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ISOLATION OF PHOSPHOLIPASE A_2 FROM THE VENOM OF THE GREEN TOAD *Bufo viridis*

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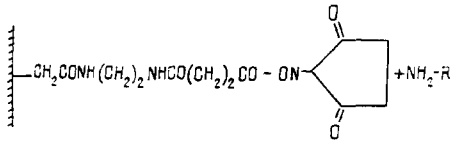
Phospholipase A_2 has been isolated fromthe venom of he Central Asian green toad Bufo viridis in the electroophoretically homogeneous state for the first time by biospecific absorption chromatography. The affinity sorbent used was Affi-Gel 10 with covalently bound phosphatidylserine (PDS). The chemical inertness of the bound PDS to the hydrolytic action of phospholipase A2 and its high tendency to form biospecific complexes have been shown. Some biochemical characteristics of the enzyme isolated have been studied: influence of the pH and of the Ca^{2+} concentration and dependence of activity on substrate concentration.

At the present time, the majority of phospholipases from natural sources have been obtained in the pure form and their basic properties have been studied, and a structural-functional analysis has been made for this class of enzymes [1, 2]. The secretions of various toads belong to this class of enzymes. Thus, the secretion of toads of the genus *Bufo* elaborated by the suprascapular glands and known under the name of "toad venom" is a natural source of many physiologically active substances: alkaloid-like bases derived from indole — serotonin, tryptamine, bufotenin, etc. — and cardiostimulating steroids (free and bound genins — bufodienolides and bufotoxins) [3]. Among the protein components of toad venom only phospholipase A has been detected in the secretion. The aim of the present work was to purify this component from the venom of the Central Asian green toad *Bufo viridis* and to study some of its biochemical and catalytic properties.

Known methods of isolating and purifying phospholipases from many sources are based on their preliminary concentration (salting out, treatment with organic solvents, etc.), followed by gel filtration and ion-exchange chromatography [4-7]. However, the majority of these methods are not very effective, have a large numbe of stages, and give a low yield of the purified enzyme.

In recent years, highly efficient affinity sorbents permitting the isolation of enzymes of this class in a single stage and with good yield have been obtained [8-10]. The ligands used in these sorbents are fatty acid residues, hydrophobic ligands of various structures, epoxy derivatives of substrates, and antibodies to the phospholipases. The most effective among such sorbents have proved to be those containing as ligands phosphatidylethanolamine and phosphatidylserine covalently attached to an insoluble matrix [8]. We made use of just this method of biospecific chromatography with covalently immobilized phosphatidylserine on an insoluble matrix.

For the synthesis of the biospecific adsorbent we used as the insoluble support the highly stable Affi-Gel 10, which has a polyatomic "stalk." Unlike other known polysaccharide supports, it permits the spatial separation of the ligand from the surface of the support, which decreases the influence of steric factors on its interaction with the enzyme to be isolated [11]. The N-hydroxysuccinimide-activated carboxy groups of this support rapidly and with good yield form amide bonds with the primary amino groups of the ligand.



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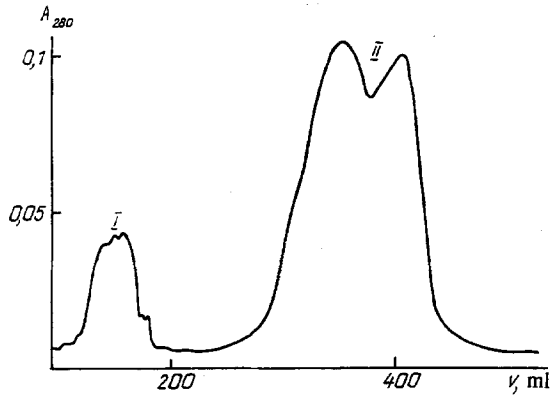

Fig. 1. Gel chromatography of the whole *Bufo viridis* venom on a column (2.5 × 100 cm) of Ultragel AcA 54; 0.1 M ammonium acetate buffer, pH 5.6.

