Journal of Chromatography, 260 (1983) 522–526
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,660

Note

Fractionation of *Echis carinatus* venom by affinity chromatography on immobilized lectins

H. FOŘTOVÁ*, J. E. DYR and Z. VODRÁŽKA
Institute of Haematology and Blood Transfusion, Prague (Czechoslovakia)
and
F. KORNALÍK
Institute of Pathophysiology, Charles University, Prague (Czechoslovakia)
(Received December 30th, 1982)

The saw-scaled viper *Echis carinatus* accounts for most of the deaths due to snake bite in vast areas of Africa and Asia[1]. It is still not clear to what extents the procoagulation enzyme ecarin and the fibrinolytic enzymes present in the venom contribute to the defibrination and haemorrhage. Ecarin was isolated by a multi-step procedure[2] in a poor yield; isolation of the fibrinolytic enzymes has not yet been reported. The ecarin isolated by the original method[3] exhibited fibrinogenolytic activity. As ecarin is a glycoprotein[2,4], we have attempted to isolate it by affinity chromatography on immobilized lectins.

EXPERIMENTAL

Affinity chromatography was carried out on concanavalin A (Con A)–Sepharose, wheat germ lectin–Sepharose 6MB, lentil lectin–Sepharose 4B and *Helix pomatia* lectin–Sepharose 6 MB (all from Pharmacia, Uppsala, Sweden).

The glycoproteins were eluted with methyl α-D-glucopyranoside (Koch-Light, Colnbrook, Great Britain) for Con A-Sepharose and lentil lectin–Sepharose 4 B, N-acetyl-D-glugosamine (Serva, Heidelberg, G.F.R.) for wheat germ lectin–Sepharose 6 MB and N-acetyl-D-galactosamine (Serva) for *Helix pomatia* lectin–Sepharose 6 MB.

The protein concentrations in the individual fractions were determined from the absorbances at 280 nm, assuming an extinction coefficient ($E_1^{1\%}_{cm}$) of 10.0. The coagulation activity was determined according to Shieck *et al.*[3].

The rates of hydrolysis by the isolated fractions (100 μl) of the chromogenic substrates for thrombin (T.S.; Tos–Gly–Pro–Arg–pNaHCl), for plasminokininogenase (P.S.; Bz–Pro–Phe–Arg–pNA AcOH) (both products of Pentapharm, Basle, Switzerland) and for S-2251 (H-D-Val–Leu–Lys–pNA 2HCl) (Kabi, Stockholm, Sweden) were measured spectrophotometrically[5] at 405 nm.

The fibrinogenolytic activity was tested by means of SDS electrophoresis of reduced and non-reduced samples of human prothrombin-free fibrinogen (Kabi), 1.5 mg/ml, in the presence of Trasylol, 15 kallikrein inhibitor units (K.I.U.) (Bayer, Leverkusen, G.F.R.). Fibrinogen was treated with the individual chromatographic fractions (0.1 μg/ml) for periods of 0–24 h[5,6].

0021-9673/83/$03.00

Electrophoresis in polyacrylamide gel containing Tris–glycine buffer (pH 8.3) was carried out according to Davis[7] and SDS electrophoresis in 5% gel according to Weber and Osborn[8].

RESULTS AND DISCUSSION

Four different lectins immobilized on Sepharose (see Experimental) were tried for fractionation of *Echis carinatus* venom. The non-bound fractions [referred to as fraction(s) I] and the fraction(s) eluted with the saccharides [fraction(s) II] were tested for coagulation activity, fibrino(geno)lytic activity and activity towards some chromogenic substrates, chosen on the basis of our previous experiments[5].

