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Note

Isolation and purification of a fibrinogenolysin from the venom of the saw-scaled viper (*Echis carinatus*) by high-performance liquid chromatography

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Traditionally, isolation of biologically active proteins from snake venoms has been achieved by open column ion-exchange chromatography. More recently, the venom of *Echis carinatus* (saw-tailed viper) has been fractionated by chromatographic and electrophoretic techniques, affinity chromatography and fast protein liquid chromatography[1–3]. Attempts at utilizing reversed-phase high-performance liquid chromatography (HPLC) have often resulted in denaturation of the venom proteins by the organic mobile phase with concomitant loss of biological activity[4,5]. Schaeffer *et al.*[6] have recently fractionated *E. carinatus* venom by gel permeation HPLC. Protein separations by means of hydrophobic interaction HPLC have been reported by Goheen and Englehorn[7]. This paper describes the successful fractionation of crude snake venoms by hydrophobic interaction HPLC, with retention of biological activity. A fibrinogenolysin, with its biological activity intact, was isolated from crude *E. carinatus* venom with use of a hydrophobic interaction column (TSK-Phenyl 5PW). After further purification by ion-exchange and reversed-phase HPLC, fourteen amino acid residues of its N-terminal sequence were determined.

EXPERIMENTAL

Materials

Lyophilised snake venoms were obtained from Sigma (St. Louis, MO, U.S.A.). "Centricon" micro-concentrators (10 000 M.W. cut off) were obtained from Amicon (Melbourne, Australia). Thrombin, topical-bovine origin (Parke-Davis & Co., De-

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troit, MI, U.S.A.) and human fibrinogen (Commonwealth Serum Laboratories, Melbourne, Australia) were used for assaying biological activity. CM-Trisacryl M gel (LKB) was purchased from Linbrook (Sydney, Australia).

HPLC instrumentation and conditions

Both Kortec and Waters Assoc. HPLC systems were used. The Kortec system consisted of a Rheodyne Model 7125 syringe-loading injector with a 200-μl sample loop, two single piston pumps (Model K45M), a Model K45 gradient controller and a Model K95 variable-wavelength detector set at 254 nm. A TSK-Phenyl 5PW column (LKB, 75 mm × 7.5 mm) was used for hydrophobic interaction separations, with a linear ammonium sulphate gradient over 30 min. The gradient was run at room temperature and started with 2 *M* ammonium sulphate and 0.1 *M* ammonium phosphate buffer at pH 7.0, and ended with the same buffer without the ammonium sulphate.

A TSK-DEAE 5PW column (LKB, 75 mm × 7.5 mm) was used for ion-exchange chromåtography. A linear gradient, run at room temperature over 30 min, started with 0.1 *M* ammonium acetate at pH 8.0 and ended with 0.5 *M* ammonium acetate at pH 6.0.

The Waters Assoc. HPLC system consisted of a Model U6K syringe-loading injector and 200-μl sample loop, Model M600 and M45 pumps, Model 720 gradient controller and Model 441 UV detector with a fixed wavelength of 254nm. It was used with a μBondapak C_{18} column (Waters Assoc., 30 mm × 3.9 mm) for reversed-phase chromatography. A linear gradient, run at room temperature, over 30 min, started with 0.05% (v/v) trifluoroacetic acid (TFA) in water and ended with 0.05% (v/v) TFA in acetonitrile–water (60:40). A flow-rate of 1 ml/min was used for both HPLC systems. A CM-Trisacryl open column (12 cm × 1.5 cm) was packed at room temperature. An elution gradient, starting with 0.05 *M* ammonium acetate, pH 7.0, and finishing with final buffer of 0.05 *M* ammonium acetate, pH 7.0 containing 2 *M* sodium chloride, with a 25–30 ml/h flow-rate was used. Detection was carried out at 280 nm at 2 a.u.f.s. using a Pharmacia single path monitor, Model UV1. The amino acid sequence was determined with a gas-phase protein sequencer, Model 470A (Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS AND DISCUSSION

A fibrinogenolytic protein was isolated from 250 mg of crude *E. carinatus* venom using a CM-Trisacryl open column (Fig. 1). Detection of fibrinogenolytic activity was by the thrombin time test[8] after incubation of fractions with fibrinogen (2 mg/ml) for 60 min at 37°C. Prolonged thrombin times were considered indicative of fibrinogenolytic activity. The active fractions (Fig. 1) were pooled and lyophilised prior to HPLC analysis.

Separations of crude *E. carinatus* venom were carried out by HPLC, using TSK-Phenyl 5PW, TSK-DEAE 5PW and μBondapak C_{18} columns. Injection of the fibrinogenolytically-active material from the open column chromatogram into each of these columns enabled the peak corresponding to active material to be identified by retention time (Fig. 2).

