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A PHOSPHOLIPASE A_2 WITH ANTICOAGULANT ACTIVITY

I. ISOLATION FROM *VIPERA BERUS* VENOM AND PROPERTIES

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Summary

An anticoagulant protein has been isolated by DEAE cellulose chromatography and gel filtration from the venom of the *Vipera berus orientale* (Eastern Europe).

Purification has been completed by elution on carboxymethyl cellulose with continuous gradient at constant pH. The inhibitor of coagulation was separated from the other venom enzymes, e.g. procoagulant, fibrinogenolytic, aminoesterase and amino acid oxidase activities. It was also separated from other phospholipase components which were not related to the anticoagulant property.

The inhibitor appeared as a single polypeptidic chain protein, formed by 119 amino acid residues, with a molecular weight of 13 400 and an isoelectric point of 9.2. At low saline molarity, a monomer-trimer transition of this protein was observed. Both forms had the same amino acid composition. There were six disulfide bridges without free SH groups per phospholipase molecule.

Deprived of any proteolytic activity, the clotting inhibitor displayed a high phospholipase activity in the presence of calcium. Activity did not appear with EDTA buffer deprived of cation. Finely dispersed micellar suspensions were found suitable for obtaining the highest phospholipase activity. High sodium cholate concentration or methanol/chloroform/ether solvent were effective without loss of enzymatic activity. As characteristic of phospholipase A_2 (EC 3.1.1.4), the degradation products identified on thin-layer chromatography induced hemolysis of human erythrocytes. The apparent K_m value $1.25 \cdot 10^{-3}$ M was determined on phosphatidylcholine isolated from ovolecithin.

This purified *berus* inhibitor would be of value for investigating the involvement of phospholipids in the clotting mechanism.

Introduction

Phospholipase A_2 (EC 3.1.1.4.) catalyses the specific removal of the fatty acyl group from the position 2 of an *Sn*-3-phosphoglyceride.

This enzyme has been detected in numerous animal tissues and fluids and in bacteria but pancreatic secretion and snake and bee venoms have provided the major sources of enzyme [1,2]. Phospholipase activity and molecular parameters have been studied in various venomous snake species from: *Elapidae*, *Crotalidae*, *Viperidae* and *Hydrophiidae*. In several venoms, the phospholipase was found to consist of a group of isoenzymes as described the first time in *Naja naja* venom [3].

Various biological effects of phospholipase A_2 on human blood phospholipid components have been investigated, from ghost or native red blood cells [4], platelets [1] and serum lipoproteins [5—7].

Modifications of normal blood coagulation have been described as a general property of many snake venoms [8]. Up to now, the action of purified phospholipase A_2 on phospholipid involved in blood coagulation has not been reported.

Several phospholipases A_2 have been found in the venom of *Vipera berus orientale* [9,10]. Among these, a toxic phospholipase A_2 showed anticoagulant activity [11].

In this work we describe the isolation process of the anticoagulant factor from the crude venom and investigations of its physicochemical and enzymatic properties.

Experimental procedures

1. Venoms. Dried venom from *V. berus* (Eastern Europe) was provided by l'Institut Sérothérapique et Vaccinal, Bern, Switzerland. Crude venom was powdered and solubilized in 0.01 M Tris · HCl buffer (pH 8.), dialysed overnight at 4°C and centrifuged in order to discard insoluble material.

2. Chromatographic procedures. Venom fractionation was performed on DEAE cellulose, on carboxymethyl cellulose (Whatman DE 32, CM 32), and by gel filtration either on Biogel A 0.5 M, 100—200 mesh (Bio Rad Lab.) and Sephadex G-75 or on Sephadex G-75 and G-200 (Pharmacia).

Ultrafiltration (Diaflo membranes P.M. 10 or U.M. 2) was operated at a N_2 pressure of 4 kg/cm^2, under constant stirring at 4°C. Protein content was measured by absorbance at 280 nm and by the method of Lowry et al. [12] using crystallized human serum albumin as standard.

3. Polyacrylamide gel analysis. Electrophoresis on polyacrylamide gel electrophoresis (9%) was carried out according to the technique of Weber and Osborn [13].

