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Proteinase Inhibitors from the Tropical Sea Anemone *Radianthus macrodactylus*: Isolation and Characteristic

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Abstract—Two new serine proteinase inhibitors (RmIn I and RmIn II) from the tropical sea anemone *Radianthus macrodactylus* have been isolated and characterized. The purification procedure includes polychrome-1 hydrophobic chromatography, Superdex^{TM} Peptide 10/30 FPLC, and Nucleosil C_{18} reverse-phase HPLC. The molecular masses of RmIn I, RmIn II, and the complexes RmIn II/trypsin and RmIn I,II/α-chymotrypsin have been determined. The K_i values of RmIn I and RmIn II for trypsin and α-chymotrypsin have been determined. The polypeptides RmIn I and RmIn II are shown to be nontoxic and to exhibit antihistamine activity. The N-terminal amino acid sequences of RmIn I (GICSEPIVVGPCKAG-) and RmIn II (GSTCLEPKVVGPCKA-) have been determined. A high homology of the amino acid sequences is demonstrated for the proteinase inhibitors produced by such evolutionarily distant species as coelenterates, reptiles, and mammals.

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Key words: sea anemone, serine proteinase inhibitor, trypsin, chymotrypsin, inhibition constant, amino acid sequence

Interest in natural inhibitors of serine proteinases is due to their direct involvement in a number of important processes (decomposition of physiologically active peptides, complement system functioning, defense of tissues from destruction by endogenous proteinases, control of formation of digestive enzymes, etc.). The properties of plant and animal serine proteinases have been studied in detail [1-6].

Proteinase inhibitors of sea anemones are less studied. It is known that they exist in the producing organism together with proteolytic enzymes and neuro- and cytotoxins. Proteinase inhibitors of polypeptide nature were first found while isolating neurotoxins from aqueous-alcoholic extracts of sea anemones. Inhibitors of serine proteinases from *Anemonia sulcata* [7, 8], *Radianthus macrodactylus* [9, 10], *Actinia equina* [11, 12], *Stichodactyla helianthus* [13, 14], and *Anthopleura aff. xanthogrammica* [15] have been characterized to date. It was found that Kunitz-type proteinase inhibitors from sea anemones (SHPI-1 and SHPI-2 from *S. helianthus* [13, 14]) inhibit cysteine and aspartic proteinases as well as serine proteinases, or block the binding of dendrotoxin to potential-sensitive K^+-channels. The latter were isolated from *A. sulcata* and were named kalicludines [16]. Kalicludines were shown to be the first representatives of the bifunctional inhibitors isolated from sea organisms.

The biological role of serine proteinase inhibitors in sea anemones is unclear. It is supposed that the inhibitors defend sea anemones from the proteinases of their victims (i.e. perform the same function as the inhibitors of land animals), and also play an important role in the regulation of digestion mechanisms including self-digestion by their own enzymes or by the enzymes of the symbiotic microorganisms [17, 18].

The present work describes isolation, investigation of physicochemical and biological properties, and determination of partial amino acid sequence of two new inhibitors of serine proteinases from the tropical sea anemone *R. macrodactylus*.

MATERIALS AND METHODS

The following chemicals were used in this work: polychrome-1 (Olaine, Latvia); N-benzoyl-D,L-arginine

Abbreviations: BAPNA) N-benzoyl-D,L-arginine *p*-nitroanilide; BSA) bovine serun albumin; BTEE) N-benzoyl-L-tyrosine ethyl ester; HPLC) high performance liquid chromatography; TFA) trifluoroacetic acid.

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p-nitroanilide (BAPNA), N-benzoyl-L-tyrosine ethyl ester (BTEE), α-chymotrypsin, histamine, protein standards, and sodium dodecyl sulfate for SDS-PAGE (Sigma, USA); trypsin (Spofa, Czechoslovakia); Brilliant Blue G (Serva, Germany); acetonitrile (Cryochrom, Russia); trifluoroacetic acid (TFA) (Merck, Germany); bovine serum albumin (BSA) (Reanal, Hungary). Other chemicals were of domestic production and of analytical grade purity.

