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Resolution of isoforms of natural and recombinant fibrolase, the fibrinolytic enzyme from *Agkistrodon contortrix contortrix* snake venom, and comparison of their EDTA sensitivities

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Abstract

Fibrolase, the fibrinolytic enzyme from *Agkistrodon contortrix contortrix* snake venom, is a zinc metalloproteinase with a molecular mass of 23 kDa. We report a method to isolate two isoforms of natural fibrolase (fib1 and fib2) and three isoforms of recombinant fibrolase (r-fib1, r-fib2 and r-fib3) using CM 300 cation-exchange high-performance liquid chromatography. Utilizing mass spectrometry we characterized differences in molecular masses of the isoforms of r-fibrolase. These findings suggest that the isoforms differ by minor sequence variations at their amino-termini. Since the stability of fibrolase is exquisitively sensitive to the removal of zinc, we examined the EDTA sensitivity of the isoforms of fibrolase and r-fibrolase to determine if their different chromatographic behavior is related to differences in their zinc affinities. All of the isoforms examined appear to have similar zinc binding affinities. Thus, the IC_{50} (concentration of EDTA to produce 50% inhibition of enzymatic activity) for fib1 is 160 μM. For the closely related r-fib1, the IC_{50} is 180 μM. Similarly, r-fib3 has an IC_{50} of 140 μM.

1. Introduction

Fibrolase has been isolated from *Agkistrodon contortrix contortrix* (southern copperhead snake) venom and characterized [1–5]. The enzyme is a zinc metalloproteinase (one mol zinc per mole enzyme) with a molecular mass of 23 kDa [5]; it contains 203 amino acids with the amino-terminus blocked by a cyclized glutamine residue [6]. It has a p*I* of approximately 6.8 [5]. The enzyme possesses direct-acting fibrinolytic activity and does not activate plasminogen [7]. *In vitro*, fibrolase degrades both fibrin and fibrinogen through hydrolysis of their alpha and beta chains. The gamma chain appears to be resistant to the enzyme [8]. Fibrolase has possible therapeutic applicability as a thrombolytic agent [9]. For potential clinical use of the enzyme it is important to understand the relationship between structure and function of the enzyme and examine potential destabilizing influences on structure that may lead to loss of function.

Earlier observations, including sequence heterogeneity [6] and the occurrence of double bands on immobilized pH gradient isoelectric focusing gels using an ultra narrow pH interval

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(pH 6.7–7.0) [10], revealed that natural fibrolase exists in two isoforms. More recently it was possible to detect and quantitate isoforms of fibrolase and r-fibrolase using high-performance capillary electrophoresis [11].

Studies on the effect of acidic pH and chelating agents on fibrolase revealed that removal of zinc led to dramatic structural changes in the molecule [12,13]. Therefore we were interested in determining whether there are differences in the zinc binding affinities between the isoforms of fibrolase and r-fibrolase. In this report we describe a method to isolate the isoforms of fibrolase and r-fibrolase using a weak cation-exchange-based HPLC procedure. We have used mass spectrometry to characterize differences between the three isoforms of r-fibrolase. We also examined the effect of EDTA concentration on enzymatic activity of the isoforms of fibrolase and r-fibrolase.

2. Experimental

2.1. Materials

Agkistrodon contortrix contortrix snake venom was purchased from Biotoxins (St. Cloud, FL, USA). Recombinant fibrolase was prepared by Chiron Corp. (Emeryville, CA, USA).

High-performance liquid chromatography (HPLC)-grade solvents and chemicals were purchased from Fischer Chemical (Fair Lawn, NJ, USA). HPLC-grade trifluoroacetic acid was purchased from Pierce (Rockford, IL, USA). Electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Q Sepharose fast flow (strong anion-exchange), phenyl Sepharose 6 fast flow (hydrophobic interaction chromatography), and Sephacryl S-100 high solution (gel permeation) chromatography resins were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ, USA). All other reagents were of the highest grade available.

For hydrophobic interaction chromatography (HIC), a Poly Propyl A column (250 × 21 mm I.D., Poly LC) was purchased from Western Analytical Products Co. (Temecula, CA, USA). For hydroxyapatite chromatography (HAP), a SynChropak HAP-5 column (100 × 21.2 mm I.D.) was obtained from SynChrom (Lafayette, IN, USA). Anion-exchange chromatography was performed on a monoamine quaternary (MONO Q) high resolution fast protein liquid chromatography (FPLC) column (100 × 16 mm i.d., Pharmacia LKB). Weak cation-exchange HPLC was performed on a SynChropak carboxymethyl (CM) 300 (250 × 10 mm I.D.) column obtained from SynChrom.

HPLC purification of fibrolase was performed using a Bio 410 HPLC system equipped with a LC-95 UV/Vis detector (Perkin-Elmer, Norwalk, CT, USA) connected to a Frac-100 fraction collector (Pharmacia LKB), and a LC-100 integrator (Perkin-Elmer).

Concentration of protein samples for HPLC was performed with either a stirred cell (Amicon, Beverly, MA, USA) containing a YM 10 membrane (Amicon, 43 mm and 25 mm) or a Centricon-10 concentrator (Amicon). For dialysis, Spectrapor membrane tubing, 6000–8000 molecular mass cut-off, was used (Spectrum Medical Industries, Los Angeles, CA, USA).

2.2. Protein concentration determination

Two methods were used to determine protein concentrations. The method of Warburg and Christian was used to determine concentrations of chromatographic fractions based on absorbance at 280 nm ($A_{280} = 0.88$ for a 1 mg/ml fibrolase solution) [5,14]. Protein concentrations were also measured using the BCA assay (Pierce) [15,16]. Bovine serum albumin (BSA) was used as a standard. Protein concentrations were determined in 96-well microtiter plates (Corning Glass, Corning, NY, USA) and absorbance at 600 nm was analyzed with a manual Bio-Tek microplate reader (Fisher Scientific, Pittsburg, PA, USA).

2.3. Proteolytic activity assays

Enzymatic activity of fibrolase was assessed by azocasein hydrolysis as described by Charney

and Tomarelli [17]. To measure proteolytic activity, 0.75 ml of azocasein (Sigma Chemical Co., St. Louis, MO, USA) solution (50 mg/ml in 1% sodium bicarbonate, pH 8.5) was added to a 2.0-ml microcentrifuge tube. The fibrolase solution to be tested (50 μl) was added and the tubes were incubated at 37°C for 30 min. Reactions were stopped by the addition of 0.75 ml of 1.16 *M* perchloric acid and the precipitate was removed by 10 min centrifugation at 10 000 *g*. Hydrolysis of azocasein was measured as increased absorbance at 390 nm in the supernatant, after subtracting the absorbance obtained from a sample treated in an identical manner except with bicarbonate buffer in place of fibrolase. A standard curve was obtained by using varying concentrations of fibrolase. The linear section of the curve was used to estimate the specific activity (absorbance units 390 nm/mg enzyme).

Fibrinolytic activity of fibrolase was measured using the fibrin plate clearance assay described by Bajwa *et al.* [18].

2.4. One dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The Mini-Protean II dual slab cell (Bio-Rad) was used. A 14% gel was prepared for SDS-PAGE according to the method of Laemmli [19]. Standard molecular mass markers (Sigma Chemical Co.) were used. SDS-PAGE gels were stained with 0.15% Coomassie blue R-250 (Sigma Chemical Co.) in 40% methanol, 10% acetic acid.

2.5. Purification of natural fibrolase

Fibrolase was purified from *Agkistrodon contortrix contortrix* snake venom by a combination of HIC, HAP, Mono Q and CM 300 HPLC. All operations were carried out at 4°C. For the first step of purification, ten grams of venom was applied to HIC HPLC in five applications. The venom (two grams) was dissolved in 9 ml of HIC buffer A (O.1 *M* phosphate, 1.0 *M* $(NH_4)_2SO_4$, 0.02% NaN_3, pH 6.8) and centrifuged at 9000 *g* for 30 min at 4°C. The supernatant was filtered using a 0.2-μm membrane and applied to the column. The following gradient was employed to elute venom proteins: (a) 50 min isocratically at 100% HIC buffer A; (b) 90 min linear gradient to 100% HIC buffer B (0.1 *M* phosphate, 0.02% NaN_3, pH 6.8); and (c) 60 min isocratically at 100% HIC buffer B. A flow-rate of 5 ml/min was used and fractions of 5 ml were collected. For all HPLC procedures, protein concentration was monitored by measuring absorbance at 280 nm, fibrinolytic activity was also determined and SDS-PAGE was performed on selected fractions.

