THE SEARCH FOR POTENTIAL HYPOTENSIVE PEPTIDES IN THE AMINO ACID SEQUENCE OF HUMAN RENALASE AND THEIR IDENTIFICATION IN PROTEOLYTIC FRAGMENTS OF THIS PROTEIN

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Renalase (RNLS) is a secretory protein discovered in 2005. It plays an important role in the regulation of blood pressure. Studies by two independent laboratories have shown that administration of purified recombinant RNLS reduced blood pressure in experimental animals. However, the mechanisms of the antihypertensive effect of RNLS still remain unclear, especially in the context of the shift in the catalytic paradigm of this protein. In addition, there is growing evidence that endogenous plasma/serum RNLS, detected by enzyme immunoassay, is not an intact protein secreted into the extracellular space, and exogenous recombinant RNLS is effectively cleaved during short-term incubation with human plasma samples. This suggests that the antihypertensive effect of RNLS may be due to peptides formed during proteolytic processing. Based on the results of a bioinformatics analysis of potential RNLS cleavage sites (Fedchenko et al., Medical Hypotheses, 2022; DOI: 10.1016/j.mehy.2022.110895), a number of short peptides have been identified in the RNLS sequence that show similarity to fragments of known peptide inhibitors of angiotensin-converting enzyme. Some of them were found as a part of larger RNLS peptides, formed during RNLS cleavage by chymotrypsin and, and to a lesser extent, by trypsin.

Key words: renalase; proteolytic processing; renalase peptides; biological activity; peptide inhibitors of angiotensin-converting enzyme

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INTRODUCTION

Renalase (RNLS) is a secretory protein discovered in 2005, which plays different roles inside and outside cells [1-5]. Intracellular RNLS is a FAD-dependent oxidoreductase (EC 1.6.3.5) [5, 6], which oxidizes isomeric forms of β-NAD(P)H reduced at the 2 or 6 position of the nicotinamide ring instead of the metabolically active 4 position [7]. In this case, FAD can only be “accommodated” in a full-length protein containing an N-terminal peptide [8, 9], which is cleaved during the secretion of this protein into the extracellular space [10]. This explains why extracellular RNLS, lacking the N-terminal peptide, is unable to bind FAD and perform catalytic FAD-dependent functions [9]. It exhibits various protective effects on the cell through interaction with receptor proteins [11–13]. However, the absence of an intramolecular fragment, corresponding to amino acid residues 100–116 of the RNLS sequence [14, 15], indicates that it is not the full-length protein (lacking the secretory N-terminal peptide) that circulates in the blood, but it represents the product(s) of the proteolytic cleavage of extracellular RNLS. Short-term incubation of recombinant RNLS with blood plasma preparations leads to a significant decrease in the level of full-length protein [16]. All this obviously indicates that RNLS entering the bloodstream undergoes proteolytic processing [16], and the resulting RNLS peptides have their own biological activity.

In this regard, the well-known experimental fact of a decrease of blood pressure in laboratory animals induced by administration of recombinant RNLS (and demonstrated in two independent laboratories), requires reinterpretation [1, 17]. Taking into account the change in the catalytic paradigm (RNLS is not a catalytically active amine oxidase circulating in the blood [1, 2], but is an intracellular oxidoreductase unrelated to the degradation of pressor amines), it becomes increasingly clear that the hypotensive effect of exogenous RNLS may be determined by some peptide fragments of this protein. Bioinformatic analysis performed using the Peptide Cutter and Pro cleave programs identified potential cleavage sites, as well as proteolytic enzymes capable (or not capable) of RNLS processing [16].

The aim of this study was to search for potential antihypertensive peptides in the amino acid sequence of human RNLS and their mass spectrometric identification in proteolytic fragments of recombinant human RNLS obtained as a result of cleavage of this protein with trypsin or chymotrypsin.

