

## A STUDY OF PROTEASES AND PROTEASE-INHIBITOR COMPLEXES IN BIOLOGICAL FLUIDS\*

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In view of the many important processes that are catalyzed and regulated by specific proteases, the study of extracellular proteolytic reactions is of increasing and general interest. The characterization of these reactions may present difficulties which vary depending on the reaction conditions and the degree of purity of the system under examination. Impure and complex mixtures such as native plasma or serum contain many proteases, zymogens, and inhibitors capable of interacting either to amplify or to terminate a particular reaction of interest; dilution of plasma or serum reduces the concentration of all protein species and may thereby initiate a variety of artefactual reaction, owing either to the dissociation of normally inactive protein complexes or to the reduction of inhibitor concentrations, or both; finally, reactions limited to highly purified species may be associated with extensive autolysis of proteases, promiscuous rather than limited proteolysis of substrates, and a variety of other nonphysiological hydrolytic events.

These considerations emphasize the need for procedures that could (a) reveal the number and identity of catalytically active species appearing in proteolytic reactions, (b) permit analysis of events taking place at short time intervals, and (c) be applicable to impure systems such as plasma and other body fluids. With this in mind, we have made an initial effort to explore the potential usefulness of sodium dodecyl sulfate (SDS)<sup>1</sup>-polyacrylamide gel electrophoresis as a general method for separating extracellular proteinases. The rationale for this approach is based on two facts: (a) the activity of most vertebrate proteinases is abolished in the presence of SDS; hence, suitable concentrations of this detergent could be expected rapidly to terminate any sequence of proteolytic reactions. (b) The inactivation by SDS of at least one specific proteinase-plasminogen activator is reversible, since the activity is recovered on removal or dilution of detergent (1, 2); this enzyme, like most other extracellular and neutral proteinases is a serine enzyme, and it seemed possible that other serine enzymes might be reactivated in the same way. We have therefore surveyed the behavior of a series of serine proenzymes, proteases, and protease inhibitor complexes after SDS-polyacrylamide gel electrophoresis.

In previous work (1), plasminogen activator was located after electrophoresis

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<sup>1</sup> Abbreviations used in this paper:  $\alpha_2$ -M,  $\alpha_2$  macroglobulin; MEF-MSV, mouse embryo fibroblast transformed with MSV; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; REF-SV40, rat embryo fibroblast transformed with SV40; SDS, sodium dodecyl sulfate.

by assaying individual slices of the SDS gel for enzyme activity. Under these conditions the SDS in each slice was diluted by the relatively large volume of the assay solution to a concentration at which the enzyme was no longer inhibited. This procedure yields reproducible results, but it is both laborious and time-consuming. To retain the advantages of SDS-polyacrylamide gel electrophoresis (PAGE) under conditions permitting more rapid, precise, and economical detection of proteases, we have applied the method of Converse and Papermaster (3) and have used solutions of nonionic detergents for extracting SDS after completion of electrophoresis. This procedure efficiently removes SDS but does not extract significant quantities of protein; the electrophoretic slab gel can then be layered onto a second indicator gel consisting of fibrin and agar. Zones of fibrin degradation, corresponding to the position of proteases in the SDS-gel overlay, can be seen as clear areas in an opaque background on dark field illumination, or as clear areas in a dark blue background after fixation and staining with Coomassie Blue or amido black. The results presented below indicate that this procedure is a versatile one and can be applied to study a broad spectrum of proteolytic events.

### Materials and Methods

**Materials.** Reagents were obtained as follows: acrylamide, bis-acrylamide, *N,N,N',N'*-tetramethylethylenediamine and ammonium persulfate (Eastman Kodak Co., Rochester, N.Y.); chymotrypsin, trypsin, factor Xa, Lubrol PX Triton X-100, hemoglobin, cephalin, and Coomassie Brilliant Blue were all from Sigma Chemical Co., St. Louis, Mo. heparin (Bio-Heprin, Ries Biological, Inc.); SDS, specially pure (BDH Chemicals, Ltd., Poole, England); purified agar (Difco Laboratories, Detroit, Mich.); urokinase (Leo Pharmaceutical Co., Ballerup, Denmark) elastase (purified elastase from swine pancreas 72.7 U/mg, Worthington Biochemical Corp., Freehold, N.J.); thrombin topical (bovine origin, Parke-Davis Co., Detroit, Mich.); purified human thrombin was the kind gift of Dr. P. Harpel, Cornell Medical School, New York; ampholine (LKB Products, Bromma, Sweden).

