Biochimica et Biophysica Acta, 613 (1980) 542—555

BBA 68988

PURIFICATION AND CHARACTERIZATION OF A PLASMINOGEN ACTIVATOR FROM MOUSE CELLS TRANSFORMED BY AN ONCOGENIC VIRUS

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(Received September 13th, 1979)

Key words: Plasminogen activator; Transformation; Proteolysis ; (Mouse cell)

Summary

On the basis of cellular morphology, a subline of mouse sarcoma virus-infected 3T3 cells was selected which released a 48 000-dalton plasminogen activator at an approx. 40-fold higher rate than those of the parent line, and which continued to do so for several months when the cells were maintained in serum-free culture medium. Culture medium (3.5 l) containing 0.6 mg plasminogen activator per l was used to purify 620 μg of the enzyme 130-fold with a yield of 32% by affinity chromatography followed by anion exchange chromatography and gel filtration. Crucial for the yield was the use of a non-ionic detergent and of inhibitors of proteolysis to prevent adsorption and degradation, respectively. The purified enzyme was homogeneous as evaluated by SDS-polyacrylamide gel electrophoresis and had an isoelectric point of pH 9.2. The purified enzyme showed characteristics of a trypsin-like serine protease (labeling with [^{3}H]diisopropylphosphorofluoridate which was prevented by *p*-nitrophenyl-*p'*-guanidinobenzoate) and converted the single chain of human plasminogen into two chains of plasmin with electrophoretic mobilities identical to those of the chains formed by non-purified enzyme and by human urokinase. In the absence of inhibitors, solutions of purified enzyme were stable for 24 h at 4° C at pH 3—9.

Introduction

Plasminogen activators comprise a class of serine proteases catalyzing the limited hydrolysis of the plasma zymogen, plasminogen, thus converting it into

Abbreviations: MSV, mouse sarcoma virus; SDS, sodium dodecyl sulphate.

plasmin — a serine protease with trypsin-like specificity that attacks most proteins. Plasminogen represents approx. 0.3% of the plasma protein in many species and thus constitutes an abundant source of potential proteolytic activity that is locally available to amplify the proteolytic action of plasminogen activators.

It has long been supposed that the main function of this enzyme system was to participate in thrombolysis. More recently it has been found that plasminogen activators are synthesized and released by a variety of cell types. These include many types of cultured malignant neoplastic cells [1—6], stimulated macrophages [7], polymorphonuclear granulocytes [8], granulosa cells during rupture of Graafian follicles in ovulation [9], trophoblasts in their invasive phase [10] and epithelial cells during involution of mammary glands after termination of lactation [11]. It is likely that in some or all of these cases the cellular plasminogen activators play a role in localized tissue destruction, and the release of plasminogen activators may be an important part of the malignant phenotype [12].

Pure preparations of plasminogen activators are essential for the molecular characterization of these enzymes, for the study of the kinetics of the activation reaction and its control and for the immunochemical and immunocytochemical detection of plasminogen activators in body fluids and tissues. Murine plasminogen activators are of special interest in this connection, since many of the original observations on the biological function of these enzymes were made in this species [2,5,7,10,11].

Our initial observations indicated that the purification of murine plasminogen activator presented a number of technical difficulties: available starting materials contained enzyme in low concentrations with low specific activity, the enzyme had hydrophobic characteristics leading to severe losses by adsorption, and as purification proceeded enzyme was also lost by proteolytic degradation.

We have overcome these obstacles by selection of a subline of oncogenic virus-infected cultured murine fibroblasts that release relatively large amounts of a 48 000-dalton plasminogen activator and that survive for prolonged periods in serum-free medium, by the use of the detergent Triton X-100 to counteract losses by hydrophobic interactions and by the use of $ZnCl_2$ and low pH to prevent proteolytic degradation.

This paper presents a procedure which allows purification of 600 μg of the 48 000-dalton murine plasminogen activator to homogeneity as assessed by SDS-polyacrylamide gel electrophoresis. A preliminary report on part of these results has been published previously [14].

Materials and Methods

Chemicals. Dulbecco's modified Eagle's medium and foetal bovine serum were obtained from Grand Island Biological Co., Grand Island, NY, U.S.A.; 4-aminobenzamidine aminododecyl cellulose, L-arginine, *N,N,N',N'*-tetramethylenediamine, ammonium persulfate and dimethyl sulfoxide were from Merck, Darmstadt, F.R.G.; Sephadex G-25 (medium), QAE-Sephadex A-25 and Pharmalyte were from Pharmacia Fine Chemicals, Uppsala, Sweden; sodium

[^{125}I] iodide (carrier-free) and [^{3}H]diisopropylphosphorofluoridate (3.4 Ci/mmol) from The Radiochemical Centre, Amersham, U.K.; bovine fibrinogen (plasminogen-free) from Poviet Producten, B.V., Amsterdam, Netherlands; benzamidine and Triton X-100 from Sigma Chemical Co., St. Louis, MO, U.S.A.; Sea Plaque agarose from Marine Colloids, Rockland, ME, U.S.A., Bio-Gel P60 (100—200 mesh) from Bio Rad Laboratories, Richmond, CA, U.S.A., Aquacide III from Calbiochem, La Jolla, CA, U.S.A., bovine thrombin from Leo Pharmaceutical Industries, Ballerup, Denmark; *p*-nitrophenyl-*p'*-guanidinobenzoate was a gift from Dr. E. Shaw; all other reagents were as described previously or of the best commercially available grade.

