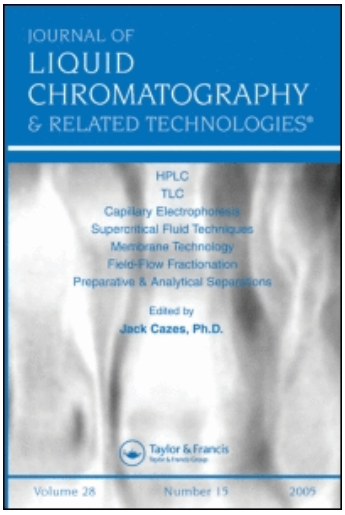


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# RESOLUTION OF ISOFORMS OF NATURAL AND RECOMBINANT FIBRINOLYTIC SNAKE VENOM ENZYME USING HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS

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## ABSTRACT

Prior studies have indicated that highly purified samples of natural fibrolase, a fibrinolytic metalloproteinase from southern copperhead snake venom, contains two isoforms. The isoelectric points of these isoforms differ by 0.01 to 0.03 pH units. In this study we show that these isoforms can be resolved by capillary zone electrophoresis (CZE) using non-treated capillaries. CZE analyses can be performed on as little as 5ng of protein. Needle-like crystals of natural fibrolase were harvested and their composition analyzed by CZE which revealed the same ratio of isoforms in the crystals as was found in the sample of natural enzyme. Recombinant fibrolase was shown to contain at least two isoforms which could be resolved by cation-exchange HPLC. Analysis by CZE revealed that the early eluting fraction from cation-exchange HPLC contained only one isoform and this isoform corresponded to one of the isoforms of natural fibrolase. The late eluting fraction contained two components when analyzed by CZE.

CZE and enzymatic activity measurements were used to assess the effects of metal replacement on fibrolase following EDTA titration. To stabilize the enzyme, 4M urea

was added to the buffers used for electrophoresis. This minimized intermolecular interactions which appeared to occur after removal of zinc. It was shown that a 30 minute incubation in 10mM EDTA completely removed zinc from the enzyme.

CZE has been shown to be a powerful tool for the analysis of protein isoforms and is useful in assessing the interactions between proteins and metal ions.

Key words: capillary electrophoresis, HPCE, CZE, fibrolase, metalloproteinase, isoforms

## INTRODUCTION

Fibrolase is a fibrinolytic enzyme that has been purified from southern copperhead (*Agkistrodon contortrix contortrix*) snake venom. The enzyme is a zinc metalloproteinase containing one mole of zinc per mole of enzyme. Fibrolase has a molecular weight of 23 kDa (1) and is not glycosylated. The sequence of the enzyme is known and it contains 203 amino acids with an amino-terminus blocked by a cyclized glutamine residue (2). The enzyme possesses direct-acting fibrinolytic activity and does not activate plasminogen. *In vitro* studies reveal that the enzyme degrades clots made from purified fibrinogen or from whole blood (3). Interestingly, the primary structure of fibrolase shows extensive sequence homology with several small snake venom hemorrhagic metalloproteinases (2, 4-7). However, fibrolase possesses no hemorrhagic activity. Thus when fibrolase is injected under the skin of the backs of experimental animals, it does not induce rupture of capillary endothelium leading to leakage of blood components into the surrounding tissue (3). Despite this apparent difference in activity profile, these two classes of enzymes, the fibrinolytic and hemorrhagic venom metalloproteinase, appear to be structurally very similar. Perhaps subtle evolutionary changes in protein structure have produced these two (or more) enzyme subgroups with different inherent biological activities. On this basis alone there is strong rationale for determining the three-dimensional structure of one of the venom metalloproteinases. Additionally, sequence similarity exists in the zinc-binding region between fibrolase, the venom hemorrhagic metalloproteinases, and mammalian matrix-degrading metalloproteinases (MMP's) (2). There is also evidence of similar substrate preference between small venom metalloproteinases and MMP's (8,9).