4. Molecular weight determination. The molecular weight was estimated according to Andrews' method [14] on Sephadex G-75 column and by (12%) polyacrylamide gel electrophoresis in sodium dodecyl sulphate 0.1% and 2-mercaptoethanol (120 min) [13].

5. Amino acid analysis. Amino acid analysis was carried out according to the procedure of P.B. Hamilton [15]. Determination of free SH groups was per-

formed after protein labelling with iodo[2-^{14}C]acetic acid (spec. act.: 21 Ci/mol) (Radiochemical Centre, Amersham) in the presence of 10 M urea or 8 M guanidine chlorhydrate. Enzymatic digestion was performed by pepsin and trypsin. Labelled peptides [16] were detected by autoradiography after high voltage electrophoresis at pH 3.5.

6. Determination of isoelectric point by electrofocusing. Isoelectric focusing was carried out in the 110 ml LKB 8 121 apparatus using 1% Ampholine concentration with a pH ranging from 1 to 13 and from 7 to 10. 200 μg of *berus* inhibitor (100 μl) were incorporated in the sucrose density gradient with a voltage power supply from 600 to 1000 V for 48 h. Elution was carried out at a 1.5 ml/min flow rate.

pH values were measured at 5°C. Phospholipase activity and anticoagulant activity were determined on fractions submitted to Sephadex G-25 gel filtration in 0.10 M NaCl/0.05 M Tris buffer pH 7.3.

7. Assay for amino acid esterase activity. The activity was measured by pH-stat titration using α-*N*-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) (Mann), at pH 7.7, 37°C under N_2 stream on a 3 μmol/ml solution in deionized water (3 ml).

8. Phosphorus determination. Phosphorus digestion (10—100 μg) was carried out on an electrical heated Kjeldahl rack (160—180° C) by adding 0.5 ml of 72% perchloric acid [17]. After adding 1 ml distilled water and 1 ml of 1% ammonium molybdate solution, the samples were heated at 100°C. After the appearance of yellow colour in the highly concentrated sample of phosphorus standards, 1 ml of 0.005% hydrazine sulphate was added. Tubes were heated (100°C, 10 min), then cooled before spectrometrical determinations at 830 nm [18].

9. Phospholipase A_2 assay. Measurements were performed as already described [19] by potentiometric titration according to the technique of de Haas et al. [20]. Ovolecithin (Merck) or phosphatidylcholine (8—10 mg/ml) isolated by thin-layer chromatography were irradiated in a water cholate/calcium medium [21] by repetitive exposures of 20—30 s with MSE 150 Watts ultrasonic desintegrator under a 20 kHz/s frequency at 2°C.

Titration using a 0.25 ml autoburette was carried on with 0.01 M NaOH, pH 8.0, 37°C under a stream of N_2.

K_m was determined on phosphatidylcholine with 2 μg *berus* inhibitor according to the Lineweaver-Burk experimental procedure [22].

10. Thin-layer chromatography. Thin-layer chromatography was performed on precoated plate silica gel F 254 (layer thickness: 0.25 mm) (Merck). A chloroform/methanol/acetone/acetic acid/water (10 : 2 : 4 : 21, by vol.) solvent was used for developing chromatograms [23].

Hydrolysed products were obtained with 1 mg of phosphatidylcholine in 1 ml buffer mixed on Whirlimixer apparatus with 100 μl of diethyl ether and 10 μg *berus* inhibitor at 30°C. The following buffer was used: saline solution (0.001 M EDTA, 0.01 M $CaCl_2$)/methanol/diethyl ether (100 : 5 : 5, by vol.) adjusted to pH 9.0 by 1 M NH_4OH. After 15 min, 100 μl of chloroform/methanol (v/v, 2 : 1) were added before the next 15 min incubation. Phospholipids were extracted by chloroform/methanol (v/v, 2 : 1) and the aqueous phase removed by centrifugation. Hydrolysed phospholipids were dried under nitrogen and 40 μm of the chromatographic solvent were added. 10 μl of the

mixture were incorporated on silica gel plates. Lipid spots were detected under ultraviolet light (366 nm), after Rhodamine 6-G spraying, or directly by iodine vapors. All chemicals used were reagent grade.

