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ISOLATION OF THE PROTHROMBIN-CONVERTING ENZYME FROM FIBRINOGENOLYTIC ENZYMES OF *ECHIS CARINATUS* VENOM BY CHROMATOGRAPHIC AND ELECTROPHORETIC METHODS

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SUMMARY

Chromatography of crude *Echis carinatus* venom revealed four enzymes with fibrinogenolytic activity and activity to chromogenic substrates, specific for proteases of the coagulation and fibrinogenolytic systems. By employing three-step chromatography on DEAE-Sephacel and Sephacryl S-200, this venom afforded the procoagulation enzyme Ecarin in good recovery (73%) and purification (53-fold). This highly purified preparation has proved to be a single-chain glycoprotein, occurring in two isomers differing in electrical charge and having a low fibrinogenolytic activity.

INTRODUCTION

The crude venom of *Echis carinatus* contains some proteolytic enzymes and a procoagulation enzyme, called Ecarin[1]. Ecarin catalyses the conversion of prothrombin into the so-called Ecarin-thrombin[2,3], having a thrombin-like activity. Application of Ecarin *in vivo* rapidly leads to afibrinogenemia without thrombogenic effects[4]. It remains to be determined whether the harmless course of afibrinogenemia is due to the formation of fibrin, induced by Ecarin-thrombin, or whether imperfect fibrin formation resulting from proteolytic cleavage of fibrinogen by the Ecarin preparation used has contributed to this effect. Ecarin prepared by two-step ion-exchange chromatography[1] split the Aα and Bβ chains of fibrinogen, and very slowly the γ chains, thus exhibiting a fibrinogenolytic rather than a fibrinolytic activity[5]. However, Ecarin prepared, in a very poor yield, by a five-step combination of chromatographic methods was reported to have no fibrinogenolytic activity[6].

The aim of this work was to characterize the proteolytic enzymes of crude *Echis carinatus* venom by chromatographic methods and to find a suitable procedure for isolation of Ecarin.

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MATERIALS AND METHODS

The lyophilized crude venom of *Echis carinatus* was dissolved in, and dialysed against, 0.05 *M* Tris–HCl buffer, pH 8.0. The insoluble portion was removed by centrifugation at 6000 *g* for 10 min. The concentration of eluted protein(s) in the individual chromatographic fractions was determined from the absorbances at 280 nm, assuming an extinction coefficient, $E^{1\%}_{1cm}$, of 1.0 for 1.0 mg/ml solution.

The chromatographic media used were: Sephacryl S-200 Superfine, Sephadex G-100 and G-200 Superfine and DEAE-Sephacel (Pharmacia, Sweden), Ultrogel AcA-44 (LKB, Sweden) and Bio-Gel P-150 (Bio-Rad Labs., U.S.A.). The columns were calibrated with a low-molecular-weight gel filtration calibration Kit (Pharmacia). Fibrinogen was a commercial product (Kabi, Sweden); IgG was prepared in our laboratories.

Coagulation activity was tested according to Shieck *et al.*[1]. The rates of hydrolysis of chromogenic substrates for thrombin (Tos-Gly-Pro-Arg-pNA · HCl, TS; Pentapharm, Switzerland), plasminokininogenase (Bz-Pro-Phe-Arg-pNA · AcOH,PS; Pentapharm) and for S-2251 (H-D-Val-Leu-Lys-pNA · 2HCl, Kabi) by the isolated fractions (100 μl) were measured spectrophotometrically at 405 nm under the following conditions: substrate concentration 0.1 m*M*; ionic strength 0.15; 37°C; total volume 2.5 ml; Tris imidazole buffer pII 7.4 (S-2251) and pH 8.4 (TS, PS).