Fibrinogenolytic activity, although present in the crude venom, could not be

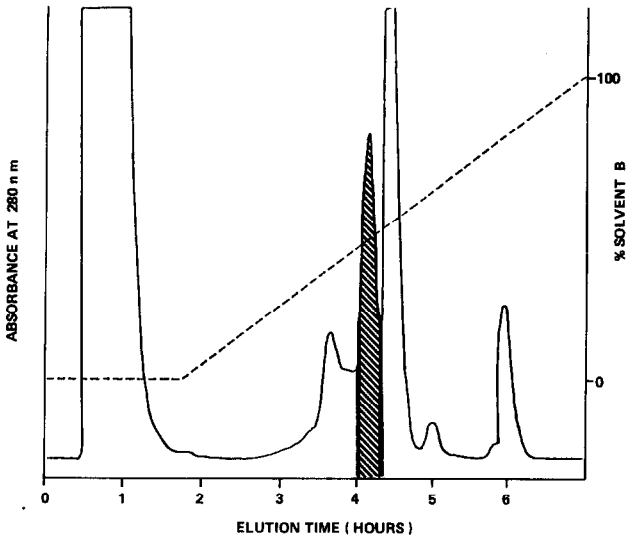

Fig. 1. Elution profile for crude *E. carinatus* venom. Crude venom (250 mg) was dissolved in 5 ml of starting buffer. After centrifugation, the supernatant was loaded on to a CM-Trisacryl open column (12 cm × 1.5 cm). Buffers and elution conditions are outlined in the Experimental section. Fractions of 10 ml were collected. The fraction showing the fibrinogenolytic activity is shaded. The gradient profile is indicated by the dashed line. a.u.f.s. = 2.

measured prior to isolation of the active component due to interference by coagulant enzymes. Activity was retained in the fractions collected from the TSK-Phenyl 5PW and TSK-DEAE 5PW columns. Material recovered from the reversed-phase μBondapak C_{18} column had lost all activity. Separations obtained by injection of crude venoms of four other species of snakes with these three columns are included for comparison (Fig. 3).

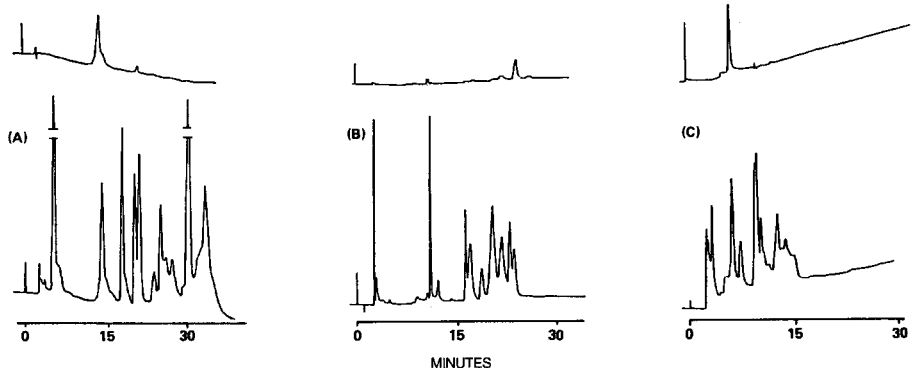

Fig. 2. Crude *E. carinatus* venom (1 mg) was dissolved in 200 μl of starting buffer. After centrifugation, the supernatant was injected into the HPLC chromatograph (lower profile). A volume of 200 μl of fibrinogenolytic material of the CM-Trisacryl fraction (Fig. 1) was injected directly into the HPLC chromatograph (upper profile). HPLC system: (A) TSK-Phenyl 5PW column, a.u.f.s. = 1; (B) TSK-DEAE 5PW column, a.u.f.s. = 0.125; (C) μ-Bondapak C_{18} column, a.u.f.s. = 0.5.