Active fractions from HIC HPLC were pooled, concentrated using 80% ammonium sulfate, and dialyzed against HAP buffer A (0.01 *M* phosphate buffer, 0.02% NaN_3, pH 6.8). Dialysis was completed as quickly as possible using several changes of buffer to avoid protein precipitation. The dialyzed sample from the HIC HPLC run was concentrated using the Amicon concentrator to about 10 ml and applied to the HAP HPLC column. Proteins were eluted according to the following gradient: (a) 15 min isocratically at 100% HAP buffer A; (b) 130 min linear gradient to 60% HAP buffer B (0.35 *M* phosphate, 0.02% NaN_3, pH 6.8); and (c) 35 min linear gradient to 100% HAP buffer B. Fractions of 2 ml were collected at a flow-rate of 2 ml/min.

Anion-exchange HPLC with a Mono Q column was utilized to remove several minor contaminants prior to resolution of the isoforms of fibrolase. Fractions with fibrinolytic activity following HAP HPLC were pooled, concentrated using the Amicon stirred cell, and dialyzed against several changes of Mono Q buffer A (20 m*M* Tris-HCl, pH 8.0) overnight. Approximately 18 mg of the HAP pool was filtered through a 0.2-μm membrane and applied to the Mono Q FPLC column. The following gradient was used: (a) 10 min isocratically at 100% Mono Q buffer A; (b) 90 min linear gradient to 20% Mono Q buffer B (20 m*M* Tris-HCl, 500 m*M* NaCl, pH 8.0); (c) 5 min linear gradient to 100% Mono Q buffer B; and (d) 5 min isocratically at 100% Mono Q buffer B. The flow-rate was 4 ml/min and 2 ml fractions were collected.

To resolve the isoforms of fibrolase, a weak

cation-exchange HPLC procedure was employed. Approximately 9 mg of the Mono Q pool was dialyzed against CM 300 buffer A (30 m*M* 2-[N-morpholino] ethanesulfonic acid, sodium salt [NaMES], pH 6.4) and concentrated using the Amicon stirred cell. The sample was filtered (0.2-μm membrane) and applied to the CM 300 HPLC column. Fractions were eluted according to the following gradient: (a) 10 min isocratically at 100% CM 300 buffer A; (b) 95 min linear gradient to 30% CM 300 buffer B (30 m*M* NaMES, 500 m*M* NaCl, pH 6.4); (c) 40 min linear gradient to 100% CM 300 buffer B; and (d) 10 min isocratically at 100% CM 300 buffer B. The flow-rate was 2.4 ml/min and fractions of 1.2 ml were collected.

To completely resolve the isoforms (fib1 and fib2), pooled fractions (containing fib1 and fib2) after CM 300 HPLC were dialyzed against CM 300 buffer A and reapplied to the CM 300 column. The isoforms were eluted using identical conditions to those employed during the initial CM 300 HPLC application. Fractions containing fibrinolytic activity were pooled and stored at −80°C until use.

2.6. Purification of recombinant fibrolase (r-fibrolase)

Recombinant fibrolase from yeast was purified using a 4-step chromatographic procedure. For the first step anion-exchange chromatography was performed using Q Sepharose (Pharmacia LKB Bio Process BP 113 column, 12 × 11 cm, 1.244 l bed volume). The gel was equilibrated with Q Sepharose buffer A (15 m*M* triethanolamine-HCl, 0.5 m*M* $ZnCl_2$, pH 7.5). Four liters of yeast broth containing r-fibrolase was divided into four equal aliquots and each aliquot was activated with an equal volume of 8 *M* urea in Q Sepharose buffer A. The mixture was incubated 30 min with gentle stirring at room temperature before loading onto the column. Four such Q Sepharose runs were performed so that the yeast broth was not exposed to urea for more than 30–60 min. The column was washed immediately with Q Sepharose buffer B (20 m*M* triethanolamine-HCl, 0.5 m*M* $ZnCl_2$, pH 7.5) at a flow-rate of 50 ml/min. Active and partially active r-fibrolase was eluted stepwise with 100 and 350 m*M* NaCl in Q Sepharose buffer B, respectively. Fractions of 600 ml were collected during loading and washing the column and fractions of 100 ml during stepwise elution. Protein concentration was monitored by measuring absorbance at 280 nm. Proteolytic activity was measured using the azocasein assay.

For the second step hydrophobic interaction chromatography was employed using phenyl Sepharose 6 (350 × 50 mm I.D., bed volume 570 ml). The resin was equilibrated in Q Sepharose buffer B containing 1.1 *M* ammonium sulfate. Q Sepharose fractions that contained active r-fibrolase were pooled and adjusted to 1.1 *M* ammonium sulfate. This material was loaded onto the column and washed with Q Sepharose buffer B containing 0.9 *M* $(NH_4)_2SO_4$. r-Fibrolase was eluted with Q Sepharose buffer B containing 0.4 *M* $(NH_4)_2SO_4$. Fraction size was 100 ml in the flow-through and 12 ml after the gradient. A flow-rate of 15 ml/min was maintained throughout the run. Protein concentration and proteolytic activity were measured and selected fractions were analyzed by SDS-PAGE to determine which fractions contained r-fibrolase.

Gel permeation chromatography was then employed using Sephacryl S-100 (Pharmacia LKB XK 26/150 column, bed volume 466 ml). Sephacryl S-100 was equilibrated with Sephacryl buffer A (30 m*M* NaMES, 0.1 m*M* $ZnCl_2$, pH 6.4). Phenyl Sepharose fractions with proteolytic activity were pooled and concentrated using 80% ammonium sulfate. The sample was dialyzed against Sephacryl buffer A and 200 mg of protein was applied to Sephacryl S-100. Fractions of 12 ml were collected at a flow-rate of 1.5 ml/min and protein concentration was monitored at 280 nm. Activity was measured using fibrin plate assay and selected fractions were analyzed by SDS-PAGE.

To separate isoforms of r-fibrolase, weak cation-exchange HPLC was employed. Fractions containing fibrinolytic activity from the Sephacryl S-100 run were pooled, concentrated using an Amicon stirred cell, dialyzed against

CM 300 buffer A (30 m*M* NaMES, 0.1 m*M* $ZnCl_2$, pH 6.4), filtered and applied to the CM 300 HPLC column. Elution employed the following gradient: (a) 10 min isocratically at 100% CM 300 buffer A; (b) 85 min linear gradient to 30% CM 300 buffer B (30 m*M* NaMES, 500 m*M* NaCl, 0.1 m*M* $ZnCl_2$, pH6.4); (c) 40 min linear gradient to 100% CM 300 buffer B; and (d) 10 min isocratically at 100% CM 300 buffer B. The flow-rate was 2.4 ml/min and fractions of 1.2 ml were collected. Protein concentration was monitored by measuring the absorbance at 280 nm. Fibrinolytic activity was measured by the fibrin plate assay and SDS-PAGE was carried out on selected fractions.

To purify the individual isoforms, appropriate fractions were pooled, concentrated, dialyzed and rerun on the CM 300 HPLC column. Identical conditions were employed as for the original CM 300 run. CM 300 HPLC resulted in the separation of three isoforms of r-fibrolase, henceforth called r-fibrolase 1 (r-fib1), r-fib2 and r-fib3.

2.7. Mass spectrometry of r-fibrolase

Approximately 150 μg of r-fib1, r-fib2 and r-fib3 (after CM 300 cation-exchange HPLC) were desalted by reversed-phase (RP) HPLC in preparation for mass spectrometry. A C_{18} HPLC column (250 × 4.6 mm I.D., 218TP54 column, Vydac, Hesperia, CA, USA) was utilized. RP-HPLC was performed using a Spectra-Physics 8800 HPLC equipped with a Spectra-Physics 8450 variable wavelength UV/Vis detector and Spectra-Physics WINner data acquisition module. The C_{18} column was equilibrated with 90% of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 10% of 80% acetonitrile in 0.1% TFA in water (solvent B). Elution was achieved using the following conditions: (a) isocratic at 90% solvent A and 10% solvent B for 5 min, (b) linear gradient to 80% solvent B over 70 min. Absorbance at 215 nm was measured and peak fractions were collected manually. Recombinant isoforms eluted at approximately 68% solvent B. Samples were dried in preparation for mass spectrometry.

Electrospray ionization (ESI) mass spectra were obtained by dissolving the samples in water–acetonitrile–acetic acid (50:50:0.1, v/v), and injecting 5-μl aliquots of the resulting solutions into the infusion line connected to an electrospray ion source attached to a quadruple mass spectrometer (Sciex API IIIR, Toronto, Canada).

