PHOSPHATASE ACTIVITIES OF FRACTIONS OF THE VENOM OF RENARD'S VIPER *Vipera ursini renardi*

Sh. M. Khamudkhanova and D. N. Sakhibov

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The separation of the venom of Renard's viper by filtration through Sephadex G-75 gel has been performed and phosphatase activities have been determined in the fractions and in the course of separation. The bulk of the activity of the phosphatases investigated was concentrated in the high-molecular-weight fractions I and II. A correlation has been found in the distribution of the DNase and ATP-pyrophosphatase activities with the phosphodiesterase activity in the venom fractions. There is no correlation in the distribution of the RNase and phosphodiesterase activity.

Various enzymes belonging to three groups of phosphatases — phosphomonoesterases, phosphodiesterases, and polyphosphatases — have been found in snake venoms. Their mutual interference prevents the use of the whole venum as sources of an enzyme. We have made an attempt to separate and purify the phosphatases with the aid of gel filtration.

In the separation of Renard's viper venom by filtration through Sephadex G-75 gel, we used a procedure developed previously [1]. The activities of the phosphatases — 5'-nucleotidase and nonspecific alkaline monophosphatase from the group of monophosphatases; phosphodiesterase, DNase, and RNase from the group of phosphodiesterases; and ATP-pyrophosphatase, which is a polyphosphatase — were determined in the fractions and during the course of separation on Sephadex G-75 (Fig. 1).

In the process of gel filtration, the viper venom was separated into five fractions, the separation being between fractions I and II being the most satisfactory. Analysis of the enzyme activities during the course of separation showed a concentration of the 5'-nucleotidase in fractions I and II; the phosphodiesterase and nonspecific monophosphatase were distributed over the whole of the separation with a very low activity; a small "peak" of phosphodiesterase activty was found in fractions I and II, and a peak of phosphomonoesterase activity in fractions I and II and at the point of junction of fractions III and IV. More detailed characteristics of the fractions are given in Table 1.

Fractions I and II were the main ones quantitatively, making up about 56-57% of the total weight of the whole venom, 37-39% being represented by the other three fractions, and the losses on fractionation amounting to 4-7%. The protein contents in the fractions reached high values — about 100%, and only for fractions IV and V, containing the low-molecular-weight components of the venom, was it substantially lower, not exceeding 37%. The bulk (with respect to activity) of the phosphatases investigated, with the exception of the ribonuclease, was concentrated in the high-molecular-weight fractions I and II.

Attention is attracted by the absence of a correlation in the distribution of the activities of the RNase and the phosphodiesterase when the fractions of Renard's viper venom that we investigated are compared (see Table 1). Conversely, the DNase and the ATP pyrophosphatase were separated into the fractions of viper venom studied in almost the same way as the phosphodiesterase. The losses of phosphodiesterase, DNase, and ATP pyrophosphatase activities in the process of fractionating the venom on Sephadex were practically the same, amounting to 60%, and in the distribution of the activities between the fractions again a parallelism was observed, which was possibly due to their molecular identity [sic] and is confirmed by some literature statements [2].

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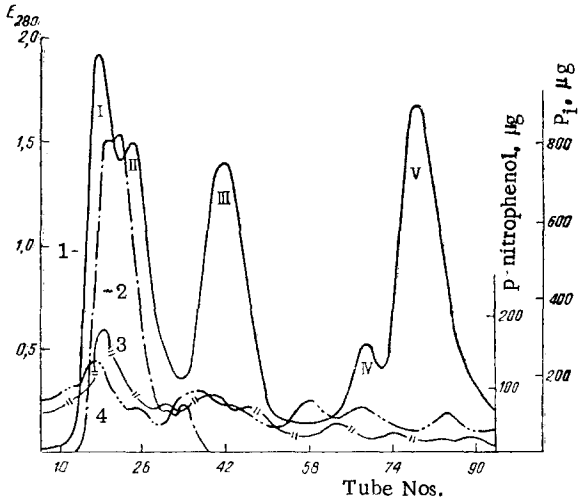

Fig. 1. Graph of the gel filtration of Renard's viper venom on Sephadex G-75. Column 25 × 980 nm; rate of elution 30 ml/h; fraction volume 6 ml; 1) protein content (absorption at 280 nm); 2) 5'-nucleotidase activity; 3) nonspecific alkaline monophosphatase activity (μg P_i); 3) phosphodiesterase activity (μg of p-nitrophenol): I-V) fractions combined according to the protein "peaks."

EXPERIMENTAL

The venom of Renard's viper *Vipera urseni renardi* Ch., dried in a desiccator over calcium chloride, was obtained from the Central Asian zonal Uzzookombinat.

Fractionation through Sephadex gel of the whole Renard's viper venom was carried out on a column of Sephadex G-75 (25 × 980 nm). The preparation of the gel for use in the filling of the column was czrried out in accordance with L. M. Ginodman's recommendations [3]. The column was equilibrated and the material was eluted with 0.05 M ammonium bicarbonate buffer, pH 8.2. The collection of the fractions and the automatic recording of the protein concents of the fractions were performed on a Uvicord-II instrument.

Nonspecific alkaline monophosphatase activity was determined by the method of Dixon and Purdom [4] from the splitting out of inorganic phosphate from the substrate, the amount of inorganic phosphate being measured by the method of Ya. Kh. Turakulov et al. [5]. The nonspecific alkaline phosphatase was investigated through its action on sodium β-glycerophosphate at pH 9.3. The incubation mixture contained 0.1 ml of the material (fraction) under investigation and 4.4 ml of buffer and substrate (0.05 g of β-glycerophosphate and 0.424 g of barbital sodium were added to 100 ml of distilled water) with pH 9.3. The reaction mixture was incubated at 37°C for 2.5 h. The reaction was stopped by the addition of 0.01 ml of 50% TCAA. Experimental samples were centrifuged, and the amount of inorganic phosphate in the supernatant was measured. The optical density in the isobutanol layer was determined at a wavelength of 390 nm on a VSU-2P spectrophotometer.