Because of the potential therapeutic value of fibrolase as a thrombolytic agent, there is considerable interest in its 3-dimensional structure and the relationship between

structure and function. Unfortunately only needle-like crystals have been obtained from our attempts to prepare crystals suitable for x-ray diffraction analysis. It is likely that the presence of two isoforms, as revealed by earlier studies (10), has frustrated our efforts to crystallize the enzyme. In this report we describe a rapid and reproducible method for the detection and quantification of these two isoforms using high performance capillary electrophoresis (HPCE). The method has been applied to native fibrolase, recombinant fibrolase, and fibrolase crystals. Additionally we studied the effects of EDTA on the electrophoretic mobility and stability of the metalloenzyme and apoprotein.

## METHODS AND MATERIALS



### Preparation of fibrolase

Southern copperhead venom was obtained from Bio-Toxins Inc. Fibrolase, the fibrinolytic enzyme from southern copperhead venom, was purified either by a four step open column procedure as previously described (1), or by a three step procedure including hydrophobic interaction, hydroxyapatite, and anion exchange HPLC in a method similar to that described by Retzios and Markland (11). The purity of these preparations was assessed by SDS PAGE, IEF, and reversed phase HPLC as previously described (1,11).

### Preparation of recombinant fibrolase

Recombinant fibrolase, prepared from a yeast expression vector, was provided by Chiron Corporation (Emeryville, CA). The enzyme was further purified by cation exchange high performance liquid chromatography (CE-HPLC) using a TSK SP-5PW column (Toya Soda) and a System Gold HPLC (Beckman Inst.) equipped with a 406 interface, 114M pumps and a model 168 diode array detector (see Figure 2). An early eluting 280 nm absorbing peak did not contain protein as evidenced by IEF with silver staining. Fibrolase eluted in the first of two protein peaks as demonstrated by IEF using natural fibrolase as a marker.

### Isoelectric focusing

CE-HPLC fractions were analyzed by IEF using precast polyacrylamide gels containing ampholytes (PAG Plates, pH range 5.5-8.5, Pharmacia LKB Biotechnology).

Electrophoresis conditions were as described in the manufacturers instruction booklet (12). Proteins were visualized by silver staining using a Bio-Rad kit (13).

**Capillary electrophoresis**

Natural fibrolase, EDTA inhibited fibrolase, enzyme treated with zinc, cobalt or cadmium, recombinant fibrolase, and crystals of the natural enzyme were all analyzed by capillary zone electrophoresis (CZE). Analyses were performed on a Beckman PACE 2100, high performance capillary electrophoresis (HPCE) instrument using non-treated capillaries. Capillaries used had an effective length of 50 cm (to detector) and a 50 micron inner diameter. The buffer electrolyte for all separations was 100mM Tris-HCl, pH 7.6, unless otherwise noted. A stock solution of 200mM EDTA was prepared from the sodium salt and its pH adjusted to neutral to minimize any pH change that may occur when it was added to be buffers used in some of the experiments. Sample loading onto the capillary was accomplished by a 3 second pressure injection from 5 ul of a solution containing 0.25 - 0.5 mg/ml protein. Electrophoresis was carried out at 15, 20, or 25 kV, and absorbance was monitored at 200nm. Data was collected and analyzed using System Gold software (Beckman Instruments).



**Azocaseinolytic and Fibrinolytic Activity**

Azocaseinolytic and fibrinolytic activity of natural and recombinant fibrolase were determined using a previously described procedure (11).

## RESULTS

**Natural fibrolase**

Preliminary CZE experiments were monitored at 280nm, however the high protein concentrations required to produce an adequate signal caused band broadening, and may have resulted in overloading of the capillary. Because of these difficulties subsequent experiments were monitored at 200nm, which improved sensitivity, eliminated the need for concentrated protein solutions, and reduced sample consumption.

CZE analysis of a preparation of natural fibrolase, purified as previously described, clearly revealed the presence of two isoforms (Figure 1A). The early eluting isoform is

