https://dx.doi.org/10.22161/ijreh.1.3.6 *ISSN: 2456-8678*

Isolation of biologically active and anticoagulant components from the venom of the snake Macroipera lebetina obtusa and honey bee Apis mellifera Caucasica

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Abstract*—The paper presents experimental data on the separation, identification and isolation of anticoagulant components of the snake venom and of the honey bee Apis mellifera Caucasica, harvested from the ecologically clean zone of Azerbaijan.*

The protein components of venom snake and bee venom with molecular masses 20 kD, 32kD, 35, 79 , 101, 132 kD and 41kD, 20 kD, 15 kD, corresponding to glycoprotein, toxic component, growth factor, protease that activates the clotting factor of the blood, glycoproteins and hyaluronidase and phospholipase was isolated from venom by the method of gel chromatography on a column with Sephadex G-750 eluting with 0.4 M sodium phosphate buffer, followed by spectrophotometric measurement of the unit optical density of the fractions at λ=280 nm on a Hitachi-557 spectrophotometer.

Keywords*— *snake, venom, Macroipera lebetina obtuse, honey bee, Apis mellifera Caucasica, anticoagulant.

I. INTRODUCTİON

There are more than 300 species of venomous snakes in the world, and their venoms have numerous molecules with diverse biomedical functions.

Snake procoagulant molecules, especially from the Viperidae family, have been used in medical applications and as diagnostic tools. A procoagulant protein, Batroxobin from Bothrops atrox is useful for fibrinogen level assays [1, 2, 3](Aronson, 1976; Bell, 1997; Van Cott et al., 2002). Another known procoagulant from Russell's viper venom (RVV), RVV-factor X activator (RVV-X) is useful for measuring a lupus anticoagulant [4, 5] (Lo et al., 1989; Derksen and de Groot, 2004).

Procoagulant and anticoagulant properties are widely studied from the Viperidae family. Only a few anticoagulants have been isolated from snake venoms of the Elapidae family [6, 7, 8, 9] (Sundell et al., 2003; White, 2005; Gowda et al., 2006; Kumar et al., 2010).

The activated clotting time (ACT) and clot rate were used for screening procoagulant and anticoagulant properties of 28 snake venoms. Crude venoms from Daboia russellii siamensis, Bothrops asper, Bothrops moojeni, and one Crotalus oreganus helleri from Wrightwood, CA, had procoagulant activity. These venoms induced a significant shortening of the ACT and showed a significant increase in the clot rate when compared to the negative control. Factor X activator activity was also measured in 28 venoms, and D. r. siamensis venom was 5–6 times higher than those of B. asper, B. moojeni, and C. o. helleri from Wrightwood County. Russell's viper venom-factor X activator (RVV-X) was purified from D. r. siamensis venom, and then procoagulant activity was evaluated by the ACT and clot rate. Other venoms, Crotalus atrox and two Naja pallida, had anticoagulant activity. A significant increase in the ACT and a significant decrease in the clot rate were observed after the addition of these venoms; therefore, the venoms were considered to have anticoagulant activity. Venoms from the same species did not always have the same ACT and clot rate profiles, but the profiles were an excellent way to identify procoagulant and anticoagulant activities in snake venoms [10].

Despite the presence of a large arsenal of hormonal drugs, antibiotics and other new potent chemotherapeutic drugs, bee venom remains among the most effective medicines, the use of which is expanding.

The mechanism of toxic effect of bee venom is very complex and is the result of a complex effect of its components on various organs and systems. Bee venom increases the amount of hemoglobin and blood leukocytes, reduces its viscosity and coagulability and dilates capillaries and small arteries, increasing the flow of blood to the organs [18].

Separate components of bee venom can be used to achieve certain biological effects. Bee venom also affects

https://dx.doi.org/10.22161/ijreh.1.3.6 *ISSN: 2456-8678*

the central and peripheral nervous system and can be used to treat patients with heart disease. In the literature, data on the use of bee venom for the treatment of patients with various degenerative diseases of the nervous system, such as multiple sclerosis, Alzheimer's disease and Parkinson's disease and others [12, 13, 14, 15, 11, 16] have been published.

H. Zolfagharian, M. Mohajeri, M. Babaie revealed that the bee venom increases the clotting time. By the authors, the honey bee venom were divided into fractions by using gel filtration and chromatography on Sephadex G-50 and their molecular weight was determined by using electrophoresis using sodium dodecyl sulfate in a polyacrylamide gel. Column gel chromatography isolated F1 fraction containing hyaluronidase, F2 and F3 containing phospholipase and F4 containing melittin with molecular masses of 3, 15, 20 and 41 kDa, respectively. It was noted that fractions F2, F3 and F4 had a greater anticoagulant activity than fraction F1.