The same solvent was pumped through the infusion line at a rate of 10 μl/min. Data were collected by scanning m/z from 400 to 2000, and scans containing the ions of interest were summed and background subtracted. Myoglobin was used as a standard. The raw data were mass measured using the multiply charged ion series from a separate introduction of polypropylene glycol for calibration. In this form the data are obtained with the abscissa presenting the mass/charge (m/z) ratio. Because ESI spectra typically show for each component several ions of differing charge state, it aids interpretation to transform the data into a true molecular mass spectrum in which the abscissa is in mass units. This was achieved by the software package supplied with the spectrometer. Under normal conditions the accuracy of the mass measurement carried out in this manner is typically ± 0.01% of the molecular mass (or 2.3 mass units for fibrolase).

2.8. Zinc-binding studies

Two methods were employed to investigate the relative binding affinity of zinc to the isoforms of natural and recombinant fibrolase. In the first method, the time course of EDTA binding to the different isoforms was examined. In the second procedure the effect of varying concentrations of EDTA on proteolytic activity was measured. Isoforms of natural and recombinant fibrolase were dialyzed against 0.1 *M* HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffer, pH 7.8, to insure the initial conditions were identical for all isoforms.

In the first method, natural or recombinant fibrolase (0.15 mg/ml) was incubated with 500-fold or 1000-fold molar excess of EDTA for varying periods of time. Reactions were carried

out in 0.1 *M* HEPES buffer, pH 7.8, in a volume of 0.25 ml at room temperature. Reactions were quenched by adding 10-fold molar excess of $CaCl_2$ over the chelating agent. Aliquots were removed for assay of proteolytic activity using the azocaseinolytic assay. All measurements were in duplicate.

In the second method, the individual isoforms (0.15 mg/ml) were incubated for 30 min with varying concentrations (from 0- to 10 000-fold molar excess) of EDTA in a volume of 0.25 ml. Reactions were stopped and azocaseinolytic activity was determined as described above. For both methods, enzyme incubated under identical conditions but in the absence of EDTA served as a positive control to determine percentage of activity remaining.

3. Results

3.1. Purification of isoforms of natural fibrolase

Fig. 1 presents the elution profile obtained when 2 g of *Agkistrodon contortrix contortrix* venom were purified by HIC HPLC. SDS-PAGE revealed that the pooled fractions contained a major band at 23 kDa, corresponding to fibrolase, plus several minor bands. The pooled fractions were concentrated and dialyzed against HAP buffer A. For the second step of purification HAP HPLC was employed. A single sharp peak with fibrinolytic activity was observed (Fig. 2). SDS-PAGE revealed that several contaminants were still present. Mono Q FPLC was employed to remove these contaminants. Chromatography of the HAP HPLC pool by Mono Q FPLC resulted in one major peak eluting at approximately 20 m*M* NaCl, well separated from several contaminants (Fig. 3). Although this step did not resolve the isoforms of fibrolase, the enzyme was essentially homogeneous. SDS-PAGE revealed one band of identical mobility to a fibrolase standard at 23 kDa.

In order to resolve the isoforms of fibrolase CM 300 weak cation- exchange HPLC was employed (Fig. 4). Each peak, representing an individual isoform, was pooled separately. The earlier eluting peak was designated fib1 and the later peak as fib2. Both proteins possessed fibrinolytic activity and each migrated as a single band at the same molecular weight on SDS-PAGE. When mixed, the two proteins co-migrated on SDS-PAGE.

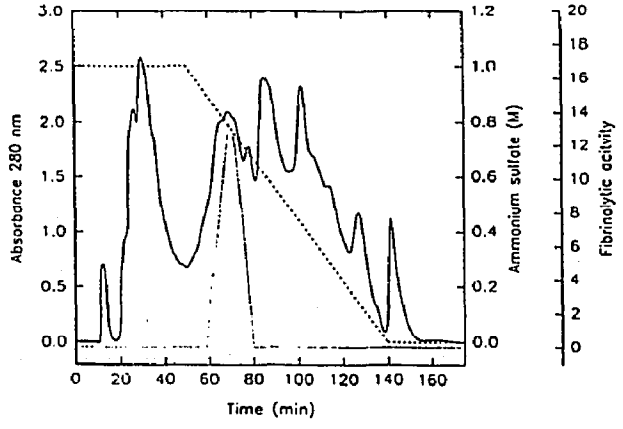

Fig. 1. Hydrophobic interaction HPLC of fibrolase from *A. c. contortrix* venom. Two grams of venom were applied to the HIC HPLC column. Elution conditions are described in Experimental. The solid line indicates absorbance at 280 nm, the dotted line indicates fibrinolytic activity (fibrin plate assay, diameter of fibrin clearance in mm), and the dashed line is the ammonium sulfate gradient.

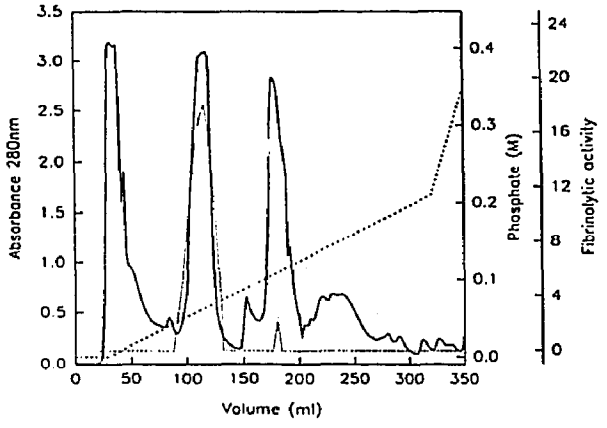

Fig. 2. Hydroxyapatite HPLC of natural fibrolase. Fibrolase (791 mg) from HIC HPLC was applied to the hydroxyapatite HPLC column. Elution conditions are described in Experimental. The solid line indicates absorbance at 280 nm, the dotted line indicates fibrinolytic activity (as in Fig. 1), and the dashed line is the phosphate gradient.

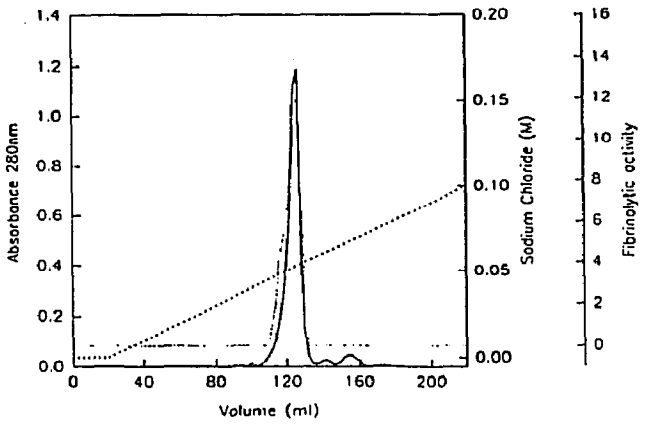

Fig. 3. Anion-exchange FPLC (Mono Q) of fibrolase pool from HAP HPLC. Approximately 18 mg of fibrolase was applied to the column. Elution conditions are described in Experimental. The solid line indicates absorbance at 280 nm, the dotted line indicates fibrinolytic activity (as in Fig. 1), and the dashed line is the sodium chloride gradient.

When an aliquot of either isoform was reapplied to the CM 300 HPLC column, a single peak eluted at the same position as the initial CM 300 HPLC run. Furthermore, reapplication of fib1 on CM 300 did not yield fib2 and *visa versa*. This indicates that the isoforms could be completely resolved and isolated in a homogeneous form by this semipreparative procedure, and that they are not interconvertible. Fig. 5A and Fig. 5B present the elution profiles corresponding to fib1 and fib2, respectively. As expected, their elution times are very similar.

Fig. 6 presents the SDS-PAGE profile of natural fibrolase at various stages during purification of the isoforms.

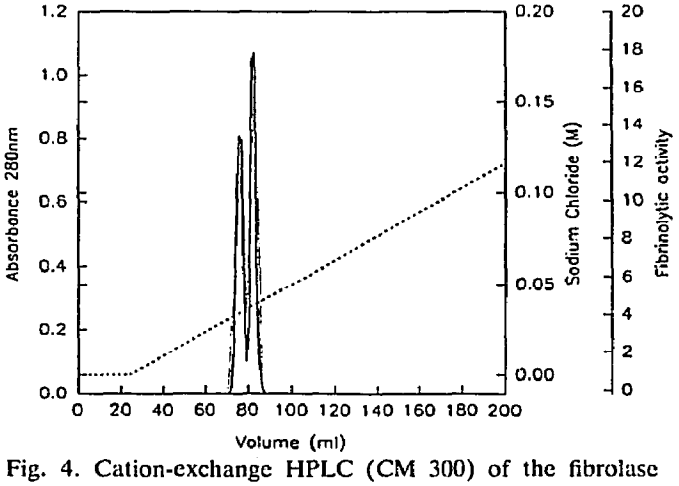

Fig. 4. Cation-exchange HPLC (CM 300) of the fibrolase pool after Mono Q FPLC. Approximately 9 mg of the pool was applied to the CM 300 column. Elution conditions are described in Experimental. The solid line indicates absorbance at 280 nm, the dotted line indicates fibrinolytic activity (as in Fig. 1), and the dashed line is the sodium chloride gradient.

