Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 8292–8295, December 1987
Biochemistry

Preparation and purification of microplasmin

(plasmin/plasmin autolysis)

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Contributed by Myron L. Bender, August 18, 1987

ABSTRACT **A catalytically active, human microplasmin was produced by incubation of [Lys]plasmin in buffer at pH 11.0 for up to 12 hr. The microplasmin was purified by affinity chromatography that used lysine-Sepharose and soybean trypsin inhibitor-Sepharose columns. It is homogeneous and pure by electrophoretic analysis in $NaDodSO_4$/polyacrylamide gels and by gel filtration on a Superose 12 column. The molecular weight of the microplasmin determined by $NaDodSO_4$ gel electrophoresis is 29,000 and 26,500 under reducing condition, whereas the molecular weight of native plasmin is 76,500. Microplasmin consists mainly of the light (B) chain of native human plasmin and possesses one active site per protein molecule when titrated with *p*-nitrophenyl *p'*-guanidinobenzoate. Microplasmin hydrolyzes the peptide substrate NH_2-D-Val-Leu-Lys-*p*-nitroanilide (S-2251) with a K_m of 0.361 ± 0.017 mM and a k_{cat} of 40.3 ± 3.3 s^{-1} at pH 7.4 and 37°C, whereas native plasmin has a K_m of 0.355 ± 0.002 mM and a k_{cat} of 27.9 ± 0.3 s^{-1} under the same conditions.**

Native human plasminogen has a molecular weight of 92,000 (1–3); when activated by streptokinase or urokinase, the zymogen is converted into plasmin, which consists of heavy (A) and light (B) chains. The proteolytic active center, serine and histidine residues, of human plasmin is located in the B chain (4–6). The A and B chains are connected by two disulfide bonds (4, 7–10). The isolation of a series of lower molecular weight fragments of plasminogen and plasmin led to structure–function relationship studies (11). A peptide of 76 amino acids can be cleaved from the NH_2 terminus of [Glu]plasminogen yielding [Lys]plasminogen of M_r 84,000 (12, 13). A plasminogen of M_r 50,000–55,000 has been obtained by cleavage of two peptides from the NH_2 terminus of [Glu]plasminogen by plasmin (14, 15). One other small plasminogen fragment of M_r 38,000 containing kringle (domain) 5 and B chain ([Val^{442}]neoplasminogen) was isolated by limited digestion of human plasminogen with elastase (8, 16, 17). Catalytically active plasmins of molecular weight smaller than [Lys]plasmin were obtained by activating these last two degraded forms of plasminogen (8, 14–19). A functionally deficient human B chain of plasmin (from Val-561 to Asn-790) of M_r 26,000 with its active site 9% diisopropyl fluorophosphate titratable has been isolated from a partially reduced and alkylated plasmin. It was converted to a "rigid" enzyme in a B chain–streptokinase complex with its active site 70% titratable (20, 21).

In this study we have prepared a fully functional microplasmin that was purified by two affinity columns, lysine-Sepharose and soybean trypsin inhibitor-Sepharose, and characterized its amidolytic activities.

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MATERIALS AND METHODS

Proteins. Forms 1 and 2 of native human plasminogen were separated by affinity chromatography on lysine-Sepharose columns (22, 23). Form 2 of plasminogen was used exclusively in these studies. The starting materials, human plasma Cohn fraction III and pooled plasma, were generously provided by Cutter Laboratories (Berkeley, CA) and Blood Donating Center (Tainan, Taiwan).

Protein and Enzyme Concentration. The active site concentration of plasmin or microplasmin was determined by the *p*-nitrophenyl-*p'*-guanidinobenzoate burst titration of Chase and Shaw (24). In the determination of protein concentration, the following values of $\varepsilon_{1\,cm}^{1\%}$ (280 nm) and of molecular weights were used: for [Glu]plasminogen, 17.0 and 92,000 (3), respectively, and for microplasmin, 16 and 29,000 (21), respectively.

Preparation of Protein-Substituted Sepharoses. Soybean trypsin inhibitor (40 mg) or urokinase (45,000 units) was coupled to CNBr-activated Sepharose 4B (2 g) in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3. The gel was washed repeatedly with 100 ml of 0.1 M acetic acid/1 M NaCl, pH 4.0, alternating five times with 100 ml of 0.1 M sodium borate buffer, pH 8.5, and stored in 0.1 M sodium phosphate buffer, pH 8.0.