11. Hemolytic activity of the lysoderivatives. 1 mg of phosphatidylcholine in 1 ml of 0.05 M Tris/0.15 M NaCl/0.02 M $CaCl_2$ buffer, pH 7.25, were submitted to 10 µl (10 µg) of *berus* inhibitor or buffer, and incubated at 37°C. The hemolytic activity was assayed on a suspension of washed human red blood cells and measured at 540 nm after centrifugation.

12. Blood clotting assays. Plasmas were prepared from human blood as described previously [11]. Polystyrene tubes were used in all preparative steps, glass tubes in clotting assays. Veronal buffer (0.026 M barbital sodium, 0.026 M sodium acetate, 0.1 M NaCl), pH 7.3, was used. Clotting times were measured at 37°C. The venom fractions were diluted or not in veronal buffer according to their protein concentration. For each assay, a control system was applied using veronal buffer instead of venom for diluted fraction, or chromatographic buffer for undiluted fraction.

Recalcification times (0.025 M $CaCl_2$) were performed on platelet-poor plasma to follow the elution of procoagulant and anticoagulant factors. Crude cephalin : chloroform extract from human thromboplastin [24] was or was not added to the calcium. Fibrinogenolytic activity was detected as previously for *Vipera aspis* venom [19] by thrombin times (bovine thrombin, Hoffmann-La Roche, Basel, 5 units/ml in veronal buffer) on a platelet-poor plasma, incubated (1 h, 37°C) with the venom fraction.

Factor V activation was measured by specific one stage assay with factor-V deficient plasma [25]. Platelet-poor plasma was incubated (3 min, room temperature) with the venom fraction before serial dilution.

Results

Separation of the anticoagulant protein from the other enzymatic activities of the venom

V. berus venom was first fractionated into five main fractions on DEAE-cellulose chromatography (Fig. 1). The anticoagulant activity was found with the more basic proteins (fraction A), separated from the procoagulant activity (maximum in fraction C) and from the slight fibrinogenolytic effect eluted in fraction E. The highest peak of phospholipase activity was present in fraction A but no esterase activity on Bz-Arg-OEt was detected in this fraction. A very weak activation of factor V (1.5 units) was induced by fraction B.

After dialysis and concentration, fraction A was submitted to recycling gel filtration on Sephadex G-75, G-200 (Fig. 2). Three protein peaks were obtained. The anticoagulant and the phospholipase activities were recovered in the second peak. The active fractions were pooled (Fig. 2), concentrated and dialysed before next purification step.

A chromatography on carboxymethyl cellulose CM 32 was then performed (Fig. 3). A small protein peak without activity was eluted with the starting buffer (peak I). In the experimental conditions, specific phospholipase activity remained constant over the two following protein peaks, peak II eluted with 0.1 M NaCl in phosphate buffer and peak III eluted with a gradient 0.1—0.25 M