3.2. Purification of isoforms of recombinant fibrolase: anion-exchange chromatography (Q Sepharose)

Southern copperhead snake venom gland mRNA was isolated and a full length fibrolase cDNA clone was prepared at Chiron Corporation. Using proprietary expression vectors (Doris Coit and Pablo Valenzuela, unpublished results), secretion of fibrolase from yeast (*S. cerevisiae*) has been achieved via a plasmid-mediated expression system. Fibrolase yields of 30–60 mg per liter are obtained using a protease deficient yeast strain in a defined medium containing zinc. After concentration and diafiltration of yeast broth, r-fibrolase was activated by dilution of the broth with an equal volume of 8 *M* urea. The activated enzyme solution was then rapidly applied to Q Sepharose. Fig. 7 represents the elution profile from Q Sepharose. Three major protein fractions were obtained. After washing with the equilibration buffer (Q Sepharose buffer A), peak 1, containing inactive fibrolase was eluted. Peak 2, containing active fibrolase, was obtained after eluting with one bed volume of 100 m*M* NaCl in Q Sepharose buffer A. Peak 3, containing inactive fibrolase, was eluted after washing with approximately 2 bed volumes of 350 m*M* NaCl in Q Sepharose buffer A. SDS-PAGE under reducing conditions showed that all peaks contained proteins with molecular masses of 23 kDa identical to that of fibrolase.

3.3. Hydrophobic interaction chromatography (phenyl sepharose)

Fig. 8 presents the elution profile following the application of Q Sepharose peak 2 onto phenyl Sepharose. Flow-through fractions were col-

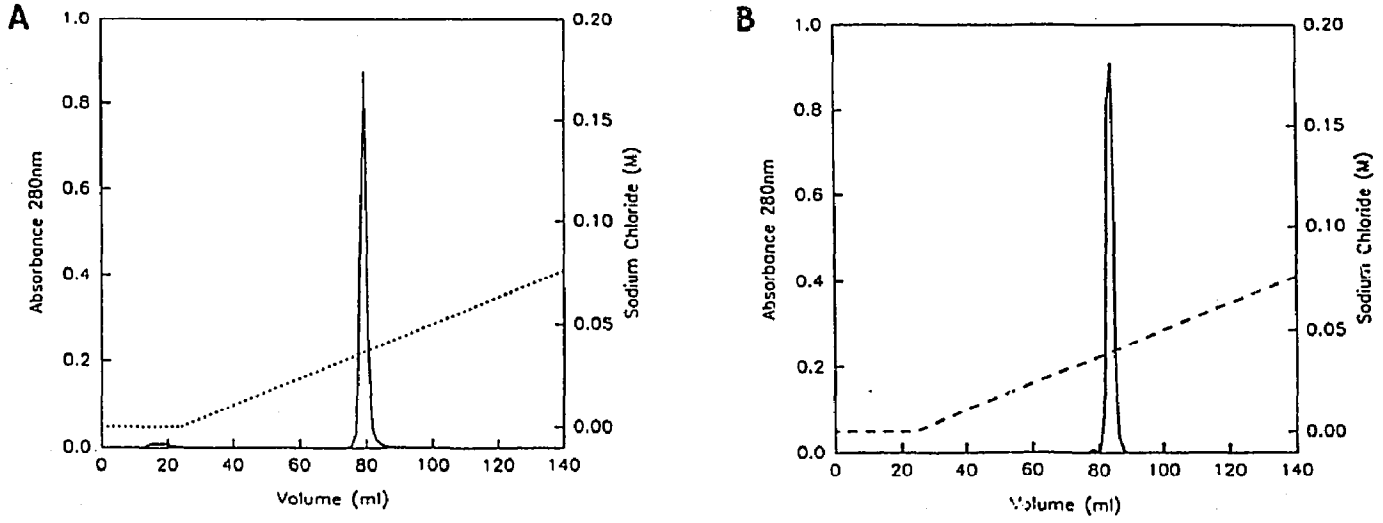

Fig. 5. Rechromatography using cation-exchange HPLC (CM 300) of individual pools after the first CM 300 HPLC application. (A) fib1; (B) fib2. The elution conditions are identical to those described in Fig. 4. The solid line indicates absorbance at 280 nm and the dashed line is the sodium chloride gradient. Peak fractions were pooled and fibrinolytic activity was determined.

lected in four bed volumes of phenyl Sepharose buffer B containing 0.9 *M* $(NH_4)_2SO_4$. This fraction contained a large amount of protein but no fibrinolytic activity. Three peaks were obtained following elution with phenyl Sepharose buffer B containing 0.4 *M* $(NH_4)_2SO_4$.

According to SDS-PAGE analysis, phenyl Sepharose peak 1 contained an inactive form of r-fibrolase plus contaminants of higher molecular mass. Peak 2 did not contain fibrolase. Peak 3 contained the major proteolytic activity which on SDS-PAGE had identical mobility to a r-fibrol-

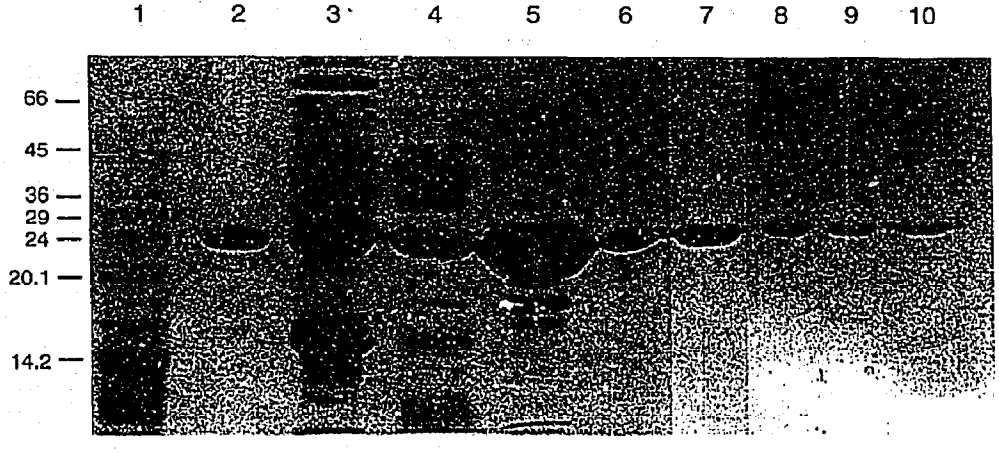

Fig. 6. SDS-polyacrylamide gel electrophoresis of natural fibrolase purified by the five-step HPLC procedure from *A. c. contortrix* venom and of the r-fibrolase isoforms. Lane 1, molecular mass standards: from top to bottom: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), 36 kDa; carbonic anhydrase (bovine erythrocyte), 29 kDa; trypsinogen (bovine pancreas), 24 kDa; trypsin inhibitor (soybean), 20 kDa; and α-lactalbumin (bovine milk), 14 kDa (molecular masses shown to the left of the standard proteins); lane 2, natural fibrolase control; lane 3, crude venom; lane 4, pool from HIC HPLC; lane 5, pool from HAP HPLC; lane 6, pool from Mono Q HPLC; lane 7, fib2 from CM 300 (fib1 had identical mobility); lanes 8-10, r-fib1, r-fib2 and r-fib3, respectively, after CM 300 HPLC.