Bovine fibrinogen (60% clottable) was purchased from Sigma Chemical Co. and further purified by the method of Laki (4) to >95% clottability. The fibrinogen was then freed of plasminogen by the method of Mosesson (5), and stored at  $-20^{\circ}\text{C}$  at a concentration of 10 mg/ml in  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -free isotonic saline containing sodium phosphate buffer (0.001 M, pH 7.4) (PBS).

Human plasminogen was prepared by two cycles of affinity chromatography from outdated human plasma as described elsewhere (6), lyophilized, and stored at  $-20^{\circ}\text{C}$ .

$^{125}\text{I}$ -plasminogen and  $^{125}\text{I}$ -trypsin were prepared by iodinating the respective enzymes with ICl and  $\text{Na}^{125}\text{I}$  as described by Helmkamp et al (7); a three to fivefold molar excess of ICl relative to protein was used, and the yield of incorporated radioactivity was routinely 50-60%, based on the total initial  $^{125}\text{I}$ .

Human plasmin was generated by incubating plasminogen (7.5 mg/ml) with urokinase (100 Plough U/ml) in 0.1 M Tris Cl buffer, pH 8.1 containing 50% glycerol for 24 h at  $30^{\circ}\text{C}$ . Plasma samples were always obtained and prepared immediately before use. Freshly drawn, heparinized blood was centrifuged, sequentially, for 10 min at 800 *g*, and for 15 min at 12,000 *g* in the cold, both pellets were discarded. Serum free conditioned medium was prepared exactly as described elsewhere (8).

Crude bovine prothrombin was kindly provided by Dr. Y. Nemerson, State University of New York, Stony Brook, N.Y., in the form of the eluate from the barium citrate precipitate of fresh bovine plasma.

$\alpha_2$  Macroglobulin was prepared from human plasma as described by Harpel (9).

**Methods.** Plasminogen activator was assayed by using the  $^{125}\text{I}$ -fibrin plate method previously described (10). 1 U of enzyme was defined as the amount that catalyzed the solubilization of 5% of the total radioactivity in 2 h under standard conditions.

**SDS-PAGE.** Gels and buffers were prepared according to Laemmli (11).  $9 \times 15\text{-cm}$  slab gels

with stacking gels of 4% acrylamide were used. Samples contained a final concentration of 2.5% SDS. Electrophoresis was performed at constant current (8 mA) until the dye front reached the bottom of the gel, approximately 18 h. After completion of the run the gel was sliced longitudinally between the appropriate lanes and washed, by gentle rocking for 1 h, in a solution of 2.5% (wt/vol) Triton X-100 or Lubrol-PX. The gel was then rinsed thoroughly with distilled water and applied to the surface of a fibrin-agar indicator gel.

*Fibrin-Agar Indicator Gel Plates.* The fibrin-agar gels were prepared by using the following solutions: (a) a 2.5% solution of agar in water, boiled for 10 min before use and kept at 42°C; (b) PBS supplemented, where desired, with plasminogen (18  $\mu\text{g}/\text{ml}$ ) and thrombin (0.2 U/ml), warmed to 42°C; (c) fibrinogen (10 mg/ml) in PBS. 3.2 ml of solution (a) was added to 1.9 ml of solution (b) and, after mixing, 1.3 ml of solution (c) was added. The solutions were mixed rapidly and then poured into the 14  $\times$  9-cm plastic covers of Linbro multiwell dishes (Linbro Chemical Co., New Haven, Conn.) (FB16-24TC), care was taken to avoid bubbles. The solution was spread evenly on the surface of the dish, allowed to form a firm gel, and used immediately.

The washed electrophoretic gel was laid carefully onto the indicator gel, the unit sealed with plastic wrap, and incubated at 37°C in a humid environment. At intervals the plates were removed for observation in dark-field immunodiffusion viewer (Dade Div., American Hospital Supply Corp., Miami, Fla.). The lysis zones were easily seen as clear areas in the cloudy background provided by the unlysed fibrin. Depending on the concentration of enzyme in the electrophoretic gel lysis zones may become visible at times ranging from 1 to 36 h. After the reaction had progressed to the desired extent, the electrophoretic gel was removed, the fibrin layer was stained for 10 min with a solution containing 0.1% amido black in 70% methanol, 10% acetic acid, and destained in 70% methanol, 10% acetic acid. Alternatively, gels were also stained with 0.25% Coomassie brilliant Blue in 50% methanol, 7% acetic acid, and destained in 30% methanol, 10% acetic acid.