Cell culture. 3T3 cells infected with mouse sarcoma virus (MSV) were obtained from Dr. H.M. Temin, University of Wisconsin. From these cells a subline 3T3/MSV-LO was isolated which produced high amounts of plasminogen activator (see Ref. 14 and Results). Cell cultures were prepared and maintained essentially as described previously [5] using disposable plastic Petri dishes (100 or 150 mm diameter, Falcon Plastics, Oxnard, CA, U.S.A.) at 37°C in atmospheric air supplemented with 5% of CO_2. Trypsinized cells from subconfluent cultures were seeded at a density of $1 \cdot 10^6$ or $2.5 \cdot 10^6$ cells in 100 and 150 mm dishes, respectively. The cells were grown to subconfluency ($1.0—1.5 \cdot 10^5$ cells/cm^2) in 4—5 days in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. The cultures were then washed three times with a buffer containing 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and incubated in 10 or 25 ml (100 and 150 mm dishes, respectively) serum-free medium with intervals of 2 or 3 days between medium changes. For routine preparative purposes, seeding of the cells, washing of the cultures and medium changes were performed automatically with a specially modified Petrimat (Struers K/S, Copenhagen, Denmark). Primary cultures of mouse embryonal fibroblasts were prepared and infected with MSV, and serum-free culture medium from the transformed fibroblasts was prepared as described previously [5].

Assay for plasminogen activator. Plasminogen activator was assayed using a modification of the ^{125}I-labelled fibrin plate method [3]. Multiwell Disposo Trays (Linbro Scientific Co., New Haven, CT, U.S.A.) were coated with 20 μg (80 000 cpm) fibrin per well. Each assay well contained 0.5 ml 0.1 M Tris-HCl, pH 8.1, and 1 μg of human plasminogen. A standard preparation of medium from 3T3/MSV-LO cells containing 4.2 kU/ml of plasminogen activator (see Ref. 5 for the definition of one unit) was used to calibrate each assay. The assays were linear with enzyme concentration over the range 0.3—2 U. All samples and the standard were diluted to contain an amount within these limits in 25—50 μl and this volume was added to the assay mixture. Dilutions were performed with a digital diluter (Hamilton, Reno, NV, U.S.A.) in a 0.1 M Tris-HCl buffer, pH 8.1, containing 0.25% gelatine and 0.1% Triton X-100. All assays were performed in duplicates. Human plasminogen and ^{125}I-labelled bovine fibrin were prepared as described previously [3].

Purification procedures. All purification procedures were performed at 4°C. Chromatography columns were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and the required flow rates were maintained with Pharmacia multi-channel peristaltic pumps; these were also used to mix fluids immediately

before passage through the columns, to adjust the pH and to add $ZnCl_2$. The absorbance of the eluates at 280 nm was monitored with a Uvicord II (LKB Instruments, Bromma, Sweden). All glassware used for the purification was siliconized and columns for gel filtration were pretreated with a solution of ovalbumin (5 g/l) for 16 h and then extensively washed. Polystyrene plastic ware (Nunc, Roskilde, Denmark) was used for highly-purified enzyme preparations (fractions III and IV).

Affinity chromatography on 4-aminobenzamidine aminododecyl cellulose. A 30-ml column (50 × 15 mm) of 4-aminobenzamidine aminododecyl cellulose was equilibrated with a buffer containing 0.01 M acetate, pH 5.5, 0.2 M NaCl, 0.01 M $ZnCl_2$. Culture fluid was adjusted to pH 5.5, made 0.01 M with respect to $ZnCl_2$ and applied to the column (260 ml/h). The column was washed with the equilibrating buffer (260 ml/h; 3—4 l) until the absorbance of the eluate reached the base line; it was then washed with 200 ml (260 ml/h) of a buffer which was identical to the equilibrating buffer, except that the NaCl concentration was 0.5 M. Enzyme was eluted with a buffer containing 0.01 M acetate, pH 5.5, 0.5 M benzamidine and 0.01 M $ZnCl_2$ at a flow rate of 55 ml/h. All enzymatic activity was consistently eluted in the first 80 ml after the breakthrough of protein and benzamidine. This fraction was collected and the pH adjusted to 3.0 by mixing with 1/30 vol. of 1 M glycine-HCl, pH 2.3.

Buffer exchange was performed on a 300 ml (26 × 535 mm) Sephadex G-25 column equilibrated with a solution consisting of 0.05 M acetic acid, pH 3.0, 0.1 M NaCl, and 0.01 M $ZnCl_2$, and developed with the same buffer at a flow rate of 170 ml/h. The eluted protein peak was mixed with 1/23 vol. 1 M NaOH and 1/4 vol. 0.02 M sodium citrate, pH 5.5, to adjust the pH to 5.5.

The resulting solution was applied to a second column of 4-aminobenzamidine aminododecyl cellulose (5.4 ml, 26 × 11 mm), which was treated identically to the first affinity column, except that buffer volumes and flow rates were reduced to 1/6 and that elution was performed with a buffer consisting of 0.01 M acetate, pH 5.5, 0.6 M arginine and 0.01 M $ZnCl_2$. The pH of the eluate was adjusted to 3.0. The protein-containing fraction was collected (fraction II).

