4528, 4819, 8603	0.068
Bos taurus Tauris L. pigment epithelium	4303, 4532, 4819	0.0017
Bovine serum	1666, 1812, 1915, 2016	0.072
Fetal bovine serum	4301, 8601	0.100
Bos taurus Tauris L. liver	2112, 2170, 2866, 2940, 3009, 3151, 5026, 5237	1.370
Wistar rat liver	3649, 5025	1.000
Wistar rat brain	2820, 3481, 4300, 4331, 4403, 4671, 4801, 9945	1.690
Wistar rat heart	3049, 3262, 7565, 7684, 8463, 8581, 8766, 8967, 9094, 9951, 10404	1.520
Wistar rat bone	4301	0.050

search again with the mass value corresponding to the loss of the N-terminal methionine, taking into account the possibility of the proteins' posttranslational modifications.

Statistical analysis. For the forming of the intermediate tables, implementation of the elementary calculations, descriptive statistics, and plotting, we used the Microsoft Office Excel 2003 software.

RESULTS AND DISCUSSION

In the present research, we explored 15 fractions of MHTBs obtained from the different tissues of mammals, and also 18 fractions of the bioregulators of this group and 13 species of plants and the bioregulators obtained from the 2 species of fungi (Tables 1, 2). As shown earlier, salting out in the saturated solution of the ammonium sulfate gives the precipitation of all the doped proteins [4, 6]. Precipitated proteins did not show membrane tropic activity; therefore, this fraction was not explored further. In the supernatant, were the bioregulators rendering the membrane tropic action in minute doses. Therefore, it seemed urgent to implement the comparative research of the peptide composition of bioregulators isolated from the different objects on this stage of purification and also for the identification of the minor components entering into the composition of bioregulators [6, 13]. Concentration of the total protein in the explored fractions of the supernatants varied from 2 μg/ml to 1.7 mg/ml for the proteins of the animal origin (Table 1) and from 60 μg/ml to 3 mg/ml for the proteins of the plant and fungal origin (Table 2). We used the standard protocol of mass spectrometry with use of matrices and parameters of reading the mass spectrums, allowing us to register predominantly protein molecules. We analyzed three of the most used matrices for the ionization of the sample of MALDI-TOF mass spectrometry. As a result of an evaluation of mass spectrums, repeatability, resolution of signals, ratio of signal to the noise, number of signals and their intensity, and the range of values of mass/charge registered through the analysis, we chose α-cyano-4-hydroxycinnamic acid for the analysis. The read of the mass spectrums was implemented in the range of 1000–20000 *m/z*; in general, the signals of regulatory peptides were found in the range of 2000–10000 *m/z*, which correlates to the data obtained in the study of MHTBs with other methods [11, 13, 14]. Most informational signals were in the range of 2000 to 5000 *m/z* (Tables 1, 2).

The analysis of the values of the mass spectrums of the peptides contained in the supernatants of the extracts of the different tissues of rat and bull revealed that in some tissues of organs, for example the brain and bone (tibia) of rat, fetal bovine serum (FBS), retina, lens, vitreous body, iris, ciliary body, pigment epithelium, and sclera and cornea of bull, a peptide with a molecular weight of 4301 ± 2 Da was present (Table 1). This peptide was not found in the bioregulators obtained from the serum and liver of mature bulls and liver and heart of rats (Table 1). We have to note that in the bioregulators obtained from the bull eye tissues

Table 2. Signals of the mass spectra of peptides identified in the plants and fungi tissue supernatants