*Quantitation of the Assay.* For quantitative comparison of proteolytic activity, washed electrophoretic gels were placed onto fibrin-agar gels that had been coated on the surface of 2  $\times$  14-cm glass slides. These slides were incubated as described above for coated plastic surfaces. After appropriate incubation the polyacrylamide gel was removed, the fibrin-agar gel fixed with destaining solution, and dried. The lysis zone was then analyzed by densitometry in a Joyce-Loebl model E12 MK3 densitometer, and the size of the peak was measured by cutting out the area on the graphic representation of the scan and weighing it.

## Results

The gel assay procedure described below was developed for screening the plasminogen activator content of large numbers of samples and for unmasking cryptic or inhibited forms of the enzyme. To identify plasminogen activators duplicate SDS-gels were tested on two fibrin-agar indicators, one of which had been supplemented with plasminogen. As shown in Fig. 1 for crude samples of unpurified urine and cell culture fluids the presence of plasminogen permitted the development of distinct zones of lysis. For all of the samples tested no lytic bands were observed in the absence of plasminogen, indicating that the major source of SDS-resistant proteolytic activity in these fluids was due to plasminogen activator. Several bands of plasminogen activator were present in each specimen, and the molecular weights of the human enzymes differed significantly from those of the rodent species. A more comprehensive survey of plasminogen activators secreted by a variety of cell lines and strains gave the results summarized in Table I. In general, the predominant molecular weights from the plasminogen activators found in cell culture media were lower than those of the plasma enzymes from the same species, suggesting that the cell culture enzymes might be partial degradation products derived from a larger molecule. However, we do not yet have any direct evidence to substantiate this inference.

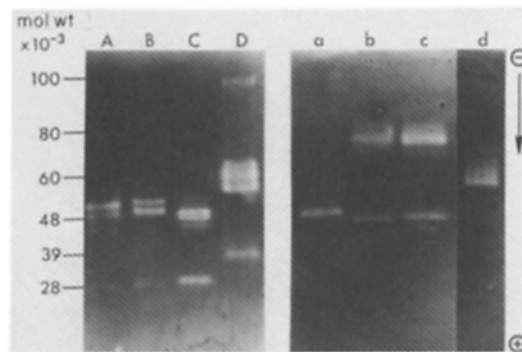


FIG. 1. Identification of plasminogen activator after SDS-PAGE of urine and conditioned media from cultured cells. Urine ( $2 \mu\text{l}$ ) from A: hamster, B: mouse, C: rat, D: human; and samples of conditioned medium from cultures of a: hamster-SV40 (18 U); b: mouse F26 melanoma (20 U); c: rat embryo fibroblast transformed with SV40 (REF-SV40) (20 U); d: human osteosarcoma (18 U) were electrophoresed in SDS-11% polyacrylamide slab gel and the gel processed for detection of fibrinolytic activity in the presence of plasminogen as described in Materials and Methods. The figure is a photograph of the amido-black stained film in the fibrin-agar indicator gel.

The spectrum of plasminogen-dependent and independent proteolysis was somewhat different in plasma from various species (Fig. 2). Both types of indicator gels revealed multiple bands, showing the presence of plasminogen activators as well as other proteases whose electrophoretic mobilities placed them in the same region of the gel. Detailed analysis of these patterns for human plasma has shown (unpublished observations, W. D. Schleuning, A. Guha, P. Aiyappa, and E. Reich) that all of the plasminogen-independent lytic zones were due to different electrophoretic forms of plasmin, whereas the plasminogen-dependent lysis was produced either by a newly isolated plasminogen activator, or, occasionally, by kallikrein.

To explore the possibility of performing the assay under conditions permitting quantitation, the effect of several variables was assessed by densitometric scanning of the dried indicator gels (Figs. 3-5). Regions of good linearity were obtained in the kinetics of fibrinolytic activity (Fig. 3), and in the dose-response curves reflecting the changing concentrations of plasminogen activator and plasminogen, respectively, in the electrophoretic (Fig. 4) and indicator gels (Fig. 5). These results showed that the sensitivity of the assay could be adjusted within a wide range, by varying the plasminogen concentration and time of incubation to meet the demands of a particular experimental framework. In addition, good quantitation was possible provided that the method was calibrated by using known and purified reagents.