Fibrinogenolytic activity was tested by means of sodium dodecyl sulphate (SDS) electrophoresis[7] of reduced and non-reduced samples of human fibrinogen (concn.1.5 mg/ml, Kabi, in the presence of Trasylol 15 KIE/ml; Bayer, G.F.R.) incubated with the individual chromatographic fractions (0.1–5 μg/ml) for 0 24 h[5]. Prothrombin removal from fibrinogen was achieved by its adsorption on barium sulphate. Fibrinogenolytic activity under the above conditions was classified as (a) high if 50% or more of the A α chain was split in 30 min (b) medium, if this extent of degradation occurred in 5 h, and (c) low, if a measurable amount of the A α or B β chain was split in 24 h.

Electrophoresis in polyacrylamide gel (PAGE) was carried out using a Tris glycine buffer, pH 8.3 (ref. 8); electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was conducted in 5% polyacrylamide gel[7]. The molecular weights of proteins were estimated using SDS-PAGE with the aid of electrophoresis calibration kits (Pharmacia) and from the relative mobilities on PAGE gels of various concentrations from 3.5 to 10.5% (ref. 9). The staining of glycoproteins in PAGE was carried out according to Glossmann and Neville[10].

RESULTS

Gel chromatography of crude *Echis carinatus* venom has been attempted on several chromatographic materials (Sephadex G-100, G-200, Sephacryl S-200, Bio-Gel P-150 and Ultrogel AcA-44). Best results were obtained with Sephacryl S-200 Superfine (Fig. 1). The fraction having the maximum coagulation activity (C.F.) had a lower elution volume than the fractions exhibiting maximum activity with the chromogenic substrates. The several maxima of the substrate activities suggest that the crude venom contains several (at least four) proteolytic enzymes. The recovery of the coagulation fraction was 85%, the purification being 6.2-fold. All these data,

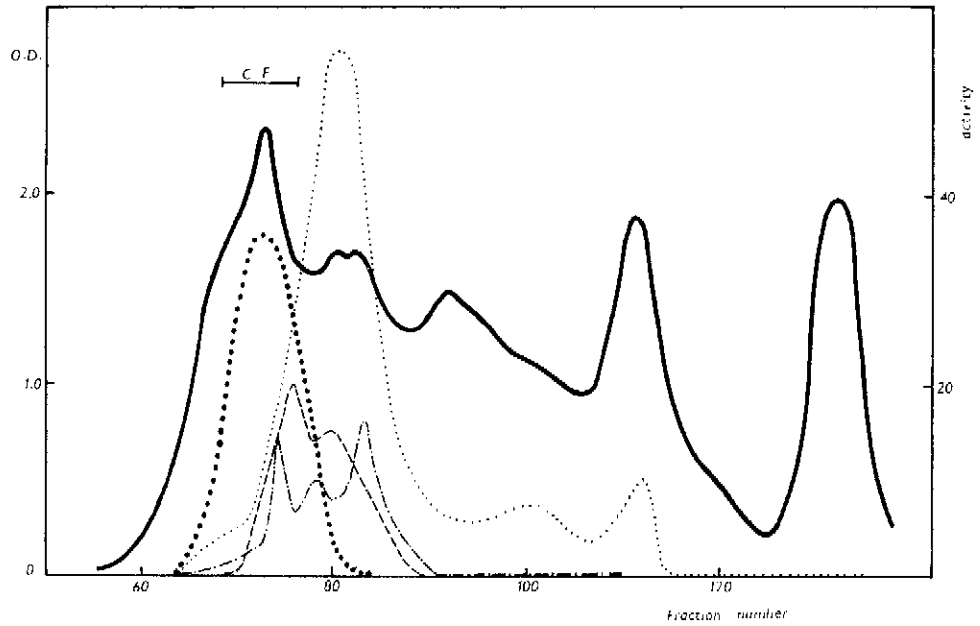

Fig. 1. Elution profile (—) of crude *Echis carinatus* venom on Sephacryl S-200. Column; 90 × 1.6 cm. Eluent: 0.05 *M* Tris–HCl pH 8.0.Flow-rate: 6.6 ml/h (1.1-ml fractions). Temperature: 4°C. ● ● ● Coagulation activity × $4 \cdot 10^{-2}$ (units per ml). Activities (O.D./min) to chromogenic substrates: TS (· · ·) × 10^3; S-2251 (– –), × 10^3; PS (- · - ·), × 2.

as well as data from further separation experiments, are average values from three independent experiments. The coagulation fraction had a medium fibrinogenolytic activity and proved active with all of the three chromogenic substrates tested. All the fractions that were highly active to the chromogenic substrates also had high fibrinogenolytic activity. Fractions with zero activity to the chromogenic substrates also lacked fibrinogenolytic activity.