Anion-exchange chromatography. Fraction II (60 ml) was made 0.1% with respect to Triton X-100 and passed at 170 ml/h through a 300 ml (26 × 535 mm) Sephadex G-25 column equilibrated with 0.2 M glycine-HCl, pH 3.0, 0.001 M $ZnCl_2$, 0.1% Triton X-100. Fractions containing the eluted protein peak were pooled and adjusted to pH 9.0 by mixing with 1/17 vol. 1 M NaOH and 1/17 vol. of a 0.2 M glycine-NaOH buffer, pH 9.0. This solution was then applied at a rate of 450 ml/h to a 75 ml QAE-Sephadex column (26 × 141 mm), which was equilibrated and eluted with 0.2 M glycine-NaOH, pH 9.0, 0.001 M $ZnCl_2$, 0.1% Triton X-100. The eluate was mixed with 1/5 vol. 1 M glycine-HCl, pH 2.3, 0.05 M $ZnCl_2$ and 0.1% Triton X-100 to yield a final pH of 3.0. The eluate was assayed for plasminogen activator and the active fractions were pooled (fraction III).

Gel filtration on Bio-Gel P60. Fraction III (90 ml) was made 0.5 M with respect to arginine (pH 3.0) and concentrated by ultrafiltration in a dialysis bag against a solution of highly polymerized polyethyleneglycol (Aquacide III, 275 g/l) in a buffer consisting of 0.2 M glycine-HCl, pH 3.0, 0.5 M arginine, 0.01 M $ZnCl_2$ and 0.1% Triton X-100. The concentrated fraction III (2 ml) was applied

to a 120-ml Bio-Gel P60 column (16 × 600 mm) equilibrated and developed (2 ml/h) with the above buffer. The eluate was assayed for plasminogen activator and the active fractions were pooled (fraction IV).

Gel electrophoresis. Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS) was performed in a stacking system of slab gels as described previously [5].

Isoelectric focusing was performed by a modification of the method described by Davies [15] in slab gels (1.2 × 110 × 150 mm) containing 6% acrylamide, 10% sorbitol, 0.1% Triton X-100 and 6% of a carrier ampholyte solution (Pharmalyte) to give a pH gradient of pH 7.5—10.

Trypsin-like serine proteases were detected by labeling with [^{3}H]diisopropylphosphorofluoridate in the presence and absence of non-labeled *p*-nitrophenyl-*p'*-guanidino benzoate followed by SDS-polyacrylamide gel electrophoresis and fluorography as described previously [5] except that Triton X-100 in a final concentration of 0.1% was added during dialysis and incubation with [^{3}H]diisopropylphosphorofluoridate.

Plasminogen activators in polyacrylamide gels were detected by a modification of the method of Granelli-Piperno and Reich [16]. Gels were washed with 2.5% Triton X-100 and sandwiched between two agarose gels coated on glass. One agarose gel (10 ml, 120 × 190 × 0.4 mm) contained 1% Sea Plaque agarose, 0.5 N.I.H. units bovine thrombin, 19 mg bovine fibrinogen and 200 μg human plasminogen. The other agarose gel was identical except that plasminogen was omitted. The sandwich was incubated at 37°C for 3—12 h. The agarose gels were stained with amido black.

Miscellaneous procedures. Protein was estimated by the method of Lowry et al. [17]. When Triton X-100 was present, SDS was added during protein determination according to the method of Bonsall and Hunt [18] to prevent precipitation.

Intracellular plasminogen activator was extracted as described previously [3] with 0.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.1, from 3T3/MSV-LO cells 100 μl buffer per 10^6 cells) and homogenized mouse lung tissue (10 μl per mg tissue) prepared immediately after killing of the animal.

Human plasminogen (160 μg/ml) was converted into plasmin by incubation for 1 h at pH 8.1 with murine plasminogen activator (2000 U/ml) in the presence of bovine pancreatic trypsin inhibitor (600 Kallikrein inhibitor units/ml) and the single chain of plasminogen was separated from the two chains of plasmin by SDS-polyacrylamide gel electrophoresis under reducing conditions as described previously [19,20].

Results

Production of plasminogen activator by cell cultures

A variety of cultured mouse cell types were tested for production and release of plasminogen activator into the culture medium. Among these, the highest activity was found in the medium from a line of 3T3 cells transformed by MSV. Serum-free medium from confluent cultures of these cells contained after 48 h approx. 125 units of plasminogen activator per ml (corresponding to approx. 15 μg/l, see below). By phase-contrast microscopy these cultures