Isolation and purification of proteinase inhibitors. The sea anemones, collected on the Seychellois islands during the 11th expedition of the research ship "Akademik Oparin" (in 1990), were homogenized in three volumes of distilled water. After 12 h, the homogenate was filtered and centrifuged at 10,000*g* on a K-24 centrifuge (Janetzki, Germany). The aqueous extract of the sea anemones was lyophilized and stored at –20°C. Hydrophobic chromatography of the polypeptides from *R. macrodactylus* was performed using a polychrome-1 column (2.6 × 15 cm). The proteinase inhibitors were eluted with 40% ethanol at 1 ml/min (fraction volume, 8 ml). The proteins were purified by gel filtration using an AKTA system (Pharmacia, Sweden) on a Superdex™ Peptide 10/30 column (1 × 30 cm; Amersham Pharmacia Biotech AB, Sweden). The proteins were eluted with 10% acetonitrile in 0.1% TFA (pH 2.2) at 5 ml/min (fraction volume, 1.5 ml). Then the polypeptides were purified by HPLC using an Agilent 1100 chromatograph (Germany) on a reverse-phase Nucleosil C_{18} column (4.6 × 250 mm; Sigma-Aldrich, USA). The polypeptides were eluted with a 5-60% acetonitrile gradient in 0.1% TFA (pH 2.2) at 1 ml/min during 60 min. Protein concentration was determined by the Lowry method [19] using BSA as the standard.

Amino acid analysis. Amino acid composition of the polypeptides was determined using a Biochrom 30 amino acid analyzer (England). Samples of proteinase inhibitors (2.5-3 nmol) were hydrolyzed for 24, 48, and 72 h in 5.7 M HCl at 110°C.

The N-terminal amino acid sequence of the inhibitors was determined using a Procise 492 automatic protein sequencer (Applied Biosystems, USA) according to the manufacturer's instructions.

Mass spectrometry. MALDI TOF MS analysis of proteinase inhibitors was performed using a Vision 2000 time-of-flight mass-spectrometer (Thermo, England) in the Institute of Bioorganic Chemistry of the Russian Academy of Sciences. The time-of-flight mass spectra were recorded in the straight flight and in the reflector mode.

Mass-spectrometric analysis of the complexes of the proteinase inhibitors with trypsin and α-chymotrypsin was performed using a BIFLEX III time-of-flight mass-spectrometer (Bruker Daltonic, Germany) in the group of mass-spectrometry of Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences. The time-of-flight mass spectra were recorded in the straight flight and in the reflector mode.

Complexes of proteinase inhibitors with enzymes. One microliter of trypsin or α-chymotrypsin 5 mg/ml in 0.001 M HCl was supplemented with 5 μl of 0.1 M Tris-HCl buffer, pH 8.1, and 1 μl of an aqueous solution of the proteinase inhibitor (15 mg/ml). The reaction mixture was incubated for 5 min at 37°C and analyzed by mass spectrometry.

Inhibition constants for trypsin and α-chymotrypsin were determined by Dickson's method [21] using BAPNA and BTEE as the substrates.

Biological activity. Trypsin-inhibiting activity was determined using the substrate BAPNA: 50 μl of trypsin solution (100 μg/ml in 0.001 M HCl) and 250 μl of 0.1 M Tris-HCl buffer, pH 8.1, were added to 50 μl of aqueous solution of the inhibitor. After incubation for 5 min at 37°C, 250 μl of the substrate solution (4 mg of BAPNA in 0.3 ml of dimethylformamide diluted 10-fold with 0.1 M Tris-HCl buffer, pH 8.1) were added. The amount of the formed *p*-nitroaniline was determined spectrophotometrically at 410 nm.

The α-chymotrypsin-inhibiting activity of the inhibitors was determined using BTEE as the substrate: 50 μl of α-chymotrypsin solution (100 μg/ml in 0.001 M HCl) and 250 μl of 0.1 M Tris-HCl buffer, pH 8.1, were added to 50 μl of an aqueous solution of the inhibitor. After incubation for 5 min at 37°C, 250 μl of the substrate solution (4 mg of BTEE was dissolved in 0.3 ml of dimethylformamide, and the volume was adjusted to 10 ml with 0.1 M Tris-HCl buffer, pH 8.1) was added. The amount of tyrosine formed was determined spectrophotometrically at 250 nm.

The toxicity of the isolated inhibitors was evaluated from the lethal dose after intraperitoneal injections to white outbred mice of 20 ± 2 g and expressed as LD_{50} (the amount of a protein (mg) per kg of weight resulting in the 50% mortality of the mice during 1 h).

