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Simultaneous isolation of protein C activator, fibrin clot promoting enzyme (fiprozyme) and phospholipase A_2 from the venom of the southern copperhead snake

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Abstract

The simultaneous isolation of three enzymes from the southern copperhead snake venom (*Agkistrodon contortrix contortrix*; ACC) is described. The first step is a chromatography of crude venom on a Mono S cation-exchange column at pH 6.5. A fibrin clot promoting enzyme (fiprozyme) that preferentially releases fibrinopeptide B from fibrinogen is isolated from the fraction not binding to the Mono S by a further three-step process. The procedure involves affinity chromatography on Blue Sepharose, gel chromatography on Sephacryl S-200 and metal–chelate chromatography on Chelating Sepharose. Protein C activator and phospholipase coelute from the Mono S column. They are separated by a gel chromatography on Sephacryl S-200. After this step two enzymes are obtained: a highly purified protein C activator applicable in methods for determination of functional level of protein C (a plasma regulator of hemostasis) and an electrophoretically pure enzyme with the activity of phospholipase A_2.

Keywords: Enzymes; Protein C activator; Fiprozyme; Phospholipase A_2; Fibrin clot promoting enzyme

1. Introduction

A variety of proteinases that either promote or inhibit blood coagulation have been isolated from snake venoms. The southern copperhead snake venom of *Agkistrodon contortrix contortrix* (ACC) contains at least five different factors influencing the plasmatic coagulation system. It has been one of the most intensively studied snake venoms [1]. The complexity of the crude venom makes it a really difficult-to-separate mixture. To obtain an enzyme of desirable purity employing common separation methods a multistep separation is usually necessary. The isolation of only one enzyme from the crude venom is uneconomical because a large amount of expensive material is not utilized.

Of the proteins interfering with human hemostatic system, the venom of ACC contains, among others, protein C activators (PCA) [2–6], fibrin clot-forming enzymes [7–10] and several phospholipases [1].

Fiprozyme (fibrin clot promoting enzyme) catalyses a preferential release of fibrinopeptide B from fibrinogen. It can be used in clinical investigations of inherited or acquired defects of fibrinogen and in the studies of the mechanism of fibrinogen polymerization. Dye–ligand affinity chromatography –

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proven to be a reliable method for protein purification – was used for the isolation of a similar thrombin-like enzyme – fibrinogen-converting enzyme (ficozyme, EC 3.4.21.29) – from the snake venom of *Bothrops asper* [11].

The importance of protein C in the regulation of hemostasis leads to a strong interest in the measurement of its level and activity. Protein C is a vitamin K-dependent glycoprotein that circulates in blood as a precursor of a serine proteinase. Physiologically, protein C is converted to activated protein C by a complex of thrombin and a tissue cofactor; thrombomodulin. Protein C is also activated by other, non-physiological activators. A protein C activator has been isolated from the venom of ACC (Protac®, Pentapharm, Switzerland) and is used in protein C determination [6].

Phospholipase A_2 (PLA_2) is useful in the study of biological membranes.

In this paper we describe the simultaneous isolation of three enzymes from the snake venom of ACC: fiprozyme, protein C activator and phospholipase A_2.

2. Experimental

The lyophilized crude venom of ACC was dissolved in 20 m*M* imidazole buffer (pH 6.5, 0.08 *M* NaCl) and equilibrated on a Sephadex G-25 column with the same buffer. Human fibrinogen was obtained from Kabi (Stockholm, Sweden). The chromatographic media were: Sephadex G-25 Fine, Sephacryl S-200 Superfine, Blue Sepharose, Chelating Sepharose 6B, Mono S column 5/5, all products from Pharmacia (Uppsala, Sweden).

The protein concentration in the individual fractions was determined from the absorbances at 280 nm assuming an absorbance of 10.0 for a solution of 10 mg/ml and 1 cm light path.

Clotting activity was determined from the time of the visible clot formation of a mixture consisting of: 50 μl of a fraction tested, 50 μl of 50 m*M* Tris–HCl buffer (pH 7.4, 0.1 *M* NaCl), 100 μl of human fibrinogen (3 mg/ml) and 20 μl of 25 m*M* $CaCl_2$.

The release of fibrinopeptides was estimated using the reversed-phase HPLC method on CGC C_{18} (10 μm, 150×3 mm) column according to Suttnar et al. [12].

Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was run on 5–15% gradient polyacrylamide gel essentially according to Laemli and Favre [13].

Protein C activator potency was tested according to Martinolli and Stocker [6].

The activity of phospholipase A_2 (PLA_2) was estimated using the slight modification of the method of Vignon et al. [14].

Molecular mass determination was measured by electrospray ionization combined with quadrupole mass spectrometry on Lasermat mass analyzer system (Finnigan Mat).