Using ion-exchange chromatography the best fractionation of crude *Echis carinatus* venom was achieved on DEAE-Sephacel, employing a linear elution gradient with increasing concentration of the Tris HCl buffer pH 8.0 (Fig. 2). The fractions are designated with Roman numerals in the order of their elution from the column. The first fraction III eluted after application of the gradient was yellow, had an absorption peak at 460 nm and very probably contained L-amino acid oxidase. The coagulation activity was found in fraction V (recovery 95%, purification 7.6-fold). This fraction exhibited a medium fibrinogenolytic activity. High fibrinogenolytic activity was again found in fractions with high activity to the chromogenic substrates (fractions IV and VII).

In a two-step resolution of crude *Echis carinatus* venom by combination of gel chromatography and ion-exchange chromatography, the purification of the coagulation fractions depended on their order of use. When the coagulation fraction (CF, Fig. 1) was purified on DEAE-cellulose as a second step the recovery was 78% and purification 16.3-fold. This fraction had a low fibrinogenolytic activity, activities of $50 \cdot 10^{-6}$ and $4.4 \cdot 10^{-3}$ O.D./min · μg to TS and PS respectively, and no activity to S-2251.

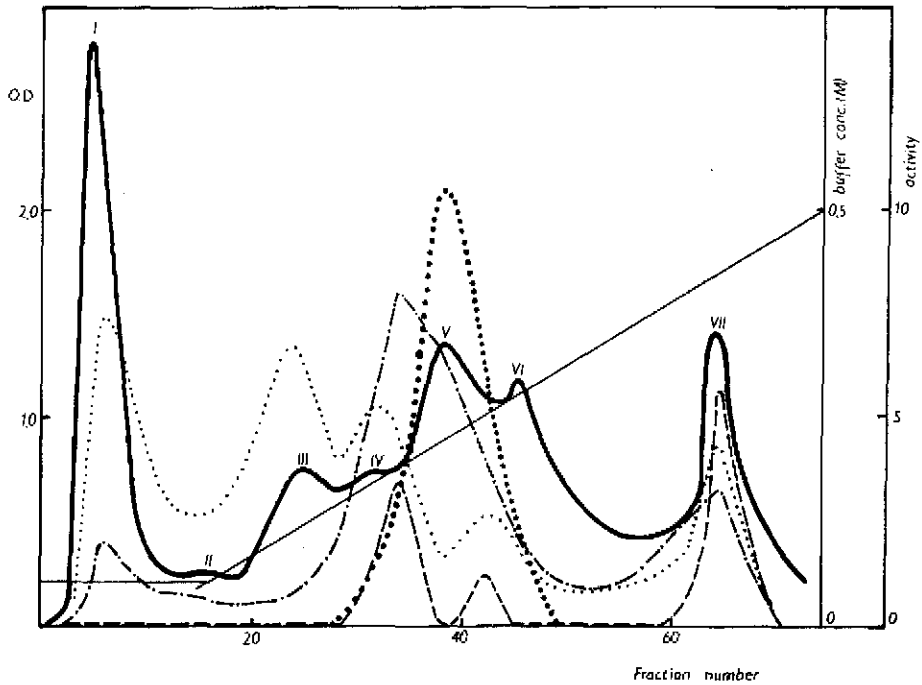

Fig. 2. Elution profile (—) of crude *Echis carinatus* venom on DEAE-Sephacel. Column: 12 × 0.9 cm. Eluent: linear gradient (0.05 to 0.5 *M*). Flow-rate: 18 ml/h (2.2-ml fractions). Temperature: 4°C. ● ● ●, Coagulation activity × 10^{-2} (units per ml). Activities (O.D./min) to chromogenic substrates: TS (· · ·), × 0.5 · 10^3; S-2251 (- - -), × 10^3; PS (- · - · -), × 10.