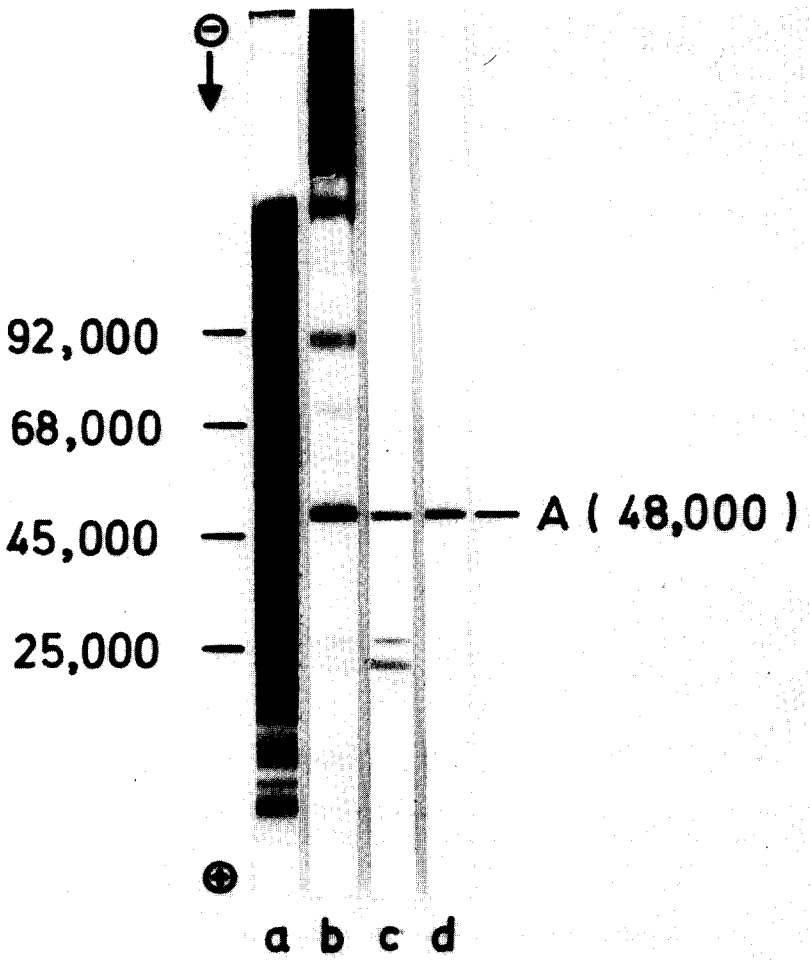

Fig. 1. SDS-polyacrylamide gel electrophoresis of plasminogen activator preparations. Samples were electrophoresed on a gel with a linear gradient of 6—16% polyacrylamide, and the gel was stained with Coomassie blue. (a) Serum-free medium from 3T3/MSV-LO cells (fraction I). (b) Eluate from 4-aminobenzamidine cellulose column 2 (fraction II). (c) Eluate from QAE-Sephadex column (fraction III). (d) Eluate from Bio-Gel P60 column (fraction IV). The content of plasminogen activator was in sample (a) 16 800 U, and in all the other samples 42 000 U. The localization of marker proteins and the estimated molecular weight of band A based on its electrophoretic mobility are indicated.

appeared heterogeneous with a monolayer of flat adherent cells and areas with multilayered spherical cells which tended to aggregate and detach in clumps from the surface of the culture dish. In other cell types a similar morphology has been correlated with high levels of plasminogen activator production [21]. A clump of 100—200 of these cells was isolated by means of a Pasteur pipette and used to establish a subline: 3T3/MSV-LO. Serum-free culture fluid from confluent cultures of these cells contained after 48 h approx. 40 times more plasminogen activator than did medium from the parent cell line.

Although 3T3/MSV-LO cultures were dependent on the presence of foetal calf serum in the medium for cell growth, subconfluent cultures maintained

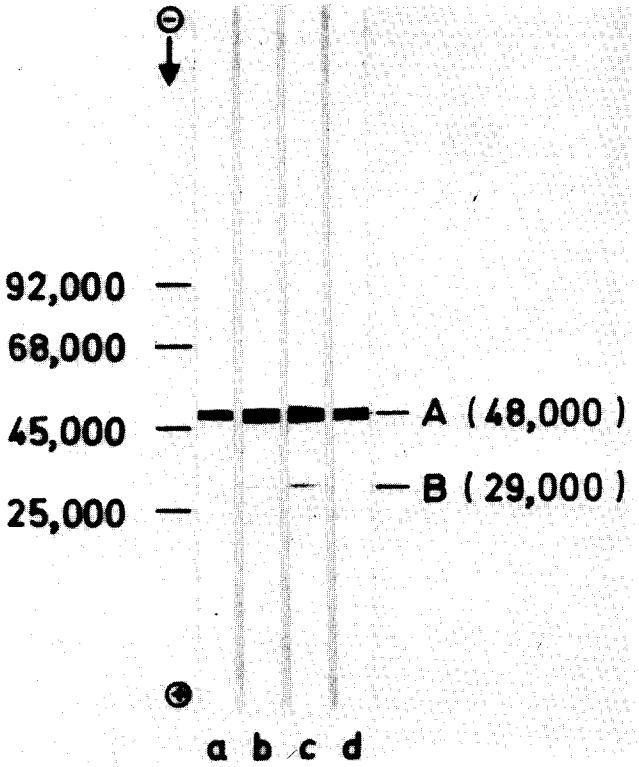

Fig. 2. Fluorogram after SDS-polyacrylamide gel electrophoresis of [^{3}H]diisopropylphosphorofluoridate-labeled plasminogen activator preparations. The samples were labeled with [^{3}H]diisopropylphosphorofluoridate and electrophoresed on a gel with a linear gradient of 6—16% polyacrylamide, and fluorography was performed for 6 days. (a) Serum-free culture medium from 3T3/MSV-LO cells (fraction I). (b) Eluate from 4-aminobenzamidine cellulose column 2 (fraction II). (c) Eluate from QAE-Sephadex column (fraction III). (d) Eluate from Bio-Gel P60 column (fraction IV). The content of plasminogen activator was in all samples 2100 U. When parallel samples were treated identically except that the incubation with [^{3}H]-diisopropylphosphorofluoridate was preceded by incubation for 1 h with 100 μM *p*-nitrophenyl-*p'*-guanidino benzoate, no bands were observed in any of the fluorograms.

a healthy morphology and continued to produce high levels of plasminogen activator when kept in serum-free medium. The concentration of plasminogen activator in the medium increased linearly with time for 48 h. Routinely, medium was changed at two intervals of 48 h followed by one interval of 72 h. This cycle was then repeated. The cultures continued to produce high amounts of plasminogen activator for more than 4 months. Occasionally a slow, gradual decrease in plasminogen activator production was observed due to detachment of cells from the culture dish. When the medium was supplemented with 10% foetal calf serum for 16 h, the cultures again grew to near confluency and the original high levels of plasminogen activator production were restored. When the interval between changes of medium was increased to 96 h, the enzyme concentration dropped by more than 90% in subsequent collections of medium.