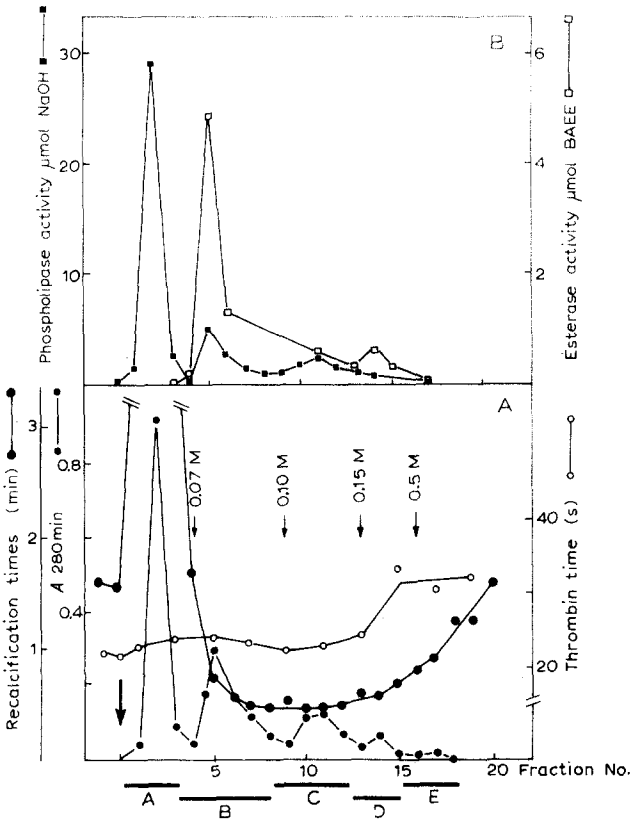


Fig. 1. Distribution of different activities present in *Vipera berus* venom after ion exchange chromatography on DEAE-cellulose. The DEAE column (0.9 X 8 cm) was loaded with 42.5 mg of dialysed *Vipera berus orientale* venom (2.4 ml). Elution was performed with discontinuous gradient buffer obtained with progressively increasing NaCl molarities in 0.01 M Tris · HCl buffer (pH 8.0) as indicated by the arrows. Flow rate, 20 ml/h; fraction volume, 4.5 ml. The eluted volume was divided into five pools A, B, C, D and E represented under the base line corresponding to the main protein peaks. They were eluted respectively with equilibrium buffer and with 0.07, 0.10, 0.15 and 0.5 M NaCl in Tris · HCl buffer. (A), protein concentration was measured as absorbance at 280 nm. Recalcification times were performed in the presence of cephalin diluted 1/200 in 0.025 M $CaCl_2$. Clotting system: 0.2 ml of plasma + 0.04 ml of venom fraction or buffer + 0.2 ml of cephalin/$CaCl_2$. Plasma was not clotted after 24 h in the interval of the interrupted lines. Fibrinogenolytic activity was studied by thrombin times after incubation. Clotting system: 0.4 ml of plasma was incubated 1 h, 37°C, with 0.08 ml of venom fraction and 0.1 ml of thrombin (5 units/ml) was added. Clotting activities were measured with an equal volume of venom fraction, not considering the protein concentration. Control times with buffer are represented by the first point of the curves in front of the protein elution tubes. (B), arginine esterase activity was measured on Bz-Arg-OEt with 0.04 ml of fraction. It is expressed in µmol of substrate hydrolysed in 4 min. Phospholipase A_2 activity was determined with 0.01 ml of fraction on 3 ml egg phosphatidylcholine suspension in 0.05 M cholate/0.001 M EDTA/0.02 M $CaCl_2$ as described in Experimental procedures. It is expressed in NaOH µmol/4 min.

NaCl in the same buffer. Anticoagulant activity measured with 8 µg/ml of protein was found only in the fraction eluted with the gradient (peak III). This peak was considered as the clotting *berus* inhibitor. Peak II consisted of a phospholipase without inhibitor activity.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the crude

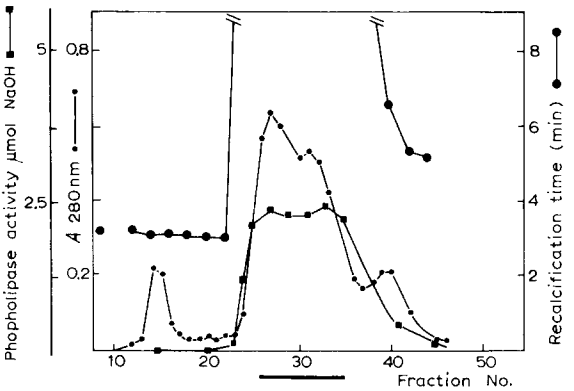


Fig. 2. Distribution of the anticoagulant and phospholipase A_2 activities after recycling gel filtration of fraction A. 19 mg of protein from dialysed fraction A (9.5 mg/ml) were filtered through Sephadex G-75 (68 X 1 cm) and Sephadex G-200 (35 X 2.5 cm) columns in 0.15 M NaCl, pH 7.5. Flow rate, 15 ml/h; Fraction volume, 7 ml. Specific anticoagulant activity was measured by recalcification time performed with $CaCl_2$ without cephalin. Clotting system as in Fig. 1. Specific phospholipase A_2 activity was expressed as the uptake of NaOH µmol/min using protein concentration of 25 µg/ml. Eluted volumes from tubes 25—35 were pooled for subsequent chromatographic step.

venom and of the material obtained in step 2 and 3 are compared in Fig. 4. Analysis of eluted fractions from sliced polyacrylamide gel showed the presence of both anticoagulant and phospholipase activities associated with the single polypeptide chain band.