The antihistamine activity of the proteins was determined by Gatsura's method [22] on white outbred female rats of 52 ± 2 g (three animals in each group) using 0.1% solution of histamine in physiological saline. The control group of the animals was given histamine resulting in a 2-min micro-shock. The shock was characterized by an enhancement of the respiratory movements of the abdominal wall of the animal with subsequent involvement of all muscles in the respiratory act, and by fast forward-and-back movement of the head.

RESULTS AND DISCUSSION

Isolation of serine proteinase inhibitors. Previously, trypsin inhibitors Jn-IV, In-2B, In-4B, and In-5B of 6170, 13500, 14100, and 14800 daltons, respectively, were isolated from the sea anemone *R. macrodactylus* and

characterized [9, 10]. For Jn-IV, the complete amino acid sequence was determined [9]. RmIn I and RmIn II are new trypsin inhibitors isolated from *R. macrodactylus* using the combination of hydrophobic, gel-penetrating, and reverse-phase liquid chromatographies (Table 1).

Chromatography of the aqueous extract of the sea anemones on a polychrome-1 column yielded a fraction of polypeptides possessing trypsin-inhibiting activity. The fraction was eluted with 40% ethanol. After evaporation of ethanol and lyophilization, the fraction was dissolved in water and used for subsequent purification. Gel chromatography on a Superdex™ Peptide 10/30 column yielded four polypeptide fractions with trypsin-inhibiting activity (Fig. 1a). The polypeptides of fraction 2 possessed the highest specific activity, and their molecular weight estimated by SDS-PAGE [20] constituted ~6000 daltons. The polypeptides of fraction 2 were accumulated, pooled, lyophilized, and then rechromatographed (Fig. 1b). Fraction 1 obtained after rechromatography was purified using HPLC on a Nucleosil C_{18} column, yielding two homogeneous polypeptides (RmIn I and RmIn II) exhibiting trypsin-inhibiting activity (Fig. 2).

Amino acid composition. The inhibitors RmIn I and RmIn II have similar amino acid composition. Similarly to the trypsin inhibitors from the sea anemones *A. aff. xanthogrammica* [15], *A. sulcata* [7], and *S. helianthus* [13], the inhibitors from *Radianthus* contain six cysteine residues that form three intramolecular disulfide bonds and have neither methionine nor tryptophan residues (Table 2).

Table 1. Scheme of isolation of proteinase inhibitors from *R. macrodactylus*

Purification stage	Protein, mg	Yield, %
Original aqueous extract	1000	100
Hydrophobic chromatography on a polychrome-1 column		
fraction of polypeptides with trypsin-inhibiting activity	58.0	5.8
Gel filtration on a Superdex™ Peptide 10/30 column		
fraction 1	2.5	0.25
fraction 2	20.0	2.0
fraction 3	17.5	1.75
fraction 4	6.0	0.6
Rechromatography of fraction 2 on the Superdex™ Peptide 10/30 column		
fraction 1	19.5	1.95
HPLC of fraction 1 on a Nucleosil C_{18} column		
RmIn I	1.61	0.16
RmIn II	1.19	0.12

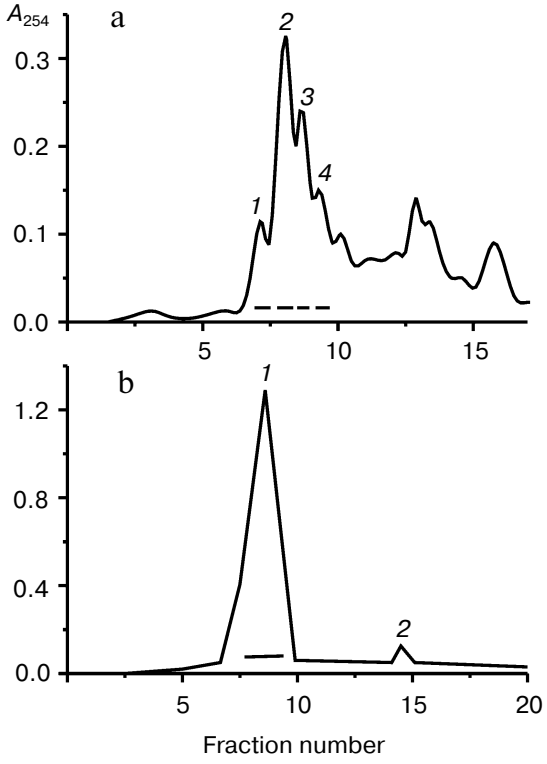

Fig. 1. a) Gel chromatography of the inhibitors obtained after hydrophobic chromatography on a Superdex™ Peptide 10/30 column (1 × 30 cm). Proteins were eluted with 10% acetonitrile in 0.1% TFA, pH 2.2, at 0.5 ml/min. Fraction volume was 1.5 ml; *1-4*) polypeptide fractions with trypsin-inhibiting activity. b) Rechromatography of fraction 2 inhibitors (a) under the same conditions; *1*) polypeptide fraction with trypsin-inhibiting activity; *2*) polypeptide fraction without trypsin-inhibiting activity. The boundaries of the pooled active fractions are shown by dashed line.