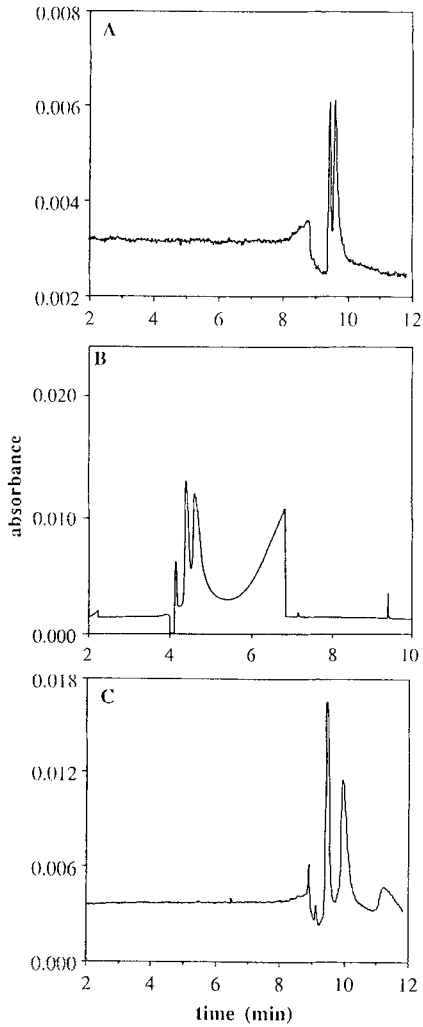


**Figure 1.** Analysis of natural fibrolase by CZE. **A**. Highly purified natural fibrolase (0.5 mg/ml) was diluted with an equal volume of 100mM Tris-HCl buffer (pH 7.6) and analyzed by CZE at 15kV in the Tris-HCl buffer. Absorbance at 200nm was monitored. The ratio of the areas of peak 1 to peak 2 was 44:56 **B**. Analysis of needle-like crystals which were obtained using the material in '**A**'. Crystals were grown from a 10µl hanging drop by vapor diffusion against a reservoir containing 0.1M MOPS (pH 7.2) with 21% saturated ammonium sulfate. The crystals from one drop were removed and washed three times with 33% saturated ammonium sulfate and dissolved in 50mM Tris-HCl buffer for analysis. CZE was performed in Tris-HCL buffer at 25kV and absorbance monitored at 200nm. The ratio of peak 1 to peak 2 was 37:63. **C**. Recombinant fibrolase analyzed under the conditions described in '**A**' above.

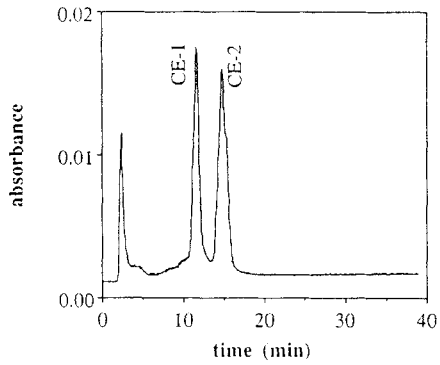


**Figure 2.** Purification of recombinant fibrolase by high performance cation-exchange chromatography using a TSK SP-5PW column (7.5 x 75 mm). The solvent system used for purification was: 30mM MES, pH 6.4 (buffer A), and 30mM MES, pH 6.4, with 0.5M NaCl (buffer B). Both buffers contained 0.1mM $ZnCl_2$ to protect the enzyme from inactivation. The column was equilibrated in buffer A and approximately 35 mg of recombinant fibrolase (17.7 mg/ml) applied to the column. The column was then eluted at 1 ml/min using the following gradient: isocratic at 100% A for 1 min; a linear gradient from 0% to 50% B in 15 min; a linear gradient from 50% to 100% B in 5 min and hold at 100% B for 5 min. Absorbance at 280nm, azocaseinolytic and fibrinolytic activity of selected fractions were measured to identify the fibrolase containing fractions. Both CE-1 and CE-2 had azocaseinolytic and fibrinolytic activity, but only CE-1 co-migrates with fibrolase on an IEF gel.



the minor one comprising 34-45% of the total mass, as determined by absorbance at 200nm. Attempts to crystallize natural fibrolase resulted in the formation of needle shaped crystals, which is often caused by heterogeneity of the protein sample. Analysis of these needle shaped crystals, which were harvested, washed with 33% saturated ammonium sulfate, and then solubilized with 100mM Tris, revealed the presence of the same two isoforms in roughly the same proportions (Figure 1B). It should be noted that CZE analysis of the crystals was performed at 25kV rather than 15kV used in the analysis of the natural protein. The negative peak observed in the analysis of the crystals at 4.0 minutes and the rise in baseline from about 5.5 to 7 minutes (Figure 1B) have been attributed to the effect of residual ammonium sulfate in the sample and these assignments confirmed by injection of 33% ammonium sulfate in Tris buffer (data not shown).