3. Results and discussion

The scheme of the simultaneous isolation of three enzymes from the crude ACC venom is shown in Fig. 1.

Using strong cation-exchange chromatography performed on Mono S at pH 6.5, only proteins with isoelectric points higher than about 7.0 are attached to the matrix. Protein C activator isoenzymes with isoelectric points 7.8, 8.0 and 8.8 [15] and some other proteins (PLA_2) bind to Mono S and can be separated. The majority of proteins from the crude venom ACC are eluted with the starting buffer

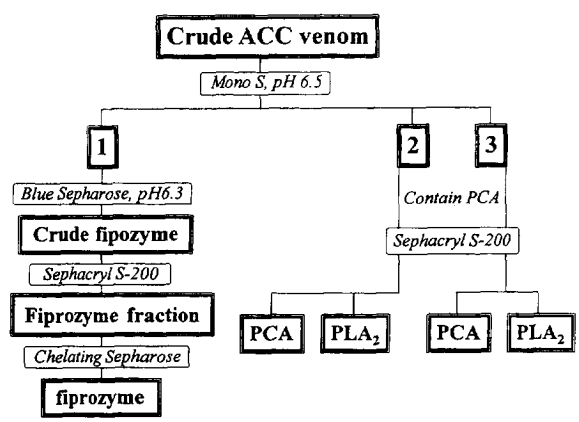

Fig. 1. Scheme of the simultaneous isolation of protein C activator, fiprozyme and phospholipase A_2 from the crude *Agkistrodon contortrix contortrix* venom.

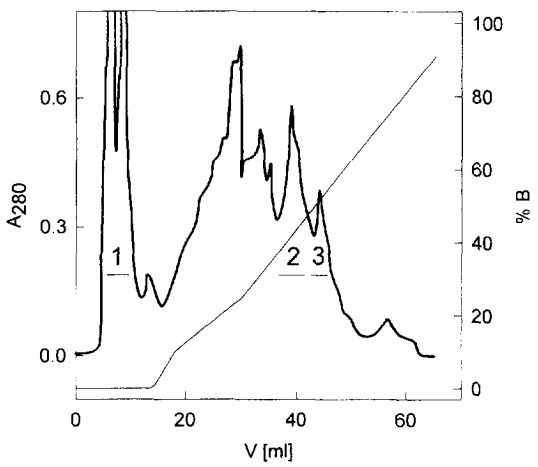

Fig. 2. Elution profile of crude ACC venom (50 mg/ml) on strong cation exchanger Mono S. Column, Mono S 5/5; flow-rate, 1 ml/min; eluent: buffer A: 20 m*M* imidazole, pH 6.5, 0.08 *M* NaCl; buffer B: 20 m*M* imidazole pH 6.5, 0.4 *M* NaCl. Fractions 2 and 3 contain protein C activator, fraction 1 contains fiprozyme.

(Fraction 1, Fig. 2). Fractions 2 and 3 in Fig. 2 are used as a source of protein C activator (PCA), which is further purified using gel chromatography on a Sephacryl S-200 column (Fig. 3). The co-product of

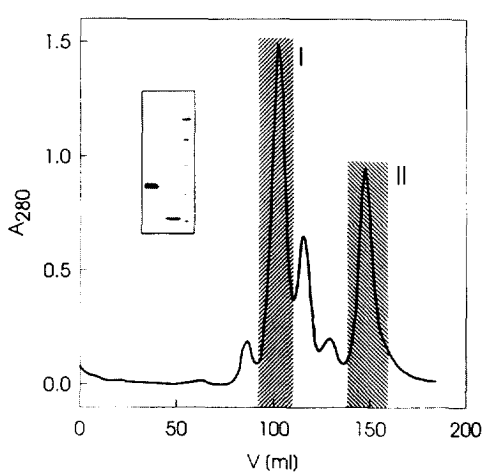

Fig. 3. Elution profile of fraction 2 (from Fig. 2, 35 mg/ml) on Sephacryl S-200. Column, 86×1.6 cm I.D.; flow-rate, 0.2 ml/min; eluent: 50 m*M* Tris–HCl, pH 7.0, 0.1 *M* NaCl. Fraction I: protein C activator (PCA); fraction II: phospholipase A_2(PLA_2). The insert shows SDS–PAGE (gradient 5–15%) of reduced samples stained with Coomassie Brilliant Blue R-250: protein C activator (left), phospholipase A_2 (middle), molecular mass markers (94 000, 67 000, 43 000, 30 000, 21 000, 14 400) (right).

gel chromatography is an electrophoretically pure fraction with relative molecular mass of 16 000 and exhibiting the activity of basic phospholipase A_2 [1].