Isoelectric point

The determination of the isoelectric point was carried out by electrofocusing technic. As seen on Fig. 5, the peak of phospholipase activity was found to coincide with that of anticoagulant activity at a pH of 9.2.

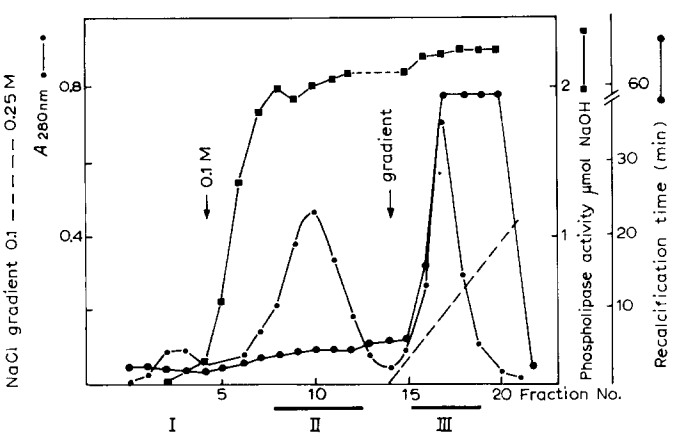
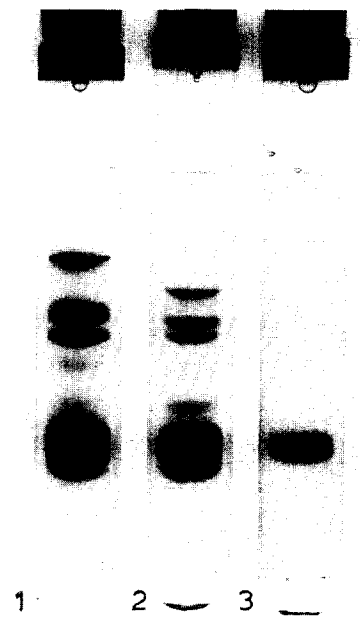


Fig. 3. Chromatography on carboxymethyl cellulose CM-32. The pooled fraction (12.9 mg of protein) after dialysis and concentration (2.5 ml) was applied to a column (diameter 5.5 mm) filled with 1.3 ml of cellulose equilibrated in 0.01 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.5. Elution was carried out by addition of NaCl to the buffer to obtain first a final concentration of 0.1 M then a continuous gradient from 0.1 to 0.25 M NaCl. Specific phospholipase A_2 activity was measured with 10 µg of the venom fraction and specific anticoagulant activity with 0.4 µg. Eluted volumes from tubes 7—13 were pooled (peak II) and from tubes 15—19 (peak III or clotting inhibitor).



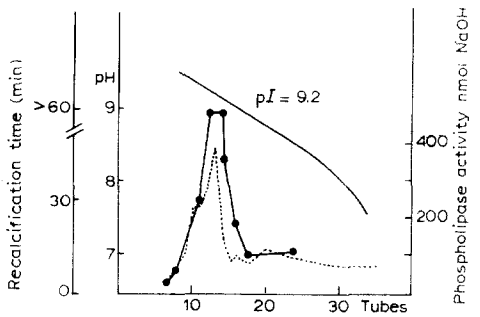


Fig. 4. Polyacrylamide gel electrophoresis. 1—40 μg of *Vipera berus* venom. 2—25 μg of Sephadex eluate (step 2). 3—10 μg of *berus* inhibitor (peak III, step 3). Samples were incubated 2 h, 37°C with 1% sodium dodecyl sulphate and 1% β-mercaptoethanol.

Fig. 5. Determination of isoelectric point by electrofocusing as described in Experimental Procedures at 5°C. Fraction volume: 2.2 ml. pH range (———) from 9.5 to 7.5. Phospholipase activity (. . . . . .) measured as described on 0.01 M phosphatidylcholine in calcium/water/cholate expressed in nmol/min per 50 μl of each fraction. Anticoagulant activity was measured by recalcification times (●———●) with 50 μl of each fraction. The isoelectric point corresponding to both maximum activities was equal to 9.2.