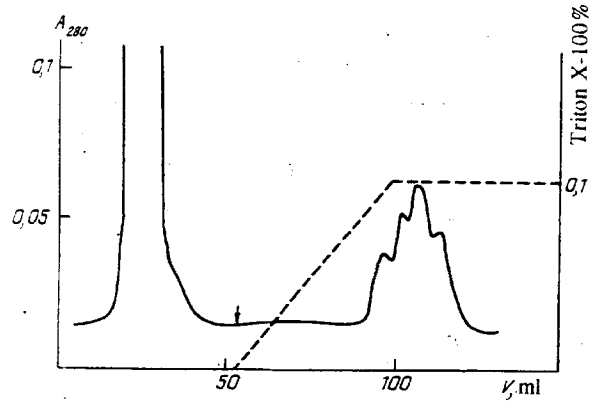

Fig. 2. Affinity chromatography of fraction 1 of *Bufo viridis* venom on a column (1 × 10 cm) of Affi-PDS; 0.01 M Tris-HCl buffer, pH 8.2.

The activated carboxy groups of the support remaining free after the addition of the ligand were neutralized by the action of an excess of monoethanolamine (MEA), which permitted the hydratability of the sorbent in solutions to be increased.

To prevent nonspecific sorption of the components of the whole venom of *B. viridis* we first eliminated low-molecular-mass substances by gel filtration (Fig. 1). The first fraction, which possessed phospholipase activity, was used for further purification by biospecific absorption chromatography.

Then fraction 1 was sorbed on a column of the synthesized affinity sorbent Affi-PDS equilibrated with the initial buffer in the presence of 10 mM $CaCl_2$, and incubation was carried out for 18 h (circulation), after which the sorbent was washed until unbound proteins had been eliminated completely.

We first convinced ourselves of the stability of the immobilized PDS to the hydrolytic action of the phospholipase A_2 under the conditions of biospecific chromatography, for which purpose we performed control experiments. The unhydrolyzability of the immobilized ligand can be explained by the well known hypothesis of the two-point binding of phospholipase A_2 with phospholipids. This was also to be expected on the basis of our previous investigations [10], in which it was shown experimentally that the binding of the amino alcohol moieties of phospholipids with a polymeric support leads to a loss of hydrolyzability by the latter.

The desorption of the enzyme from he adsorbent was carried out under various conditions, using as the desorbing solutions: 1) 10 mM cetyltrimethylammonium bromide; 2) 10 mM Na-EDTA; 3) 1 M NaCl; 4) a concentration gradient (0-0. 1%) of Triton X-100. The best results were obtained with by desorption in the Triton X-1oo concentration gradient (Fig. 2) As can be seen from Fig. 2, the enzyme fraction was eluted in several peaks, which is possibly connected with the presence

TABLE 1.

Stage of purification	FFAs, μM	A_{sp}, μM/mg/min	Degree of purification, times	A_{tot}	Protein, mg
Whole venom	1.00	0.03	1	1.65	55
Gel filtration	1.52	0.05	1.55	1.45	31
Biospecific chromatography	2.04	6.80	206	1.36	0.2

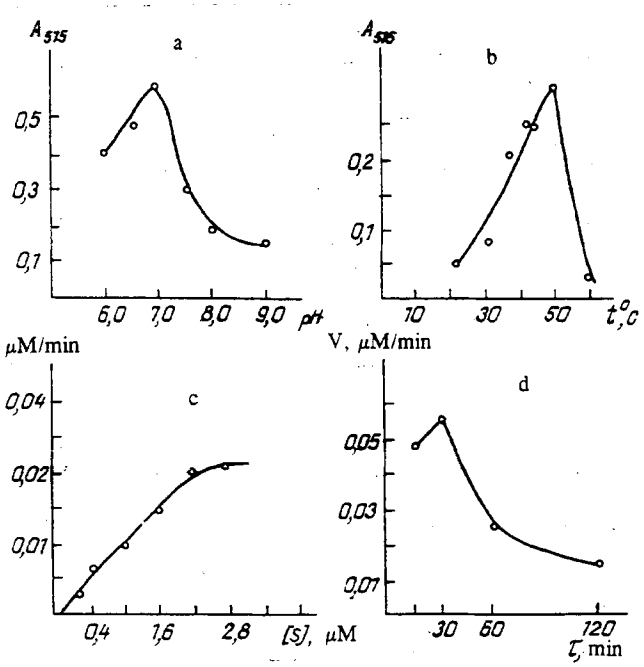

Fig. 3. Catalytic characteristics of the phospholipase A_2 fom *Bufo viridis*: *a, b, c*) pH, temperature, and substrate dependence, respectively; d) kinetics of the hydrolysis of egg phosphatidylcholine.

of several isoforms of phospholipase A_2 in the venom of the Central Asian green toad *Bufo viridis*. Furthermore, elution of the enzyme at the very end of the gradient points to fairly powerful hydrophobic interactions of the enzyme with its substrate.

