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Note

Isolation of a fibrinogen-converting enzyme ficozyme from the venom of *Bothrops asper* by one-step affinity chromatography on Blue Sepharose

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Based on the conversion of fibrinogen to fibrin, very specific enzymes with limited action, cleaving off only fibrinopeptides from fibrinogen, are widely used for the determination of the amount and functional properties of fibrinogen. Such enzymes should meet the following requirements: they must catalyse the splitting off of fibrinopeptides, they must not be inhibited by plasmatic inhibitors including heparin and they must have neither fibrinogenolytic nor prothrombin-converting activity. The main drawbacks to the use of thrombin (the physiological activator in the conversion off fibrinogen to fibrin) for the determination off fibrinogen are that it is inhibited by plasmatic inhibitors, including heparin, and also activates prothrombin [1]. Some snake venoms contain enzymes which specifically cleave of fibrinopeptides from fibrinogen [2]. For example, bathroxobin, isolated from the venom of *Bothrops atrox*, releases fibrinopeptides A only and is used in the kinetic method for the determination of fibrinogen [3].

Snake venoms represent a very complex mixture, including about 80 protein components [4] of various biological activities: procoagulant [2], fibrinogenolytic [2], prothrombin-converting [2,5] and many others [2,6]. To obtain an enzyme of desirable purity using common separation methods, multi-step procedures are usually necessary. This paper describes a one-step dye-ligand affinity chromatographic method for the isolation of a fibrinogen-converting enzyme (ficozyme) from the snake venom of *Bothrops asper*.

EXPERIMENTAL

Affinity chromatography was carried out on Blue Sepharose and gel filtration chromatography on Superose 12 HR 10/30 using the fast protein liquid chromatographic (FPLC) system (all from Pharmacia, Uppsala, Sweden).

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

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Fibrinogen-converting activity was tested as follows: 50 μl of tested fraction, 50 μl of buffer [50 m*M* Tris–HCl (pH 7.4)–0.1 *M* NaCl] and 100 μl of human fibrinogen (3 mg/ml) (Kabi, Sweden) were mixed in a test-tube and the clotting time was recorded as the time of visible clot formation.

Fibrinogenolytic activity was determined as the time necessary to dissolve the fibrin gel prepared in the same way as for the determination of the fibrinogen-converting activity.

Prothrombin-converting activity and the influence of plasmatic inhibitors were tested using a slight modification of the method for the kinetic determination of fibrinogen [3]. A 100-μl volume of the test fraction were placed in a 1-cm spectrophotometric cuvette and diluted with 2 ml of buffer [0.1 *M* Tris (pH 7.5)–polyethyleneglycol 6000 (2 g per 100 ml)–Brij 35 (1 g per 100 ml)–$CaCl_2$ (50 mg per 100 ml)] and 50 μl of human plasma were added. The absorbance at 340 nm was monitored and the increase in absorbance per minute was determined from the linear part of the curve. For the testing of prothrombin-converting activity either hirudin (1 U/ml, final concentration) or oxalate plasma free from coagulation factors [removed by adsorption on $BaSO_4$ (100 mg per 100 ml of plasma)] were added. For the testing of the influence of plasmatic inhibitors either 50–200 μl of serum or 50 μl of heparin (0.1–20 IU/ml, final concentration) were added.

The release of fibrinopeptides was determined using reversed-phase high-performance liquid chromatography (RP-HPLC) on a CGC C_{18} (10 μm) (150 × 3 mm I.D.) according to Suttnar *et al.* [7]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 5–15% gradient polyacrylamide gel essentially according to Laemmli and Favre [8].

The rates of hydrolysis of chromogenic substrates for thrombin (TS), kallikrein (PK) and plasmin (S2251) by the isolated fractions (100 μl) were measured spectrophotometrically under the following conditions: substrate concentration, 0.1 m*M*; ionic strength, 0.15 *M*; temperature, 25°C; total volume, 2.5 ml; Tris–imidazole buffer, pH 7.4 (S2251) and pH 8.4 (TS, PK).