Molecular weight

Molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel and gel filtration. The molecular weight was estimated by the first method to 13 400 ± 650. After gel filtration on Sephadex G-75, two peaks with similar specific anticoagulant activity were eluted. Both also exhibited high phospholipase activity. As seen on Fig. 6, *V. berus* inhibitor appeared under two forms: a small monomeric unit of 13 800 ± 1400 and a trimeric unit of 38 750 ± 1000. The monomeric unit had been detected with a very close molecular weight in the dissociating sodium dodecyl sulfate medium.

Amino acid analysis

The amino acid composition of the clotting inhibitor (Table I) showed a high content of cystein. The protein was not alkylated by iodo[^{14}C]acetic acid in the presence of 10 M urea or 8 M guanidin chlorhydrate at acidic pH. This suggests a very compact molecular structure, maintained by means of 6 disulfur bridges. The trimer form of the clotting inhibitor did not differ from the monomer according to their amino acid content.

Phospholipase A_2 activity

V. berus inhibitor showed a marked phospholipase activity in the presence of

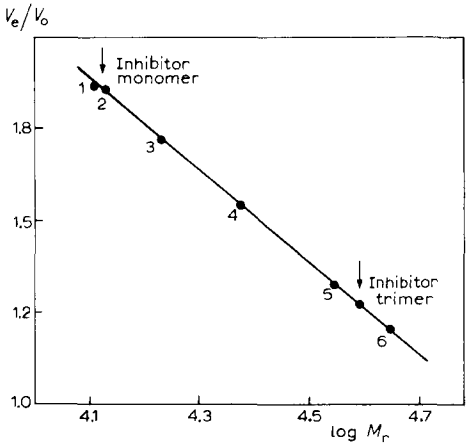


Fig. 6. Molecular weight determination by gel filtration on Sephadex G-75 in 0.15 M NaCl. Column (0.9 × 52 cm) Flow rate, 10 ml/h; fraction volume, 0.25 ml; protein incorporation, 0.25 ml of 1 mg/ml solution. V_0 was determined by dextran blue at 620 nm. 1, cytochrome C (M_r 11 830); 2, ribonuclease (13 700); 3, myoglobin (17 305); 4, chymotrypsinogen (26 076); 5, pepsin (35 000); 6, ovalbumin (45 000). For the inhibitor, the monomeric form corresponded to 13 800 ± 1400, the trimeric form to 38 750 ± 1000.

calcium ions, completely inhibited by EDTA. Michaelis constant was determined on phosphatidylcholine in calcium/water/cholate. The apparent K_m value at 37°C was $1.25 \cdot 10^{-3}$ M. Storage under lyophilizated form did not alter phospholipase activity of the inhibitor.

The hemolytic effect of lysoderivatives was measured on red cells after an

TABLE I

Amino acid composition of *V. berus* inhibitor. Determination from acid hydrolysates of 200 μg of protein. Values average of 10 runs were calculated in mol of amino acid per 13 400 g of residues recovered and corrected by extrapolation to zero time. Tryptophan was estimated spectrophotometrically. Monomers and trimer forms of *berus* inhibitor gave the same results.

Amino acid	Residues/mol
Asp	13.80
Thr	6.15
Ser	4.73
Glu	7.79
Pro	6.12
Gly	10.64
Ala	4.81
Cys	12.23
Val	2.08
Met	1.84
Ile	3.71
Leu	6.41
Tyr	9.90
Phe	5.96
His	2.67
Lys	12.33
Arg	6.11
Trp	1.00
Number of total residues	119

overnight incubation at room temperature. The two controls were: phosphatidylcholine (2 mg) or inhibitor (20 μg) in the red cell suspension. The absorption of the supernatant at 540 nm was respectively: 0.20, 0.17 and 3.82, showing the high hemolytic activity of the lysoderivatives.

Thin-layer chromatographic analysis of purified phosphatidylcholine showed lysophosphatidylcholine after hydrolysis by *V. berus* inhibitor. Hemolytic and chromatographic properties of hydrolysed products were in agreement with the specific enzyme activity on the β-acyl bond.

Discussion

During the last years, phospholipases A_2 have been isolated from various sources: pig pancreas [20], bee [26] and several snake venoms. In the *Viperidae* family, phospholipases have been purified from the venom of four species: *Vipera Russellii* [27], *Vipera aspis* [28], *Vipera berus* [10] and *Vipera palestinae* [29].