The culture fluid from 3T3/MSV-LO cells contained 75—125 mg/l of total

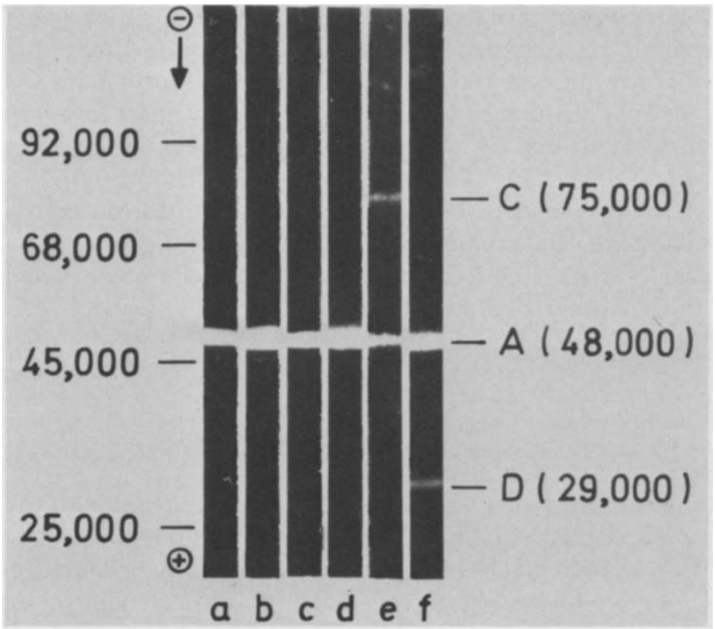

Fig. 3. Identification of plasminogen activators after SDS-polyacrylamide gel electrophoresis. Samples were electrophoresed as described in Fig. 1, except that a gel with 11% polyacrylamide was used. The gel was washed with Triton X-100 and layered over an agarose gel containing fibrin and plasminogen. The plasminogen activators diffused into the agarose gel and activated plasminogen which produced visible fibrinolysis zones. (a) Serum-free culture medium from 3T3/MSV-LO cells (fraction I). (b) Eluate from Bio-Gel P60 column (fraction IV) (c) Triton X-100 extracts from 3T3/MSV-LO cells (25 μl). (d) Serum-free medium from a tertiary culture of mouse fibroblasts transformed with MSV. (e) Triton X-100 extract from mouse lung tissue (75 μl). (f) Mouse urine (10 μl). All samples contained 42 U plasminogen activator. An agarose gel, identical to the above except that plasminogen was omitted, was applied to the other side of the polyacrylamide gel. No fibrinolysis zones were observed in this gel.

protein and an analysis by SDS-polyacrylamide gel electrophoresis showed that it contained a variety of distinct protein species (Fig. 1a). The content of serine enzymes in the culture medium was analysed by labeling with [^{3}H]diisopropylphosphorofluoridate followed by SDS-polyacrylamide gel electrophoresis and fluorography [5]. This showed that the culture fluid contained only one labeled species in detectable amounts (Fig. 2a). This protein was identified as a trypsin-like serine protease by complete inhibition of [^{3}H]diisopropylphosphorofluoridate labeling by preincubation with the active site reagent *p*-nitrophenyl-*p'*-guanidino benzoate. The [^{3}H]diisopropylphosphorofluoridate-labeled band comigrated with a distinct protein band visible in the stained gel and having an apparent molecular weight of 48 000 (Fig. 1a). When culture medium was analysed by SDS-polyacrylamide gel electrophoresis and the gel was layered onto an agarose gel containing plasminogen and fibrin, a lysis zone was observed which was dependent on the presence of plasminogen (Fig. 3a). The electrophoretic mobility of this plasminogen activator was identical to that of the [^{3}H]diisopropylphosphorofluoridate-labeled protein.

These data allowed us to conclude that the culture medium contained only one serine protease in detectable amounts and that this protease was a plas-

minogen activator with an apparent molecular weight of 48 000. In this respect it was identical to a plasminogen activator found in extracts from 3T3/MSV-LO cells, to a plasminogen activator released from a tertiary culture of mouse fibroblasts transformed by MSV, to one of two plasminogen activators found in extracts from mouse lung and to one of two plasminogen activators found in mouse urine (Fig. 3d—e). The extent of labeling with [^{3}H]diisopropylphosphorofluoridate (see Ref. 5) provided a measure of the concentration of the enzyme in the culture medium. By comparison with the labeling of known amounts of bovine trypsin, it was estimated that the culture medium contained 0.5—1.0 mg/l of the 48 000-dalton plasminogen activator.

The crude culture fluid (fraction I) could be stored at −20°C for more than 2 years without detectable loss of enzymatic activity. At 4°C, 5—10% of the activity was lost in 24 h.