MATERIALS AND METHODS

The search for potential peptides in the RNLS sequence that can exhibit an antihypertensive effect was carried out using the AHTPDB: Database of Antihypertensive Peptides (http://crdd.osdd.net/raghava/ahtpdb/index.php). For this

purpose, the entire RNLS sequence was divided into tetrapeptides, starting from the first amino acid residue with a shift of one residue. The resulting tetrapeptides were used as a query.

Except where specifically stated, all reagents were from Sigma-Aldrich (USA); protein molecular weight markers PageRuler™ PrestainedProteinLadder from 2 kDa to 250 kDa were from Bio-Rad (USA).

The nucleotide coding sequence of the full-length human RNLS gene was obtained using the exon method, described in detail previously [18–20]. RNLS was synthesized in *E. coli* cells as a protein containing a C-terminal hexahistidine tag, which was used for protein purification on Ni-agarose [18–20].

Recombinant human RNLS (0.2 μg) was incubated with trypsin (proteomics grade, Promega, USA) or α-chymotrypsin for 30 min at 37°C in a volume of 20 μl. In the case of trypsin, incubation was carried out in standard trypsin buffer (Sigma-Aldrich) containing trypsin (1 U). In the case of α-chymotrypsin, RNLS was incubated in 100 mM Tris-HCl buffer (pH 7.8) containing 10 mM $CaCl_2$ and α-chymotrypsin (1 U). Incubations were stopped by heating at 90°C for 5 min, and samples were them used for mass spectrometric analysis of RNLS digestion products.

Mass Spectrometry Analysis

The samples were separated using an Acquity H-Class UPLC system (Waters, UK) and loaded in a volume of 3 l onto an Acquity™ UPLC BEH C18 (2.1 × 50 mm, 1.7 μm particle size; Waters) column with the pre-installed in-line 0.2 μm filter at a flow rate of 0.3 ml/min. The column was heated at 50°C. Peptides were separated at a flow rate of 0.3 ml/min in a gradient of mobile phase A (water with 0.1% formic acid and 0.015% trifluoroacetic acid) and mobile phase B (acetonitrile with 0.1% formic acid and 0.015% trifluoroacetic acid), using the following gradient scheme: 0–2.5 min 3% B, then raising B to 17% at 31.5 min, then raising B to 37% at 45 min and to 97% at 47.5 min. The washing was maintained in the isocratic mode for up to 51 min at a flow rate of 0.45 ml/min, then phase B was gradually reduced to the initial gradient conditions for 53.5 min and equilibrated for the next 6 min at a flow rate of 0.3 ml/min.

Proteomic analysis was performed on a Xevo G2-XS high-resolution time-of-flight mass spectrometer (Waters) with a Z-spray electrostatic ionization source in the positive ionization mode at a capillary voltage of 2.8 kV and a focusing voltage of 85 V with shift to 115 V. The rate of desolvation gas flow was set to 720 l/h at a temperature of 410°C, the flow rate of the focusing gas was 50 l/h at a temperature of 150°C. Parent ion scanning were performed in the *m/z* range of 300–1250 with a full working cycle of 235 ms. Fragment ions were obtained in the dissociation mode with argon with an increase in activation energy in the range of 14–42 eV. Active mass calibration *m/z* = 556.27 (leucine enkephalin at a concentration of 100 pg/μl, constant injection at a flow rate of 5 μl/min) was performed every 30 s.

Raw data files were uploaded to the PLGS search engine (Protein Lynx Global Server, version 3.0.3, Waters). The search was conducted in UniProt KB (*Homo sapiens*, May 2021 release). To assess the rate of false positive results, a database was generated automatically using reversed amino acid sequences. The search was performed with a parent ion mass tolerance of 20 ppm (±10 ppm tolerance window) and a fragment ion mass tolerance of 0.008 Da (±4 mDa tolerance window). Methionine oxidation and Q/N deamidation were included in the search algorithm as possible modification variables. The minimum peptide length was set to six amino acid residues. A false positive rate (FDR) of 1% was used as a criterion for peptide identification.