Chromatography of the coagulation fraction V (described in Fig. 2) on Sephacryl S-200 as a second step (Fig. 3) gave a recovery of 80% and purification was 40-fold. This fraction (designated E) had a fibrinogenolytic activity, which was not apparent until after prolonging the incubation with fibrinogen over 24 h, a very low activity to TS (0.5 · 10^{-6} O.D./min · μg) and an activity to PS (0.6 · 10^{-3} O.D./min · μg), but no measurable activity to S-2251.

In the rechromatography of fraction E on Sephacryl S-200 the recovery was 73% and purification 53-fold. This highly purified preparation of the procoagulation enzyme (Ecarin) still exhibited some fibrinogenolytic activity on prolonged incubation with fibrinogen and had a measurable activity to PS only. It behaved as a single component in SDS-PAGE, giving only one zone and identical mobilities for a reduced and a non-reduced sample. This suggests that the enzyme is a single-chain protein.

In PAGE at pH 8.3 this preparation of Ecarin gave two, very close and diffuse zones (Fig. 4), both stainable for glycoproteins with a periodic acid–Schiff reagent, in gels of concentrations 3.5–10.5%. The two zones were separated by preparative PAGE at pH 8.3 and a gel concentration of 7.5%. The isolated zones retained their electrophoretic mobility in PAGE at pH 8.3; in SDS-PAGE they had the same electrophoretic mobility as the starting material. Both of the isolated fractions had a very low fibrinogenolytic activity. Evaluation of the PAGE at pH 8.3 and different