Chromatography on 4-aminobenzamidine-cellulose

Benzamidine is a strong inhibitor of plasminogen activator and binds to the active site of this enzyme [5,20]. As shown in Table I, a 20-fold purification of plasminogen activator from crude culture medium was obtained by chromatography on 2 columns of 4-amino-benzamidine covalently bound to cellulose by an aminododecyl spacer, and at the same time the enzyme preparation was concentrated considerably.

The yield in this purification step was about 50%. In initial experiments the loss of enzymatic activity was more than 90%. This could be attributed to adsorption and proteolytic degradation of the enzyme, which was counteracted by the measures described in the Materials and Methods section.

It should be noted that $ZnCl_2$, which is a strong reversible inhibitor of both plasminogen activator and plasmin [20], did not prevent the binding of plasminogen activator to the benzamidine columns. This is in agreement with the finding that $ZnCl_2$, in contrast to many other inhibitors (e.g. benzamidine and L-arginine), does not compete with [^{3}H]diisopropylphosphorofluoridate for

TABLE I

PURIFICATION OF MURINE PLASMINOGEN ACTIVATOR BY CHROMATOGRAPHY ON 4-AMINOBENZAMIDINE AMINODODECYL CELLULOSE

Fraction	Volume (ml)	Protein (mg)	Activity (kU)	Specific activity (kU/mg)	Yield (%)
Culture fluid applied to 4-aminobenzamidine aminododecyl cellulose column 1 (fraction I)	3525	273	15 447	56	100
0.6 M benzamidine eluate from column 1	81.5	14.6	8 799	602	57
Eluate from Sephadex G-25 column	110	14.0	8 396	600	54
0.6 M L-arginine eluate from 4-aminobenzamidine aminododecyl celllulose column 2 (fraction II)	61	6.9	7 938	1150	51

binding to the active site of the enzyme (see Ref. 5, and K. Danø, unpublished data).

In the 0.6 M L-arginine eluate from the second benzamidine column (fraction II), a protein labeled by [^{3}H]diisopropylphosphorofluoridate appeared, having an apparent molecular weight of 29 000 (Fig. 2b). The amount of this protein was increased by incubation at neutral pH without $ZnCl_2$. By diffusion into an agarose gel containing fibrin and plasminogen, this protein proved to be a plasminogen activator and it probably represented a degradation product of the 48 000-dalton enzyme.

Anion exchange chromatography and gel filtration

Isoelectric focusing of Fraction II followed by detection of plasminogen activator by the agarose-fibrin plate overlay technique showed that the plasminogen activator had an isoelectric point of pH 9.2, while the isoelectric points of most of the impurities in this fraction were below pH 8. This finding suggested that conditions for anion-exchange chromatography could be obtained which allowed binding of most of the impurities to the column without binding of the plasminogen activator. As shown in Table II and Fig. 1c, this was the case with a QAE-Sephadex column at pH 9.0: only plasminogen activator and a few contaminants visible on the stained polyacrylamide gel (with apparent molecular weights 20 000—26 000) were eluted with the starting buffer (Fig. 1). This eluate (fraction III) also contained an increased amount of the [^{3}H]diisopropylphosphorofluoridate-labeled 29 000-dalton protein (Fig. 2c) probably because at pH 9.0, $ZnCl_2$ at the maximal concentration obtainable (0.001 M) did not inhibit proteolytic degradation of the plasminogen activator completely. Addition of other inhibitors of proteolysis (e.g. benzamidine and arginine) decreased the binding of the impurities present in fraction II to the QAE-Sephadex column. In initial experiments where the QAE-Sephadex column was developed without Triton X-100, more than 70% of the activity in

TABLE II

ION-EXCHANGE CHROMATOGRAPHY AND GEL FILTRATION OF MURINE PLASMINOGEN ACTIVATOR

Fraction	Volume (ml)	Protein (mg)	Activity (kU)	Specific activity (kU/mg)	Yield (%)
Fraction II applied to Sephadex G-25 column	55	6.8	7366	1083	100
Eluate from Sephadex G-25 column	68	6.6	7246	1097	98
Eluate from QAE-Sephadex column (fraction III)	120	n.d.	5253	n.d.	71
Fraction III after ultrafiltration	2.1	0.91	4615	5071	63
Eluate from Bio-Gel P60 column (fraction IV)	8.8	0.62	4534	7312	62

n.d., not determined.

fraction III (at pH 3.0) was lost in 1 h at 4°C. However, when Triton X-100 (0.1%, w/v) was added to fraction III, more than 90% of the activity was preserved after 24 h at 4°C and it could be stored frozen for more than 2 months without loss of activity. In all later experiments, therefore, 0.1% Triton X-100 was added to samples and buffers in this and the subsequent steps.

All detectable contaminants in fraction III could be removed by gel filtration on a Bio-Gel P60 column (Figs. 1d and 2d). Before the gel filtration, fraction III was concentrated 60-fold by ultrafiltration against an Aquacide solution with a recovery of about 90% (Table II). Activity was eluted from the Bio-Gel column in a symmetrical peak and the recovery at this latter step was consistently nearly 100% (Table II). Essential for the high recovery was the presence of Triton X-100 and a high concentration of L-arginine, both in the concentration step and in the gel filtration step.