RESULTS AND DISCUSSION

The search for potential antihypertensive peptides in the RNLS sequence resulted in identification of 6 tetrapeptides, whose sequences matched regions of the peptides exhibiting antihypertensive activity (Table 1S, Supplementary Material). In most cases, longer peptides containing fragments similar to RNLS tetrapeptides showed antihypertensive activity (Table 1). These tetrapeptide fragments were located in different parts of the RNLS amino acid sequence (Fig. 1). One of the tetrapeptides (VGAG) is located at the N-terminus of the sequence (7–10). Since the N-terminal peptide is an extracellular signal peptide, which must be cleaved during secretion outside the cell, it cannot contribute to changes of blood pressure in the body. At the same time, when exogenous full-length recombinant RNLS reaches circulation, its cleavage can lead to the appearance of a peptide exhibiting the antihypertensive activity due to the presence of the N-terminal fragment.

For each of the four RNLS tetrapeptides ($I^{215}TSN^{218}$, $P^{249}FGV^{252}$, $P^{275}GLP^{278}$, $V^{84}LRP^{87}$) 2 peptides with antihypertensive activity were found in the database; they contained a sequence equivalent to the tetrapeptides from RNLS. In the case of the tetrapeptide $F^{104}VAP^{107}$ 49 such peptides were found (Table 1S, Supplementary Materials). Analysis of the location of these tetrapeptides on the 3D RNLS structure has shown that the tetrapeptides $V^{7}GAG^{10}$ and $F^{104}VAP^{107}$ are located inside the protein globule (Fig. 1). Thus, their potential antihypertensive effect appears to be possible only through partial hydrolysis of RNLS or through unfolding of the protein molecule.

Analysis of the peptides identified in the database for the mechanism of their antihypertensive action has shown that they all inhibit the activity

Table 1. Peptide inhibitors of ACE, containing tetrapeptide sequences identical to fragments of the RNLS sequence

RNLS tetrapeptide	Sequence of ACE peptide inhibitor*	Peptide length	IC_{50} (μM)	Peptide source	Reference
$I^{215}TSN^{218}$	SAYPGQ**ITSN**	10	7.08	Not shown	[21]
$P^{249}FGV^{252}$	V**PFGV**G	6	336.0	Wheat (*Triticum sp. sour dough*)	[22]
$P^{275}GLP^{278}$	GA**PGLP**GP	8	29.4	Hen (*Gallus gallus*)	[23]
$V^{7}GAG^{10}$	G**VGAG**Y	6	4.07	Not shown	[21]
$V^{84}LRP^{87}$	FC**VLRP**	6	12.3	Shrimp	[24]
$F^{104}VAP^{107}$	F**FVAP**	5	6.0	Cow casein	[25]

* Tetrapeptide sequences of the ACE inhibitors, identical to fragments of the RNLS sequence, are shown in bold.