Thus, the authors consider bee bee venom as a complex of substances containing an anticoagulant factor consisting of 4 protein fractions with molecular masses of 3, 15, 20, and 41 kDa. A lethal dose of the whole LD50 venom was determined to be 177.8 μg / mouse [17].

Despite numerous studies on the study of bee and snake venom, the isolation and identification of poison components, a number of questions on the study of their effect on the coagulating blood system of experimental animals are available, the study of which is of great scientific and practical interest.

Proceeding from the foregoing, the purpose of these studies was to isolate the anticoagulant fractions from the venom of the snake Macrovipera lebetina obtusa and honey bee Apis mellifera Caucasica, collected of snake and honey bee from the ecologically clean zone of Azerbaijan.

II. MATERIAL AND METHODS

The material of the study was the whole venom of the Macrovipera lebetina obtusa and of the honey bee Apis mellifera Caucasica, collected from bees from apiaries located in the area of the ecologically clean zone of Azerbaijan, from the territory of the Ismail area. After storage, the venom was stored in a desiccator over a couple of calcium chloride.

Venom solutions were prepared immediately before the experiment. Separation of the poi-son into fractions was carried out by column chromatography on a Sephadex G-75 column measuring 15x150 mm.

To identify the protein components of bee venom, we developed a model technique for the separation of marker proteins.The molecular weights of the marker proteins were determined on a Sephadex G-75 column. For preparation of the column, the matrix G-75 gel was soaked for 48 hours. The prepared gel suspension was carefully filled into a chromatography column. After the height of the layer of the settled gel reached 5 cm, a column crane was opened and a stream of pre-prepared solvent was passed through it, observing the conditions under which the rate of solvent effluent from the column was much less than the flow rate of the solvent during chromatography. After uniform gel settling, the column was washed with a buffer solution and again left for 12 hours at the temperature of chromatography. 0.4 M Na-phosphate buffer solution with a pH value of 7.0 was selected as the eluent. The volume of the investigated solution of the venom did not exceed 1 ml. The elution was carried out with a 0.04 M Na-phosphate buffer solution at pH 7.0 and at a rate of 8 ml / hr.

III. RESULTS

At construction of the calibration curve, the protein-marcers: Cytochrome C with Mm = 12kD, trypsin with Mm = 20 kD, erythrocyte spacecraft with Mm = 30 kD and albumin lyophilized from human serum with Mm = 67 kD were used.. Further, a mixture of marker proteins of 5 mg was passed through a separating chromatographic glass column. The fractions were collected in separate 4.0 ml tubes, followed by measuring the optical density on a spectrophotometer. The quantitative data of spectrophotometric separation of marker proteins are given in table 1 .

Further, the collected fractions, separated by elution with a 0.4 M solution of Na-phosphate buffer pH 7.0, were combined into separate solutions of marker proteins, followed by measurement of their optical density (table 2).

As can be seen from these tables, the marker proteins were arranged in descending order of elution volume - VR, corresponding to an increase in the molecular mass of proteins.

Based on the data presented in figure 1, it can be seen that the direct proportional dependence of the marker proteins is in the range 12-67 kD.

Thus, on the basis of experimental data, the separation conditions of the marker proteins were determined by column chromatography using Sephadex G75 followed by spectrophotomet-ric determination of molecular weights, the isolated components in the range of 12-67 kD.

For the separation and identification the proteins of zootoxin, we sampled 10 mg of bee and snake venoms, which were dissolved in 1 ml of bidistilled water and pipetted onto the Sephadex G-75 surface by means of a pipette.

Elution of the snake venom or bee venom proteins was carried out with 0.04 M sodium phosphate buffer. The

https://dx.doi.org/10.22161/ijreh.1.3.6 *ISSN: 2456-8678*

fractions were collected in a volume of 4 ml, followed by a spectrophotometric measurement of the unit optical density of the samples at λ = 280 nm on a Hitachi-557 spectrophotometer.

The data of chromatographic separation of snake venom proteins by the gel filtration method on a column with Sephadex G-75 are presented in table 3, 4.