PCA isolated by the above described method seemed to be identical with protein C activator (ACC-C) isolated by Kisiel et al. [4]. PCA is a typical serine proteinase inhibited by phenylmethylsulphonyl fluoride, diisopropylflourophosphate, *p*-amidinophenylmethanesulfonyl fluoride, chloromethyl ketones and human antithrombin III [4] but not inhibited by hirudine, iodacetamide, *o*-phenanthroline and EDTA [5]. PCA also cleaves human prothrombin, factor X, factor IX, factor VII and fibrinogen [4], PCA is therefore proteinase with a relatively broad specificity. Primary structure of PCA from ACC was described by McMullen et al. [16] and these authors found some differences in properties of ACC-C [4] and Protac [2]. Our preliminary experiments with chromogenic substrates also showed our PCA to be somewhat distinct from Protac. PCA is almost free of PLA_2 activity and can be used in the functional coagulometric method of protein C determination [6]. The M_r of PCA, isolated by the method described above, estimated to be 37 000 (from SDS–PAGE) and 37 020 (from Lasermat) was in good agreement with other reports [2,4,5]. The isolation of PCA is only a two-step process that is comparable with other methods used for its isolation. A chromatography on SP-Sephadex in combination with gel chromatography is usually used [2,4,5]. PCA isolated by only one step process of Klein and Walker [3] was probably contaminated with PLA_2. We could remove PLA_2 by only one additional purification step: gel chromatography. The presence of PLA_2 might have caused the lower M_r (20 000) value found by the authors [3]. The contamination with PLA_2 also indicates their finding that "the venom activator appears to be a major protein constituent of venom" (8% of total venom proteins). From our experiments (see Table 1) PCA represents approximately 3.5% and PLA_2 (fraction II, Fig. 3) 3% of the total venom proteins. We could remove PLA_2 by only one additional purification step: gel chromatography.

Fraction 1 from the Mono S (Fig. 2) is used as a source of fiprozyme. Dye–ligand affinity chromatography on Blue Sepharose is used for the separation of proteins from fraction 1. Enzyme fiprozyme, the

Table 1
The protein content and specific activity in individual chromatographic fractions starting with 100 mg of soluble crude venom

Chromatography column	Fraction	Total protein[a] (mg)	Specific activity[a] (units/mg)
Mono S	Fraction 1	32	–
	Fraction 2	4	–
	Fraction 3	4	–
Blue Sepharose	Crude fiprozyme	2	–
Chelating Sepharose	Pure fiprozyme	1	–
Sephacryl S-200	PCA	3.5	445
	PLA_2	3	1568

[a] Protein and specific activity was determined as described in Section 2.

minor component of this mixture, is bound very strongly to Blue Sepharose (Fig. 4) and is eluted with a high ionic strength. Besides fiprozyme, another fibrinopeptide-releasing enzyme is eluted with lower ionic strength (Fig. 4). It releases both fibrinopeptides at approximately the same rate. The separation of two fibrinopeptide-releasing enzymes during Blue Sepharose chromatography confirms the observation of Dyr et al. [9]. Fractions not bound to Blue Sepharose contain mixture of larger proteins. The fraction eluted between fiporozyme and another fibrinopeptide-releasing enzyme exhibits high fibrinolytic activity. On SDS–PAGE, one main zone and two minor zones were found. The main zone had relative molecular mass of 24 000 and was probably identical with a fibrinolytic enzyme fibrolase. Fibrinolytic activity was inhibited by EDTA. These findings are in good correlation with [17].

The crude fiprozyme fraction is purified in the second step by gel chromatography on the Sephacryl S-200 column (Fig. 5) and final purification of fiprozyme is achieved in the third step: metal–chelate affinity chromatography, performed on the Chelating Sepharose 6B (Fig. 6). Metalloproteinases, main contaminants of fiprozyme fraction, are separated during affinity chromatography on Chelating Sepharose.

Fiprozyme, prepared by the three-step purification process described above, is a glycoprotein with activity of a typical serine proteinase. It is inhibited

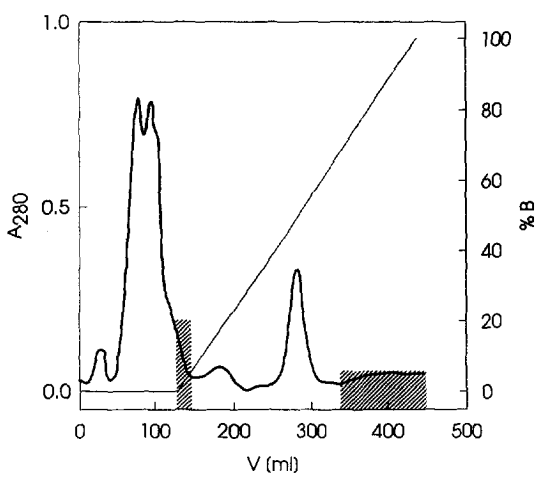

Fig. 4. Elution profile of fraction 1 (from Fig. 2, 85 mg/15 ml) on Blue Sepharose. Column, 20×2.6 cm I.D.; eluent: buffer A: 50 m*M* imidazole, pH 6.3, 0.1 *M* NaCl, buffer B: 50 m*M* imidazole pH 6.3, 1 *M* NaCl. The fractions containing fibrin clot-promoting enzymes are hatched. Crude fiprozyme is eluted with the highest ionic strength.