The preceding results suggested that slight changes in method might increase both the versatility of the assay and the scope of its application, and the following variations have been tested successfully: (a) plasminogen can be identified in the electrophoretic gel by reversing the conditions used for locating plasminogen activators, that is, by incorporating a plasminogen activator (e.g. human urokinase) into the fibrin-agar underlay. This approach could be useful for detecting plasminogen at concentrations below those required for activation

TABLE I  
*Apparent Molecular Weights of Different Plasminogen Activators*

	80,000	70,000	60,000	48,000	39,000	28,000
Chicken TS68				+		
Chicken RSV				+		
Chicken plasma	> +					
Hamster SV40				+		
Hamster urine				+		+
Hamster plasma	> +			+		
REF-SV40	< +			+		
Rat mammary carcinoma tumor	< +			+		
Rat granulosa cells	< +			+		
Rat urine				+		+
Rat uterine fluid	< +			+		
Rat plasma	> +			+		
NIH-3T3	< +					
MEF-MSV				+		
Mouse macrophages				+		+
Mouse urine				+		+
Mouse granulosa cells	< +			+		
Mouse F 26 melanoma	< +			+		
Mouse neuroblastoma	< +					
Mouse teratoma	< +			+		
Mouse epidermal cells	< +			+		
Mouse plasma	> +			+		
Human melanoma		+	+			
Human rhabdomyosarcoma			+			
Human embryo lung			+			
Human osteosarcoma			+			
Human urine			+			+
Urokinase (purified from human urine)			+	+		+
Human liposarcoma			+			
Human pancreas carcinoma			+			
Human polymorphonuclear leukocytes			+			
Human epidermal cells						
Human plasma	> +					

Molecular weight of plasminogen activator present in biological fluids and in conditioned medium from cultured cells. Samples of conditioned medium and biological fluids were electrophoresed in SDS 11% polyacrylamide slab gel and tested on the fibrin-agar indicator gel in presence of plasminogen. The apparent molecular weights were determined by using bovine serum albumin, chicken ovalbumin, chymotrypsinogen, and ribonuclease A as standards. Since the electrophoresis was not performed under reducing conditions the molecular weight values must be taken as approximate. > mol wt slightly in excess, and, > mol wt slightly lower than 80,000.

in free solution (i.e., below  $K_m$  for activators), since electrophoresis in gradient gels, which crowds the molecules into narrow bands, provides a high local concentration permitting efficient activation.

(b) General proteases such as trypsin, chymotrypsin, and elastase hydrolyse fibrin directly and they are easily detected by using plasminogen-free fibrin-agar indicators (12-13).

(c) By substituting fibrinogen for fibrin, supplementing the indicator gel

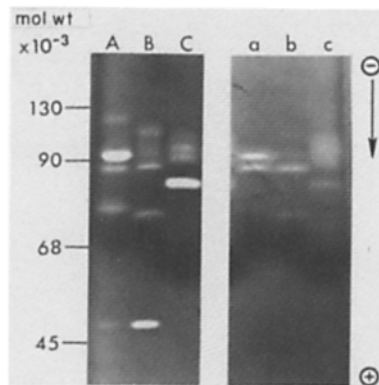


FIG. 2. Identification of plasma proteases after SDS-PAGE. Plasma samples 1-2- $\mu$ l were electrophoresed in SDS-polyacrylamide (6-13% gradient) slab gels and the gels processed for fibrinolytic activity either in presence (A, B, C) or in absence (a, b, c) of plasminogen as described in Materials and Methods. A,a: mouse plasma; B,b: rat plasma; C,c: human plasma. The figure is a photograph of the amido-black stained fibrin-agar indicator gel. The dark areas in the 68,000 mol wt region of the plates is due to the plasma albumin from the SDS gel that diffuses into the fibrin-agar gel during the assay.