Molecular weight of the inhibitors. The molecular weights of RmIn I and RmIn II calculated from the amino acid composition constitute 6322.8 and 6096.0 daltons, respectively. According to MALDI TOF MS analysis, the molecular weights of RmIn I and RmIn II constitute 6201.6 ± 100 and 6086.5 ± 100 daltons, respectively. The determined values are close to the molecular weights of the inhibitors from other species of sea anemones (Table 2).

Specificity of the inhibitors. It was demonstrated that RmIn I and RmIn II inhibited the activity of trypsin and α-chymotrypsin. The formation of stable complexes between the proteinase inhibitors RmIn I and RmIn II and trypsin and α-chymotrypsin was confirmed by MALDI TOF MS analysis (Fig. 3). The values of the inhibition constants (K_i) for RmIn I and RmIn II calculated by Dixon's method [21] were $2.4 \cdot 10^{-9}$ and $2.5 \cdot 10^{-9}$ M for trypsin and $2.3 \cdot 10^{-8}$ and $3.0 \cdot 10^{-8}$ M for α-chymotrypsin, respectively. The K_i value determined for the previously isolated inhibitor Jn-IV was $9.6 \cdot 10^{-9}$ M for trypsin. Inhibitors of serine proteinases isolated from

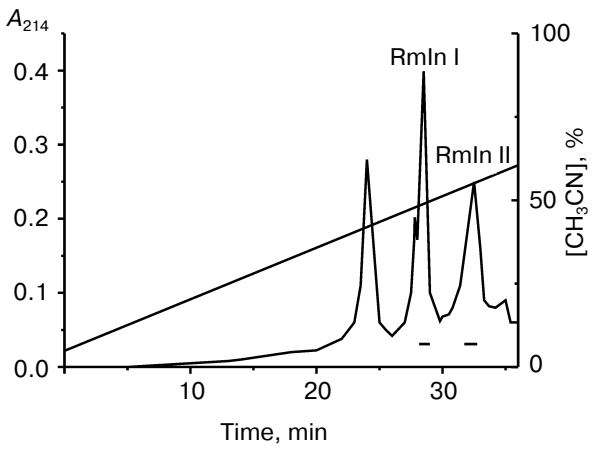

Fig. 2. Reverse-phase HPLC of the inhibitors of fraction 1 (Fig. 1b) on a Nucleosil C_{18} column (4.6 × 250 mm). The proteins were eluted with a 5-60% gradient of acetonitrile in 0.1% TFA, pH 2.2, at 1 ml/min during 60 min. The boundaries of the pooled active fractions are shown by dashed line.

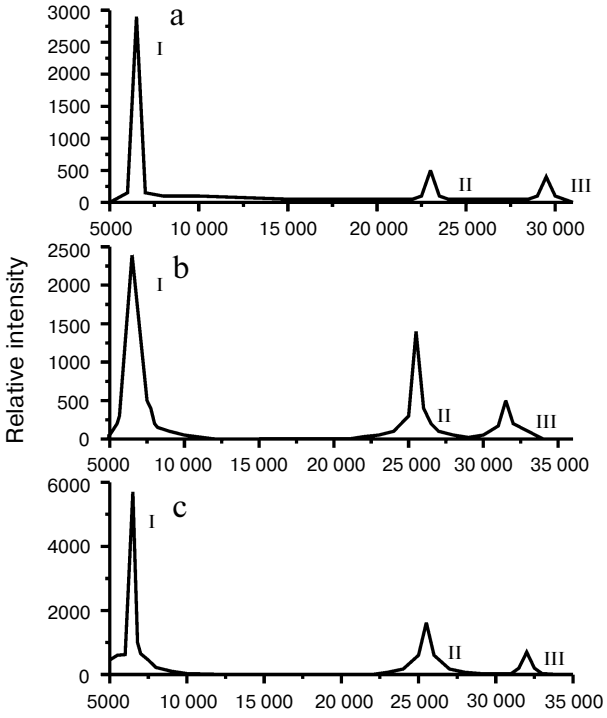

Fig. 3. MALDI TOF MS analysis of the complexes of the proteinase inhibitors RmIn I and RmIn II with trypsin and α-chymotrypsin. a) I-III: RmIn II (6079.5 daltons), trypsin (23303 daltons), and RmIn II/trypsin complex (29324.2 daltons), respectively; b, c) I-III: RmIn I and RmIn II (6171.9 and 6047.7 daltons), α-chymotrypsin (25254.7 and 25304.3 daltons), and complexes RmIn I and II with α-chymotrypsin (31462.4 and 31518.6 daltons), respectively.