**Recombinant fibrolase**

Attempts to crystalize a preparation of recombinant fibrolase also resulted in the formation of needle shaped crystals. CZE analysis of the recombinant enzyme, under conditions identical to those used to identify isoforms of natural fibrolase, revealed the presence of three peaks (Figure 1C), only one of which corresponded to an isoform of natural fibrolase. Cation exchange HPLC of the recombinant enzyme preparation produced two well separated protein peaks (Figure 2), both of which contained azocaseinolytic and fibrinolytic activities. The material isolated from each peak was examined by IEF. The first peak (CE-1) contained a protein that co-migrated with natural fibrolase and the second peak (CE-2) contained a more basic protein (data not shown).

Analysis of the CE-HPLC purified recombinant fibrolase (CE-1 in Figure 2) revealed only one component (Figure 3A). As indicated above, this protein was identical to natural fibrolase by IEF. Analysis of CE-2 (Figure 2) revealed the presence of two components (Figure 3B). When a small amount of the CE-2 protein was mixed in equal volume with the purified recombinant fibrolase (CE-1) and applied to CZE, three well separated peaks were obtained eluting at 7.46, 7.78, and 8.54 minutes, representing purified recombinant fibrolase, CE-1, and the two proteins from CE-2, respectively (Figure 3C).



**Stability and metal ions**

When fibrolase was incubated with EDTA, enzymatic activity was lost. Studies aimed at reversing the effect of EDTA on enzyme activity by dialyzing the EDTA treated enzyme against zinc, cobalt, or cadmium revealed that the time of exposure to EDTA was critical. Only minimal reactivation was observed following a 110 minute incubation. Following a 30 minute incubation with EDTA, which produced complete loss of activity, dialysis against zinc restored 63% of the activity (Table 1). Under these conditions cobalt was able to restore some activity but was only 40% as effective as zinc. Cadmium was not an effective enzyme activator.

CZE analysis of natural fibrolase after treatment with EDTA revealed a loss of recovered mass (data not shown). When EDTA treatments were carried out in the presence of 4M urea to prevent possible protein aggregation or precipitation, no loss of mass was observed. However, the two isoforms of natural fibrolase could not be resolved

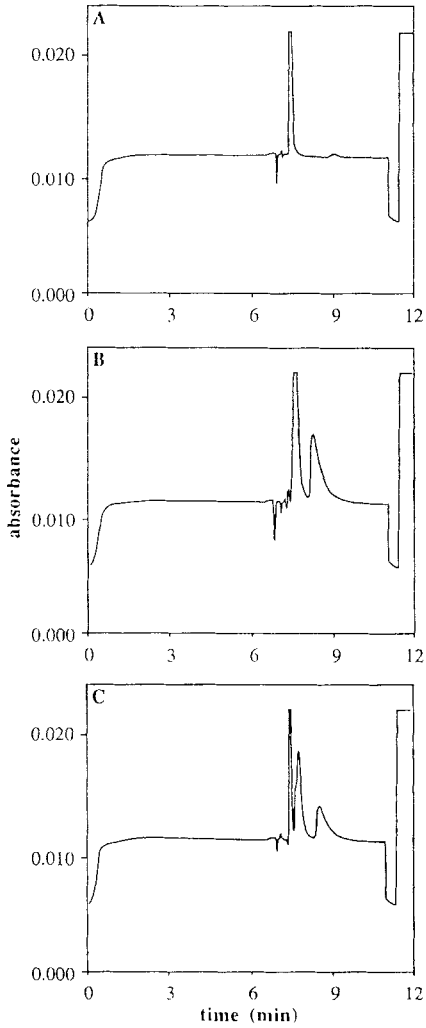


**Figure 3.** CZE analysis of recombinant fibrolase purified by cation-exchange-HPLC. The concentrated proteins CE-1 (75 mg/ml) and CE-2 (105 mg/ml) were diluted 1:50 with 100mM Tris-HCl buffer (pH 7.6). CZE was performed at 20kV in 100mM Tris-HCl buffer (pH 7.6) and absorbance monitored at 200nm. **A.** Analysis of CE-1; one peak was observed with an elution time of 7.33 minutes. **B.** Analysis of CE-2; two peaks were observed eluting at 7.52 and 8.25 minutes. **C.** Equal volumes of the diluted samples used in **A** and **B** above were mixed and the mixture analyzed by CZE. Three peaks were observed eluting at 7.46, 7.78, and 8.54 min corresponding to the 7.33, 7.52, and 8.23 min eluting peaks, respectively.