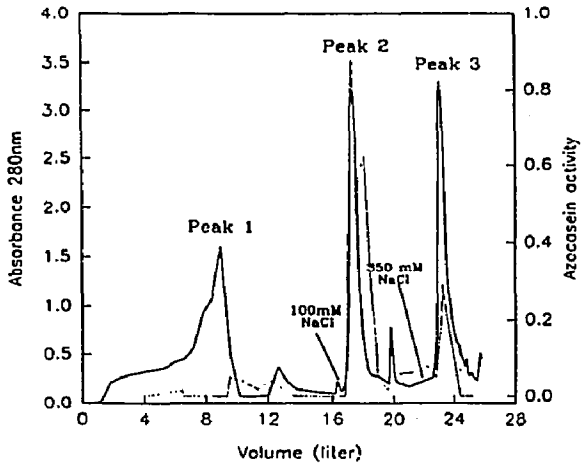

Fig. 7. Anion-exchange chromatography (Q Sepharose) of r-fibrolase. Four liters of yeast broth was activated with 8 *M* urea in 20 m*M* triethanolamine-HCl, 0.5 m*M* $ZnCl_2$, pH 7.5, and applied to the column. Elution conditions are described in Experimental. Peak 2 contained the active form of fibrolase. The protein concentration was determined by absorbance at 280 nm (solid line), and azocaseinolytic activity was determined by measuring the absorbance at 390 nm (dotted line). Stepwise elution with 100 m*M* and 350 m*M* sodium chloride are indicated

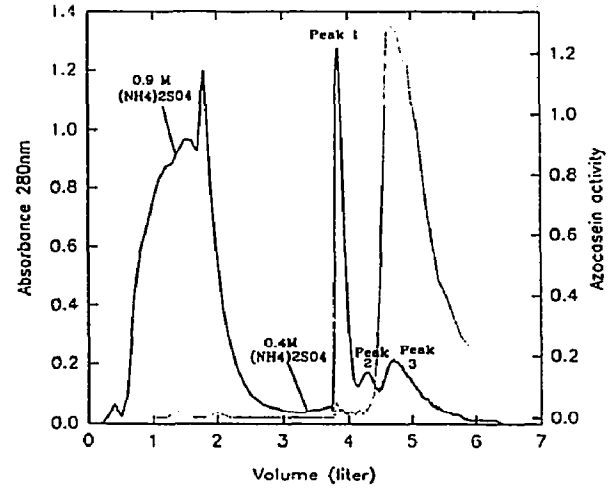

Fig. 8. Hydrophobic interaction chromatography (phenyl Sepharose FF) of the r-fibrolase pool after Q Sepharose. Elution conditions are described in Experimental. Elution during the flow-through was with buffer B containing 0.9 *M* $(NH_4)_2SO_4$. After 3.3 l elution was switched to 0.4 *M* $(NH_4)_2SO_4$ in buffer B. Peak 3 contained fibrinolytic activity and these fractions were pooled. Protein concentration was determined by absorbance at 280 nm (solid line), and azocaseinolytic activity was determined by measuring the absorbance at 390 nm (dotted line).

ase control at 23 kDa. SDS-PAGE analysis also revealed that a low level of contaminants were still present in this fraction.

3.4. Gel permeation chromatography (Sephacryl S-100 HR)

A single peak with fibrinolytic activity was obtained after molecular sieve chromatography on Sephacryl S-100 (Fig. 9). SDS-PAGE analysis of the Sephacryl S-100 fractions indicated only trace amounts of lower molecular mass contaminants. The enzyme at this stage of purification represents a homogeneous r-fibrolase preparation, as assessed by SDS-PAGE.

3.5. Cation-exchange chromatography (CM 300)

Analysis of the Sephacryl S-100 pool by narrow range IEF (pH 5.5–8.5) revealed that there were at least two isoforms of the recombinant protein [11]. The isoforms could be resolved by strong cation-exchange HPLC (TSK SP-5PW) into two well-separated protein peaks [11]. However, weak cation-exchange HPLC (CM 300) resolves recombinant fibrolase into three iso-

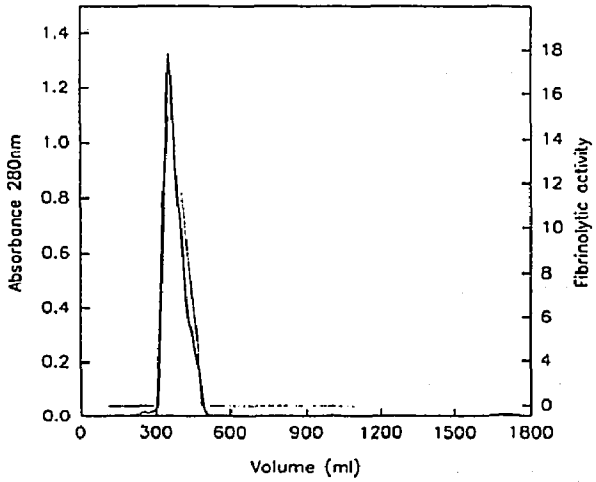

Fig. 9. Gel permeation chromatography on Sephacryl S-100. Pooled fractions from phenyl Sepharose were concentrated and applied to the column. Elution conditions are described in Experimental. The solid line indicates absorbance at 280 nm and the dotted line indicates fibrinolytic activity (as in Fig. 1).

forms. Thus, when 10.5 mg of protein from the Sephacryl S-100 pool was applied to the CM 300 HPLC column three peaks with fibrinolytic activity, corresponding to the three isoforms of recombinant fibrolase (r-fib1, r-fib2 and r-fib3), were obtained (Fig. 10). Using SDS-PAGE under reducing conditions, each isoform migrated as a single band corresponding to authentic r-fibrolase with a mass of 23 kDa (Fig. 6).

3.6. Mass spectrometry of recombinant fibrolase isoforms

Mass spectra were obtained for the three isoforms of recombinant fibrolase in order to determine their masses. Quintuplicate determination of the masses for each of the isoforms revealed that r-fib1 has a mass of 22 881.2 amu (atomic mass units) (S.D. = ± 0.4), r-fib2 has a mass of 22 896.9 amu (S.D. = ± 0.5), and r-fib3 has a mass of 22 771.3 amu (S.D. = ± 0.3).

Natural fibrolase possesses two amino-termini: <EQRFP··· and <ERFP··· (<E= cyclized glutamine) [6]. The theoretical masses for these two amino-teriminal forms are 22 885 amu and 22 757 amu, respectively. The mass of

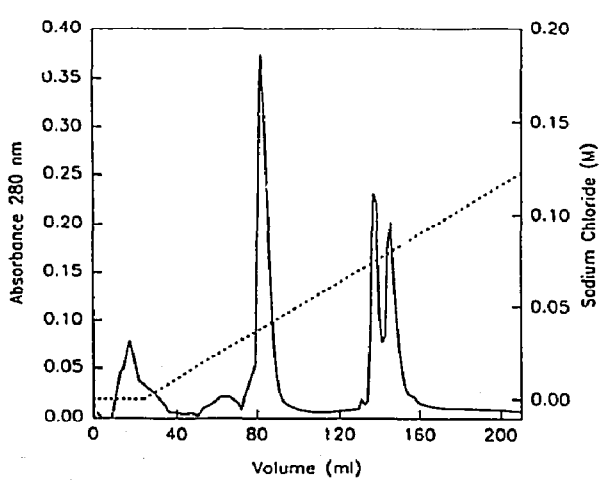

Fig. 10. Weak cation-exchange HPLC (CM 300) of r-fibrolase after Sephacryl S-100. Approximately 11 mg of the pool was applied to the column. Elution conditions are described in Experimental. Solid line indicates absorbance at 280 nm and the dashed line is the sodium chloride gradient. Fibrinolytic activity was detected in each of the three peaks eluting at 85, 135, and 145 min. The peak at 18 min did not contain protein on SDS-PAGE.

<EQRFP··· amino-terminal form of natural fibrolase is comparable to the mass of 22 881.2 amu for r-fib1. Since the recombinant protein was obtained from a single cDNA clone, these findings suggest that different isoforms of r-fibrolase may be generated due to the degree of acid exposure during yeast expression. One possible interpretation is that the smallest isoform (r-fib3) is a truncated form that is missing the amino-terminal glutamine residue and has an amino-terminal sequence of QRFP···. The theoretical mass of such an isoform would be 22 775 amu which is in excellent agreement with the measured mass of 22 771.3 amu for r-fib3. The mass of 22 896.9 for r-fib2 is also in excellent agreement with the theoretical mass of 22 903 amu for the unblocked form of natural fibrolase which would have an amino-terminal sequence of QQRFP···.

The mass of r-fib1 is less than the mass of r-fib2 by 16 amu due to the loss of an amide group as a result of cyclization of the amino-terminal glutamine. Thus r-fib1 appears to be identical to natural fibrolase with a cyclized amino-terminal glutamine having a sequence of <EQRFP··· which is consistent with their similar mobilities on IEF [11]. r-Fib2 would appear to be fibrolase with an uncyclized amino terminal glutamine residue, and r-fib3 would appear to be a form of fibrolase missing the amino-terminal glutamine residue.

3.7. Zinc-binding studies

The time course experiments showed that fib1 and r-fib1 lost between 85% to 90% of azocaseinolytic activity when the enzymes were incubated 20 min with either 500-fold or 1000-fold molar excess of EDTA (Figs. 11A and 11B). Fib2 lost approximately 75% of its activity at both concentrations of EDTA, whereas r-fib3 lost 76 to 81% of its proteolytic activity (Figs. 11C and 11D). On the basis of these results 30 min was chosen as the optimum incubation time for further studies.