other species of anemones exhibit similar K_i values (Table 3).

N-Terminal amino acid sequences of the inhibitors. The amino acid sequences of the N-terminal fragments of RmIn I and RmIn II were determined (GICSEPIVVGPC-KAG- and GSTCLEPKVVGPCKA-, respectively). Comparison of the N-terminal fragments of the inhibitors RmIn I and RmIn II with the analogous sequence of Jn-IV isolated from the same source showed a high extent of homology (80%). Evidently, these polypeptides are isoforms. Figure 4 presents the alignment of the amino acid sequences of the proteinase inhibitors and the N-terminal fragments of RmIn I and RmIn II. The number and positions of the cysteine residues of the inhibitors isolated from different organisms was found to be the same. It was demonstrated that the identity of complete amino acid sequences of the proteinase inhibitors of coelenterates, reptiles, and mammals is rather high (from 37 to 78%) (Table 4).

Thus, based on the comparison of physicochemical characteristics, the polypeptides RmIn I and RmIn II can be referred to Kunitz-type serine proteinase inhibitors.

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RmIn I       -----GICSEPIVVGPCKAG...
RmIn II      ----GSTCLEPKVVGPCKA..
Jn-IV        ----GSICLEPKVVGPCTAYFPRFYFDSETGKCTPFIYGGCEGNSYVDEKLHACRAICRA
SHPI-1       -----SICSEPKKVGRCKGYFPRFYFDSETGKCTPFIYGGCGGNGNNFETLHQCRAICRA
SHPI-2       -----SFCLEPKRVGRCKGYFPRFYFDSKTGKCTPFIYGGCGGNGNNFETLHQCRAICRA
SA5 II       ---INGDCELPKVVGPCRARFPRYYYNSSSKRCEKFIYGGCGGNANNFHTLEECEKVCGVRSV
AXPI-I       -APVNEDCLLPKKVGPCRAAVPRFYYNSDSGKCEGFTYGGCHANANNFKTKDECKNACH
VBPI I       -QDHPKFCYLPADPQRCKAHIPRFYYDSASNKCNKFIYGGCPGNANNFKTWDECRQTCGASA
CBPI         FQTPPDLCQLPQARGPCKAALLRYFYNSTSNACEPFTYGGCGGNNBNFETTEMCLRICEPPQQTDKS

Consensus    FQ     C ePk vgpCka fpRfyydS sgkC pFiYGGCggN nnfetl  Cr iC a   TDKS
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Fig. 4. Comparison of amino acid sequences of proteinase inhibitors. RmIn I, RmIn II, and Jn-IV (Swiss-Prot, P16344) [9] are serine proteinase inhibitors from the sea anemone *R. macrodactylus*; SHPI-1 and SHPI-2 are proteinase inhibitors from the sea anemone *S. helianthus* (Swiss-Prot, P31713 and P81129, respectively) [13, 14]; SA5 II, serine proteinase inhibitor from the sea anemone *A. sulcata* (Swiss-Prot, P10280) [8]; AXPI-I, proteinase inhibitor from the sea anemone *A. aff. xanthogrammica* (Swiss-Prot, P81547) [15]; VBPI I, serine proteinase inhibitor from the venom of the viper *Vipera ammodytes ammodytes* (Pir, TIVIT1) [23]; CBPI, trypsin inhibitor from the colostrum of the cow *Bos taurus* (Swiss-Prot, P00976) [24]. The identical amino acid residues in all sequences are highlighted. The alignment was performed using the CLUSTALW program [25].