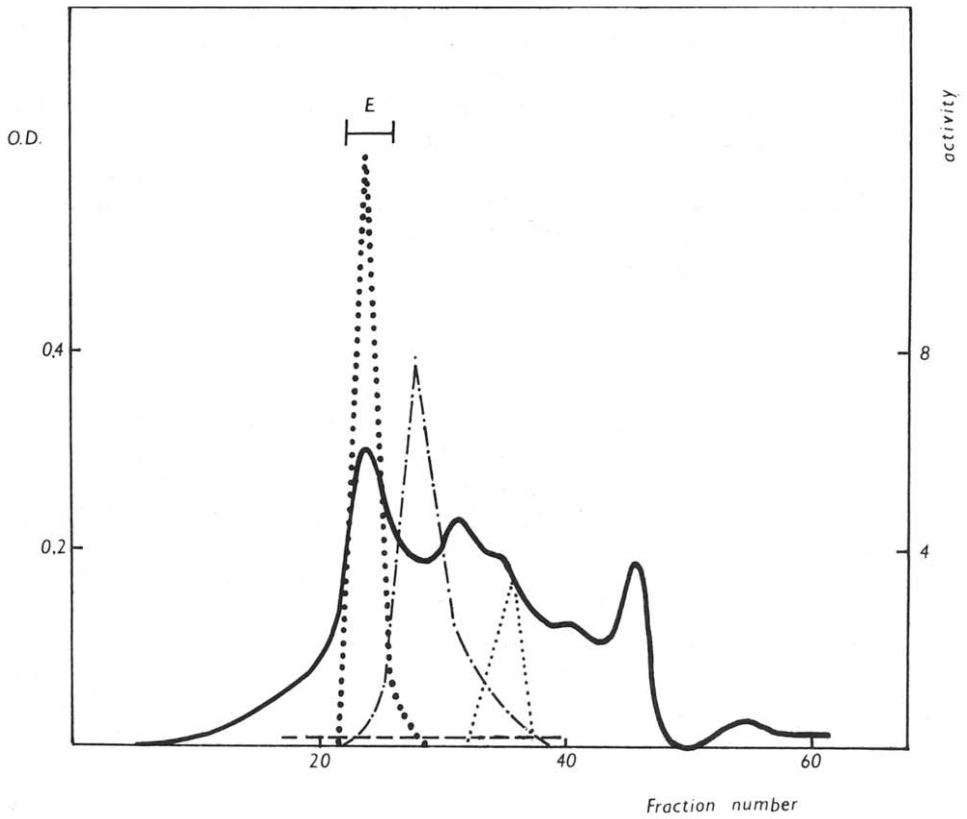

Fig. 3. Elution profile (—) of fraction V (eluted from DEAE-Sephacel, Fig. 2) on Sephacryl S-200. Flow-rate: 17.8 ml/h (2.2-ml fractions). For other details see Fig. 1. ● ● ●, Coagulation activity × 10^{-2} (units per ml). Activities (O.D./min) to chromogenic substrates: TS (· · · ·), × 10^3; S-2251 (— — —), × 10^3; PS (- · - · -) × 20.

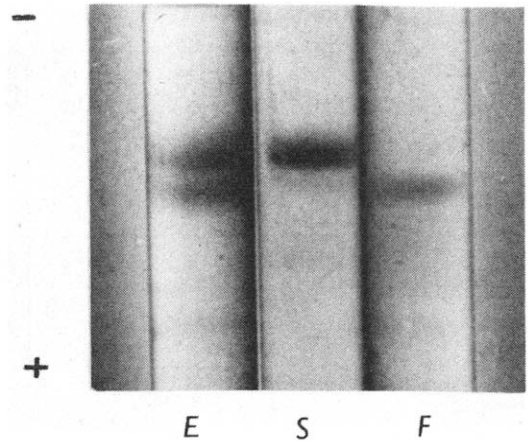

Fig. 4. PAGE of a highly purified Ecarin (E) and its fast (F) and slow (S) zones obtained by preparative PAGE.

gel concentrations by use of the Ferguson plot of log M_R *vs.* gel concentration (Fig. 5) yielded two parallel lines. This demonstrates that the two components had the same molecular weight, and suggests that they were charge-isomers.

The molecular weight was 63,000 from the Ferguson plot, 85,000 from SDS-PAGE and 84,000 from the elution volume in chromatography on Sephacryl S-200. After incubation of our preparation of Ecarin in 0.05 *M* Tris-HCl buffer pH 8.0 at 37°C for 3 days its molecular weight was unchanged (SDS-PAGE, gel chromatography on Sephacryl S-200). Its fibrinogenolytic activity and activity to the chromatogenic substrates were also unchanged.

DISCUSSION

Our results demonstrate that crude *Echis carinatus* venom contains several proteolytic enzymes (four or more), with activity to chromogenic substrates specific for proteases of the coagulation and fibrinolytic systems. These enzymes have greater elution volumes in gel chromatography than the procoagulation enzyme Ecarin, and are responsible for most of the fibrinogenolytic activity of the crude venom. Ecarin can be separated from these enzymes in good yield and purification by two-step chromatography DEAE-Sephacel and Sephacryl S-200. The puzzling fact that the quality of the separation depends on whether gel chromatography is followed by ion-exchange chromatography or *vice versa* may have several causes. In the crude venom the proteolytic enzymes are present in high concentrations, and using gel