The results given that gel filtration on a column with Sephadex G-75 is supposed to have a protein with an activity of acid proteinase: a glycoprotein with a molecular weight of 20,000 D. A polypeptide with molecular masses Mm ~ 32 kD is a toxic component, whereas a polypeptide with molecular masses Mm ~ 35kD, possibly a growth factor of the neural tissue, and a protein with a molecular weight of 79,000 D - a protease that activates the clotting factor of the blood. The isolated polypeptides with molecular masses Mm ~ 101 and 132.5k D, in probability, also glycoproteins.

The obtained data on the separation of venom proteins can be practically used to obtain pure samples with the goal of creating new medicinal and diagnostic drugs on their basis.

The data of chromatographic separation of bee venom proteins by the gel filtration method on a column with Sephadex G-75 are presented in Table 5, 6 and in figure 2.

As can be seen from Table 6, as a result of bee venom fractionation by gel chromatography on a column with Sephadex G-75, the investigated venom samples were separated into fragments of fractions of 3 proteins with molecular weights from 15 to 41 kD.

From these tables, it can be seen that the components of the bee venom are arranged in order of increasing elution volumes-VR, which correspond to the decrease in the molecular masses of proteins. Comparing the obtained data with the data of published sources, it can be stated that the isolated components of the bee venom with molecular masses of 41kD correspond to hyaluronidase and 20 kD, 15 kD to phospholipase.

Thus, by the method of column chromatography elution with 0.04M Na-phosphate buffer, optimal conditions for the fractionation of the venom of the snake and honey bee were determined by gel chromatography on a column with Sephadex G-75.

IV. FIGURES AND TABLES

Table.1: Data of spectrophotometric determination of the unit of optical density of protein-marker fractions separated by gel filtration on a column with Sephadex G-75

No. of fractions	The unit of optical density of fractions	No. of fractions	The unit of optical density of fractions	No. of fractions	The unit of optical density of fractions	No. of fractions	The unit of optical density of fractions
1	2	3	4	5	6	7	8
1	0.01	15	0.041	29	0.026	43	0.038
2	0.015	16	0.038	30	0.022	44	0.036
3	0.032	17	0.036	31	0.020	45	0.032
4	0.035	18	0.031	32	0.018	46	0.028
5	0.038	19	0.033	33	0.032	47	0.042
6	0.040	20	0.035	34	0.034	48	0.046
7	0.048	21	0.032	35	0.036	49	0.060
8	0.052	22	0.034	36	0.040	50	0.063
9	0.055	23	0.34	37	0.050	51	0.069
10	0.060	24	0.068	38	0.085	52	0.052
11	0.064	25	0.045	39	0.060	53	0.042
12	0.075	26	0.032	40	0.048	54	0.032
13	0.046	27	0.030	41	0.035	55	0.021
14	0.054	28	0.028	42	0.033	56	0.010

Table.2: The separation of marker proteins by gel filtration on a column with Sephadex G-75

No. of fractions	Protein Markers	VR, ml	M, thousand Daltons
1	Albumen	48	67
2	KA Erythrocyte	96	30

https://dx.doi.org/10.22161/ijreh.1.3.6 *ISSN: 2456-8678*

3	Trypsin	172	20.1
4	Cytochrome C	204	12

Table.4: The optical density data of the snake venom fractions separated by gel filtration on a Sephadex G-75 column

No. of fractions	The unit of optical density of fractions	No. of fractions	The unit of optical density of fractions	No. of fractions	The unit of optical density of fractions
1	0.220	41	0.020	81	0.021
2	0.250	42	0.021	82	0.024
3	0.200	43	0.019	83	0.021
4	0.370	44	0.022	84	0.022
5	0.240	45	0.019	85	0.128
6	0.220	46	0.018	86	0.020
7	0.320	47	0.020	87	0.022
8	1.190	48	0.021	88	0.028
9	1.395	49	0.160	89	0.108
10	0.680	51	0.024	90	0.022
11	0.899	51	0.085	91	0.020
12	0.721	52	0.027	92	0.022
13	0.620	53	0.025	93	0.024
14	0.540	54	0.020	94	0.089
15	0.460	55	0.081	95	0.018
16	380	56	0.019	96	0.020
17	0.348	57	0.021	97	0.019
18	0.321	58	0.025	98	0.021
19	0.248	59	0.025	99	0.018
20	0.120	60	0.028	100	0.020
21	0.322	61	0.027	101	0.018
22	0.280	62	0.024	102	0.101
23	0.199	63	0.022	103	0.108
24	0.140	64	0.020	104	0.089
25	0.101	65	0.024	105	0.019
26	0.080	66	0.148	106	0.020
27	0.042	67	0.026	107	0.014
28	0.029	68	0.020	108	0.019
29	0.025	69	0.025	109	0.020
30	0.020	70	0.028	110	0.024
31	0.018	71	0.024	111	0.028
32	0.020	72	0.026	112	0.030
33	0.024	73	0.023	113	0.662
34	0.016	74	0.069	114	0.027
35	0.018	75	0.021	115	0.020
36	0.016	76	0.025	116	0.019
37	0.020	77	0.022	117	0.020
38	0.019	78	0.098	118	0.019
39	0.021	79	0.019		
40	0.025	80	0.018		