Characterization of pure plasminogen activator

The apparent molecular weight of the pure plasminogen activator as estimated by SDS-polyacrylamide gel electrophoresis was 48 000 (Fig. 1d, 2d and 3b), and its electrophoretic mobility was indistinguishable from that of the enzyme in the culture fluid used as starting material for the purification (Figs. 1—3) and that of plasminogen activator extracted from the producer cells (Fig. 3).

The specific activity of the pure preparation of plasminogen activator was 7300 kU/mg as based on determination of activity by the radiolabeled fibrin plate assay and an estimation of protein by the method of Lowry et al. (Table II). From this value it can be calculated that the concentration of the enzyme in the medium from the 3T3/MSV-LO cells was approx. 15 nM and that the sensitivity of the iodinated fibrin plate assay used in this study was approx. 10^{-15} mol of the enzyme.

The fraction IV enzyme (10 μg/ml) retained its activity when kept at 4°C for 24 h in the buffer used for elution from the Bio-Gel P60 column. Removal of arginine and $ZnCl_2$ from the buffer did not affect the stability of the enzyme under these conditions even at pH 5.5, 7.2 and 9.0. No loss of activity was observed after frozen storage of fraction IV for 3 months.

Isoelectric focusing of 1 μg fraction IV enzyme followed by detection of the plasminogen activator by diffusion into an agarose gel containing plasminogen and fibrin showed a single band of activity corresponding to an isoelectric point of pH 9.2.

Activation of human plasminogen by the purified mouse plasminogen activator in the presence of bovine pancreatic trypsin inhibitor was monitored by electrophoresis in SDS-polyacrylamide gels under reducing conditions. It was found that the pure enzyme converted the single chain of plasminogen into heavy and light chains of plasmin with electrophoretic mobilities identical to those previously obtained by activation with crude preparations of 48 000-dalton murine plasminogen activator or with human urokinase [19].

Discussion

This is the first study on the purification of a murine plasminogen activator. Partial or complete purification of plasminogen activators has most often been

described for human urinary urokinase or its degradation products [22—26]. Human plasminogen activators have also been purified from uterus, cell cultures and blood vessel perfusates [27—30], and plasminogen activators have been purified from chicken and hamster cells transformed by oncogenic viruses [3,4] and from pig heart [31,32]. In most previous reports, the plasminogen activators were present in the starting material in a very low concentration and/or with a low specific activity, and yields of purified intact enzyme have usually been low. For this study, we selected a subline of virus-transformed mouse cells which released the plasminogen activator into serum-free medium in relatively high concentrations (approx. 500 μg/l) and with a high specific activity, such that less than a 150-fold purification was needed, and a simple purification procedure was developed which allows purification with an acceptable yield (approx. 30%) of the milligram amounts necessary for chemical and immunological studies. The purified enzyme was homogeneous as evaluated by SDS-polyacrylamide gel electrophoresis (Fig. 1d). Its electrophoretic mobility was identical to that of the enzyme in the starting material, indicating that the purification procedure is sufficiently gentle not to induce major changes in the molecule.

The pure plasminogen activator was labeled by [^{3}H]diisopropylphosphorofluoridate and this labeling could be prevented by *p*-nitrophenyl-*p'*-guanidino benzoate. This identifies the enzyme as a trypsin-like serine protease in agreement with previous findings for crude preparations of the enzyme from MSV-transformed mouse fibroblasts [5], and findings reported for other plasminogen activators including urokinase [5,7]. A further resemblance to other plasminogen activators was the conversion by the purified enzyme of the single polypeptide chain of human plasminogen to plasmin molecules consisting of two chains with the same electrophoretic mobilities under reducing conditions as those formed by conversion with urokinase [19,33].

As determined by SDS-polyacrylamide gel electrophoresis, the apparent molecular weight of the purified plasminogen activator was 48 000. Plasminogen activators with identical electrophoretic mobility were found in culture medium from a MSV-transformed tertiary culture of mouse embryonal fibroblasts, in mouse urine and in extracts from mouse lungs, and a similar electrophoretic mobility has been reported for plasminogen activators from stimulated mouse macrophages [7], mouse mammary gland cells during postlactation involution [11], mouse trophoblasts during their invasive phase [13], and a variety of cultured mouse cells of malignant neoplastic origin [16]. It is likely that all these plasminogen activators are identical to the enzyme purified in this study, and it should be noted that in several cases these plasminogen activators have been implicated in degradation of tissues [12]. Plasminogen activators with molecular weights of approx. 48 000 have also been found in hamster and rat cells [16], while all reported molecular weights of human plasminogen activators have been significantly different [8,16,22—26,30,32].

A 29 000-dalton plasminogen activator has previously been reported in mouse urine [16] and in culture medium from MSV-transformed mouse embryonal fibroblasts [5]. In the present study, a 29 000-dalton plasminogen activator appeared during the purification. These three plasminogen activators are probably identical molecules and our findings suggest that they may be

degradation products of the 48 000-dalton enzyme.

A plasminogen activator with an apparent molecular weight of 75 000 was found in extracts from mouse lungs. Plasminogen activators with a similar molecular weight are also present in mouse plasma [16] and in mouse embryonal ectodermal cells [13]. The relationship between these enzymes and the 48 000-dalton enzyme is uncertain. One possibility is that the 48 000-dalton and the 75 000-dalton enzymes are independent plasminogen activators which might have different functions, e.g. tissue degradation and thrombolysis, respectively. An alternative possibility that the 48 000-dalton enzyme is a conversion product of the 75 000-dalton enzymes seems less likely, as in this study plasminogen activators with molecular weights higher than approx. 48 000 were not detected either in the culture medium from 3T3/MSV-LO cells or in extracts from these cells.