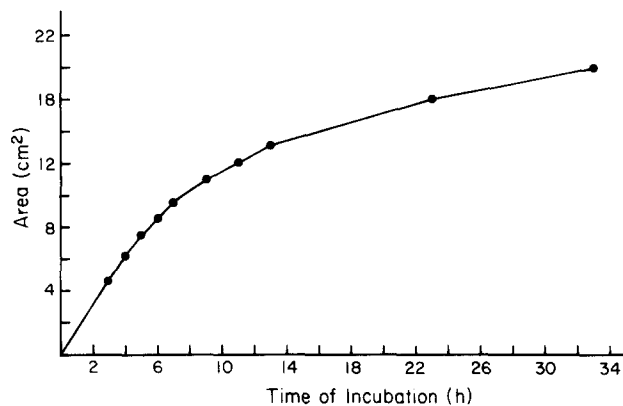


FIG. 3. Kinetics of the fibrinolytic assay. Conditioned medium from hamster-SV40 cells containing 34 U of plasminogen activator were electrophoresed in SDS-11% polyacrylamide slab gel. Single lanes of the electrophoretic gel were cut and incubated on fibrin coated glass slides (Materials and Methods). At different times the slides were fixed and dried and analyzed by densitometry. Each point represents the average of duplicate determinations.

with appropriate cofactors, and observing the reaction under dark-field illumination, it was possible to use clotting rather than fibrinolysis as an end-point and thereby to locate enzymes of the coagulation pathway in the SDS-PAGE. Examples showing such application to prothrombin and thrombin are illustrated in Fig. 6.

(d) Because the method yields an estimate of molecular weight for each band of proteolytic activity detected, it appeared to be advantageous for analyzing some properties of complexes formed between proteases and various inhibitors, especially those found in plasma. Examples of such applications to the com-

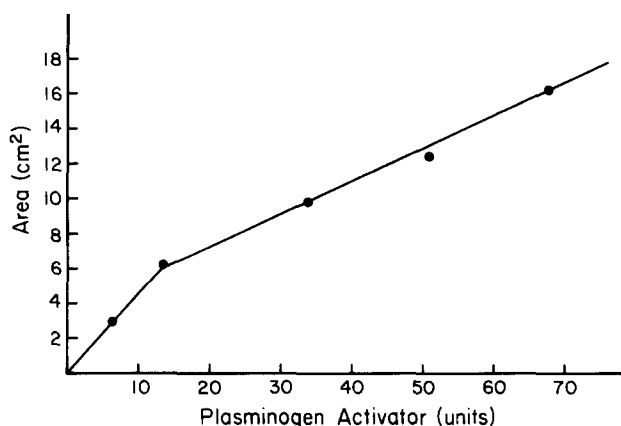


FIG. 4. Dose-response curve of fibrinolytic assay: area of lysis zones as a function of increasing plasminogen activator concentration. Conditioned medium from hamster-SV40 cells containing 6.8, 13.6, 34, 51, and 68 U of plasminogen activator were electrophoresed in SDS 11% polyacrylamide slab gel. Single lanes were cut and incubated for 5 h on glass fibrin-coated slides (Materials and Methods). The fibrin slides were then fixed, dried, and analyzed by densitometry. Each point represents the average of duplicate determinations.

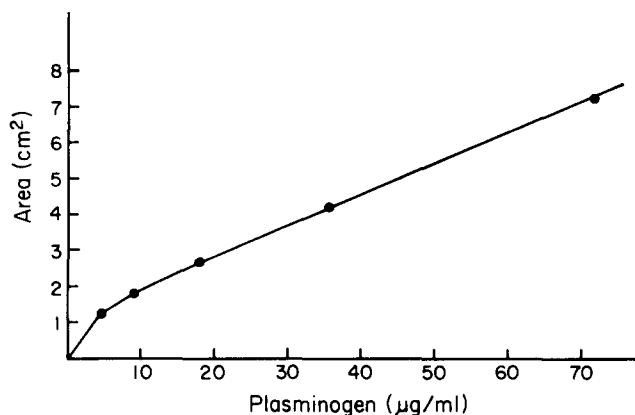


FIG. 5. Dose-response curve of fibrinolytic assay: area of lysis zones as a function of plasminogen concentration in the fibrin-agar indicator gel. Conditioned medium from hamster-SV40 cells containing 34 U of plasminogen activator were electrophoresed in SDS-11% polyacrylamide slab gel. Single lanes were cut and incubated on glass fibrin-coated slides prepared with increasing concentrations of plasminogen: 0 µg; 4.5 µg; 9 µg; 18 µg; 36 µg; 72 µg. After 7 h the slides were fixed, dried, and analyzed by densitometry. Each point represents the average of duplicate determinations.

plexes involving  $\alpha_2$ -macroglobulin ( $\alpha_2$ M) and trypsin or plasmin, respectively, are presented in Figs. 7 and 8.