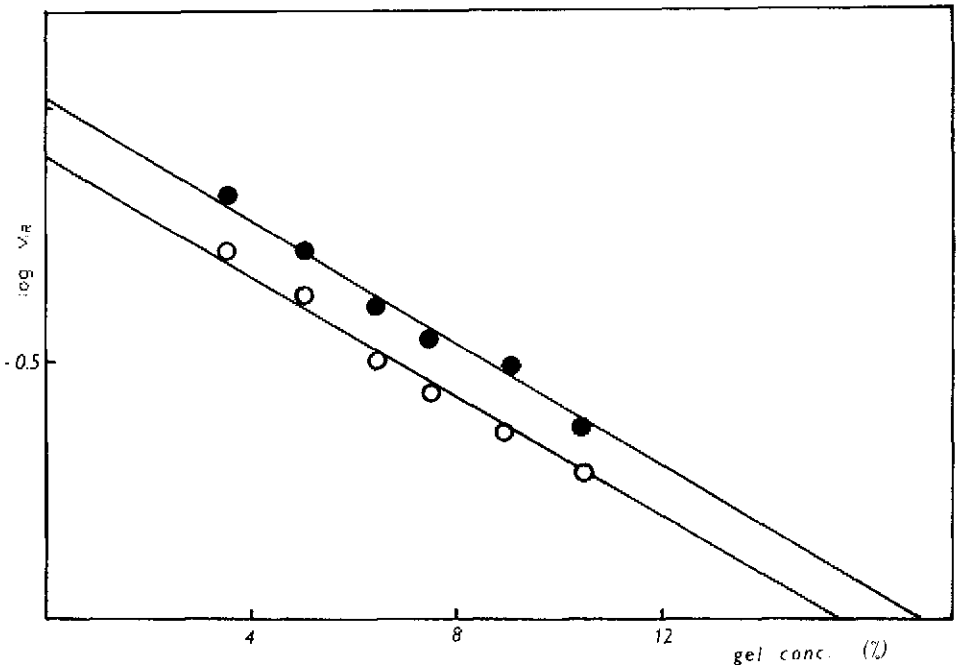

Fig. 5. Ferguson plot of PAGE mobilities of a highly purified Ecarin *vs.* gel concentration (%). The mobilities are expressed as relative mobilities, M_R, with respect to bromphenol blue as marker. ○, Slow zone (S); ●, fast zone (F).

chromatography as the first step they cannot be completely separated from Ecarin. Besides, gel chromatography takes too long compared to the ion-exchange chromatography, so that the long contact of the proteolytic enzymes with Ecarin may result in its cleavage. It is also possible that the proteins present in the venom interact; this would reduce their elution volume in gel chromatography, to values close to that of Ecarin. However, using ion-exchange chromatography as the first step the separation from Ecarin is effective.

The three-step chromatography on DEAE-Sephacel, Sephacryl S-200 and Sephacryl S-200 afforded an Ecarin preparation in 73% recovery and 53-fold purification, which is comparable with the 56.7-fold purification reported by Morita and Iwanaga[6] who used five-step chromatography. The electrophoretic methods have shown that Ecarin is a single-chain glycoprotein, homogenous as regards the molecular weight, but occurring as at least two isomers differing in charge. This charge heterogeneity has been reported for a number of enzymes, including the thrombin-like[11].

In gel chromatography on all supports used (Sephadex G-100, G-200, Sephacryl S-200, Bio-Gel P-150, Ultrogel AcA44) Ecacrin exhibited the elution volume corresponding to a higher molecular weight (84,000) than that calculated from the electrophoretic mobility in PAGE in gels of different concentrations. The molecular weight obtained from the calibration curve for SDS PAGE was also higher (85,000). Discrepant molecular weights for Ecarin had already been reported[6] and ascribed to its glycoprotein nature. Glycoproteins behave anomalously in the binding of SDS, since only the protein moiety is capable of binding it[12]. They have rather expanded (hydrated) molecules which is supposed to affect their behaviour in gel chromatography[13,14]. An autolysis of Ecarin can be ruled out, since prolonged incubation of Ecarin at 37°C did not lead to any change in its molecular weight.

The fibrinogenolytic activity of Ecarin, prepared by the above three-step procedure, was not manifested until after prolonged incubation with fibrinogen. The fact that both charge isomers (obtained by preparative PAGE) retained the fibrinogenolytic activity suggests that the procoagulant enzyme itself has this activity and there are no contaminating proteases. However, in the incubation of Ecarin with prothrombin and fibrinogen, when the fibrinopeptides A and B were liberated quantitatively, less than 1% of the Bβ 15-42 immunoreactivity was released. This shows that the procoagulation activity of Ecarin is by far the prevailing one[15].

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