The 5'-nucleotidase activity was also determined by Dixon and Purdom's method [4] in relation to the splitting out of inorganic phosphates from the substrate, which was again determined by the method of Ya. Kh. Turakulov et al. [5]. In view of the simultaneous presence of nonspecific alkaline monophosphatase, which interferes with the 5'-nuclease in the material under investigation, the activity of the latter was investigated from the hydrolysis of 5'-AMP at pH 7.5, a correction to the results obtained being made for the hydrolysis of the β-glycerophosphate at the same pH. Two experimental samples were prepared containing 0.1 ml of the material under investigation and 4.4 ml of a mixture of buffer and substrate. The 5'-nucleotidase activity was established in a similar manner to the determination of the activity of the nonspecific alkaline monophosphatase.

The phosphodiesterase activity was determined spectrophotometrically from the accumulation of p-nitrophenol split off from the substrate — calcium bis(p-nitrophenyl phosphate) — by the method of R. I. Tartarskaya et al. [6].

TABLE 1. General Characteristics of the Fractions Obtained by the Gel Filtration of Renard's Viper Venom

Venom and fraction	Yield, % by weight	Protein content, %	Enzyme activity, units/mg of venom					
			μg of P_i			μg of p-nitrophenol, PDe	μg of standard enzyme	
			NAP	5'-ND	ATPase		DNase	RNase
Venom	100	86	22,5	250,0	80,0	68,0	88,0	0,8
Fraction I	30	99–100	9,2	315,7	37,0	32,0	44,2	0,2
II	26–27	99	4,0	90,9	40,9	27,0	31,8	2,7
III	22	98	5,0	—	20,0	30,0	30,0	—
IV	5–6	30	1,5	—	10,0	15,0	15,0	—
V	10–11	30						

The incubation medium contained 1.4 ml of Tris-HCl buffer (pH 8.8), 0.1 ml of a 0.1 solution of magnesium chloride, 0.5 ml of a 0.1 solution of the substrate — calcium bis(p-nitrophenyl phosphate) — and 0.1 ml of a solution of the material (fraction) under investigation. The total volume of the sample was about 3 ml. The samples were incubated at 37°C for an hour and were then subjected to spectrophotometry at 400 nm.

The RNase activity was determined by the method of Dickman and Trupin [7], according to whom the products of the hydrolysis of RNA after their separation from the unhydrolyzed RNA are determined spectrophotometrically from the absorption at 260 nm. The experimental samples contained 0.5 ml of a 2% solution of sodium salt of yeast RNA, 0.4 ml of 2 M acetate buffer (pH 5.0), and 0.1 ml of a solution of the fraction. The samples were incubated at 37°C for 150 min. The reaction was stopped by the addition of 3 ml of a 0.8% solution of uranyl acetate in 0.85% $HClO_4$ which led to the precipitation of the unhydrolyzed RNA. The optical density was measured spectrophotometrically at 260 nm on a VSU-2P spectrophotometer.

The DNase activity was determined spectrophotometrically at 600 nm from the adsorption of the deoxyribonucleotides liberated under the action of the enzyme on a substrate by the method of Allfrey and Mirsky [8] and cited by Orolovskii [3]. Cattle spleen DNA ("Reakhim," USSR) was used as the substrate. The incubation medium contained 1 ml of a 0.2% solution of the substrate in 0.05 M magnesium sulfate, 1.9 ml of barbital/barbital sodium buffer, pH 7.5, and 0.1 ml of a solution of the fraction under investigation. The mixture was incubated at 37°C for 4 h, and then the reaction was stopped by the addition of 1 ml of 3 M TCAA. The samples were centrifuged at a speed of 10,000 rpm for 15 min, 1.5 ml of supernatant liquid was taken and it was treated with 3 ml of diphenylamine reagent (1 ml of concentrated sulfuric acid + 40 ml of a 1% solution of diphenylamine in glacial acetic acid). The test tubes with the samples were placed in the boiling water bath for 20 min and were then left at room temperature for 20 min and photometered at 600 nm.

ATP pyrophosphate activity was determined from the amount of inorganic phosphate split off from the substrate — the sodium salt of ATP — under the action of the enzyme. The amount of inorganic phosphate was measured by the method of Ya. Kh. Turakulov et al. [4]. The incubation mixture contained 0.2 ml of a 0.65% solution of the sodium salt of ATP, 0.1 ml of a solution of the material under investigation, and 4.1 ml of 0.1 M barbital/barbital sodium buffer (pH 8.8). The samples were incubated at 37°C for 1 h, the reaction was stopped by the addition of 50% TCAA, and the amount of organic phosphate in the supernatant liquid was determined.

The amounts of proteins in the samples were measured by Lowry's method [9] and spectrophotometrically from the absorption at 280 nm.

SUMMARY

1. A high-molecular-weight fraction characterized by the concentration in it of the 5'-nucleotidase and of the bulk (with respect to activity) of the other phosphatases, with the exception of RNase, has been obtained from Renard's viper venom with the aid of gel filtration.

2. A quantitative estimate of the phosphoesterase activities in the fractions of Renard's viper venom has been given.

3. Parallelism has been established in the distribution of the phosphodiesterase, DNase, and ATP-pyrophosphatase over the fraction of Renard's viper venom, while the RNA had a different distribution and is separated from the above-mentioned enzymes in the gel filtration of the viper venom on Sephadex.

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