Phenylmethanesulphonyl fluoride (PMSF) (Sigma, St. Louis, MO, U.S.A.) and hirudin (Sigma) were used as received.

RESULTS AND DISCUSSION

Dye-ligand affinity chromatography of the crude venom of *Bothrops asper* was performed on a column of Blue Sepharose (20 × 2.6 cm I.D.) equilibrated with buffer A [50 m*M* imidazole (pH 6.3)–0.1 *M* NaCl). A 500 -mg amount of lyophilized crude venom of *B. asper* was dissolved in 5 ml of buffer A; insoluble material was removed by centrifugation (15 000 *g* for 20 min) and the soluble portion was applied to the column. During isocratic elution the venom was separated into three peaks (Fig. 1). The fraction containing ficozyme (indicated by the hatched area) constituted about 2.0% of the venom proteins. It exhibited a specific activity of 300 batroxobin units [9] per mg (BU/mg). The separation resulted in an overall 55-fold purification at a 90% yield. Proteins that were more strongly adsorbed on Blue Sepharose were eluted at a higher ionic strength [buffer B: 50 m*M* imidazole (pH 6.3)–1 *M* NaCl].

The crude ficozyme split off only fibrinopeptides A from fibrinogen as indicated by RP-HPLC. Only the peak of fibrinopeptide A was detected by HPLC of the

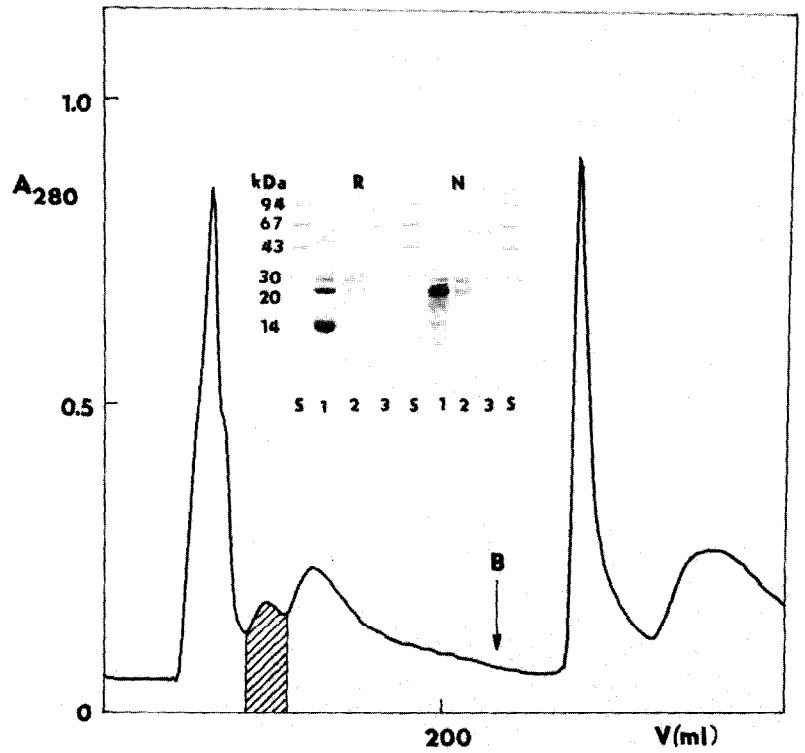

Fig. 1. Elution profile of crude *Bothrops asper* venom (500 mg in 5 ml) on Blue Sepharose. Column 20 × 2.6 cm I.D.; flow-rate, 1.5 ml/min; starting buffer, 50 m*M* imidazole (pH 6.3)–0.1 *M* NaCl; buffer B: 50 m*M* imidazole (pH 6.3)–1 *M* NaCl. The fraction containing ficozyme is hatched. The inset shows SDS gradient (5–15%) PAGE of the crude *B. asper* venom (1), the hatched crude fraction (2) and ficozyme further purified (3) by gel filtration on Superose 12. R = reduced, N = non-reduced samples; S = molecular mass markers. The gel was stained with Coomassie Brilliant Blue R-250.

supernatant of a sample taken after incubation of the ficozyme with fibrinogen for a time equal to ten times the clotting time. SDS-PAGE of the reduced precipitate of the sample showed more than 95% of intact α-, β- and γ-chains of fibrinogen, indicating a negligible fibrinogenolytic activity of ficozyme.