All affinity chromatographic experiments were carried out with Pharmacia columns (12 × 0.9 cm I.D.) using 0.05 *M* Tris–HCl buffer (pH 8.0) containing 0.2 *M* sodium chloride. An increase in ionic strength [[0.05 *M* Tris–HCl (pH 8.0) containing 1.0 *M* sodium chloride] did not cause any release of bound proteins from any of the chromatographic materials used. The glycoprotein fractions were eluted by a 10% concentration of the corresponding saccharide in 0.05 *M* Tris–HCl (pH 8.0) contain-

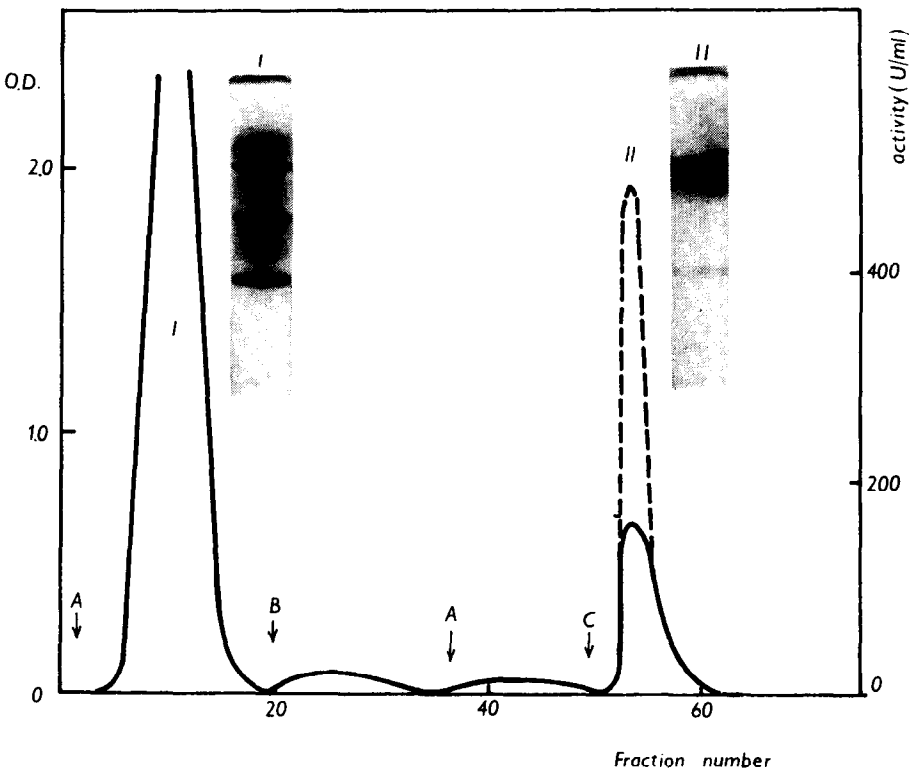

Fig. 1. Chromatography of crude *Echis carinatus* venom on wheat germ lectin–Sepharose 6 MB. Column, 12 × 0.9 cm I.D. Eluent: A, 0.05 *M* Tris–HCl (pH 8.0), 0.2 *M* NaCl; B, 0.05 *M* Tris–HCl (pH 8.0), 1 *M* NaCl; C, 0.05 *M* Tris–HCl (pH 8.0), 0.2 *M* NaCl, 10% N-acetyl-D-glucosamine. Flow-rate, 30 ml/h (1-ml fractions). Coagulation activity is expressed in units/ml (– – –). Electrophoresis was performed in 7.5% polyacrylamide gel.

TABLE I

ACTIVITIES OF FRACTIONS SEPARATED BY AFFINITY CHROMATOGRAPHY

*Affinity material**	*Fraction I***					*Fraction II***				
	C.A.	*T.S.*	*S-2251*	*P.S.*	*F.A.*	*C.A.*	*T.S.*	*S-2251*	*P.S.*	*F.A.*
WGL	0	0	0	0.002	±	8	31.6	10	0.9	+
LL	0.6	33.2	27	1.64	+	2	40	14.4	1.44	+
Con A	1.1	56	13.2	1.72	+	0.8	13.2	0	0.59	+

* WGL = wheat germ lectin–Sepharose 6 MB; LL = lentil lectin–Sepharose 4 B; Con A = Con A–Sepharose.