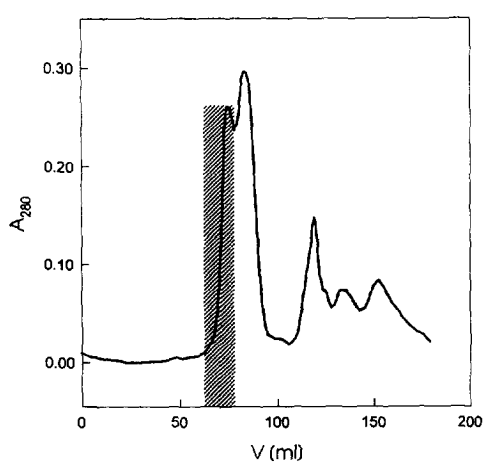

Fig. 5. Elution profile of crude fiprozyme (17 mg/ml) on Sephacryl S-200. Column, 86×1.6 cm I.D.; flow-rate, 0.2 ml/min; eluent, 50 m*M* Tris–HCl, pH 7.0, 0.1 *M* NaCl. The fraction containing fiprozyme is hatched.

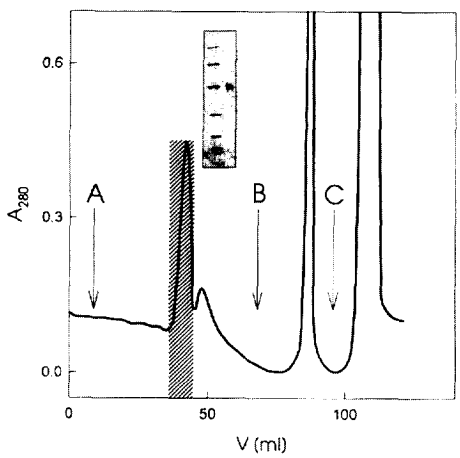

Fig. 6. Elution profile of fiprozyme fraction (Fig. 5, 1.9 mg/ml) on Chelating Sepharose. Column, 10.5×0.9 cm I.D.; flow-rate, 1 ml/min. Eluent: buffer A: 50 m*M* Tris–acetate, pH 8.0, 0.5 *M* NaCl, buffer B: 50 m*M* Tris–acetate pH 3.0, 0.5 *M* NaCl, buffer C: 50 m*M* EDTA, 1 *M* NaCl. Fiprozyme fraction is hatched. The insert shows SDS–PAGE (gradient 5–15%), reduced samples stained with Coomassie Brilliant Blue R-250, molecular mass markers (94 000, 67 000, 43 000, 30 000, 21 000, 14 400) (left), fiprozyme (right).

by common inhibitors of serine proteinases, e.g., phenylmethylsulphonylfluorid. For fiprozyme prepared by the above described method a relative molecular mass of about 38 000 (SDS–PAGE) and 37964 (Lasermat) was found. Fiprozyme releases fibrinopeptides from fibrinogen, fibrinopeptides B with much faster rate than fibrinopeptides A. It has no other fibrinolytic activity. The role of carbohydrate moiety of fiprozyme upon the release of fibrinopeptides from fibrinogen is subject of further investigation and will be published elsewhere.

In 1 g of crude lyophylized venom ACC there is about 0.85 g of soluble proteins (an average value from six experiments). Amounts of fractions and isolated products in various stages of isolation process and specific activities of purified enzymes are summarized in Table 1. Data are average values from six experiments. It was impossible to determine the *x*-fold purifications and yields because the crude venom apparently contains two or more different Protein C activators [3], more enzymes releasing fibrinopeptides [10] and several phospholipases. Moreover, the crude venom contains a number of activities that interfere with the enzyme assays used [1,3,10] which cause the initial velocity of a reaction catalyzed by the crude venom to be not directly proportional to the concentration of the venom – thus the principal assumption of enzyme activity determination which should be valid is not fulfilled.

In conclusion, an effective utilization of ACC venom is described. Three purified enzymes are obtained: a protein C activator, fiprozyme and phospholipase A_2. They are of good quality for further use.

Acknowledgments

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