The fraction was not significantly inhibited with either plasmatic inhibitors or heparin. Its prothrombin-converting activity was also negligible and the presence of neither serum nor hirudin had any significant effect on the kinetic assay for fibrinogen determination.

However, gel filtration chromatography on Superose 12 performed in 50 m*M* Tris–HCl (pH 7.4)–0.1 *M* NaCl and SDS-PAGE revealed the size heterogeneity of the ficozyme fraction. The maximum degree of fibrinogen-converting activity (1800 BU/mg) was found in a fraction with a molecular mass of 34 kilodalton (calculated from the elution volume). SDS-PAGE showed that the fraction having the highest activity consisted of a major protein band of higher molecular mass (Fig. 1). The discrepancy between the results obtained with gel filtration and SDS-PAGE may indicate either

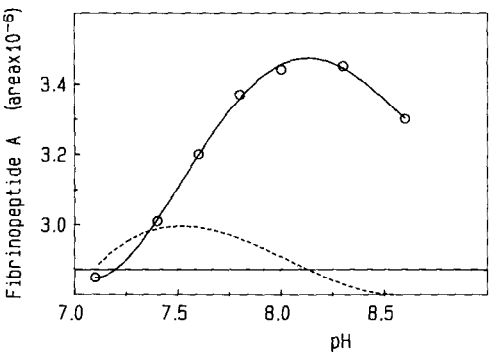

Fig. 2. pH dependence of the initial release of fibrinopeptide A (full line, expressed as the area of the peak of fibrinopeptide A) from fibrinogen at high fibrinogen concentration (20 μM). Reaction conditions: 50 mM Tris–HCl, ionic strength 0.15 M adjusted with NaCl; temperature, 30°C. A ficozyme concentration corresponding to 0.01 BU was used. The broken line is the first derivative of the pH dependence curve.

an unusual shape of the molecule or protein–Superose matrix interaction. The crude fraction had activities towards the low-molecular-mass chromogenic peptide substrates TS and PK of 0.57 and 0.39 μmol/s · mg, respectively, and the purified ficozyme 6.75 and 4.83 μmol/s · mg, respectively. Both fractions were free from S-2251 activity.

The fibrinopeptide-releasing activity of purified ficozyme was inhibited by the serine protease inhibitor PMSF. Incubation of ficozyme with PMSF (at a final concentration of 0.26 mg/ml, *i.e.*, 1.5 mM) for 30 min caused a decrease in its activity to 60% of the original level and at a final concentration of 0.52 mg/ml, *i.e.*, 3 mM, to 40%. Repeated addition of PMSF (with an increase in its concentration of 1 mM each time) at 30-min intervals for 3 h resulted in the complete inhibition of the activity of ficozyme. Ficozyme was not inhibited by antithrombin III (0.05–0.25 μM), antithrombin III (0.25 μM) simultaneously with heparin (5 IU/ml), iodacetamide (1–5 mM), 5.5'-dithiobis(2-nitrobenzoic acid) (0.25–5 mM), EDTA (2.5–12.5 mM) or *o*-phenantroline (5–10 mM). The results show that ficozyme was not inhibited by thiol protease and metalloprotease inhibitors, suggesting that it was a serine protease.

The effect of changing pH on the fibrinopeptide A-releasing activity of ficozyme is shown in Fig. 2. The optimum pH under the present consitions was found to be 8.1.

The results show that by using very simple one-step affinity chromatography on Blue Sepharose it is possible to isolate a fibrinogen-converting enzyme that has suitable characteristics for the determination of the concentration and functional properties of plasma fibrinogen.

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