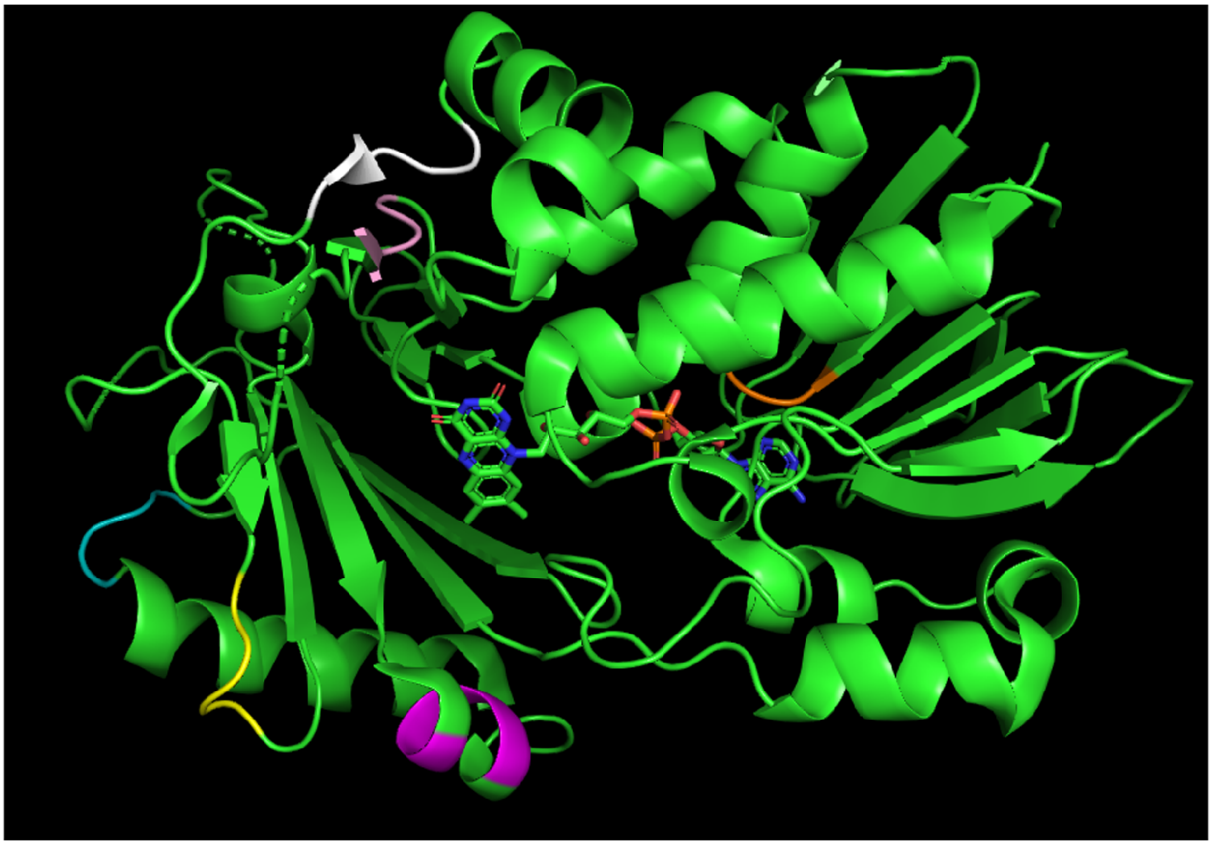

Figure 1. The location of tetrapeptide fragments identical to the tetrapeptide fragments of ACE inhibitors in the spatial structure of RNLS. Peptides: $V^{7}GAG^{10}$ – orange, $V^{84}LRP^{87}$ – white, $F^{104}VAP^{107}$ – pink, $I^{215}TSN^{218}$ – yellow, $P^{249}FGV^{252}$ – magenta, $P^{275}GLP^{278}$ – blue.

of angiotensin-converting enzyme (ACE) with varying effectiveness. Thus, it is reasonable to suggest that the antihypertensive effect observed during RNLS administration may be due to ACE inhibition by peptides formed during RNLS cleavage by proteases.

Incubation of recombinant RNLS with trypsin is accompanied by formation of several zones, characterized by different frequencies of peptide detection (Table 2). Among the seven zones, areas with a higher frequency of peptides are identified in five zones. In this case, only one zone contains an intact tetrapeptide sequence corresponding to the peptide ACE inhibitors. In the case of chymotrypsin, there are nine zones with different occurrences of the resulting peptides, and in three of them intact tetrapeptides corresponding to peptide ACE inhibitors have been found (Table 3). Taking into consideration the fact that trypsin is absent in the blood under physiological conditions, and chymotrypsin-like activity is characteristic of mast cell chymase [16], there are reasons to believe that the cleavage of RNLS by chymase will contribute to the formation of peptide ACE inhibitors. Further experiments are needed to test this assumption.