In these experiments the proteases were first labeled with <sup>125</sup>I to permit their detection by autoradiography, and then reacted with  $\alpha_2$ -macroglobulin; several samples of each reaction were subjected to SDS-PAGE and duplicate lanes from the gels were then analyzed further either by autoradiography to locate all labeled species, or by fibrin-agar gels to identify those that retained proteolytic

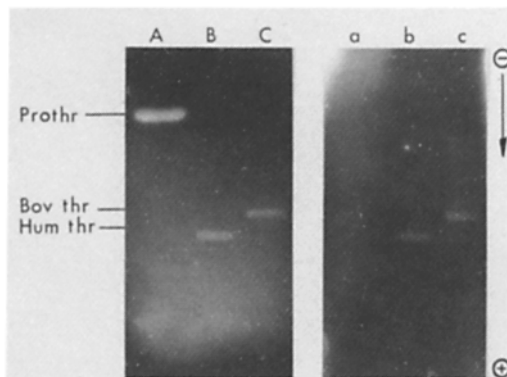


FIG. 6. Identification of prothrombin (prothr) and thrombin (thr) after SDS-PAGE. (A,a) 100  $\mu\text{g}$  of  $\text{BaCl}_2$  eluate; (B,b) 5  $\mu\text{g}$  of purified human thrombin; (C,c) 1 U of bovine thrombin were electrophoresed in SDS-11% polyacrylamide slab gel and the gel processed for the detection of clotting activity either in presence (A,B,C) or in absence (a,b,c) of cephalin (1 U), factor Xa (2.5 U).  $\text{Ca}^{++}$  (5 mM) was present in both fibrinogen-agar indicator gels. The figure is a photograph of the fibrinogen-agar layer seen with dark field illumination. The fibrinogen used for this experiment was treated with 10 mM di-isotropyI fluorophosphate at 37°C for 1 h and dialyzed against PBS ( $2 \times 100$  vol) for 24 h at 22°C before use.

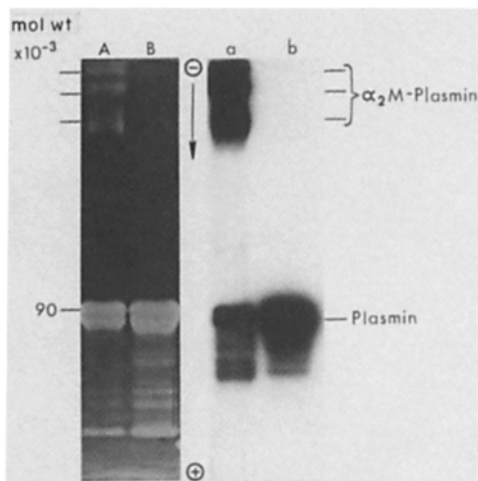


FIG. 7. Comparison of fibrinolytic and autoradiographic patterns of  $\alpha_2$ -macroglobulin-plasmin complexes.  $^{125}\text{I}$ -Plasmin (2  $\mu\text{g}$ ) (B,b) and a complex prepared by incubation (1 min, 37°C, 0.1 M Tris HCl pH 8.1) of 2  $\mu\text{g}$   $^{125}\text{I}$ -plasmin with 50  $\mu\text{g}$   $\alpha_2$ -M (A,a) were electrophoresed in duplicate on an SDS-polyacrylamide (4-13% gradient) slab gel. The gel was processed for detection of fibrinolytic activity and for autoradiography. The left panel of the figure is a photograph of the amido-black stained fibrin-agar indicator gel; the right is a photograph of the autoradiogram.

activity. The two patterns thus obtained revealed the following: (a) in each reaction mixture multiple forms of enzyme were separated by SDS-PAGE. For both plasmin and trypsin there were several distinct bands in the very high molecular weight region of the gel, corresponding to the position of  $\alpha_2$ -macroglobulin, and some radioactivity migrated at a rate indistinguishable from the free, uncomplexed enzymes.