Amidolytic Activities. The enzymatic activity of each plasmin preparation was measured with the peptide substrate, NH_2-D-Val-Leu-Lys-*p*-nitroanilide (S-2251), at 37°C and in 50 mM Tris·HCl, pH 7.4/0.1 M NaCl. The substrate concentration was varied between 0.2 and 4 K_m. The initial rate and substrate concentration data were analyzed on a Lineweaver–Burk plot. The $\varepsilon_{1\,cm}^{1\,M}$ (405 nm) employed for *p*-nitroanilide was 9559.

Preparation of Urokinase-Free Human [Lys]Plasmin. Urokinase-free human plasmin was prepared by activation of human plasminogen with Sepharose-bound urokinase. One milliliter of plasminogen (20 mg/ml) in 0.05 M sodium phosphate/0.02 M L-lysine/0.1 M NaCl/0.001 M EDTA, pH 7.0, was incubated with 0.3 ml of packed gel of urokinase-substituted Sepharose at 30°C in a reaction vial (Pierce), with slow stirring. When maximum plasmin activity was attained (≈3 hr) the activation mixture was forced through a tight glass-wool plug at the end of a 3-ml plastic syringe by centrifugation. In all cases at least 80% active plasmin was obtained as determined by *p*-nitrophenyl *p'*-guanidinobenzoate titration.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. The basic techniques of gel electrophoresis were done as described by Laemmli (25).

Reagents. Urokinase, soybean trypsin inhibitor, and the tripeptide substrate (S-2251) were purchased from Sigma; Superose 12 (HR 10/30) and CNBr-activated Sepharose 4B

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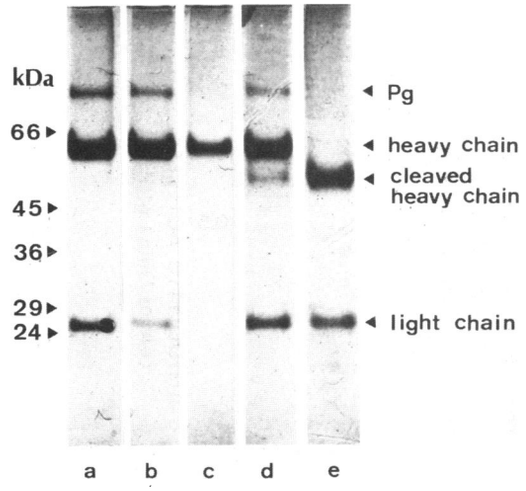

FIG. 1. $NaDodSO_4$/7.5% polyacrylamide gels of plasmin. Lanes: a, native plasmin; b, plasmin incubated in 0.1 M sodium phosphate (pH 6.5) for 1 hr; c, plasmin in the same buffer as in lane b for 6 hr; d, plasmin in 0.1 M glycine (pH 11.0) for 1 hr; e, plasmin in the same buffer as in lane d for 12 hr. Pg, plasminogen.

were purchased from Pharmacia. All other reagents were analytical grade.

RESULTS

Plasmin undergoes cannibalistic denaturation in aqueous solution as do other proteases (26–28). The number of hydrolytic sites in the autolytic reaction of plasmin varies with pH. At near neutral pH (pH 6.5), the catalytic activity of plasmin declined 50% in 2 hr; $NaDodSO_4$ gel electrophoresis results indicate that the amount of intact B chain of M_r 26,500 decreased during incubation. However, most of the A chain of M_r 63,000 remained unchanged (Fig. 1, lanes a, b, and c). The catalytic activity of plasmin in buffer at pH 11.0 decreased 10–20% after 12 hr at 25°C. The amount of protein corresponding to the B chain of M_r 26,500 remained largely unchanged. The A chain was degraded, and a new main protein band (M_r 58,000) was observed (Fig. 1, lanes d and e).