Table 2. Amino acid composition of proteinase inhibitors of sea anemones

Amino acid	*R. macrodactylus* [9]*			*S. helianthus* [13, 14]		*A. sulcata* [8]	*A. aff. xanthogrammica* [15]
	RmIn I	RmIn II	Jn-IV	SHPI-1	SHPI-2	SA5 II	AXPI I
Asx	4	3	3	4	4	5	9
Thr	4	3	3	3	3	3	1
Ser	4	3	3	3	2	3	2
Glx	4	6	4	4	3	2	3
Pro	6	7	4	3	3	4	4
Gly	8	6	6	8	8	5	5
Ala	4	3	4	2	2	2	6
$^1/_2$ Cys	6	6	6	6	6	6	6
Val	3	3	3	1	1	2	3
Met	0	0	0	0	0	2	0
Ile	2	2	3	3	2	3	0
Leu	2	2	2	1	2	4	2
Tyr	2	3	4	3	3	2	3
Phe	3	3	4	5	6	0	3
Lys	4	2	3	4	4	2	6
His	1	1	1	1	1	1	2
Arg	3	3	3	4	5	2	2
Trp	0	0	0	0	0	0	0
Number of amino acid residues**	60	56	56	55	55	48	57
M_r, daltons***	6322.8	6096	6170.2	6116.5	6204.2	5132.9	6240.5
M_r, daltons****	6201.6 ± 100	6086.5 ± 100					

* Mean values of two experiments obtained after hydrolysis for 24, 48, and 72 h.
** Number of amino acid residues per mol protein.
*** Molecular weight calculated from the amino acid composition.
**** Molecular weight according to MALDI TOF MS.

Table 3. Inhibition constants of proteinase inhibitors from sea anemones

Source	Inhibitor	K_i, M	
		trypsin	α-chymotrypsin
R. macrodactylus	RmIn I	$2.4 \cdot 10^{-9}$	$2.3 \cdot 10^{-8}$
R. macrodactylus	RmIn II	$2.5 \cdot 10^{-9}$	$3.0 \cdot 10^{-8}$
R. macrodactylus	Jn-IV	$9.6 \cdot 10^{-9}$	n.d.
A. sulcata [8]	SA5 II	$3.0 \cdot 10^{-10}$	n.d.
R. rhodostoma [18]	n.d.	$9.5 \cdot 10^{-10}$	$3.3 \cdot 10^{-8}$
S. helianthus [13]	SHPI-1	$1.1 \cdot 10^{-10}$	$2.3 \cdot 10^{-9}$

Note: n.d., not determined.

Table 4. Degree of homology of the amino acid sequences of proteinase inhibitors from different organisms

Source	Proteinase inhibitor	Homology of the amino acid sequences, %
R. macrodactylus	Jn-IV	100
S. helianthus	SHPI-1	78.5
S. helianthus	SHPI-2	77.0
A. sulcata	SA5 II	46.0
A. aff. xantho-grammica	AXPI I	46.0
Vipera ammodytes ammodytes	VBPI I	43.0
Bos taurus	CBPI	37.5

Table 5. Antihistamine activity of proteinase inhibitors from *R. macrodactylus*

Concentration, mg/g (intravenously)		Latent period before the development of the microshock, min	Desensitization period, min
histamine	RmIn I, RmIn II (1 : 1)		
0.02	—	2 ± 1	90 ± 4
0.02	0.01	5 ± 1	54 ± 4
0.02	0.02	7 ± 1	25 ± 4

Toxicity. It was demonstrated that the inhibitors in concentrations 10-100 μg per kg body weight were nontoxic for the investigated animals.

Antihistamine activity. It has been known that serine proteinases participate in the processes connected with the immune response and functioning of the complement system. For example, the enzymes tryptase and chymase related to serine proteinases are released by mast cells while allergic reactions together with such mediators as histamine, serotonin, chondroitin sulfate, leukotrienes, prostaglandins and cytokines, resulting in the dilation and increase in the permeability of the vessels, pain, itch, bronchospasm, etc. Presumably, the allergic reaction (its clinical manifestation) must weaken or be suppressed after injection of serine proteinase inhibitors.

The inhibitors were assayed for the antihistamine activity using Gatsura's method [22]. Female white outbred rats were injected with 0.1% solution of histamine into the tail vein. It was demonstrated that RmIn I and RmIn II weakened the clinical manifestation of the allergic reaction, i.e. exhibited antihistamine activity. The effect of the inhibitors on the development of the allergic reaction depended on the dose (Table 5).

The results of the present work suggest further investigations that will be directed to the determination of the primary structure and spatial organization of the inhibitors to elucidate the localization of their sites of binding with proteinase and to study the mechanisms of different biological activities of these polypeptides.

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