The effect of EDTA on the proteolytic activity of fibrolase and recombinant fibrolase isoforms was dependent on the concentration of EDTA

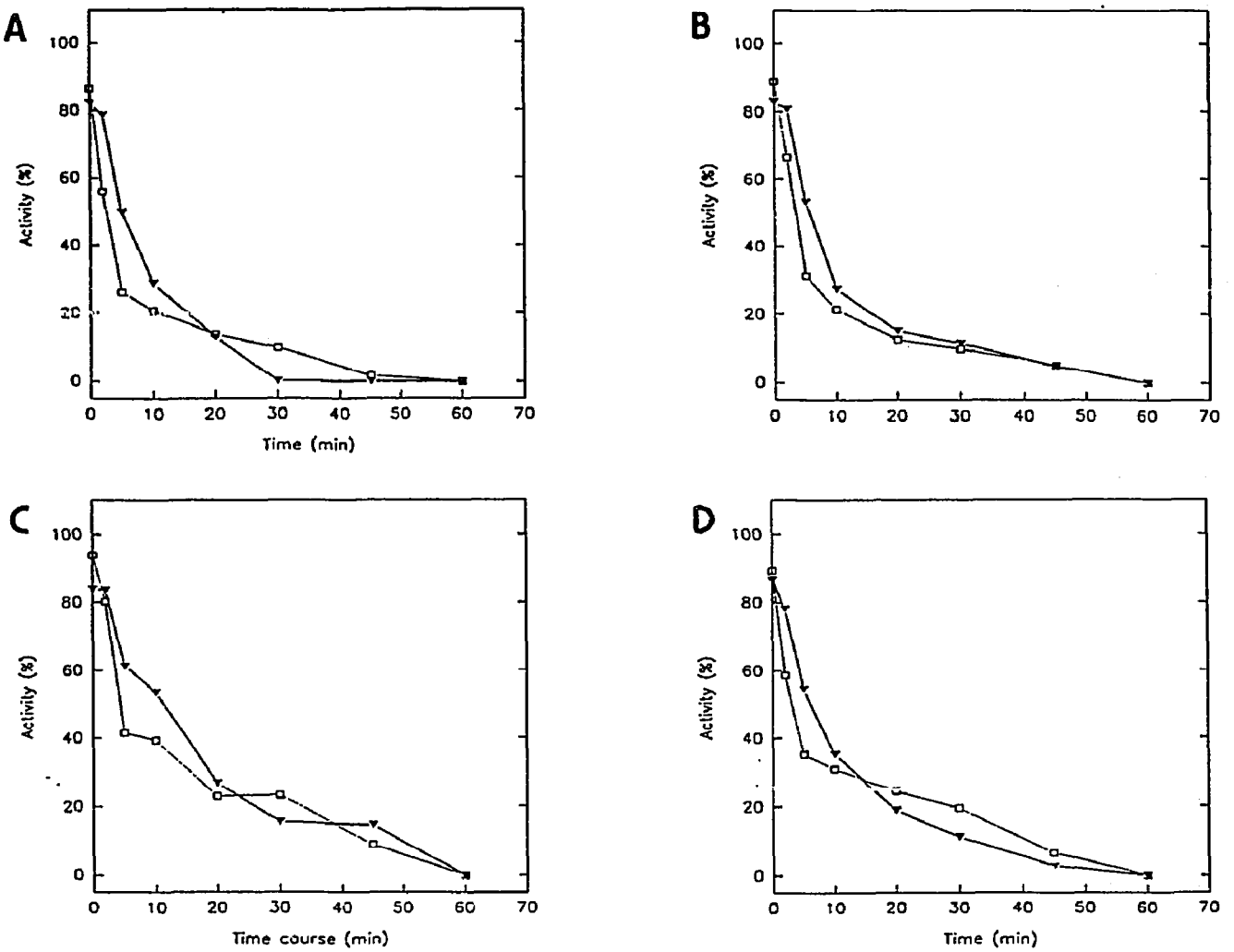

Fig. 11. Time dependent inhibition of fibrolase by EDTA. (A) fib1; (B) r-fib1; (C) fib2; (D) r-fib3. Enzymes (0.13 nmol) were incubated with EDTA at either 500-fold molar excess (▼) or 1000-fold molar excess (□). The reaction was quenched by adding 10-fold molar excess of $CaCl_2$ over the chelating agent following which proteolytic activity was determined by azocasein assay as described in Experimental. Azocaseinolytic activity was measured in duplicate and the average values are plotted. Enzyme incubated under identical conditions but in the absence of inhibitor served as a positive control. HEPES buffer, pH 7.8, incubated with EDTA was used as a negative control.

added and did not seem to vary significantly from one isoform to another. Low concentrations of EDTA from 1- to 20-fold molar excess did not produce any significant inhibitory effect on enzyme activity of any isoform of fibrolase or r-fibrolase during the 30 min incubation. However, partial inhibition of fibrinolytic activity of all isoforms was achieved at EDTA concentrations ranging from 50- to 100-fold molar excess. With 50-fold molar excess of EDTA, fib1 was inhibited 45%, r-fib1 was inhibited by 36% and r-fib3 was inhibited 50%. Fibrolase and r-fibrolase isoforms were inhibited from 70% to 80% by 100-fold molar excess of EDTA. Almost complete inhibition was achieved when fibrolase and r-fibrolase isoforms were incubated with EDTA concentrations equal to or greater than 300-fold molar excess (Fig. 12).

The IC_{50} (concentration of EDTA to produce 50% enzyme inhibition) for fib1 is 160 μM, for

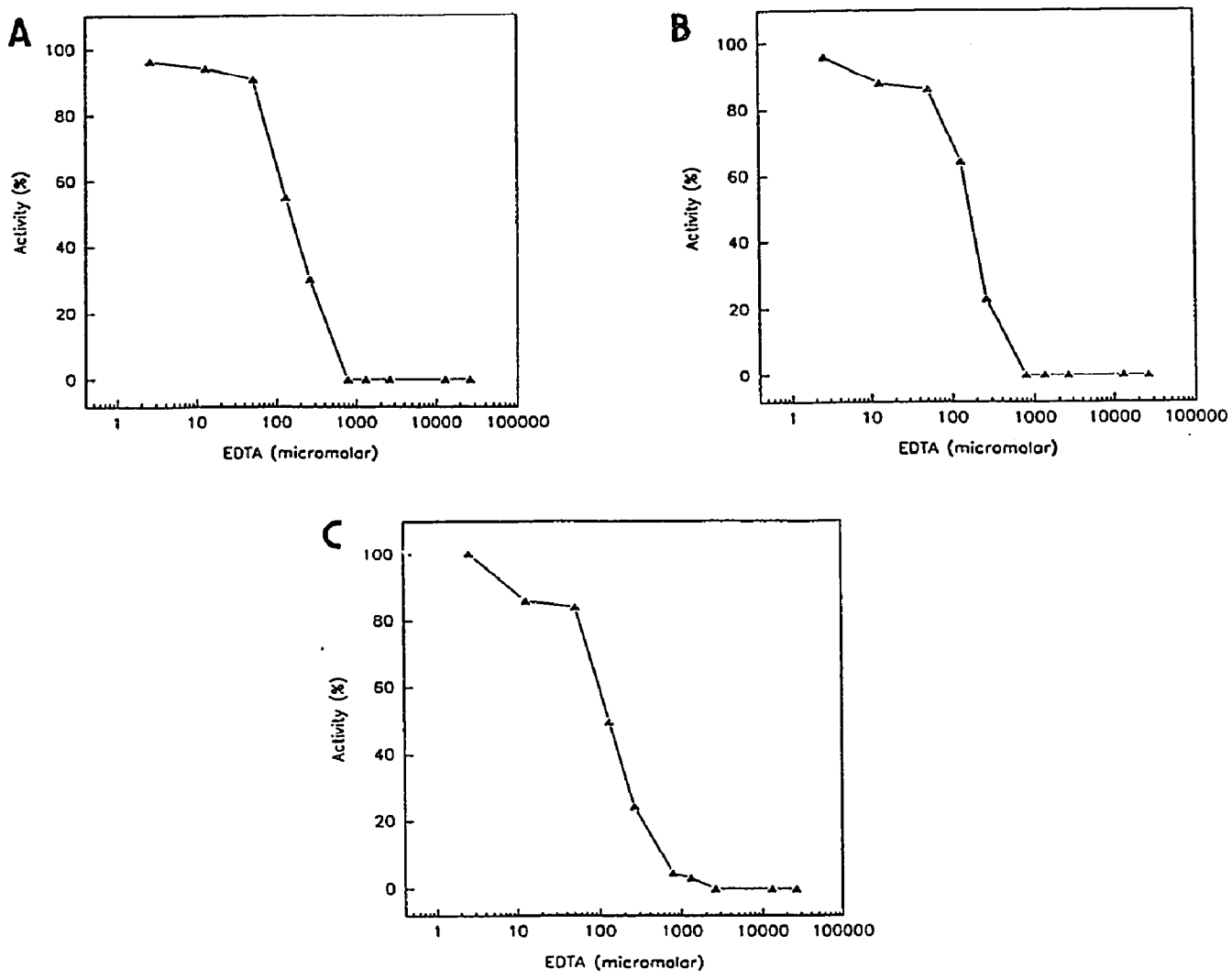

Fig. 12. Effect of varying concentrations of EDTA on fibrolase azocaseinolytic activity. (A) fib1; (B) r-fib1; (C) r-fib3. The enzyme (0.13 nmol) was incubated with varying concentrations of EDTA (from 0- to 10 000-fold molar excess) for 30 min. The reaction was quenched by adding 10-fold molar excess of $CaCl_2$ over the chelating agent. Azocaseinolytic activity was measured in duplicate and the average values are plotted. Reaction conditions are described in Experimental. Enzyme incubated under identical conditions but in the absence of EDTA served as a positive control. HEPES buffer, pH 7.8, incubated with EDTA was used as a negative control.

r-fib1 the IC_{50} is 180 μM, and for r-fib3 the IC_{50} is 140 μM (Fig. 13). Thus, the isoforms of fibrolase and r-fibrolase appear to have similar zinc binding affinities and EDTA susceptibilities based on the studies reported here.

