Plasminogen: Purification from Human Plasma by Affinity Chromatography

Abstract. *Plasminogen was prepared from human plasma by affinity chromatography on* L-*lysine–substituted Sepharose. Thirty milligrams of plasminogen, with a specific activity of 100 caseinolytic units (Committee on Thrombolytic Agents) per milligram of nitrogen, were obtained from 340 milliliters of plasma. This corresponds to over 200-fold purification from plasma. Disc-gel electrophoresis at* p*H 8.3 indicated seven distinct bands, all of which contained activity.*

Plasminogen is the zymogen of the proteolytic enzyme plasmin (E.C. 3.4.4.14), the enzyme responsible for the dissolution of fibrin clots in the blood. In a continuing effort to resolve problems of activity, yield, reproducibility, homogeneity, stability, and solubility at physiological *p*H, dozens of methods have been used for the preparation of human plasminogen (*1*). We have described our purification (*2*) of plasminogen from human plasma by affinity chromatography. In these initial experiments we used the properties of ε-aminocaproic acid (ε-ACA), an inhibitor of plasminogen activation to plasmin (*3*), by covalently binding the α-amino group of L-lysine to Sepharose. We now present a modified and relatively large-scale procedure for the preparation of plasminogen, by affinity chromatography, as an attractive alternative to the multistep procedures now used by others.

Agarose (Sepharose 4B) was activated with cyanogen bromide (*4*). To a 150-ml suspension (0.1*M* $NaHCO_3$ buffer, *p*H 8.9), containing 100 ml of activated agarose, 20.0 g of L-lysine monohydrochloride was added, titrated to *p*H 8.9 in 50 ml of H_2O, and the slurry was stirred for 24 hours at 5°C. The amount of lysine bound to the agarose was 55 μmole per milliliter of settled agarose, as determined by amino acid analysis of the unreacted lysine. If we assume a molecular weight of 81,000 for plasminogen (*5*), each milliliter of settled lysine-Sepharose could then theoretically bind about 4 g of plasminogen provided that all the lysine molecules joined to the Sepharose are available for plasminogen binding and a 1 : 1 stoichiometry exists between enzyme and inhibitor.

In a typical experiment, a column (2.5 by 30 cm) of agarose containing 50 ml of degassed lysine-Sepharose was equilibrated with 0.1*M* phosphate buffer (*p*H 7.4). A volume of 340 ml of outdated plasma (*6*) was diluted to 640 ml with water and passed through the lysine-Sepharose column at 75 ml/hour and the column was next washed with 0.3*M* phosphate (*p*H 7.4) at 175 ml/hour until the absorbancy at 280 nm was less than 0.01. The plasminogen was then eluted as a sharp peak with 0.2*M* ε-aminocaproic acid (*p*H 7.4) at 100 ml/hour. All of the steps in the procedure, up to this point, were performed at room temperature,

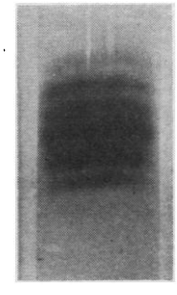

Fig. 1. Disc-gel electrophoresis of plasminogen purified by affinity chromatography. Electrophoresis of 60 μg of lyophilized product was performed at 3 ma per tube for 2½ hours at 5°C in a 7½ percent gel, *p*H 8.3. The gel was stained with amido black.

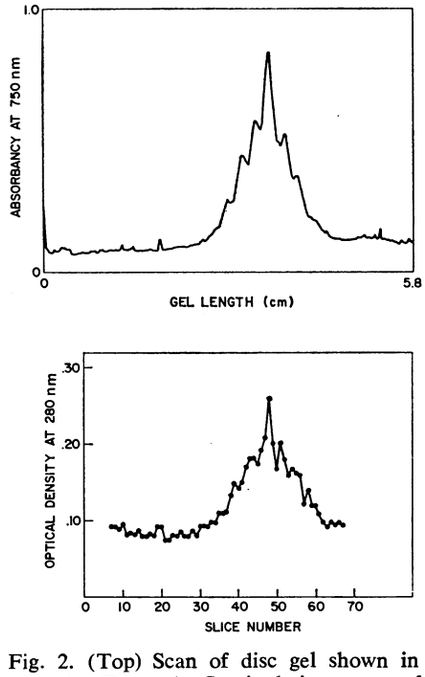

Fig. 2. (Top) Scan of disc gel shown in Fig. 1. (Bottom) Caseinolytic assay of plasminogen activity on unstained acrylamide gel. Individual slices (0.7 mm) were cut from the gel and ground in the assay buffer before activation with urokinase. The gel was scanned by a Gilford spectrophotometer with a linear transport attachment.

and 0.003*M* EDTA (ethylenediaminetetraacetate) was added to the phosphate buffers as well as to the plasma.

The ε-aminocaproic acid was removed from the plasminogen, in the cold, by gel filtration on Sephadex G-25 equilibrated with 0.005*M* phosphate (*p*H 7.4). The G-25 column was monitored with a conductivity apparatus to ensure a protein fraction free of inhibitor. The plasminogen preparation, 30 mg after correction for phosphate, was then freeze-dried and stored at −20°C. The complete procedure, as described, has also been performed with tris(hydroxymethyl)aminomethane buffers instead of phosphate, with a reduction of plasminogen yield to 5 mg and a 10 percent decrease in specific activity (*7*).