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Table.4: The optical density data of the snake venom fractions separated by gel filtration on a Sephadex G-75 column.

No. of fractions	VR, ml	M, thousand Daltons
1	18.0	149.0
2	22.0	146.5
3	42.0	132.5
4	98.0	101.0
5	102.0	99.0
6	110.0	92.5
7	134.0	79.0
8	156.0	67.0
9	170.0	56.5
10	178.0	51.5
11	188.0	45.0
12	204.0	35.0
13	208.0	32.0
14	226.0	20.0

Table.5: The optical density data of the honey bee venom fractions separated by gel filtration on a Sephadex G-75 column

No. of fractions	The unit of optical density of fractions	No. of fractions	The unit of optical density of fractions	No. of fractions	The unit of optical density of fractions
1	0.01	22	0.012	43	0.050
2	0.018	23	0.011	44	0.062
3	0.041	24	0.012	45	0.076
4	0.045	25	0.010	46	0.040
5	0.052	26	0.001	47	0.034
6	0.031	27	0.010	48	0.022
7	0. 012	28	0.010	49	0.015
8	0.011	29	0.011	50	0.012
9	0.010	30	0.012	51	0.011
10	0.012	31	0. 012	52	0.010
11	0.001	32	0.011	53	0.010
12	0.010	33	0.010	54	0.010
13	0.010	34	0.012	55	0.010
14	0.011	35	0.025	56	0.010
15	0.010	36	0.036		
16	0.010	37	0.048		
17	0.010	38	0.056		
18	0.010	39	0.078		
19	0.011	40	0.062		
20	0.010	41	0.036		
21	0.010	42	0.022		

Table.6: Data on the separation of honey bee venom by gel filtration on a Sephadex G-75 column

No. of fractions	VR, ml	Mm, kD
1	20.0	41.0
2	156.0	20.0
3	180.0	15.0

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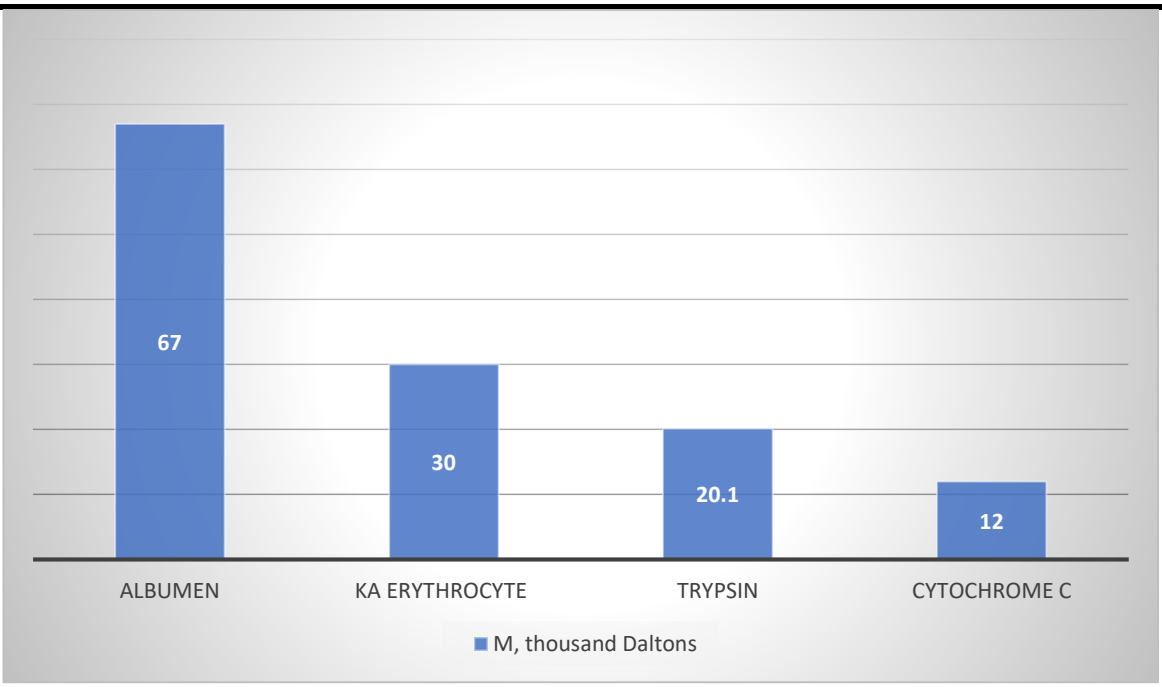