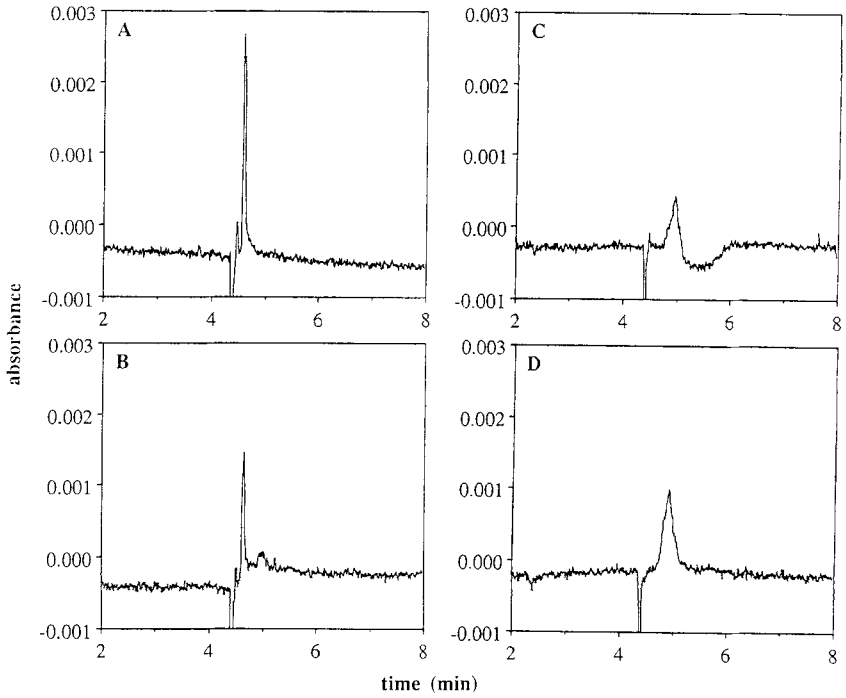


**Figure 4.** The effect of EDTA on the electrophoretic mobility of natural fibrolase. All analyses were performed at 25kV in 50mM Tris-HCl (pH 7.6) containing 4M urea to prevent protein aggregation. The fibrolase concentration was approximately 0.25 mg/ml in the Tris buffer containing urea. **A.** Natural fibrolase; note the loss of resolution between the two isoforms in the presence of 4M urea. The protein eluted at 4.63 minutes. **B.** Natural fibrolase incubated 20 min. with 1mM EDTA. Elution time is still 4.66 minutes. **C.** Natural fibrolase incubated 20 min. with 10mM EDTA. Elution time has shifted to 4.97 minutes. **D.** Natural fibrolase incubated 20 min. with 100mM EDTA. Elution time still shifted to 4.91 minutes.

under these conditions (Figure 4A). Analysis of natural fibrolase treated with EDTA, in the presence of urea, revealed a shift in the elution time from 4.62 min to 4.90 min and an increase in peak width (Figure 4C and 4D). An EDTA concentration of greater than 10mM was required to drive this shift to completion. Treatment with 1mM EDTA (Figure 4B) resulted in a partial shift in the elution time (i.e. the peak at 4.62 was still observed and a new peak at 4.90 minutes began to appear).

## DISCUSSION

Because of the potential therapeutic value of fibrolase, and its sequence homology to enzymes with markedly different biologic activities, there is considerable interest in its 3-dimensional structure. Unfortunately the presence of two isoforms has frustrated attempts to prepare crystals suitable for x-ray analysis. Initial crystallization trials using ammonium sulfate and polyethylene glycol as precipitating agents have produced only needle-like crystals that are unsuitable for structural analysis. Prior studies have suggested that natural fibrolase exists as two closely related isoforms with isoelectric points of about 6.85. This conclusion is based on the results of immobilized pH gradient isoelectric focusing (IPG) (10) and amino acid sequence analysis (2). In the present report we present convincing evidence that highly purified natural fibrolase does contain two isoforms. These isoforms, which IPG had previously shown to differ in isoelectric points by 0.01 to 0.03 pH units, were resolved by CZE using non-treated capillaries in a Tris-HCl buffer, pH 7.6. These findings suggest that our repeated inability to grow suitable crystals of fibrolase may have been due to the mixture of isoforms of the natural protein. Additional support for this conclusion was derived from CZE analysis of the protein crystals prepared from ammonium sulfate, which after washing and solubilization revealed the same two isoforms in essentially the same relative proportions as the starting non-crystalline natural protein (Figure 1).