Figure 2 and Table 1 show the results of the use of the adsorbent synthesized for the purification of phospholipase A_2 from *B. viridis* venom by biospecific chromatography. The bulk of the proteins passed through the column without retention and contained no phospholipase A_2 activity. Washing the column with a concentration gradient (0-0.1%) of Triton X-100 ensured the complete elution of the phospholipase A_2 from the adsorbent. The molecular mass of the phospholipase A_2 determined by disk electrophoresis in PAAG in the presence of SDS was 55 kDa.

An investigation of some of the catalytic characteristics of the phospholipase A_2 isolated showed that it was active over a fairly wide pH range and, as can be seen from Fig. 3, had an optimum at pH = 7.0. The activity of the enzyme increased with a rise in the temperature to 50°C (Fig. 3, *b*). A further rise in the temperature led to a fall in activity, which was possibly connected with the denauration of the enzyme. The bivalent Ca^{2+} ions exerted an activating influence on the enzyme up to a concentration of 20 mM in the reaction mixture, although it can also exert its activity in the absence of Ca^{2+} ions.

The formation of free fatty acids from egg phosphatidylcholine increased during the first 30 min of the enzyme reaction and then their amount remained constant (Fig. 3, *d*). The rate of enzymatic hydrolysis of phosphatidylcholine by the phospholipase A_2 from the the venom of the green toad depended on the concentration of the substrate. The catalytic constants of this reaction were determined in the reciproval Lineweaver-Burk coordinates: K_M = 2.77 mM and V_{max} = 0.02 μM/min; Fig. 3, *c*.

EXPERIMENTAL

We used phosphatidylcholine and phosphatidylserine from the Khar'kov chemical reagents factory, Triton X-100 from Merck (FRG), and palmitic acid and Rhodamine G from Soyuzkhimreaktiv. The salts for the preparation of the buffer solutions and the organic solvens were of ch.d.a ["pure for analysis"] grade. The whole green toad venom was obtained from the Central Asian zonal combine.

The gel fitration of the whole *Bufo viridis* venom was conducted on a column (2. 5 × 100 cm) of Ultragel Aca 54 in 0.1 M ammonium acetate buffer, pH 5.6. Here and below, detection of the optical density of the eluate at 280 nm was carried out on a Uvicord C instrument (LKB, Sweden). Fractions with a volume of 10 ml were obtained in an Ultrorac fraction collector. The fractions possessing phospholipase activity were combined and freeze-dried.

Biospecific chromatography was conducted on a column (1 × 10 cm) with the synthesized sorbent Affi-PDS.

The biospecific sorbent was synthesized in the following way: 3 ml of Affi-Gel 10 (Bio-Rad, USA) was incubated at 4°C with a solution 3 mg of PDS in a mixture of methanol and 0. 2 M borate buffer, pH 8.0, in the presence of 0.5 M NaCl. The nonmbound phospholipids were washed out with the initial buffer. The free functional groups of the sorbent were blocked with a 0.1% solution of MEA, with incubation for 6 h. The excess of MEA was washed out with the same buffer solution.

Fraction 1 of the venom of the green toad *Bufo viridis* was deposited on a column of Affi-PDS equilibrated with 0. 01 M Tris-HCl buffer, pH 8.2, containing 10 mM $CaCl_2$ with incubation by circulation for 18 h, after which the sorbent was washed until unbound proteins had been completely eliminated. The enzyme was eluted from the adsorbent Affi-PDS with a concentration gradient from 0 to 0.1% (50-50 ml) of Triton X-100. The homogeneity of the purified phospholipase A_2 was checked by electrophoresis in PAAG in the presence of SDS [13].

The protein contents of the fractions were determined by Lowry's method [14]. The concentrations of free fatty acids (FFAs) in the system were found photometrically at 515 nm in the presence of Rhodamine 6G by the method of [15]. The enzymatic activity of phospholipase A_2 was determined as in [16]. All spectrophotometric measurements were made at 40°C with the aid of a Spectromom-410 photoelectric colorimeter.

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