4. Discussion

Evidence that natural fibrolase contains two isoforms has been demonstrated previously by immobilized pH gradient isoelectric focusing (IPG) [10], amino acid sequence analysis [6], and capillary zone electrophoresis [11]. IPG analysis revealed that the two isoforms, with p*I* values differing by only 0.01–0.03 pH units, could be resolved using an ultra narrow pH gradient, pH 6.65–6.95. However, recovery of enzymatic activity was only between 10 to 30% when attempting to use IPG as a preparative procedure [10]. Therefore, we developed a semi-preparative HPLC procedure using weak cation

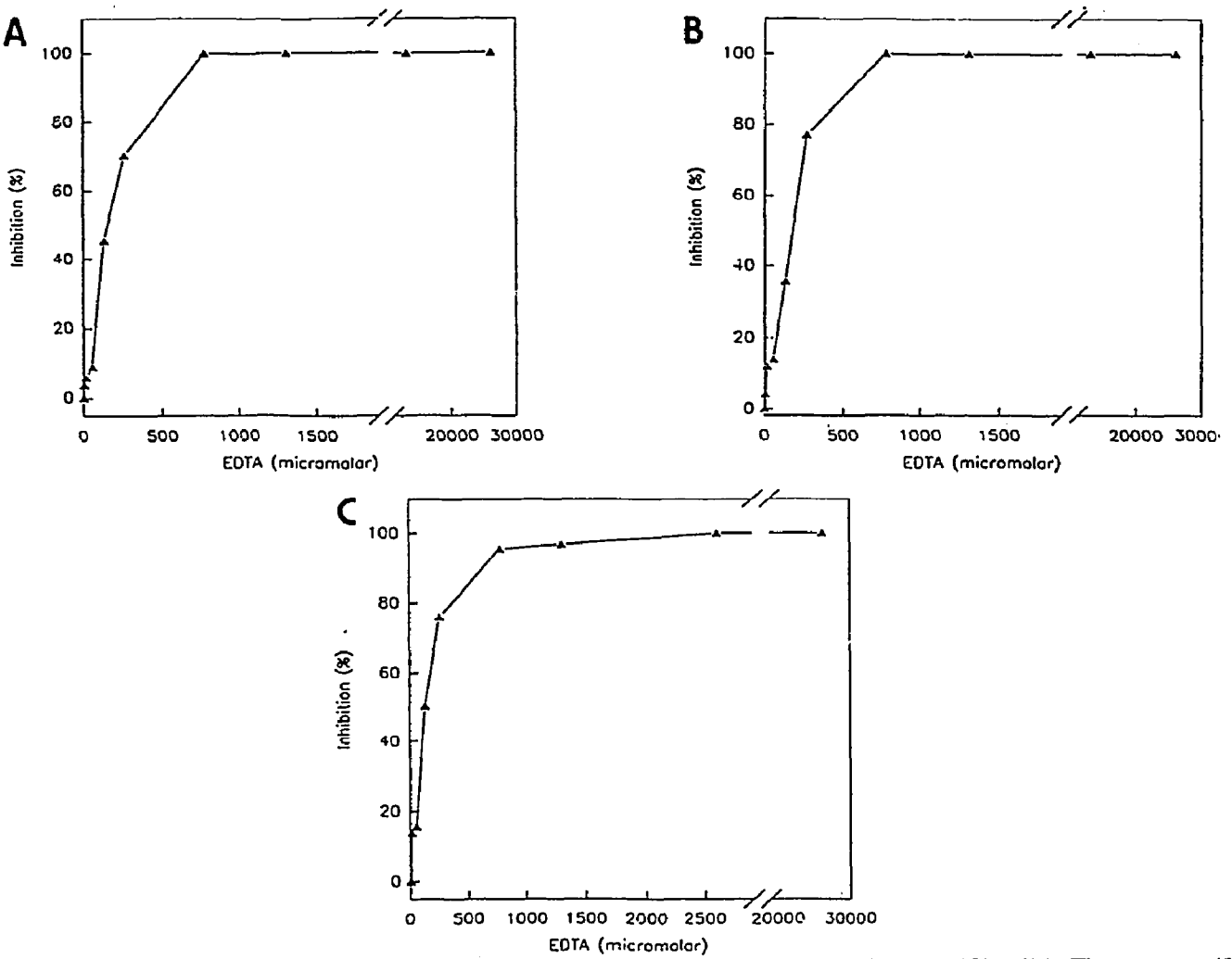

Fig. 13. IC_{50} for EDTA inhibition of isoforms of fibrolase and r-fibrolase. (A) fib1; (B) r-fib1; (C) r-fib3. The enzymes (0.13 nmol) were incubated with varying concentrations of EDTA (0- to 10 000-fold molar excess over fibrolase) for 30 min. The reaction was quenched by adding 10-fold molar excess of $CaCl_2$ over the chelating agent. Incubation conditions are described in Experimental.

exchange (carboxymethyl) HPLC to resolve the isoforms of fibrolase. We determined that the isoforms of fibrolase are stable and not artefacts derived during purification. Thus, when the isoforms were reapplied to CM 300, the same retention times were obtained as in the original CM 300 run. The isoforms were completely separated and were not interconvertible. Furthermore, when both isoforms were mixed they migrated on SDS-PAGE as a single band at 23 kDa, the known molecular mass of fibrolase. Their recovery following CM 300 HPLC was close to 80% and enzymatic activity was undiminished. Although we suspected that the presence of the fibrolase isoforms was due to genetic variance in the large pool of snakes used for milking, recent investigations of venom from individual milkings of southern copperhead snakes from different geographical locations indicated that this was not the case. Thus we found that individual milkings in all cases contained both isoforms (M. Trikha *et al.*, unpublished data), suggesting that the isoforms are not the result of population genetic differences but rather are due to the genotypes of the individual snakes.

Recent investigations (F.S. Markland and B.R. Lucchesi, unpublished data) revealed that

both natural and r-fibrolase are effective thrombolytic agents in a canine reoccluding carotid arterial thrombosis model system. However, it is not known if one isoform has an advantage over the other in thrombolytic efficacy. The present HPLC procedure will enable us to answer this question. Further, attempts to crystallize fibrolase have been hampered by the inability to separate the isoforms and needle-like crystals have been repeatedly observed (F.S. Markland and K.B. Ward, unpublished data). The availability of the individual isoforms enabled us to reproducibly grow large ($0.6 \times 0.5 \times 0.4$ mm) rhombohedral parallelipipeds, a "chunky" habit ideal for diffraction studies. These crystals were obtained using either natural fibrolase (fib1) or r-fibrolase (r-fib1).

r-Fibrolase has been produced from a single cDNA clone inserted into a yeast expression system; the mature enzyme is secreted into the culture medium. A simple and rapid three-step purification procedure was developed that provides r-fibrolase of high purity and good yield from concentrated and diafiltrated yeast broth. This procedure involves dilution of the concentrated broth with an equal volume of 8 *M* urea containing 0.5 m*M* $ZnCl_2$ to allow full activation of the r-fibrolase. r-Fibrolase, which does not bind zinc at the pH of yeast broth, pH 5.8, due to protonation of histidine residues involved in zinc binding [6], assumes an inactive conformation which can be at least partially reversed in the presence of urea and zinc. After brief exposure to urea the diluted yeast broth was applied to Q Sepharose. r-Fibrolase was eluted stepwise with a yield of about 70% of the activity. The recombinant enzyme was further purified by phenyl Sepharose hydrophobic interaction chromatography and Sephacryl S-100 gel permeation chromatography. The enzyme was homogenous as assessed by SDS-PAGE and overall recovery of activity was about 40%. However, an additional step has been added to isolate individual isoforms of r-fibrolase that were observed by narrow range isoelectric focusing (pH 5.5–8.5) after the Sephacryl S-100 step of purification [11]. We employed weak cation-exchange HPLC on a carboxymethyl (CM 300) column to isolate the major isoforms. As with the natural enzyme, the separation of isoforms of r-fibrolase is essential for suitable crystal growth. The individual isoforms have virtually identical fibrinolytic and azocaseinolytic activities, which are identical as well to those of natural fibrolase. r-Fib1 appears identical to fib1 by pH range 5.5–8.5 IEF [11]. Further, as already noted these two isoforms form single crystals of identical habit when exposed to conditions promoting crystal formation.