** The data under C.A. express coagulation activity (compared with the activity of the crude venom); T.S., S-2251 and P.S. express activity towards these chromogenic substrates (absorbance units/min × 10^3); F.A. denotes fibrinogenolytic activity.

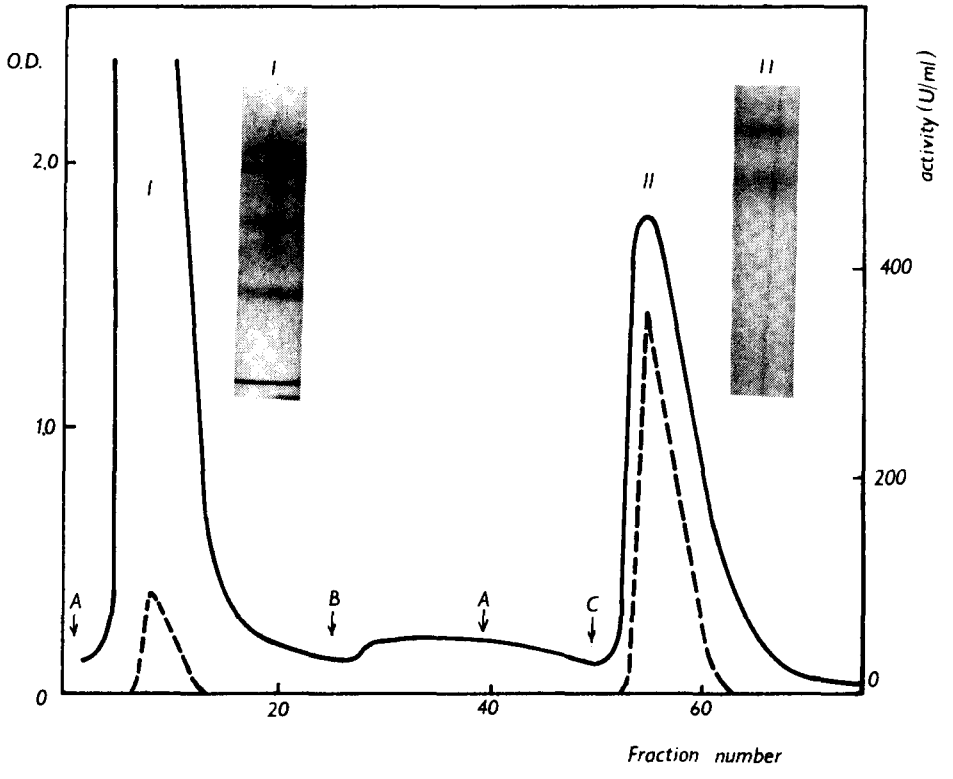

Fig. 2. Chromatography of crude *Echis carinatus* venom on lentin lectin–Sepharose 4B. Eluents: A and B as in Fig. 1; C, 0.05 *M* Tris–HCl (pH 8.0), 0.2 *M* NaCl, 10% methyl α-D-glucopyranoside. For other details, see Fig. 1.