Vipera berus anticoagulant phospholipase (*berus* inhibitor) was found as a single polypeptide chain with 119 amino acid residues and a molecular weight of 13 400. *Berus* inhibitor was also found, in the medium without dissociating reagent, as a trimeric form. Both forms were active in blood clotting. Similar molecular weight variations ranging from 8500 to 40 000 have been described in other phospholipases isolated from snake venoms. The discrepancies have been explained mostly by the presence of monomer-dimer conversion as demonstrated the first time for *Crotalus adamenteus* phospholipase [29] and also found for other venom phospholipases [26,30—32].

The phospholipase purified from *Vipera berus* venom was found cross-linked by six disulfide bridges, as are the phospholipases from pancreatic secretion [33] and from the venom of *Laticauda semifasciata* [34], *Vipera palestinae* [31], *Bitis gabonica* [35]. Seven and eight disulfide bridges have been found in other snake venom phospholipases. With four disulfide bridges, the *Apis mellifica* phospholipase appears as a less reticulated protein. The position of disulfide bridges differs markedly for pancreas [36] and bee venom phospholipases [37]. The structural relationships between phospholipases have been elucidated by the determination of amino acid sequences. They are entirely [33,38—42] or partially [10,43,44] known for some of them.

Berus inhibitor has a high isoelectric point (9.2) compared to those of venom phospholipases from *Apis mellifica* (10.5 [26]), *Agkistrodon halys blomhoffii* (10.0 [45]), *V. russellii* (9.9 [27]) or *Crotalus terrificus* (9.7 [46]). The action of these four enzymes on blood clotting has not been reported.

In this work, two phospholipases have been isolated from *V. berus* venom. With similar specific phospholipase activity, these isoenzymes differed by their action on blood clotting. One showed high inhibitory activity which has not been found in the other one. Isoenzymes have been described for several venom phospholipases. Large p*I* differences between isoenzymes have been observed, such as those of *Agkistrodon halys blomhoffii*, p*I* 4.10—10.0 [45], *V. russellii*, p*I* 4.62—9.9 [27], *V. berus*, p*I* 4.8—9.3 [10], *Naja naja*, p*I* 4.6—5.6 [27]. Too few data are actually available for determining the part played on blood clotting inhibitor by p*I* and other physicochemical properties of such enzymes.

The anticoagulant phospholipase appeared as a calcium-activated phospholipase A_2 as described for almost all other phospholipases. An exception is reported for the *Agkistrodon piscivorus* enzyme, which is active without the addition of Ca^{2+} [47]. *Berus* inhibitor was a heat-stable enzyme and very resistant to solvent. The equilibrium between monomeric and trimeric forms was independent of the presence of Ca^{2+}. Both units were able to split the β-acyl bond of phospholipids.

It is well known that most of the snake venoms possess factors able to interfere with blood coagulation [48,49]. Those factors are proteases with specific activity either on fibrinogen giving fibrinopeptides A or B or on prothrombinase components such as factor X activator, factor V activator or prothrombin activator.

Anticoagulant properties of venom are generally ascribed to fibrinolytic and fibrinogenolytic enzymes or to other enzymes which possess polyvalent activities on blood clotting protein or blood platelets. To our knowledge, two anticoagulant venom factors have been isolated, one from *Agkistrodon acutus* venom [50,51] the other from *V. aspis* venom [52]. These are both devoid of phospholipase activity. The *A. acutus* anticoagulant shows quite different characteristics from those of *berus* inhibitor. It is a larger molecule of 20 650 molecular weight and is thermolabile and negatively charged (p*I* 4.8).

The anticoagulant proteins isolated from *V. aspis* venom showed an effect on human plasma near to that of *berus* inhibitor [11]. However, it presented quite different characteristics and was more alike *A. acutus* inhibitor, as far as physicochemical characteristics were concerned (unpublished results).

It was remarkable that phospholipase and anticoagulant activities of *berus* inhibitor have always been eluted together, either from the single protein band of polyacrylamide gel, or from the electrofocusing ampholites and from the Sephadex G-150 during molecular weight determination. It appeared clearly that both activities were carried on the same molecule.

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