Mass spectrometry of the r-fibrolase isoforms indicates that r-fib1 possesses a cyclized amino-terminal glutamine residue (identical to natural fibrolase). r-Fib2 is the uncyclized intact amino-terminal form of fibrolase and r-fib3 is the truncated unblocked form of fibrolase. Since the isoforms originated from a single cDNA clone following expression in yeast, we suspect that they were derived from acid exposure during yeast expression.

The yeast expression system offers the opportunity of large scale production of fibrolase for potential clinical application as a thrombolytic agent. Since the isoforms of r-fibrolase possess identical enzymatic activity, the final purification step, weak cation-exchange HPLC, may not be necessary. However, this step removes a small amount of impurities. Furthermore, the isoforms obtained after this step exhibit high thrombolytic activity in the canine arterial thrombosis model system.

Previous reports have shown that fibrolase is a zinc metalloproteinase [5,12] since it was inhibited by metal chelators such as tetraethylenepentamine, dithiothreitol (DTT), *o*-phenanthroline and EDTA [5,13]. The presence of the zinc metal ion is critical for proteolytic activity, but it is also important to the structure of the protein. Changes in both structure and function due to treatment by chelating agents have been reported for other zinc proteases such as hemorrhagic toxins in different snakes venoms [20–23]. Some of the venom enzymes appear to be very similar to fibrolase in the amino acid sequence around the zinc binding site. For example, spectroscopic methods were used to study the structure of native hemorrhagic toxin e

(Ht-e) from *Crotalus atrox* as well as structural changes caused by zinc removal and readdition [21]. By CD spectroscopy, the native toxin Ht-e was estimated to consist of 23% α-helix and 6% β-structure. When over 90% of the zinc was removed, the α-helix content dropped from 23% to 7%. When the apotoxin was incubated with zinc, an increase in α-helix content was observed from the peptide region of the CD spectrum. Thus, there appears to be significant conformational changes in Ht-e with the removal of zinc. Pretzer *et al.* [12,13] have demonstrated that removal of zinc from fibrolase can be achieved by elevated temperature, acidic pH, and addition of chelating agents (EDTA and DTT). The loss of zinc atom leads to a rapid decrease in the enzymatic activity. Circular dichroism measurements indicate that there is a partial unfolding of an α-helical segment of the protein. A decrease from 24% to 10% amount of α-helix was determined.

Bothropasin, a 48 kDa protease from *Bothrops jararaca* venom, was inhibited by EDTA and EGTA. Mandelbaum *et al.* [23] observed a time dependent conversion of the 48 kDa molecular mass band on SDS-PAGE gels to a 38 kDa molecular mass band after incubation in 10 m*M* EDTA or EGTA for up to 30 days at 4°C. They concluded that loss of metal induces an irreversible conformational change in the structure of the enzyme.

Recently, recombinant fibrolase has been produced by a yeast expression system. In view of the necessity to expose the recombinant protein to urea in the presence of zinc to enable proper refolding, we were concerned that the r-fibrolase isoforms may have originated from improper refolding and/or zinc binding. However, the observation that all r-fibrolase isoforms possess identical activity suggests this is not the case. Further, studies reported here indicate that the zinc binding affinity, as evidenced by the concentration of EDTA to cause 50% inhibition of proteolytic activity (IC_{50}) is virtually identical for all isoforms examined including fib2, r-fib1 and r-fib3. These findings provide further support for the mass spectral data suggesting that the isoforms originate from exposure to the acid pH of the yeast broth and that the isoforms are identical in all respects except for their amino-termini. We were also interested in determining whether natural and recombinant enzymes have similar susceptibility to inhibition by chelating agents. We have chosen EDTA to study this effect since we previously showed that it was an effective inhibitor of the natural protein [5]. We found that the time course of inhibition of fib1 and r-fib1 was relatively fast, virtually complete proteolytic activity was lost in 20 min using 500-fold molar excess of EDTA. A similar inhibitory time course was observed with fib2 and r-fib3.

Earlier studies investigating the effect on proteolytic activity of exchanging zinc ions for other metals (cobalt, cadmium) have been completed. Bjarnason and Fox [24] described the exchange of cobalt for zinc in hemorrhagic toxin e (Ht-e) and the properties of the cobalt containing toxin as well as the proteolytic specificity of Ht-e and cobalt Ht-e from *C. atrox* venom. Cobalt was incorporated into Ht-e by direct exchange dialysis. The cobalt Ht-e thus prepared contained 1.1 moles of cobalt per mole of toxin and no measurable zinc. The UV spectra of Ht-e and cobalt Ht-e appeared to be identical. Likewise, the CD spectra of the cobalt containing toxin in the aromatic and peptide regions appeared identical to the spectra of the native toxin. Thus there appears to be no change in structure of Ht-e accompanying the metal exchange. However, considerable structural changes were observed by CD following formation of the apotoxin by zinc removal. Zinc removal causes large structural perturbations, whereas exchanging zinc with cobalt is accomplished without observable structural changes.

Markland *et al.* have also observed reversible changes in the activity of natural fibrolase following brief treatment by EDTA [11]. Short exposure of fibrolase to EDTA avoids irreversible conformational changes. Under these conditions, dialysis against buffer containing zinc ions produce 65% regeneration of activity. However, on prolonged treatment with high concentrations of EDTA, the enzyme aggregated and precipitated with a resultant irreversible loss of activity. This suggests that the zinc ion may bind to

several amino acids from remote sites in the protein and when the metal is removed by EDTA, these residues probably lose their structural stabilizing influence, leading to unfolding, loss of activity, and eventual denaturation and precipitation. Similar results were obtained by Pretzer *et al.* [12,13] in their attempt to reconstitute zinc into fibrolase after DTT and EDTA treatment. After EDTA or DTT treatment of fibrolase, zinc is lost leading to unfolding, aggregation and precipitation, making reconstitution very difficult. In order to maintain stability of recombinant fibrolase during purification, we added low concentrations of $ZnCl_2$ to all chromatography buffers.

We found that low concentrations of EDTA do not effect fibrolase activity indicating that zinc is bound relatively tightly to the enzyme. The finding that fib1 and r-fib1 have essentially identical EDTA inhibition profiles suggests that they are identical. This is consistent both with the results of isoelectric focusing which show that fib1 and r-fib1 have identical mobilities on narrow range isoelectric focusing gels [11], and with their identical crystal habits.

5. Conclusions

Fibrolase is an active fibrinolytic enzyme with potential for use in thrombolytic therapy. The enzyme has been purified from *Agkistrodon contortrix contortrix* (southern copperhead snake) venom. The recombinant enzyme has been prepared from a yeast expression system. Both natural and r-fibrolase are metalloproteinases; they bind one mole of zinc per mole of enzyme and the metal ion is essential for retention of activity and structural integrity. We have developed a semipreparative purification procedure employing weak cation-exchange (CM 300) HPLC to resolve two isoforms of natural fibrolase and three isoforms of recombinant fibrolase. We have also studied the zinc binding affinities of the different isoforms by characterizing their sensitivities to inhibition by varying concentrations of EDTA. All isoforms appear to have identical EDTA inhibition profiles. Therefore the differences between the isoforms do not reside in their zinc binding sites. Earlier studies using isoelectric focusing revealed that the electrophoretic mobilities of fibrolase isoform 1 (fib1) and recombinant fibrolase isoform 1 (r-fib1) are very similar. Mass spectrometry of r-fib1 indicates a mass of 22 881 amu which is in excellent agreement with the theoretical mass of 22 885 amu and an amino-terminal sequence of $<$EQRFP$\cdots$. We conclude that fib1 and r-fib1 are identical. r-Fib2 has an uncyclized amino-terminal glutamine with an amino-terminal sequence of QQRFP$\cdots$ and a mass of 22 897 amu and r-fib3 is missing the amino-terminal glutamine residue with an amino-terminal sequence of QRFP$\cdots$ and a mass of 22 771 amu. Since a single cDNA clone was used for yeast expression, we conclude that r-fib2 and r-fib3 originate from acid exposure during yeast expression.

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