In an attempt to overcome the problem of multiple isoforms, we have obtained recombinant fibrolase expressed from a single cDNA clone (Chiron Corporation, Emeryville, CA). Analysis of the recombinant protein using IEF, on a pH 5.5 to 8.5 PAG Plate, followed by silver staining, revealed two bands. These were resolved by cation-exchange HPLC and both possess fibrinolytic and azocaseinolytic activities. This suggests that the proteins in each peak are fibrolase or fibrolase-like, as fibrolase possesses azocaseinolytic activity as well as fibrinolytic activity. Using IEF it was determined that CE-1 contains a protein identical to natural fibrolase, while the proteins in CE-2 are more basic, as expected by their later elution during CE-HPLC. Using CZE under conditions which resolve the two isoforms of natural fibrolase, the recombinant protein CE-1 was shown to contain only one protein component. Preliminary crystallization studies with this homogeneous preparation of recombinant protein have produced

**Table 1.**

Inhibition of fibrolase by EDTA and restoration of activity by various metal ions. (All values represent the average of two independent determinations.)

| Conditions | Azocasein Activity |
|---|---|
| Enzyme control | 100% |
| EDTA treated | 0.5% |
| Dialyzed control | 95% |
| EDTA treated / dialyzed | |
| vs. 1mM zinc | 63% |
| vs. 1mM cadmium | 1.6% |
| vs. 1mM cobalt | 24% |



large crystals of an equidimensional habit rather the needle-like crystals obtained previously [1].

In relation to our interest in obtaining crystals of fibrolase suitable for x-ray diffraction, we have also examined reversal of EDTA inhibition of the natural enzyme (3) by different metal salts with the hope of obtaining a suitable derivative for phasing of the x-ray data. Prolonged exposure to EDTA apparently irreversibly denatures fibrolase or leads to aggregation and/or precipitation of the protein. Analysis of the EDTA-treated enzyme revealed that the apoenzyme (zinc-free form) was unstable and produced an altered and variable electrophoretic profile on CZE. Further evidence to support the formation of aggregates or precipitate was the loss of mass during a time series analysis

[1] Manuscript in preparation: Markland, F.S., Deschamps, J.R. and Ward, K.B. 1992. Preparation and initial characterization of crystals of fibrolase, the fibrinolytic enzyme from *Agkistrodon contortrix contortrix* snake venom.

of EDTA treated fibrolase and by the occurrence of excessive 'noise' in the later portion of the electropherogram, which was likely the result of protein aggregates passing through the detector. When EDTA treatment was carried out in the presence of 4M urea and CZE was run in buffers containing urea, the apoenzyme appeared to be stabilized and a reproducible and interpretable profile was obtained, but resolution of the two isoforms of natural fibrolase was lost. These findings revealed that there is a shift in the electrophoretic mobility of the apoenzyme relative to the natural zinc-associated enzyme.

We found that inhibition by EDTA can be reversed by zinc and cobalt, but not by cadmium salts (Table I). A 30 minute exposure to EDTA was chosen as the optimal time to completely inhibit the enzyme, yet still allow recovery of 65% of the activity following dialysis against buffer containing 1mM $ZnCl_2$.

These studies have demonstrated the ability of CZE to separate closely related isoforms and to serve as a useful technique to examine alterations in electrophoretic mobilities of proteins caused by structural perturbations. HP-CZE represents an exquisitely sensitive electrophoretic technique with very high resolving power. Injection volumes of 20nl are typical and as little as 5ng of protein are required for each analysis. Enhancement of the signal to achieve this sensitivity is obtained by monitoring absorbance at 200nm. We have shown with fibrolase, in the appropriate buffers and with the appropriate controls, that results obtained at 200nm reflect the same phenomenon observed as when monitoring at 280nm.



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