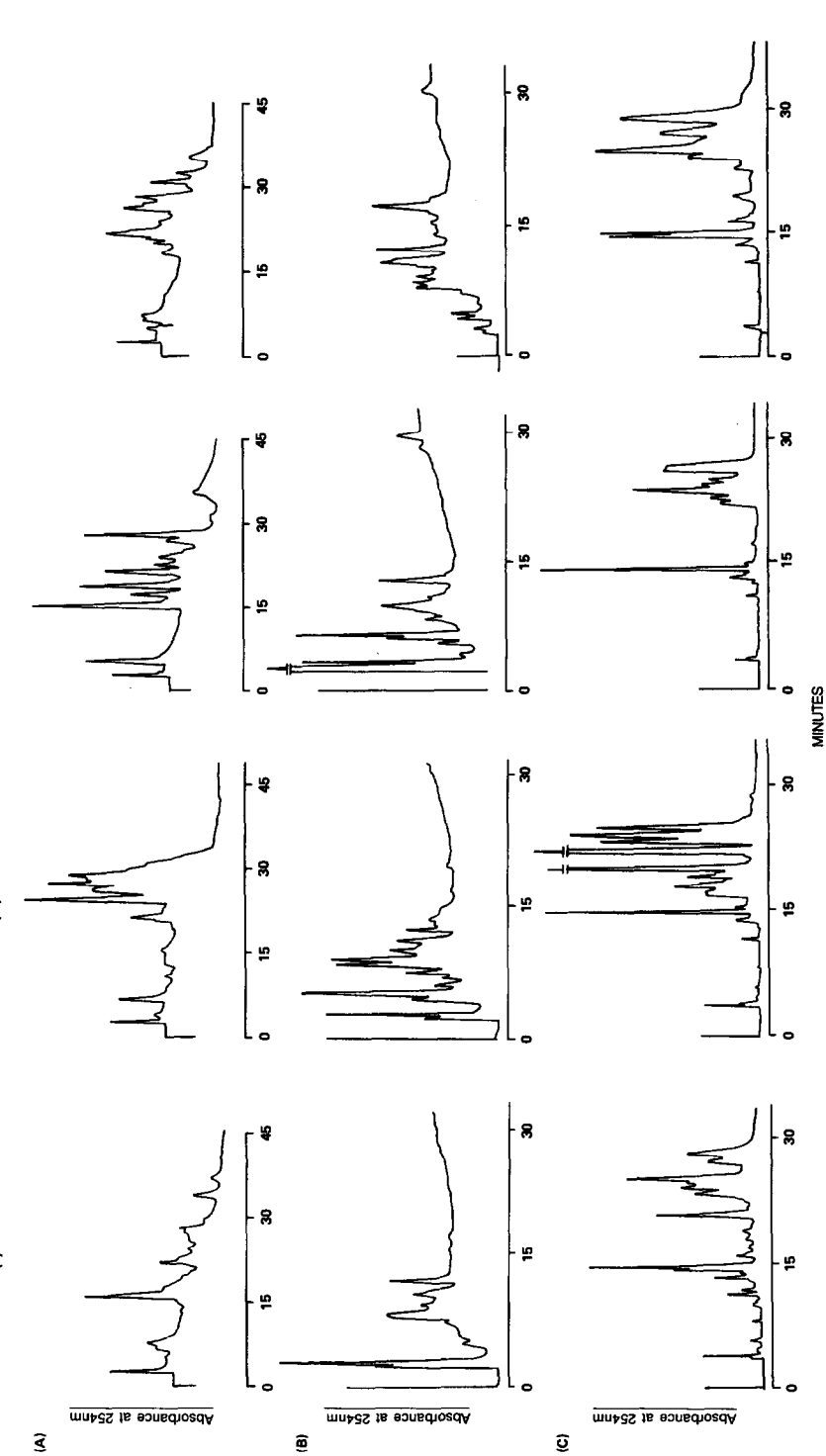

Fig. 3. HPLC separations of crude snake venoms. All crude venom was dissolved in starting buffer (1 mg/200 μl). After centrifugation, the supernatant was injected into the HPLC system. (I) *Bothrops atrox*, (II) *Crotalus scutulatus*, (III) *Agistrodon rhodostoma* and (IV) *Bothrops jaracaca* obtained with (A) TSK-Phenyl 5PW column, a.u.f.s. = 0.1; (B) TSK-DEAE 5PW column, a.u.f.s. = 0.1; (C) μBondapak C_{18} column; a.u.f.s. = 0.2.

TABLE I

SUPPLEMENTARY TABLE TO AMINO ACID SEQUENCE

Cycle	*Amino acid*	*Relative yield*[a] *(%)*
1	Gln	100
2	Arg	79
3	Phe	395
4	Asp	385
5	Pro	266
6	Arg	62
7	Tyr	480
8	Ile	95
9	Glu	371
10	Leu	340
11	Val	429
12	Val	523
13	Val	733
14	Ala	628

[a] Relative yield equals the yield of PTH-amino acid derivative produced in cycle *n* relative to yield in the first cycle, which was assigned the value of 100.

Subsequently, 30 mg of crude *E. carinatus* venom was dissolved in 200 μl 0.5 *M* ammonium acetate, pH 7.0, and injected into the TSK-Phenyl 5PW column. The peak exhibiting fibrinogenolytic activity was collected, desalted and concentrated with a "Centricon" microconcentrator. The concentrate was washed twice with the same ammonium acetate solution prior to lyophilisation. Approximately 1.5 mg (5.0%) of fibrinogenolysin was recovered from the venom by this procedure. This material was then subjected to further purification by HPLC with the μBondapak C_{18} column.

Preliminary investigations of the activity of the fibrinogenolysin by sodium dodecyl sulphate (SDS) electrophoresis[9] of the products of its reaction with human fibrinogen, reduced with mercaptoethanol and unreduced, indicated that fragmentation of the α-chain occurred initially, followed by that of the β-chain, whilst the gamma chain remained unaffected. The M_r of the fibrinogenolysin was estimated to be 20 000 by the SDS-polyacrylamide gel electrophoresis method[10]. Using gas phase Edman degradation[11], the sequence of its N-terminus was determined as: H_2N-Gln-Arg-Phe-Asp-Pro-Arg-Tyr-Ile-Glu-Leu-Val-Val-Val-Ala- (see Table I). The sequence traces showed no evidence of any inhomogeneity of the sample.

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