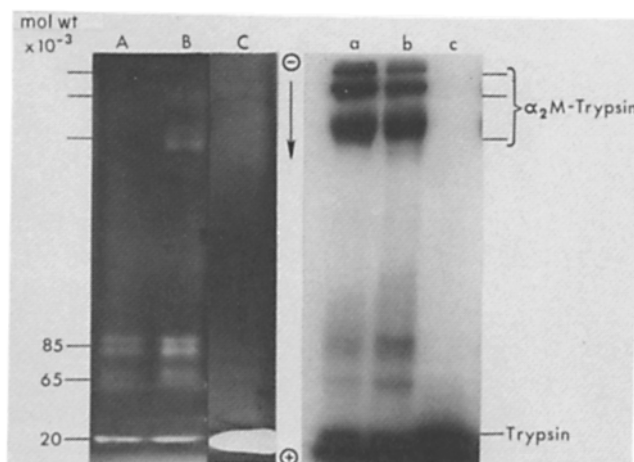


FIG. 8. Comparison of fibrinolytic and autoradiographic patterns of  $\alpha_2$ -macroglobulin-trypsin complexes.  $^{125}\text{I}$ -Trypsin ( $4 \mu\text{g}$ ) (C,c) and a complex prepared by incubation (1 min,  $37^\circ\text{C}$ ,  $0.1 \text{ M}$  Tris HCl pH 8.1) of  $4 \mu\text{g}$   $^{125}\text{I}$ -trypsin with  $50 \mu\text{g}$   $\alpha_2$ -M (A,a) and the same complex acidified to pH 3 for 1 min immediately before electrophoresis (B,b) were electrophoresed in duplicate on an SDS-polyacrylamide (4–13% gradient) slab gel. The gel was processed for the detection of fibrinolytic activity and for autoradiography. The left panel of the figure is a photograph of the autoradiogram. The trypsin  $\alpha_2$ -M-complex is active in fibrinolysis only in the specimen that had been acidified (B) before electrophoresis.

(b) Comparison of autoradiographic and fibrinolytic patterns (Fig. 7) showed that essentially all of the autoradiographically detectable forms of plasmin were catalytically active. Since it is known that native plasmin- $\alpha_2$ M complexes do not hydrolyze macromolecular substrates (14), we conclude that exposure to SDS has denatured portions of the inhibitor structure and abolished any steric obstruction to the interaction between  $\alpha_2$ M-complexed plasmin and proteins such as fibrin.

(c) The patterns found with trypsin resembled those observed with plasmin, but they differed in one respect: the high molecular weight trypsin- $\alpha_2$ M complexes were catalytically inactive after SDS-PAGE, suggesting the persistence of some structural restraints that blocked the action of complexed trypsin on fibrin. However, brief exposure of trypsin- $\alpha_2$ M complexes to low pH before SDS-PAGE abolished these restraints and unmasked the proteolytic activity of bound trypsin (Fig. 8). Addition of excess trypsin led to partial degradation of the trypsin- $\alpha_2$ M complexes, yielding forms intermediate in molecular weight between free enzyme and enzyme- $\alpha_2$ M complexes; these also retained proteolytic activity.

These observations are inconsistent with the model of protease- $\alpha_2$ M complexes proposed by Barrett and Starkey (14), according to which the proteases are thought to be trapped within a molecular cage formed by the macroglobulin. This model accounts for the fact that complexes are able to hydrolyze small synthetic substrates but not macromolecular substrates, and it predicts that conditions such as low pH or SDS, which denature the  $\alpha_2$ M component, should liberate free, native enzyme from complexes. Our results suggest an alternative interpretation.

Since the  $\alpha_2$ M complexes of both plasmin and trypsin recovered the ability to degrade fibrin after exposure to denaturing agents while retaining the electrophoretic mobility characteristics of the high molecular weight inhibitor, we conclude that the enzymes are bound to  $\alpha_2$ M by bonds that are insensitive to denaturing conditions and are thus presumably covalent; the formation of the covalent linkage is assumed to be irreversible and to involve functional groups on the enzyme that are not essential for catalytic activity. After the formation of the covalent bond, the proximity of the native  $\alpha_2$ M would sterically restrict the accessibility by the protease, thereby excluding hydrolysis of macromolecular substrates. Both the native  $\alpha_2$ M structure, and the substrate restrictions, would be abolished by denaturing agents, yielding a fully active protease still covalently bound to a random coil form of  $\alpha_2$ M.

Although a mechanism of this kind can be envisioned for other macromolecular protease inhibitors it does not apply to the complexes formed between trypsin or plasmin and more compact molecules such as soybean trypsin inhibitor or Kunitz inhibitor. These complexes are fully dissociated by exposure to SDS, and the proteases then migrate in SDS-PAGE at rates indistinguishable from those of the free parent species (data not shown).