Source	M, *m/z*	Protein concentration, mg/ml
Onion *Allium cepa* L.	4037	2.690
Garlic *Allium sativum* L.	2997, 3308, 3646, 3833, 6838, 7675, 8256, 8350, 8478	0.798
Aloe *Aloe arborescens* L.	1142, 1807, 1974, 2908, 3032, 3256, 3331, 3412, 4213, 4349	0.160
Celandine *Chelidonium* L.	4844, 9688	0.364
Beet *Beta vulgaris* L.	1616, 2154, 2611	0.057
Watermelon *Citrullus lanatus* Thunb. peel	3486	0.396
Watermelon *Citrullus lanatus* Thunb. pulp	3725, 4146, 7450	0.684
Melon *Cucumis melo* L. peel	4444, 4590, 8887, 9179	0.154
Melon *Cucumis melo* L. pulp	3894, 3978, 4148, 4673, 4714, 7958, 9348, 9436	0.422
Pumpkin *Cucurbita pepo* L. seeds	2560, 2721, 3009, 3135, 3206, 3365, 3704, 4299, 4819, 6156, 6420, 8600	0.125
Pumpkin *Cucurbita pepo* L. pulp	1233, 1284, 2940, 4556	0.705
Horseradish *Armoracia rusticana* Gilib.	3725	0.466
Lemon *Citrus limon* L. seeds	3365, 3438, 3480, 3570, 3610, 3756, 3803, 3862	0.116
Lemon *Citrus limon* L. pulp	1837, 1891, 3465, 3805, 6931	0.400
Lemon *Citrus limon* L. peel	2814, 3196, 4071, 4359, 8152	0.208
Plantain *Plantago major* L.	2374, 2963	0.064
Wormwood *Artemisia absinthium* L.	4815	0.165
Dandelion *Taraxacum officinale* Wigg.	2871, 3124, 5750, 6256, 7227, 7253, 7542, 8371, 8430, 8794, 9371, 10345	1.400
Chanterelle *Cantharellus cibarius* Fr.	3650	3.000
Tinder *Fomes fomentarius* L.	4421	1.000

were identified also the signals with the values of *m/z* of 4530 and 4818 (Table 1). Possibly, in the composition of the MHTBs of the tissues of one organ, an eye, are the peptides of the same molecular weight. Whether they are identical or not will be determined in the analysis of their primary amino acid sequence. It is important to note that data on the specific activity of MHTBs obtained from eye tissues show that it is characterized by tissue-specific but not the species-specific action [9, 20]. We can assume that tissue-specific mode of activity of MHTBs may be mostly influenced by the other peptides peculiar only to the given type of tissue. For instance, our data show that the number of such peptides was present in the supernatants of the extracts of the cornea, sclera, and vitreous body tissues (Table 1).

In the preparations of plant origin, there were no peptides with close values of molecular weights. It was shown that not only in the supernatants obtained from the plants of the one family, for example, onion and garlic (the *Alliaceae family*), watermelon, melon and pumpkin (the *Cucurbitaceae family*), or wormwood and dandelion (the *Asteraceae family*), contained peptides with different molecular weights. Only in the case of bioregulators isolated from the pulp of watermelon and melon were read the close signals of 4146 and 4148 *m/z*, respectively (Table 2). But even the peptides isolated from the different tissues of one species of plant (peel, pulp, and seeds of watermelon; melon; pumpkin; and lemon) varied by molecular weight (except the signals of 3803 and 3805 *m/z* identified in the supernatants of seeds and pulp of lemon, respectively) (Table 2). Analogous difference in the values of *m/z* signals were seen in the exploration of supernatants isolated from the extracts of fungal tissues (Table 2). It is evident that such a difference in the molecular weights of the peptides is caused by the differences among MHTBs isolated from plants and fungi.

Thus, the obtained data let us assume that peptides with the same values of molecular weight, which determine the participation of these MHTBs in the regulation of organ-tissue homeostasis, enter into the composition of bioregulators obtained from the tissue of one organ (bull's eye). Other peptides of bioregulators of eye tissues with varying values of molecular weights possibly determine the tissue-specific nature of MHTBs [4, 21–23]. After the salting out of all the fractions of supernatants obtained from different

objects, the membrane tropic action on the model of organ culture of murine liver was revealed. These data indicate that MHTBs [1, 4] with complex composition and structure of certain spatial architectonics have a component common for the whole group of bioregulators. Possibly, the peptide with molecular weight 4301 ± 2 Da, found not only in the bioregulators of bull eye tissues but also in the bioregulators isolated from the rat brain and bone tissues and isolated from fetal bovine serum, entered into its composition. This assumption is a subject of our future research.

Running an identification of obtained mass spectroscopy signals by searching for the matches of the experimental masses with the masses of the proteins annotated in the electronic databases (SwissProt/TrEMBL), we did not find any matches. This fact confirms the uniqueness of the MHTBs group that controls organ-tissue homeostasis in minute doses and is the prerequisite for further investigations and establishing of the primary structure of the proteins and peptides of this group of bioregulators.

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