Table 2. Peptide zones detected by mass spectrometric analysis of recombinant RNLS peptides formed upon incubation of this protein with trypsin

No	RNLS Peptides	Peptide position in the RNLS sequence	The presence of a fragment of the ACE inhibitor
1	RQTSGPLYLAVWDK	56–69	—
2	FYDE**LLAYGVLRPLSSPIEGMVMK**	110–133	—
3	**ESGAEVYFR**	155–163	—
4	**DDKWEVSKQ**	173–181	—
5	**QQLEAVSYSSR**YALGLFYEAGTK	217–239	—
6	**FVSIDNKK**	257–263	—
7	*EIGPSLVIHTTVPFGVTYLEHSIEDVQELVFQQLENILPGLPQP*	271–318	275–278

Here and in Table 3, peptide zones shown in bold and underlined indicate a region with a high frequency of peptide detection; peptide zones highlighted in bold only indicate a region with the average frequency of peptide detection; peptide zones shown in regular font indicate the usual frequency of peptide detection; peptide zones highlighted in italics show a region of modified peptides.

Table 3. Peptide zones detected by mass spectrometric analysis of recombinant RNLS peptides formed upon incubation of this protein with chymotrypsin

No	RNLS peptides	Peptide position in the RNLS sequence	The presence of a fragment of the ACE inhibitor
1	PLYLAVWDKADDSGGRMTTACSPHNPQCTADLGAQYITCT	61–100	84–87
2	AKKHQRF	104–110	104–107
3	**YDELLAYGVLRPLSSPIEGMVMKEGDCNF**	111–139	—
4	VAPQGISSIIKHY**LKESGAEVY**	140–161	—
5	IVLTMPVPEILQLQGDITTLISECQRQQLEAVSY	191–224	215–218
6	**YEAGTKIDVPWAGQYIT**	234–250	—
7	HSIEDVQELVF	291–301	—
8	LPQPIATKCQKW	311–322	—
9	TQSNF	354–358	—

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or the use of animals as objects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Supplementary materials are available in the electronic version at the journal site (pbmc.ibmc.msk.ru).

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ПОИСК ПОТЕНЦИАЛЬНЫХ ГИПОТЕНЗИВНЫХ ПЕПТИДОВ В АМИНОКИСЛОТНОЙ ПОСЛЕДОВАТЕЛЬНОСТИ РЕНАЛАЗЫ ЧЕЛОВЕКА И ИХ ИДЕНТИФИКАЦИЯ В ПРОТЕОЛИТИЧЕСКИХ ФРАГМЕНТАХ ЭТОГО БЕЛКА

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Реналаза (RNLS) — открытый в 2005 г. секреторный белок, которому отводят важную роль в регуляции артериального давления. По данным двух независимых лабораторий, введение очищенного препарата рекомбинантной RNLS снижало артериальное давление у экспериментальных животных. Однако механизмы антигипертензивного эффекта RNLS по-прежнему неясны особенно с учётом смены каталитической парадигмы этого белка. Кроме того, накапливается всё больше данных, что эндогенная RNLS плазмы/сыворотки крови, выявляемая при помощи иммуноферментного анализа, не является интактным белком, секретируемым во внеклеточное пространство, а экзогенная рекомбинантная RNLS эффективно разрушается при кратковременной инкубации с образцами плазмы человека. Это позволяет предположить, что антигипертензивный эффект RNLS может быть обусловлен пептидами, образующимися в ходе протеолитического процессинга. Основываясь на результатах биоинформатического анализа потенциальных сайтов расщепления RNLS (Fedchenko et al., Medical Hypotheses, 2022; DOI: 10.1016/j.mehy.2022.110895), в последовательности RNLS выявлен ряд коротких пептидов, проявляющих сходство с фрагментами известных пептидных ингибиторов ангиотензинпревращающего фермента. Часть из них была обнаружена в составе более крупных пептидов RNLS, образующихся в результате расщепления химотрипсином и в меньшей степени — трипсином.

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Ключевые слова: реналаза; протеолитический процессинг; реналазные пептиды; биологическая активность; пептидные ингибиторы ангиотензинпревращающего фермента

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