After incubation in alkaline solution, the plasmin was purified by affinity chromatography (Figs. 2 and 3), and all protein fractions were analyzed with $NaDodSO_4$ gel electrophoresis in 12% acrylamide gel (Fig. 4). A protein peak with most of the amidolytic activity of plasmin passed through the lysine-Sepharose column unadsorbed (Fig. 2). This protein peak consisted of peptides of M_rs 30,000 and 26,500 as seen in Fig. 4, lane b. A second protein peak consisting of degraded A chain with $M_r \approx 58{,}000$ was obtained by washing the column with 25- mM ε-aminocaproic acid (Figs. 2 and 4, lane c). The unadsorbed protein fractions of the first protein peak in Fig. 2 were pooled and immediately applied to a soybean trypsin inhibitor-substituted Sepharose column (0.6 × 5 cm) (Fig. 3). The unadsorbed protein fractions contained protein with M_rs 29,000–30,000 (Fig. 4, lane d). The protein in this peak had no amidolytic activity. Material was eluted from the inhibitor column with 0.1 M acetic acid, and a catalytically active protein was recovered. The recovered enzyme had 70 ± 10% of the amidolytic activity of the original plasmin. A peptide chain of M_r 26,500 was observed in this protein fraction by $NaDodSO_4$ gel electrophoresis analysis after reduction with mercaptoethanol (Fig. 4, lane e). The purified enzyme also showed only one protein peak on Superose 12 gel filtration analysis (Fig. 5). This purified, enzymatically active fragment of plasmin is named microplasmin.

The active site titration of microplasmin showed 0.9 ± 0.1 mol of active site per mol of microplasmin. Amidolytic

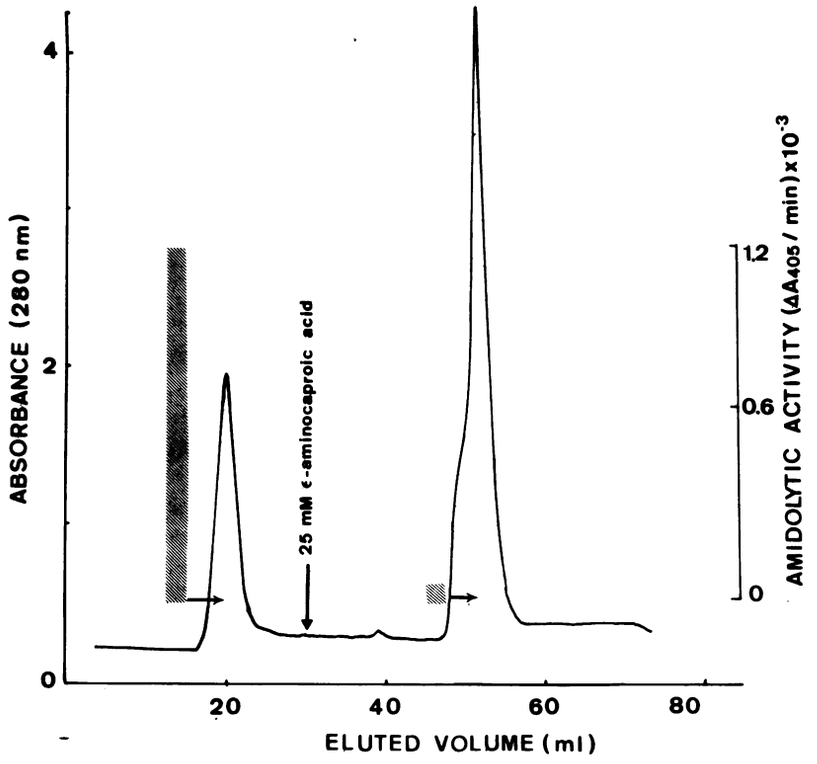

FIG. 2. Elution of the alkali-treated human plasmin. Human plasmin variant 2 (10 mg/ml), after incubation at pH 11.0 for 12 hr, was eluted with 0.1 M sodium phosphate buffer (pH 8.0) from a lysine-Sepharose column (1.5 × 20 cm) (first peak). A second peak was eluted with 25 mM ε-aminocaproic acid. The solid line is absorbance at 280 nm. The hatched bars are the enzymatic activity of the corresponding protein peak measured with S-2251.