### Discussion

The combination of procedures described in this paper, whereby reaction mixtures or biological fluids are first analyzed by SDS-PAGE and then evaluated by digestion of fibrin-agar gels, is likely to be of general usefulness for detecting and identifying proteases and zymogens of the serine enzyme type, particularly those that are active in the extracellular milieu. This conclusion is supported by the following considerations: (a) the results obtained to date indicate that nine separate serine proteases, two zymogens, and proteases complexed to six different macromolecular inhibitors retain their activity after SDS-PAGE; under appropriate conditions the enzymes (or proenzymes) can then be detected reproducibly and at low concentrations by fibrin hydrolysis in the indicator gel.<sup>2</sup> Indeed, so far none of the enzymes tested has been inactivated by these methods, at least not to the point at which they were undetectable in the nanogram range of concentrations. (b) The procedure can readily be made quantitative provided that it is appropriately standardized and calibrated by reference to known amounts of the enzyme under study. The fibrin-agar underlay has also been successfully used to identify enzymes in samples of urine after isoelectric focusing in gels. (c) The fibrinolytic zones formed in the indicator gel are sharp and narrow and permit small differences in electrophoretic mobility, e.g. molecular weight differences of the order of 2,000, to be resolved. (d) The studies described here have been limited to a few isolated proteases in addition to enzymes of the coagulation, fibrinolytic, and kinin-generating pathways. By suitable modification of the indicator gel, e.g., replacing the fibrin by sensitized erythrocytes (15), the procedure could perhaps

<sup>2</sup> The serine proteases tested were: plasminogen activator, urokinase (two forms), thrombin, kallikrein, chymotrypsin, elastase (pancreatic and leukocyte), trypsin, plasmin; the proenzymes were: prothrombin, plasminogen; and the protease complexes with the following inhibitors:  $\alpha_2$ -macroglobulin, inter- $\alpha$ -trypsin inhibitor, C, esterase inhibitor, antithrombin III, soybean trypsin inhibitor, Kunitz inhibitor.

be adapted to examining the proteolytic functions in the complement system. Since the SDS-PAGE unmasks the catalytic activity of proteases bound to inhibitors, the method might also be helpful, in combination with other reagents such as specific antibodies, both for identifying the proteases that are bound to plasma inhibitors during complex reactions such as blood clotting, and for determining the rate and sequence of such reactions.

The convenience and rapidity of the procedure can be substantially improved by reducing the dimensions of the SDS-gels, and thereby also the sample size<sup>3</sup> and the time required for electrophoresis. Under these conditions the method is useful for monitoring the fractionation of specific proteases during purification and chromatography. Other possible improvements include the substitution of fibrin by casein, hemoglobin or other chromoproteins which might provide better substrates for individual proteases.

Although we have not tested carboxyl- or thiol-proteinases in our system and therefore do not know whether the procedures can be used successfully with these enzymes, the well-known retention of pronase activity in solutions of SDS (16) suggests that the method may be applicable to several types of proteolytic and other hydrolytic enzymes.

### Summary

We have (*a*) screened a variety of cell lines and body fluids for plasminogen activators and (*b*) studied the activity of proteases bound to  $\alpha_2$ -macroglobulin after exposing the complexes to partial degradation and/or denaturing procedures to unmask proteolytic activity. The respective results show (*a*) that the plasminogen activators in urine and cell culture media are generally of lower molecular weight than those in plasma; and (*b*) that proteases bound to  $\alpha_2$ -macroglobulin recover the ability to attack macromolecular substrates after exposure to sodium dodecyl sulfate while retaining the electrophoretic mobility of the protease inhibitor complex. This indicates that the protease and inhibitor are probably linked by covalent bonds. In contrast, other complexes formed between proteases and inhibitors of lower molecular weight (such as soybean or Kunitz inhibitors) are fully dissociated by sodium dodecyl sulfate (SDS).

The experiments described were based on a new procedure for detecting proteolytic enzyme activity in SDS-polyacrylamide gels. The method relies on solutions of nonionic detergents for extracting SDS, after which the electrophoretic gel is applied to an indicator gel consisting of a fibrin-agar mixture. The method is sensitive, permitting the detection of proteinases in less than 1  $\mu$ l of fresh plasma, and it is effective for resolving small differences in molecular weight. The procedure can be quantitated and, with minor modifications appropriate to each particular system, it has been applied to a broad spectrum of serine enzymes and proenzymes, including some that function in the pathways of fibrinolysis, coagulation and kinin-generation. Other potential applications appear likely.

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<sup>3</sup> Small slab gels (7.5  $\times$  9.0  $\times$  0.1 cm) can be used to analyze 2- $\mu$ l samples by electrophoresis for 2 h at 15 mA.

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