Fig.1: Direct proportional dependence of the marker proteins is in the range 12-67 kD.

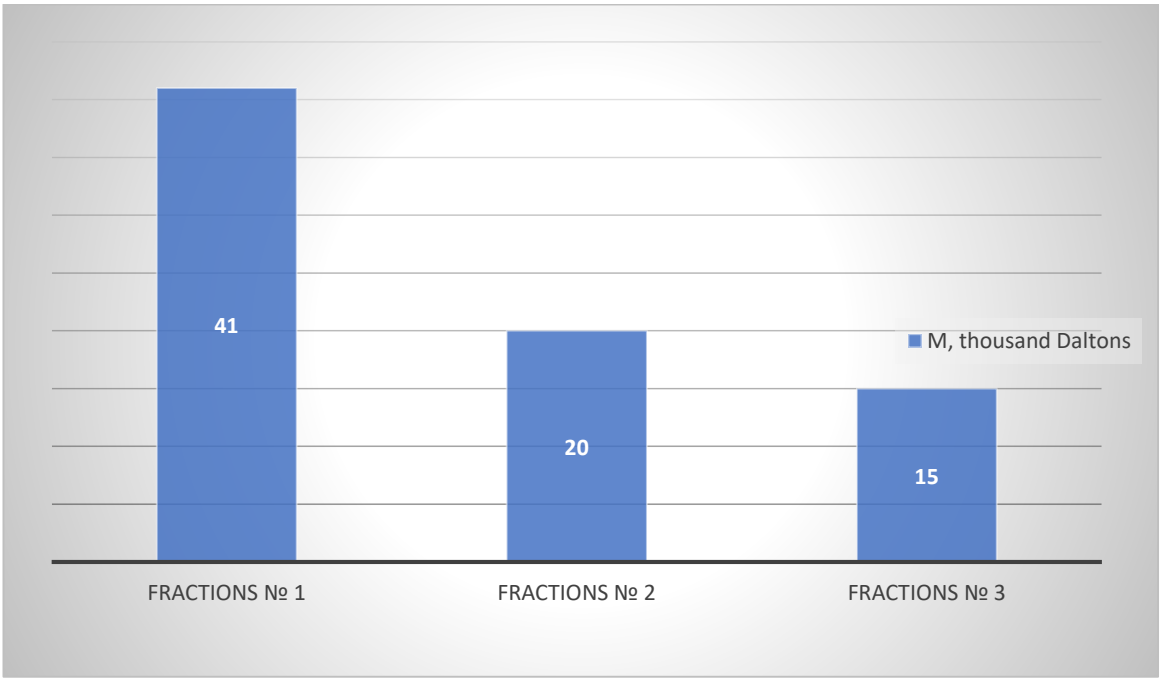

Fig.2: Direct proportional dependence of the bee venom proteins is in the range 15-41 kD.

V. CONCLUSIONS

1. Optimal conditions for the separation and identification of proteins of snake Macrovipera lebetin and honey bee venom were developed by gel chromatography on a column with Sephadex G-75 eluting with 0.04M Na-phosphate buffer.
2. On a column with Sephadex G-75 to have a protein with an activity of acid proteinase: a glycoprotein with a molecular weight of 20,000 D. A polypeptide Mm ~ 32 kD is a toxic component, a polypeptide with molecular masses Mm ~ 35kD, a growth factor of the neural tissue, and a protein with a molecular weight of 79,000 D - a protease that activates the clotting factor of the blood, polypeptides with molecular masses Mm ~ 101 and 132.5k D, in probability, also glycoproteins.
3. Hemocoagulating proteins of hyaluronidase and phospholipase with molecular masses of 41 kD and 20 kD, 15 kD, respectively, were isolated from the honey bee venom.

https://dx.doi.org/10.22161/ijreh.1.3.6 *ISSN: 2456-8678*

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