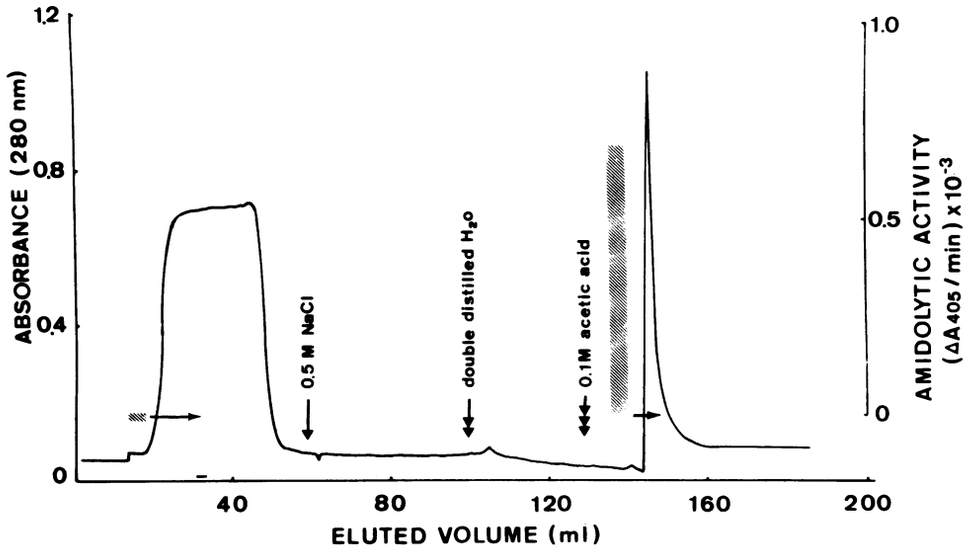

FIG. 3. Elution of unadsorbed fraction peak I in Fig. 2 from a soybean trypsin inhibitor-Sepharose column (0.6 × 5 cm). The column was equilibrated and eluted with 0.1 M sodium phosphate (pH 6.5) until absorbance at 280 nm was <0.02. The eluents were subsequently changed to 0.5 M NaCl, to distilled water, and to 0.1 M acetic acid as indicated. The solid line is absorbance at 280 nm. The hatched bars represent the enzyme catalytic activity of the corresponding protein peaks measured with S-2251.

activities of microplasmin and plasmin were determined with NH_2-D-Val-Leu-Lys-*p*-nitroanilide. Lineweaver–Burk double-reciprocal plots were used for data analysis (Table 1). Microplasmin has a K_m of 0.361 ± 0.017 mM and a k_{cat} of 40.3 ± 3.3 s^{-1} in Tris·HCl, pH 7.4, at 37°C, whereas the [Lys]-plasmin has a K_m of 0.355 ± 0.002 mM and a k_{cat} of 27.9 ± 0.3 s^{-1} under the same conditions.

DISCUSSION

Plasmin catalyzes the cleavage of peptide bonds on the COOH-terminal side of lysine and arginine residues of protein and peptide substrates (11). Plasmin can undergo an autolytic process with a cleavage site specificity that varies with pH (Fig. 1). The specific autolytic cleavage of plasmin molecule in alkaline solution leads to the formation of a low molecular weight form of plasmin, namely microplasmin. The pure microplasmin moiety was shown to have full catalytic activity.

A peptide of M_r 26,500 was detected by $NaDodSO_4$ gel electrophoresis of the reduced microplasmin. Results of gel filtration and $NaDodSO_4$ of nonreduced microplasmin showed that the molecular weight of microplasmin is ≈29,000. These results are consistent with the conclusion that microplasmin contains an intact B chain and a small peptide chain of 31 amino acids from the A chain (29).

Since microplasmin does not bind to lysine-Sepharose and consists mainly of the B chain of plasmin, the lysine-binding site is clearly absent. It is obvious that most of the A chain is not essential for the catalytic activity of plasmin, since microplasmin is slightly more effective than [Lys]plasmin in the hydrolysis of NH_2-Val-Leu-Lys-*p*′-nitroanilide. Microplasmin has a k_{cat}/K_m of 0.112 ± 0.006 $\mu M^{-1} \cdot s^{-1}$, compared to a k_{cat}/K_m of 0.079 ± 0.001 for plasmin (Table 1). The great loss of catalytic activity in the preparation of the B chain of plasmin by partial reduction seen by Summaria and Robbins (20) may be due to complete loss of structural support of the A chain (20, 21).