Plasminogen activity was measured by a standard caseinolytic assay suggested by the National Heart Institute's Committee on Thrombolytic Agents (CTA), Subcommittee for Standardization (*8*), with the specific activity expressed in CTA casein units per optical density unit. When 250 Plough units of urokinase (*9*) were used for activation of plasminogen to plasmin and when the plasminogen concentration was measured at 280 nm in 0.1*N* NaOH, the affinity chromatography preparations had an average specific activity of 10 CTA units per absorbancy unit (100 CTA units per milligram of nitrogen) with no detectable spontaneous plasmin activity under the conditions of the assay. Based on inhibitor-free plasma, this corresponds to a 250-fold purification. After the preparation was held for 5 days at room temperature in the assay buffer at *p*H 7.5, the overall specific activity did not diminish; however, about 0.7 CTA unit per absorbancy unit of spontaneous, that is, plasmin, activity developed. This remarkably stable preparation still had 73 percent of the total activity intact after 20 days at room temperature (from 10.2 to 7.6 CTA units per absorbancy unit) with 1.7 CTA units of spontaneous activity developing.

Polyacrylamide disc electrophoresis was performed according to the methods of Ornstein and Davis (*10*), except that the sample and spacer gels were omitted. Before application to the gel, the protein was dialyzed for 5 hours against two 300-ml volumes of the tris glycine buffer, *p*H 8.3. Inspection of the gel shows seven bands, of which six are visible in this reproduction (Fig. 1). The same multiband pattern was obtained when protein concentrations in

excess of 200 μg were applied. These distinct electrophoretic bands very nicely define a normal distribution curve, as shown in the scan of the gel at 750 nm (Fig. 2, top). The scan hints at an eighth and ninth band which cannot be seen visually. Figure 2 (bottom) shows the results from a caseinolytic assay of an unstained acrylamide gel in which consecutive slices were cut from the gel and assayed after activation with urokinase. The optical density at 280 nm is directly proportional to the activity and indicates that the different electrophoretic bands from the affinity chromatography plasminogen preparation are active. Since, as we have shown in parallel experiments, no activity develops unless the bands are treated with urokinase, the different forms are apparently all proenzyme. Other studies show that these plasminogen preparations form (i) a single band on polyacrylamide gel in the presence of ε-ACA at *p*H 8.3 and (ii) a single band without inhibitor (ε-ACA) present on starch gel at *p*H 2.5 (*11*). When a sample of the proenzyme is activated for 6 minutes at 25°C with the bacterial exotoxin streptokinase, 0.73 mole of active enzyme (plasmin) is formed per mole of plasminogen. The titration of plasmin is performed with *p*-nitrophenyl-*p'*-guanidino benzoate hydrochloride according to the procedure of Chase and Shaw (*12*). The titrable plasmin (73 percent active enzyme) is at the same level as that found by Chase and Shaw in a preparation of highly purified plasmin supplied by K. C. Robbins, which had a proteolytic activity of 31.7 casein units per milligram of protein (*12*).

While distinct electrophoretic forms of the proenzyme on polyacrylamide gel contradict results of some workers who have reported plasminogen preparations which give one band on disc-gel electrophoresis (*13*), others have demonstrated more than one band on starch and acrylamide electrophoresis (*14*). Summaria and Robbins have reported that polyacrylamide gel electrophoresis of human plasminogen in 8*M* urea or 0.3*M* ε-ACA (but not untreated) showed three to five bands and that the proenzyme could be separated into distinct fractions by isoelectric focusing in 7*M* urea. From other experiments, they suggest that the determinants for the multimolecular forms of the proenzyme reside in one region of the plasminogen molecule having a molecular weight of 25,700, called the light chain (*15*).

We think that the few manipulations of the proenzyme during purification by affinity chromatography and the maintenance of neutral *p*H throughout the procedure account for the unusual stability and free solubility at neutral *p*H. Because of the simplicity and speed of the method and apparent integrity of the product, this procedure will make large amounts of plasminogen readily available for clinical and structural studies.

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Collagen Molecules: Distribution of Alpha Chains

Abstract. *To ascertain the distribution of alpha chains within the collagen molecule, intramolecular cross-links were introduced into tropocollagen by reacting formaldehyde with dilute homogeneous molecular dispersions of collagen extracted from lathyritic guinea pig skin. Denaturation, chromatography on carboxymethyl cellulose, measurement of molecular weight, and analyses of the amino acids of the cross-linked product indicate that most, if not all, of the collagen molecules consist of two α1 chains and one α2 chain* $[(\alpha 1)_2(\alpha 2)_1]$.

Collagen from a number of vertebrate species is composed of twice as many α1 as α2 chains, α2 having the same molecular weight as, but different amino acid composition from, α1 (*1*). Since each tropocollagen molecule is composed of three α chains, Piez and his colleagues (*1*) suggested that the molecule be defined by the formula $(\alpha 1)_2(\alpha 2)_1$. However, the 2 : 1 ratio is only a statistical value for the bulk collagen, and it has not been demonstrated that each molecule has two α1 and one α2 chains. For example, if there were twice as many molecules of the type $(\alpha 1)_3$ as $(\alpha 2)_3$, denaturation which results in chain separation would yield the 2 : 1 ratio of α1 to α2. This report provides direct evidence for the great predominance of $(\alpha 1)_2(\alpha 2)_1$ type of collagen molecules that can be extracted from lathyritic guinea pig skin and describes a new method for revealing and quantitating molecular heterogeneity. This demonstration is based on the production of intramolecular cross-links by reaction with formaldehyde (*2*) solutions of collagen dilute enough to avoid intermolecular reactions; the cross-linked product was then isolated and

Table 1. Molecular weight distribution of cross-linked collagen at sedimentation equilibrium from concentration 0.250 to 1.021 fringes.

Type	Range
M_n	222,970 to 282,690
M_w	274,730 to 329,477
M_z	317,035 to 354,860