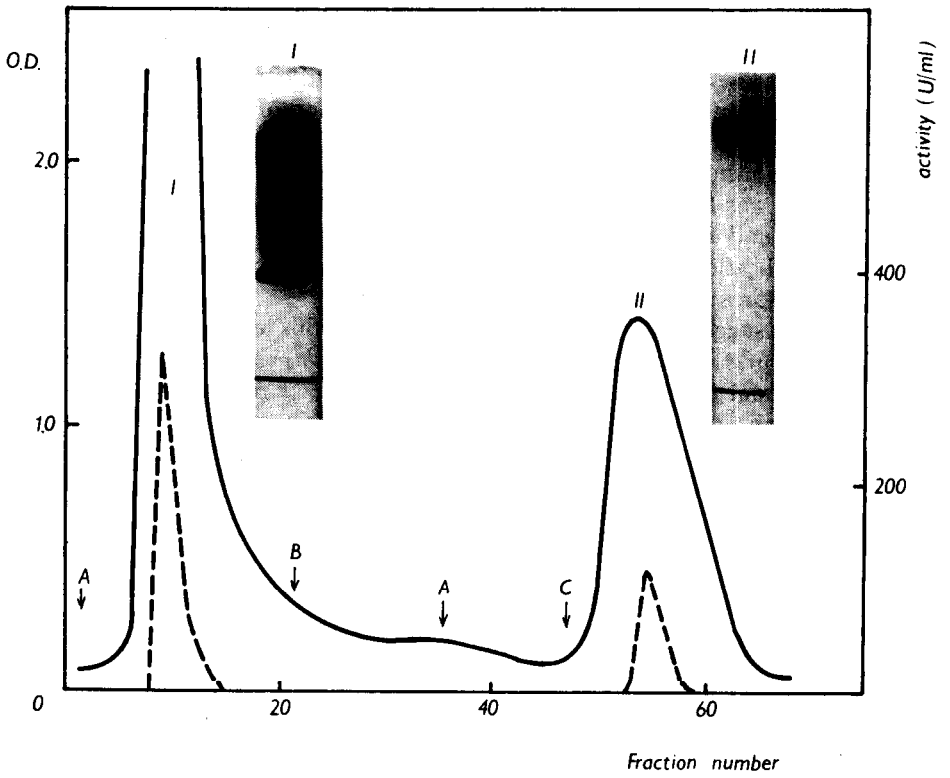

Fig. 3. Chromatography of crude *Echis carinatus* venom on Con A–Sepharose. Eluents as in Fig. 2. For other details, see Fig. 1.

ing 0.2 *M* sodium chloride. The sample applied to the column was 40 mg of the crude venom (freeze-dried, dissolved in 0.05 *M* Tris–HCl buffer containing 0.2 *M* sodium chloride; the insoluble portion was removed by centrifugation at 6000 *g* for 10 min). The flow-rate was 30 ml/h (1 ml per tube). If half the amount of the venom (20 mg) was applied, the course of the separation was the same with any of the materials used, thus proving that the binding capacity of a column was never exceeded.

Fig. 1 shows the course of affinity chromatography on wheat germ lectin–Sepharose 6 MB. All the coagulation activity (yield 96%) and all the activity towards the chromogenic substrates (Table I) but only a small activity towards P.S. were present in fraction II, representing about 20% of the amount of protein applied. An interesting finding was that only fraction I possessed the ability to dissolve fibrin under the conditions used. When highly purified ecarin[5] was applied on the column, all activity was eluted in fraction II in a yield of 97%.

In the chromatography on lentil lectin–Sepharose 4 B (Fig. 2) and Con A–Sepharose (Fig. 3), the coagulation activity and activity towards chromogenic substrates (Table I) were present in both fractions, but not in the same proportions. The distribution of the coagulation activity between the two fractions suggests that the proteolytic enzyme responsible for this activity is heterogeneous, which is in accordance with our earlier observation[5].

In all the fractions with high activity towards the chromogenic substrates, the fibrinogenolytic activity was also high. This applies to the two fractions eluted from lentil lectin–Sepharose 4 B and from Con A–Sepharose and to fraction II eluted from wheat germ lectin–Sepharose 6 MB. After 30-min digestion of fibrinogen with these fractions no intact Aα-chains were present and some Bβ-chains were also partially hydrolysed. The fibrinogenolytic activity of fraction I eluted from wheat germ lectin–Sepharose 6 MB was low but this fraction considerably degraded γ-chains of fibrinogen and fibrin and exhibited fibrinolytic activity.

In the application of the crude venom to the column packed with *Helix pomatia* lectin–Sepharose 6 MB no protein was absorbed.

Our results suggest that of the four immobilized lectins tested, only wheat germ lectin–Sepharose 6 MB may be useful in fractionating *Echis carinatus* venom. Using this affinity material it is possible to separate ecarin in a fraction of proteolytic enzymes with high activity towards chromogenic substrates from the rest of the venom containing fibrinolytic activity. For the further isolation of ecarin it is possible to apply other techniques[5].

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