Since the B chain–streptokinase complex was proven to be a better plasminogen activator than the plasmin–streptokinase complex (20, 21), it will be of great interest to compare the streptokinase–microplasmin complex with the plasmin–streptokinase complex. The availability of microplasmin should be a valuable tool for studies of streptokinase interactions. The specific fragmentation of the plasmin molecules

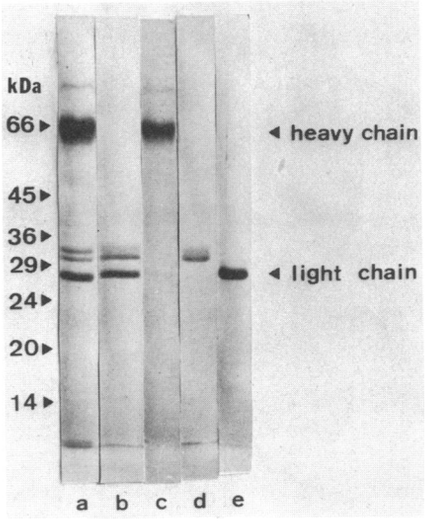

FIG. 4. $NaDodSO_4$/12% polyacrylamide gels of the plasmin sample and protein peaks in Figs. 2 and 3. Lanes: a, plasmin at pH 11.0 for 12 hr; b, protein peak of unadsorbed fractions of lysine-Sepharose column in Fig. 2; c, protein peak of adsorbed fractions of lysine-Sepharose column in Fig. 2; c, protein peak of adsorbed fractions of lysine-Sepharose column in Fig. 2; d, protein peak of unadsorbed fractions of soybean trypsin inhibitor-substituted column in Fig. 3; e, protein peak of adsorbed fractions of soybean trypsin inhibitor-Sepharose column in Fig. 3. Numbers on the left represent molecular mass ×10^3.

Table 1. Amidase parameters of [Lys]plasmin and microplasmin with NH_2-D-Val-Leu-Lys-*p*-nitroanilide at pH 7.4 and 37°C

Plasmin species	Amidase parameters		
	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, $\mu M^{-1} \cdot s^{-1}$
[Lys]Plasmin	355.4 ± 2.4	27.9 ± 0.3	0.0785 ± 0.0006
Microplasmin	360.6 ± 17.1	40.3 ± 3.3	0.1118 ± 0.0055

Values given are means ± SEM (n = 5).

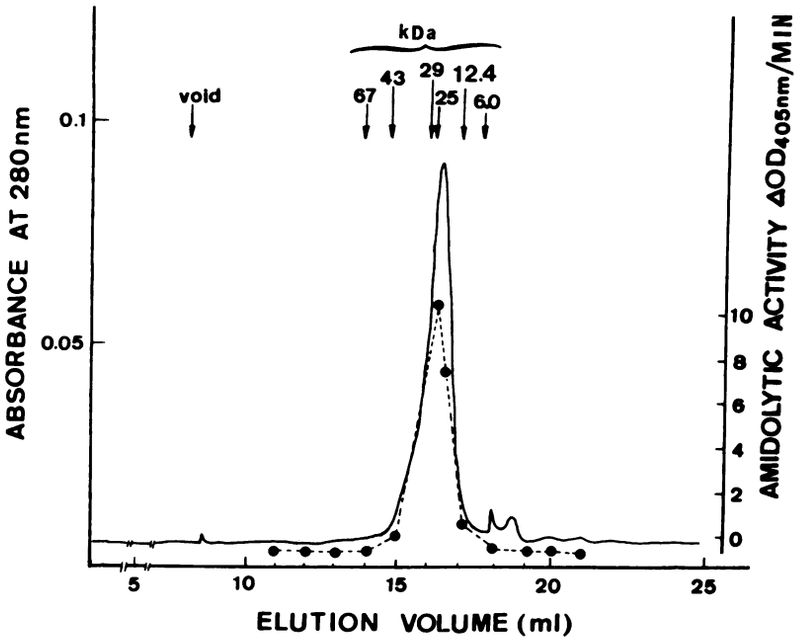

FIG. 5. Determination of molecular weight of microplasmin by gel filtration. Microplasmin (2 mg) was applied to a Superose 12 (HR 10/30) column and eluted with 0.05 M sodium phosphate/0.15 M NaCl, pH 7.4. The eluent was monitored for absorbance at 280 nm (—), and the fractions were assayed for amidolytic activity with S-2251 as substrate (– – –). The molecular weight standards used are bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (29,000), chymotrypsinogen (25,000), cytochrome *c* (12,400), and insulin (6000).

and the isolation of microplasmin with full enzymatic activity can be extremely valuable for structure–function studies of plasminogen and plasmin.

The expert technical assistance of L. F. Huang and B. I. Chang is gratefully acknowledged. This work was supported by Grant NSC-76-0412-B006-01 and NSC-76-0412-B006-20 from the National Science Council, Republic of China, and Grant N00014-86-K-0241-P01 from the U.S. Office of Naval Research.

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