It is likely that approaches similar to those presented in this paper will prove useful for the production and purification of other murine plasminogen activators and of plasminogen activators from other species, and thus contribute to the clarification of the physiological and pathological role of this class of enzymes and their mutual relationships.

Acknowledgements

We thank Drs. E. Reich, M. Ottesen and E. Dowdle for stimulating discussions during this study. We are grateful to Inge Starup, Inge Holm, Kirsten Lund Jacobsen and Lis Gøricke for excellent technical assistance. This work was supported by The Danish Medical Research Council, The Carlsberg Foundation, The Novo Foundation, The Danish Cancer Society, The NATO Science Programme, P. Carl Petersens Foundation and Ebba Celinders Foundation.

References

1 Unkeless, J.C., Tobia, A., Ossowski, L., Quigley, J.P., Rifkin, D.B. and Reich, E. (1973) J. Exp. Med. 137, 85—111
2 Ossowski, L., Unkeless, J.C., Tobia, A., Quigley, J.P., Rifkin, D.B. and Reich, E. (1973) J. Exp. Med. 137, 112—126
3 Unkeless, J., Danø, K., Kellerman, G.M. and Reich, E. (1974) J. Biol. Chem. 249, 4295—4305
4 Christman, J.K. and Acs, G. (1974) Biochim. Biophys. Acta 340, 339—347
5 Danø, K. and Reich, E. (1978) J. Exp. Med. 147, 745—757
6 Wilson, E.L. and Dowdle, E. (1978) in Regulatory Proteolytic Enzymes and Their Inhibitors (Magnusson, S., Ottesen, M., Foltman, B., Danø, K. and Neurath, H., eds.), pp. 151—160, Pergamon Press, Oxford
7 Unkeless, J.C., Gordon, S. and Reich, E. (1974) J. Exp. Med. 139, 834—850
8 Granelli-Piperno, A., Vassalli, J.-D. and Reich, E. (1977) J. Exp. Med. 146, 1693—1706
9 Beers, W.H., Strickland, S. and Reich, E. (1975) Cell 6, 387—394
10 Strickland, S., Reich, E. and Sherman, M.I. (1976) Cell 9, 231—240
11 Ossowski, L., Biegel, D. and Reich, E. (1979) Cell 16, 929—940
12 Reich, E. (1978) in Molecular Basis of Biological Degradative Processes, (Berlin, R.D., Herman, H., Lepow, I.H. and Tanzer, J.M., eds.), pp. 155—169, Academic Press, New York
13 Strickland, S. and Mahdavi, V. (1978) Cell 15, 393—403
14 Danø, K., Oronsky, A. and Gjedde, S. (1978) in Regulatory Proteolytic Enzymes and Their Inhibitors (Magnusson, S., Ottesen, M., Foltmann, B., Danø, K. and Neurath, H., eds.), pp. 113—125, Pergamon Press, Oxford
15 Davies, H. (1975) in Isoelectric Focusing (Arbuthnott, J.P. and Beeley, J.A., eds.), pp. 97—113, Butterworth, Norwich

16 Granelli-Piperno, A. and Reich, E. (1978) J. Exp. Med. 148, 223—234
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Bonsall, R.W. and Hunt, S. (1971) Biochim. Biophys. Acta 249, 266—280
19 Danø, K. and Reich, E. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B. and Shaw, E., eds.), pp. 357—366, Cold Spring Harbor Laboratory, New York
20 Danø, K. and Reich, E. (1979) Biochim. Biophys. Acta 566, 138—151
21 Ossowski, L., Quigley, J.P. and Reich, E. (1974) J. Biol. Chem. 249, 4312—4320
22 Ploug, J. and Kjeldgaard, N.O. (1957) Biochim. Biophys. Acta 24, 278—282
23 Lesuk, A., Terminiello, L. and Traver, J.H. (1965) Science 147, 880—882
24 White, W.F., Barlow, G.H. and Mozen, M.M. (1966) Biochemistry 5, 2160—2169
25 Ogawa, N., Yamamoto, H., Katamine, T. and Tajima, H. (1975) Thromb. Diathes. Haemorrh. (Stuttg.) 34, 194—209
26 Holmberg, L., Bladh, B. and Åstedt, B. (1976) Biochim. Biophys. Acta 445, 215—222
27 Rijken, D.C. and Wijngaards, G. (1977) Proc. 11th FEBS Meeting, Copenhagen, 853
28 Bernik, M.B., White, W.F., Oller, E.P. and Kwaan, H.C. (1974) J. Lab. Clin. Med. 84, 546—558
29 Wu, M., Arimura, G.K. and Yunis, A.A. (1977) Biochemistry 16, 1908—1913
30 Binder, B.R., Spragg, J. and Austen, K.F. (1979) J. Biol. Chem. 254, 1998—2003
31 Cole, E.R. and Bachmann, F.W. (1977) J. Biol. Chem. 252, 3729—3737
32 Wallen, P., Kok, P. and Rånby, M. (1978) in Regulatory Proteolytic Enzymes and Their Inhibitors (Magnusson, S., Ottesen, M., Foltmann, B., Danø, K. and Neurath, H., eds.), pp. 127—135, Pergamon Press, Oxford
33 Summaria, L., Hsieh, B. and Robbins, K.C. (1967) J. Biol. Chem. 242, 4279—4283